ISSN 2449-8955
EJBRAT 7(4) 2017

Volume 7
Number 4
October-December 2017

European Journal of Biological Research

MNiSW points 2016: **11**
Index Copernicus 2016: **95.65**

http://www.journals.tmkarpinski.com/index.php/ejbr
e-mails: ejbr@tmkarpinski.lh.pl ejbr@interia.eu

European Journal of Biological Research

ISSN 2449-8955

List of Peer-Reviewers http://www.journals.tmkarpinski.com/index.php/ejbr/pages/view/reviewers

Author Guidelines http://www.journals.tmkarpinski.com/index.php/ejbr/about/submissions

More information www.journals.tmkarpinski.com/index.php/ejbr

Publisher and Editor's office: Tomasz M. Karpiński, Szkółkarska 88B, 62-002 Suchy Las, Poland, e-mail: ejbr@interia.eu

Contents

ISSN 2449-8955

European Journal of Biological Research

Review Article

Scorpion venom: pharmacological analysis and its applications

Mukesh Kumar Chaubey

Department of Zoology, Mahatma Gandhi Post Graduate College, Gorakhpur - 273001, U.P., India;
E-mail: mgpgc@rediffmail.com

Received: 14 July 2017; **Revised submission:** 25 September 2017; **Accepted:** 27 September 2017

DOI: http://dx.doi.org/10.5281/zenodo.998076

ABSTRACT

Scorpions belong to class: Arachnida, order: Scorpionida represented now by approximately 1500 species. These are one of the most ancient group of the animals on the earth conserving their morphology almost unaltered and are the most successful inhabitants of the earth. Scorpions when stimulated secrete venom which is a cocktail of variable concentration of neurotoxins, cardiotoxins, nephrotoxins, hemolytic toxins, phosphodiesterases, phospholipases, hyaluronidase, glucosaminoglycans, histamine, seratonin, tryptophan and cytokine releasers. According to an estimate, frequency of deaths caused by scorpion sting is higher in comparison to that of caused by snake-bite. Almost all of these lethal scorpions except *Hemiscorpious* species belong to scorpion family Buthidae comprising 500 species. Scorpion venoms show variable reactions in envenomated patients. However, closer the phylogenic relationship among the scorpions, more similar the immunological properties. Furthermore, various constituents of venom may act directly or indirectly and individually or synergistically to exert their effects. Scorpion stings cause a wide range of conditions from severe local skin reactions to neurologic, respiratory and cardiovascular collapse. Lethal members of Buthidae family include *Buthus*, *Parabuthus*, *Mesobuthus*, *Tityus*, *Leiurus*, *Androctonus* and *Centruroides*. Besides their lethal properties, scorpion venoms have some unique properties beneficial to mankind. These contain anti-insect, antimicrobial and anticancer properties and thus, can play a key role in the insect pest management programmes, treatment of microbial infection and in the treatment of various cancer types.

Keywords: Scorpion venom; Envenomation; Neurotoxins; Ion channel blockers; Anticancer peptide; Antivenom.

1. INTRODUCTION

Scorpion sting is a major health problem in under developed tropical countries especially in poor communities. According to an estimate, frequency of deaths caused by scorpion sting is higher in comparison to that of caused by snake-bite [1]. Scorpions belong to class: Arachnida, order: Scorpionida. Scorpion has flattened and elongated body with four pairs of legs, a pair of claws and a segmental tail that has a poisonous spike at the end. Scorpion varies in size according to age and species from 1-20 cm in length. These can be found outside their normal territory when they accidentally crawl into luggage, boxes, containers or shoes, and are transported to home via human unwillingly. These are one of the most ancient group of the animals on

the earth represented now by approximately 1500 species conserving their morphology almost unaltered [2, 3].

Scorpions are not aggressive and do not hunt but wait for its prey. Scorpions being nocturnal in habit and capture its prey during night. They hide in crevices and burrow during day time to avoid the light. Human stinging occurs accidentally when scorpions are touched during resting and most of the stings occur on hands and feets. Scorpions are well equipped with a pair of pincer like pedipalps. Thus, peoples question why they need to produce venom at the same time. This is because most scorpions are opportunistic predators lacking the speed of its prey like insects and thus they are not choosy in their prey selection. Also obtaining relatively large prey like mouse and large beetles is a quite tough task for a scorpion. In such condition, pedipalps may prove insufficient to manage prey as quickly as possible. Thus, venom which is nature's gift provided to scorpions comes into play. A second advantage of venom is the presence of enzymes as venom's constituents with diverse activity. These enzymes initiate the process of digestion in tissue of the prey stung before consumption. Scorpion venom is an effective defensive device, which serves to deter and incapacitate the opponent. Hissing and aggressive defense posture is usually enough to deter most animals including human. When deterrence proves inadequate scorpions defend itself by injecting venom into the body of enemy. Thus, the main purpose of production of venom in scorpions is to secure food and self-protection.

Venom glands, the factory of scorpion's venom are located on the lateral side of tip of sting. These are made of different types of tall columnar cells. Of these cells, one type produces toxins while others produce mucus. Potency of scorpion's venom varies from species to species with some producing only a mild flu while other producing death. Scorpion stings cause a wide range of conditions from severe local skin reactions to neurologic, respiratory and cardiovascular collapse. Scorpion venoms exert their action mainly by affecting specific functions of the ion channels [4-6]. Among well-characterized toxins peptides from venom of the scorpion, most of them belong to family Buthidae. Buthoid venom has been reported for its severe consequences against a wide variety of vertebrate and invertebrate organisms and its toxicity is attributed to the presence of a large variety of basic polypeptides having three to four disulfide bridges [4, 7]. Due to heterogeneous nature, scorpion venoms show variable reactions in envenomated patients. However, closer the phylogenic relationship among the scorpions, more similar the immunological properties. Furthermore, various constituents of venom may act directly or indirectly and individually or synergistically to exert their effects. In addition, differences in amino acid sequences of each toxin accounts for their differences in function, pharmacology and immunology. Thus, any alteration in amino acid sequence may result in modification of function, pharmacology and immunology of toxin.

Differences in pathogenecity and level of toxicity of scorpion venom are actually due to diversity in toxin peptides and differences in amino acids in active site region of toxin peptides. This leads to diversification in their mode of action in different venomous scorpion groups in different climatic conditions and finally results into ecological adaptation in due course of evolutionary journey. Scorpions when stimulated secrete a small quantity of transparent venom called prevenom. If stimulation continues, cloudy, dense and white coloured venom is released subsequently. Prevenom contains a concentration of high K^+ salt and several peptides including some that block K^+ channels. This prevenom causes significant toxicity and scorpions use it as a highly efficacious predator deterrent and for immobilizing small prey while conserving metabolically expensive venom until a certain level of stimuli is reached [8]. That is why scorpions are known to be economical in their use of venom.

Production and storage of venom is an expensive metabolic process especially for species of extreme ecosystems. Other than antimicrobial peptides, all neurotoxins in venom are highly folded disulfide bridged molecules [9]. Low yields and reduced expression of these highly folded peptides in recombinant system indicates unique and difficult folding and storage requirement [10]. About fifty scorpion species distributed throughout the world have been proved lethal to human [11, 12]. Almost all of these lethal scorpions except *Hemiscorpius* species belong to scorpion family

Buthidae comprising 500 species. Lethal members of Buthidae family include *Buthus*, *Parabuthus*, *Mesobuthus*, *Tityus*, *Leiurus*, *Androctonus* and *Centruroides*. Common scorpions and their distributions are:

1. *Buthus*: Mediterranean area
2. *Parabuthus*: Southern and Western Africa
3. *Mesobuthus*: Asia
4. *Tityus*: Central and South America
5. *Leiurus*: Northern Africa
6. *Androctonus*: Northern Africa to South-East Asia
7. *Cetruroides*: South-West USA, Mexico, Central America
8. *Heterometrus*: Asia
9. *Pandinus*: Tropical Africa and Arabian Peninsula.

Scorpions mostly occur in temperate and tropical habitats of the world. They are well adapted to survive in extreme thermal environments, sometimes constituting a major portion of the total animal biomass in such environments. These are considered among the most successful inhabitants of the earth [13, 14]. Although numerous factors contribute to the success of scorpions, the ability to produce and deliver highly toxic venom is an important determinant of their success.

Scorpion venom is composed of water, salts, biogenic amines, peptides and enzymes. Venom of several scorpion species has been well characterized and various toxin peptides possessing the majority of biological activities have been isolated [15]. In venom mixture, there are many peptides that are specifically active against vertebrates, invertebrates or both. Toxin peptides of all these three groups are well characterized and includes peptides that target all the major ion channels such as Na^+, K^+, Cl^-, Ca^{++} and rynodine sensitive Ca^{++} channels [15, 16]. Potency of venom is mainly due to its ability to target multiple types of ion channels simultaneously resulting in a massive and recurring depolarization of nerve fibres that disables or kills prey or predators.

Generally scorpion venom possesses variable concentration of neurotoxins, cardiotoxins, nephrotoxins, hemolytic toxins, phosphodiesterases, phospholipases, hyaluronidase, glucosaminoglycans, histamine, seratonin, tryptophan and cytokine releasers. The most potent toxin is neurotoxin, which is divided in two classes viz. short chain and long chain peptides. Toxin peptides of both these classes are heat stable with low molecular weight. These are responsible for cell impairment in nerves, muscles and the heart by altering ion channel permeability. Long chain polypeptide neurotoxins cause stabilization of voltage dependent Na^+ channel in open position leading to continuous prolonged repetitive firing of somatic, sympathetic and parasympathetic neurons. These repetitive firings result in autonomic and neuromuscular over excitation preventing normal nerve impulse transmission. Further, it results in excessive release of neurotransmitters such as acetylcholine, glutamate, aspartate, epinephrine and norepinephrine.

Short chain polypeptide scorpion toxins are K^+ channel blockers. Binding of these toxin peptides is reversible but with different binding affinities. Stability of these neurotoxins is due to four-disulfide bridges that fold neurotoxin into a very compact three-dimensional structure, thus making it resistant to variation in hydrogen ion concentration and temperature. However, reagent that can break disulfide bridges can inactivate this toxin by unfolding it. Antigenicity of these toxins depends on the length and number of exposed regions out of the three-dimensional structure.

Fat tailed scorpion, *A. australis* has many toxin peptides, which are selectively lethal to mammals. This selectivity of venom can hardly be explained by food choice. This suggests a possible selective pressure for venom production against mammalian predators. It also helps to acquire other vertebrate prey as well. Also, if food acquisition is the main selective pressure for venom against vertebrates, then there should be higher composition of vertebrate toxins in large species like *P. imperator*. The hypothesis for deterrence is supported by composition of venom. Besides, other pathological and physiological effect, serotoxin, which is a constituent of scorpion venom, also causes pain similar to that caused by apamin of honey bee. In fact, immediate stimulation of pain is one of the most important properties of scorpion venom. Generally, toxin factors that initiate pain do not cause death. This certainly is the result of other components present in the cocktail of substances in venom. Now, it is a well-known fact that among all different scorpion toxins, neurotoxins are the most lethal peptides that cause high mortality in animals. Scorpions use their pincers to grasp their prey and

then arch their tail over their body to inject their venom into the prey, sometimes more than once. Scorpions regulate how much venom should be injected with each sting. The striated muscles in the sting regulates amount of venom ejected, which is usually 0.1-0.6 mg. If entire supply of venom is used, scorpion must require several days to regain venom supply.

Although poisonous scorpions are classified taxonomically into several genera, yet the mode of action of their venom is quite similar. Scorpion venoms contain neurotoxic peptides in low abundance with great diversity in their mode of action. These neurotoxin peptides are low in abundance in a complex mixture of venom having a majority of the biological effects towards the affected victim. Stings affect peripheral nervous system resulting in symptoms like intense pain at the site of sting, altered heart activity and paraesthesia [17].

In an experiment with labeled scorpion venom, amount of venom venom was found 28% in blood, 30% in muscle, 13% in bone, 12% in kidney and 11% in liver within five minutes after intravenous administration. Scorpion venom is excreted through renal and hepaticbiliary pathways. The maximum renal uptake of 32% at thirty minutes drops to 22% at three hours suggesting that excretion of venom through kidney is slow [18]. Scorpion venom in the animal body has a half-life of 24 hour indicating a slow clearance with mean residence time of 33.7 hours in the body and 26 hours in the peripheral compartments [19].

2. INSECTICIDAL PROPERTY OF SCORPION VENOM

Due to species-specific activity of scorpion toxins, efforts have been made to identify insect selective toxins that can be used to develop biopesticides as a safer alternative to replace chemical insecticides [20-22]. On the basis of mode of action, anti-insect scorpion toxins have been divided into three classes viz. (i) alpha toxins which are strictly selective for insects (ii) excitatory insect selective scorpion toxins, and (iii) depressant insect selective neurotoxins [23-25]. Anti-insect α-toxin peptides bind to voltage-dependent sodium channels with high affinity [26].

Excitatory toxin causes a repetitive firing of axon accompanied by a small depolarization [27]. On the other hand, depressant toxin produces an inhibition of excitability due to depolarization of axon. Depressant toxins cause a decrease in sodium peak current and induce a constant inward current at negative membrane potential [28]. These effects are similar to that of the beta toxins active against vertebrate systems [29]. Several insect selective toxins have been identified from scorpion venom of different geographical regions [16, 17, 30, 31]. AalT, a single chain neuropeptide isolated from *Androctonus australis*, has been proved insectotoxin by causing fast excitatory paralysis by presynaptic effect on insects's motor nerve resulting in a massive and uncoordinated stimulation of skeletal muscles. The neuronal repetitive activity is attributed to an exclusive and specific perturbation of sodium conductance as a consequence of toxin binding to external loop of insect's voltage dependent Na^+ channel and modification of its gating mechanism [32]. Three toxin peptides (AaHIT1, AaHIT2, and AaHIT3) have been isolated from *Androctonus australis* venom which act against insect and are used as potential insecticidal agents. AaHIT1 gene linked to a sendai virus has been transformed to mosquitoes by viral infections, which upon transformation express lethal toxins/ proteins and resulted in death of host [33]. The other two toxin peptides are also insect specific similar to AaHIT1 [39].

An anti-insect toxin peptide Lqh alpha IT has been isolated from *L. quinquestriatus* venom which causes a unique mode of paralysis in blowfly larvae [35]. Like excitatory and depressant insect toxins, Lqh alpha IT is highly toxic to insects but it differs from these in two important characteristics: (a) Lqh alpha IT lacks a strict selectivity for insects, highly toxic to crustaceans and also low toxic to mice. (b) It does not displace an excitatory toxin AalT from its binding site in insect neuronal membrane, which confirms that the binding site for the Lqh alpha IT is different from those imparted by excitatory and depressant toxins. Bot XIV isolated from *B. occitanus occitanus* is also an insecticidal toxin peptide but it does not show toxicity against mammals. This toxin peptide is highly antigenic in mice with the resulting antibodies having significant effectiveness in neutralizing other more toxic

proteins. Another antiinsect toxin Lqh III isolated from *L. quenquestriatus* affects sodium current in cockroach giant axon and prolongs action potential [36]. From *M. tamulus*, a short toxin peptide has been characterized which shows toxic effects against *Helicoverpa armigera* [37]. Gawade [38] has reported anti-insect toxin peptides, C56, from *Buthus* that has been shown to induce Ca^{++} dependent spontaneous excitatory activity in *Drosophila* larvae. Anti-insect toxin peptides characterized can be used in constructing genes and their *in vitro* expression product can be used as a replacement for synthetic pesticides. Albert et al. have expressed a synthetic gene encoding insecticidal neurotoxin of *A. australis* (AaIT) in NIH/3T3 mouse fibroblast cells under transcriptional control of a murine retro-viral long terminal repeat. Toxin peptides secreted in culture medium has been found toxic against yellow fever mosquito larvae but with no toxic effect on mice [20]. Genes of scorpion anti-insect toxin peptides mainly neurotoxin peptides have been used with recombinant baculovirus. These genes have been selected to avoid human and other non-target neurotoxicity as much as possible. In such aim of insect control, depressant toxin has been found more effective than excitatory toxin in recombinant baculovirus [39].

From a strict agro-technical point of view, two main points should be considered regarding the involvement of toxin peptide genes in plant protection (i) these act as a factor for genetic engineering of insect infective baculoviruses resulting in potent and selective bioinsecticides, and (ii) these must show pharmacological flexibility as a device for insecticide resistance management [32]. Indian red scorpion *M. tamulus*, known for its severe toxicity [40, 41], received little attention in this regard and only few toxin peptides have been reported for their insecticidal properties [42], while no such toxin peptides active against insects have been characterized from *Heterometrus* species till now.

3. ANTIMICROBIAL PROPERTY OF SCORPION VENOM

Since the discovery of antimicrobial peptides in invertebrates [43], more than hundreds of antimicrobial proteins have been characterized in both invertebrates [44] and vertebrates [45] with a wide phylogenic distribution including humans [46]. These have been reported in skin, epithelial cells and blood of vertebrates as well as in insect haemolymph and venomous secretions of bees, hornet, spider and scorpions [47-49]. These toxins are small basic peptides with variable length, structure and sequence. These antimicrobial peptides appear to form channels or pores in cell membrane inducing cell permeation and break down of cellular physiology [50]. These antimicrobial peptides have broad-spectrum, nonspecific activity against a wide range of microorganisms including viruses, gram-negative and gram-positive bacteria, protozoa, yeast and fungi, and may also be hemolytic and cytotoxic to cancerous cells [51, 52]. Antimicrobial peptides from scorpion venom are short peptides consisting of 10 to 50 amino acid residues with a net positive charge ranging from +2 to +9 and the proportion of hydrophobic residues are equal or more than 30% of total amino acids residues [53]. The positive amino acid residues are separated by patches of hydrophobic amino acids [54]. These antimicrobial peptides are usually cationic, amphipathic, α-helical peptides of low molecular eight (2-5 kD). Some peptides, such as hadrurin, are highly potent against both Gram-positive bacteria and Gram-negative bacteria without preference, while others show selective activity against either Gram-negative bacteria (parabutoporin) or Gram-positive bacteria (IsCT and BmKn2) [55, 56].

The pore forming antibacterial peptides of scorpion venom can be divided into two groups, depending on their primary and secondary structures: (a) linear, alpha helical peptides without cysteine residues, and (b) cysteine rich peptides that form a beta sheet or beta sheet and alpha helical structures [57]. Besides acting by destabilizing membrane structure and changing ion permeabilities, pore forming peptides can influence cell functioning by interacting with intracellular signaling molecules such as G-proteins [58]. Although many antimicrobial peptides have been described in insects [59], several antimicrobial peptides have been isolated and characterized from scorpions including several cysteine-containing peptides from haemolymph of scorpion *L. quinquestriatus hebraeus* [60] and *A. australis* [61].

The earliest peptide toxin ever studied was androctonin isolated from *A. australis* venom [62]. Androtonin shows potent antibacterial activity against both Gram-positive and Gram-negative bacteria [62]. Moerman et al. have also reported antifungal activity of androtonin [63]. Powers and Hancock have reported the antibacterial activity of parbutoporin (from *P. schlechteri*) and opistoporins (from *Opistophthalmus carinatus*). These peptide toxins target G-proteins for membrane lytic activity [64]. VpAmp1.0 and VpAmp2.0 peptides isolated from *Vaejovis punctatus* inhibit growth of both Gram-positive (*Staphylococcus aureus* and *Streptococcus agalactiae*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria, yeasts (*Candida albicans* and *Candida glabrata*) and two strains of *Mycobacterium tuberculosis* [65]. Opisin peptide isolated from *Opistophthalmus globrifrons* is a cationic, amphipathic, and α-helical molecule with 19 amino acid residues without disulfide bridges. This peptide inhibits growth of the some Gram-positive bacteria [66]. Stigmurin isolated from Brazilian yellow scorpion *Tityus stigmurus* venom gland shows antibacterial and antifungal activity [67]. Ctriporin isolated from *Chaerilus tricostatus* shows a broad-spectrum of antimicrobial activity and is able to inhibit antibiotic resistant pathogens, *Staphylococcus aureus* strains [68]. Hp1404 isolated from *Heterometrus petersii* is an amphipathic α-helical peptide with inhibitory activity against gram-positive bacteria like *Staphylococcus aureus* [69].

Buthinin, a three disulfide bridged bactericidal and fungicidal peptide, androctonin, with two disulfide bridges from *A. australis* venom and scorpine, a 75 residue antimicrobial peptide from *P. imperator* venom have been characterized [61, 70]. Alpha-helical proteins containing antimicrobial properties have been reported from *Hadrurus aztecus* venom [71] and *P. schlechteri* [72]. Pandinin 1 and pandinin 2 with antimicrobial property have been isolated from *P. impretor* venom [9]. Most of these antimicrobial peptides share some common characteristics such as their low molecular mass, presence of multiple lysine and arginine residues and their amphipathic nature. Their site of action is cytoplasmic membrane where they destabilize its lipid package and produce transient channels and disturb ion permeability across the membrane [73, 74].

Heterometrus xanthopus venom contains antimicrobial peptides like hadrurin, scorpine, Pandinin 1 and Pandinin 2. These peptides are able to kill antibiotic-resistant strains of *Bacillus subtilis* ATCC 6633, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* ATCC 14506. Two antimicrobial peptides have been identified from the venom of North African scorpion, *A. aeneas*. These peptides show antimicrobial activity against the Gram-positive bacterium, *S. aureus* and the yeast, *C. albicans*, but do not affect Gram-negative bacterium, *E. coli* [75]. Scorpion *Leiurus quinquestriatus* venom shows significant broad-spectrum antimicrobial activity against *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Candida albicans* and *Candida glabrata* [76]. Despite of their high minimum inhibitory concentration in comparison to other antibiotics, their broad spectrum of activity and speed of action makes them good candidates for drug delivery and for a number of other possible applications in pharmacological research [52].

4. ANTICANCER PROPERTY OF SCORPION VENOM

Cancer is a major health problem all over the world [77]. Treatment of cancer involves different clinical approaches including surgery, chemotherapy, radiotherapy, gene therapy, hormone therapy and immunological therapy either alone or in combinations. All of these approaches have its own advantages and disadvantages and mainly depend on the type and stage of cancer. Recent advancement in cancer therapy includes synthesis of peptides and proteins through DNA technology and production of monoclonal antibodies specific for oncoproteins. After realizing the medicinal use of anticancer proteins and peptides, many proteins of animal origin have been isolated. De Carvalho et al. isolated and characterized lectins (polyvalent carbohydrate binding proteins of non-immune origin) from snake *Bothropos jararacussu* venom which serve as an interesting tool by inhibiting tumor cells of human breast and ovarian cancer [78]. Some other group of scientists reported

anticancer effect of other lectins and toxins from some other snake venoms also [79-81].

Scorpion venom contains a number of polypeptides with diverse biological activities. It has been earlier reported that venom of some scorpions has high histopathological and necrotic effects in human and animals [82, 83]. However, for the first time, Omran has reported anticancer property of *Leiurus quinquestriatus* venom on human breast cancer cell lines [84]. According to Bruses et al. [85] some toxins bind to a specific receptor in the membrane before they can exert their action. Anticancer effects of scorpion venoms have been evaluated in various types of cancers as glioma, neuroblastoma, leukemia, lymphoma, breast, lung, prostate and pancreatic cancer. These venoms produce anticancer effects by blocking specific ion channels, inhibiting invasion and metastasis of cancer cells and activating intracellular pathways leading to cell cycle arrest and apoptosis [86-88]. Venoms from various scorpions have been reported to prevent propagation of different cell lines such as prostate cancer, human leukemia and neuroblastoma [89-91]. Venom of *A. crassicauda* inhibited proliferation of human neuroblastoma cell lines through arresting S-phase and induction of apoptosis [92]. Almaaytah et al. characterized the cytolytic peptides AamAP1 and AamAP2 from the venom of North African scorpion *A. amoreuxi* [93]. They reported that the natural peptides AamAP1 and AamAP2 show moderate antiproliferative activity against LNCaP, U251, PC3 and HMEC-1 cell lines. The venom peptide Acra3 from *A. crassicauda* induces cytotoxic effect on mouse brain tumor cells (BC3H1) through both necrotic and apoptotic pathways [90]. Venom from the Buthidae scorpions *A. bicolor, A. crassicauda* and *L. quinquestriatus* show strong anticancer activity on colorectal and breast cancer cell lines through decreasing cell motility and colony formation of cancer cells [89].

5. ION CHANNEL BLOCKING PROPERTY OF SCORPION VENOM

Scorpion venom contains several small neurotoxic peptides, which selectively act on various types of ion channels. These toxin peptides have been extensively used as valuable biochemical and pharmacological tools to characterize and discriminate various ion channel types that differ in ionic selectivity, structure and function. These neurotoxins affect victim by interfering with ionic balance and ion channel activity in excitable cells. Binding of scorpion toxins to target ion channels is known to occur through multiple interactions [94]. Numerous amino acid residues that determine the binding property to target ion channels have been characterized [15]. In addition, α-scorpion toxins are known to inhibit or slow down the Na^+ ion channel. Scorpion toxin peptides can be divided into four groups on the basis of their target ion channels. The first class belongs to toxin peptides acting on the Na^+ channel, which consist of 60-70 amino acid residues and four intermolecular disulfide bonds. These long chain toxin peptides modulate activation or inactivation of Na^+ channels [15]. These toxin peptides alter kinetics of Na^+ channel opening and closing in excitable cells [95]. Scorpion toxins affecting voltage-gated Na^+ channels have been divided into two groups α- and β-toxins on the basis of their electrophysiological effects and binding properties. Alpha-toxins bind in a voltage dependent manner and inhibit depolarization of action potential while beta-toxins bind in a voltage independent manner and modulate the activation phase of action potential [96]. Makatoxin 1 and bukatoxin, members of α-scorpion toxin family isolated from *B. martensi* venom show pharmacological action similar to other α-toxins on neuronal voltage sensitive sodium channels [97]. The toxin peptides from Buthidae family prolong Na^+ ion activation phase of action potential while toxins from Centrurinae and Tityinae venom affect Na^+ activation phase [98]. Ts-gamma, a neurotoxin of *T. serrulatus* produces very complex cardiological effects characterized by an initial reduction of both rate and contractile force followed by an increase in force and reduction of rate. This contraction finally reduces due to release of acetylcholine from vagal endings [99]. This toxin apparently produces these effects on cell currents primarily by retarding activation of cardiac sodium channels [100]. Gawade et al. [38] have isolated and characterized a toxin peptide Lqh1β1 from *Leiurus quinquestriatus hebraeus* venom. It competes with anti-insect and anti-mammalian α-toxins for its binding site on Na^+ channel. It also competes with an anti-mammalian α-toxin for its binding site.

The second class of toxins includes K^+ ion channel blockers. These toxin peptides consist of 23-40 amino acid residues having three to four disulfide bonds. Bmpo2, a 28 amino acid residues peptide from *B. mortensi* venom shows very low inhibition of apamine sensitive Ca^{++}-activated K^+ channel [101]. Neurotoxic peptides tamulotoxin (TmTx) and iberiotoxin (IbTx) from *M. tamulus* having 37 amino acid residues and three disulfide bridges have shown to cause Ca^{++}-activated K^+ channel blockage [102]. Other scorpion toxins with shorter polypeptide chain having less than 40 amino acid residues such as charybdotoxin and kaliotoxin also act on this channel [31, 103, 104]. Although numerous known scorpion toxins differ in size, sequence and biological activity, they all share a common structural motif consisting of antiparallel sheet linked to an amphipathic helix and an extended N-terminal fragment by three disulfide bridges [30]. This motif is also present in insect defensins, a family of inducible antibacterial peptides isolated from a variety of insects where they present a key element of the innate host defense against microorganisms [105].

Romi-Lebrun et al. have isolated four peptideyl inhibitors of small conductance Ca^{++}-activated K^+ channels from *B. martensi* [101]. *C. noxius* contains β-toxin which blocks voltage gated K^+ channels by binding to site different than that of other beta toxins [106]. Margatoxin of *C. margaritatus* is a potent K^+ channel blocker selecting for only one sub-type of K^+ channel. This particular K^+ channel is directly involved in lymphocytes activation and blocks lymphocyte activation and production of interleukin-2 by human T-lymphocytes [107-109]. Agitoxin of *Leirus* venom binds to external pore entry pathway of shaker K^+ channel as well as mammalian homologues [110]. This toxin is related K^+ channel neurotoxins but forms a new subclass of scorpion derived K^+ channel inhibitors [100]. Scyllatoxin (laiurotoxin1) *Leiurus* binds to high conductance Ca^{++}-activated K^+ channels [111]. *P. imperator* venom contains peptides that binds and blocks voltage-gated K^+ channels [112, 113]. Two toxin peptides viz. Imperatoxin A and Pil have been identified from *P. imperator* venom. Imperatoxin A selectively activates skeletal-type ryanodine receptor [114] and may prove a useful tool to identify regulatory domains critical for channel gating and to dissect the contribution of skeletal-type Ca^{++} release channel/ryanodine receptor to intracellular Ca^{++} wave forms generated by stimulation of different ryanodine receptor isoforms [115]. Pil toxin peptide selectively blocks shaker K^+ ion channels [116]. Many researchers have isolated short chain polypeptides like Ibtx from *B. tamulus* [117], Titustoxin V from *T. serrulatus* [118], Osk-1 from *Orthochirus scrobiculosus* [119] and Chtx from *B. martensi* [101]. These toxin peptides are potent inhibitors of voltage-gated K^+ channels. Dhawan et al. (2003) have isolated a short toxin peptide BTK-2 from *Buthus tamulus* that inhibits K^+ channel [37]. More et al. have reported a toxin peptide (PGT) fron *Palamneus gravimanus*. This toxin peptide selectively blocks the human cloned voltage-gated K^+ channel [120].

The third class of scorpion toxins acting on Cl^- channels has 35-37 amino acid residues with four disulfide bonds [121]. Chlorotoxin from *L. quinquestriatus* shows highest homology with short insect toxin [122]. The forth class includes toxins acting on Ca^{++} channels. These are short peptides with 25-35 amino acid residues [123, 124]. Kurtatoxin isolated from *P. transvaalicus* venom has been reported to inhibit voltage gated Ca^{++} channel [125].

Less than 1% of the estimated 0.1 million distinct peptides expected to exist in scorpion venom are known. It can be speculated that natural selection co-evolved distinct types and subtypes of receptors of ion channels in various groups of animals. At the same time scorpion evolved specific toxins designed to interfere with normal function of ion channels and to provide one way for scorpions to capture their prey or defend themselves from predators.

6. CARDIOTOXIC PROPERTY OF SCORPION VENOM

Scorpion venom induces complex cardiac disorders in several animal species [126-128]. When isolated hearts have given short exposure of purified or crude venom toxins, cardiac muscles show considerable increase in contractility [99, 129, 130]. These complex cardiovascular effects by scorpion venom may probably due to direct effect on

vagal and sympathoadrenal stimulation [131-133]. Isolated myocytes show a higher rate of contraction and loss of synchronous activity under the influence of *L. quinquestriatus* venom [133]. Increased deoxyglucose and Ca^{++} uptake into cardiac cells and influx of Ca^{++} into sarcoplasmic changes prevented by pretreatment with propranolol and nifedipine accompany these changes. Further, this stimulation of adrenoreceptors leads to increased influx of Ca^{++} through Ca^{++} channels which then increases contractility [133].

Venoms of all scorpion species affect cardiovascular system and cause pulmonary oedema and cardiac arrhythmias [124]. Venoms also cause cholinergic as well as adrenergic neuron hyper-stimulation by its acting on presynaptic membranes [125, 126]. These venoms have direct effect on gating mechanisms of excitable membranes [134]. As a result there is a massive release of catecholamines from synaptic nerve endings and from adrenal medulla [131, 137]. Elevations of circulating catecholamines and angiotensin result in intense vasoconstriction and cardiac stimulation [138], increased myocardial oxygen requirement and alteration in myocardial perfusions [134, 139]. Several of these mechanisms, together with a possible direct effect of toxin on myocardium may be responsible for myocarditis and focal myocardial necrosis in patients dying from envenomation [140]. Echocardiographic studies have shown severe systolic left ventricular dysfunction following envenomation [141, 142]. This is due to catechol-amine induced metabolic abnormalities in myocardium (138), increased myocardial oxygen requirements [143], myocardial ischemia [139] and direct effect of toxin [130, 140]. Scorpion venoms containing bradykinin-potentiating peptides (hypo-tensive agent) have been found in *L. quinque-striatus*, *T. serrulatus*, *B. martensii* and *B. occi-tanus*. These peptides act as bradykinin-potentiating peptides and can be used as hypotensive agents in the treatment of hypertension. Moraes et al. have reprted that *Tityus bahiensis* scorpion venom modify sodium channel gating to exert hypotension action [144].

The scorpion venom exerts its lethal action by interference with blood coagulation, either by accelerating the process or inhibits the coagulation processes. A peptide with anti-thrombotic action has been reported from *B. martensii* venom [142]. This peptide is related to the resistance against platelet aggregation and increases concentration of prostanglandin I2 in plasma [142]. *T. discrepans* scorpion venom modifies clotting times in humans. *T. discrepans* venom also affects partial thrombo-plastin time, prothrombin time and its direct clotting activity. This venom contains anticoagulant compo-nents which prolong prothrombin time and partial thromboplastic time [143].

7. OTHER PHARMACOLOGICAL ACTIVITIES OF SCORPION VENOM

Scorpion venom is known to modulate kinin pathway in animals. Kinins are peptides generated as a result of the activity of killikrekins (a group of proteolytic enzymes present in most of the tissues and body fluids) on kinogens. Once released, kinins such as bradykinin and related peptides kallikrekin (Lysbradykinin) and Met-Lys-bradykinin produce many physiological responses including pain and hyperanalgesia, in addition to contributing to inflammartory response [144, 145] *M. tamulus* venom causes increased peripheral sympathetic activity with consequent enhancement of adrenergic responses [146]. This venom also causes rhythmic fluctuation in blood pressure producing cardio-vascular collapse and death. It induces spontaneous action potential and causes prolongation of action potential duration in purkinje fibres. This venom enhanced release of acetylcholine and induced repetitive firing of nerve action potentials [147]. This effect may be due to toxins that affect opening of Na^{+} channels in nerve and muscles, which results in increased release of neurotransmitters in peripheral nervous system. It may produce cardiovascular abnormalities and respiratory paralysis also.

Venom of *B. tamulus* causes severe pancreatitis [148], increased osmotic fragility in red blood cells [149, 150], myocarditis [151], hyperglycemia and lipolysis resulting in increased free fatty acids and reduction in triglyceride level [152]. All these cardiovascular, hemodynamic and hematological alternations may be due to massive release of catecholamines, counter-regulatory hormones like glucagons and cortisol [153], angiotensin II [138], thyroxine and triodothyronine

[154] and a reduced insulin secretion [155]. *B. tumulus* venom is found to be protease inhibitors and histamine releasers [40]. Effect of *M. tamulus* and *L. quinquestriatus* venoms on noradrenergic and nitinergic transmission in rat isolated anococcygeus muscle has revealed that both venoms mediate their pharmacological effects via prejunctional mechanism involving activation of voltage sensitive Na^+ channels with consequent release of neurotransmitters mediated by other alpha scorpion toxin [156].

Radha Krishna Murthy and Zare have reported an increase in hemoglobin, mean corpuscular hemoglobin concentration, packed cell volume, plasma hemoglobin levels and increased osmotic fragility of erythrocytes during scorpion envenomation [157]. Fragility of red blood cells has also been observed when incubated with scorpion venom. Rise in packed cell volume and mean corpuscular hemoglobin concentration during scorpion envenomation may be due to hemoconcentration caused by a massive release of catecholamines [158-160] and angitensin II [138]. Phospholipase present in venom could be the agent responsible for increased hemolysis.

Certain venoms such as cobra venom contain phospholipase A, which converts lecithin to lysolecithin, a powerful hemolytic substance [161]. Chhatwal and Habermann have reported presence of phospholipoase A2 in scropion venom [40]. This enzyme is a powerful hemolytic agent and contributes to increased osmotic frgility of red blood cells [154]. Envenomation results in metabolic stress in red blood cells and pumping mechanism failure [140]. A reduction in erythrocyte Na^+K^+ ATPase has been reported in scorpion sting victim [150]. Pande and Mead have observed inhibition of Na^+K^+ATPase activity by elevated free fatty acids through their detergent properties [162]. *Hemiscorpius lepturus* venom increases circulating levels of aspartae aminotransferase, alanine aminotrans-ferase, alkaline phosphatase, creatine phosphokinase and lactic dehydrogenase in rat [163]. Similarly, Omran and Abdel-Rahman have reported elevation in serum glucose, nitrogen, creatine, glutamate oxaloacetate aminotransferase, glutamate-pyruvate aminotransferase, creatine phosphokinase and lactic dehydrogenase, while reduction in serum total protein, uric acid, cholesterol, calcium and potassium [164].

8. SCORPION ANTIVENOM

Scorpion venom is a mixture of many small polypeptides known to induce a strong immunogenic reaction from the host. Potent neurotoxins, which often are relatively small and low abundance molecule, may not always induce production of sufficient quality and quantity of antibody molecules. Therefore, the balance between injected doses, toxicity towards subject animal should be maintained for high quality antibody production. Identification of less abundant but highly potent components in purified mixture and its use as an antigen is highly advantageous in comparison to crude venom to raise antibodies for therapeutic purposes.

Severity of scorpion venom and its rapid diffusion requires appropriate treatment, which must start as soon as possible after sting. Most investigators consider antivenom as the only specific treatment of scorpion stings [127, 165,]. However, others have questioned usefulness of antivenom in eliminating cardiovascular complications of scorpion stings [160, 166].

Use of antivenom in the treatment of scorpion sting was started in 1909; and this mode of therapy, is still the only method used effectively against scorpion stings [167, 168]. Initially scorpion venom extracted from telson homogenate was used as antigen to inject in small doses in horses and sheeps to produce antivenom. After a long period of immunization, the blood of the immunized animal is obtained and the immunoglobulins are purified for use as antivenoms. Demagalhaes has claimed that toxicity of telson extract is less stable than that of pure venom [169]. Crude scorpion venom has many components, which shows poor antigenicity; therefore, other natural chemicals have been added to venom toxins to enhance antigenicity [170]. Scorpion venom is poor in antigenic composition and thus it is difficult to raise antibodies specific to neutralize lethal factor of scorpion venom. However, several attempts have been made to raise species-specific antibodies against scorpion venom. Mohammad et al.have used purified picrate venom obtained from dried telsons to prepare potent antivenom against Egyptean scorpion venom [171].

At Hoffkin Biopharmacutical Corporation Ltd. Mumbai, Kapadia et al. have attempted to prepare anti-scorpion venom serum using grounded telson extract with Freund's incomplete adjuvant [172]. The resulting antiserum, which contains antibodies against scorpion venom, has found to be inefficacious. Kankonkar et al. at Hoffkin Biopharmaceutical Corporation Ltd. Mumbai, India have used Bentonite as adjuvant for extending period of immunization and prepared potent antiserum against *Buthus tamulus* venom capable of neutralizing lethal factors of venom [173].

There are contradictory opinions about effectiveness of scorpion antivenom either in experimental animals or in scorpion sting victims. For quick neutralization of toxic effects of toxins, serotherapy is a well-tested pharmaceutical method that is used for safety of lives of many patients around the world [127, 158, 159, 174, 175]. Contrary to this, Gueron and his co-worker have reported that serotherapy is ineffective [160, 176]. Scorpions usually inject venom into interstitial spaces and not directly into blood circulation. Freire-Maia and Campos have suggested intravenous injection of antivenom to neutralize circulating venom [158]. Moreover, it is likely that antivenom administered intravenously can act on tissues later on. The best result can be achieved when antivenon is administered as early as possible and with adequate quantities to neutralize venom. Radha Kishana Murthy and Zare have reported that species-specific scorpion antiserum prepared at the Hoffkin Biopharmaceutical Corporation Ltd. Mumbai, India, reverses metabolic and hematological alterations caused by *Mesobuthus tamulus* scorpion venom [154].

Due to poor immunogenicity and vast difference in amino acid sequences in active site region, it is very tedious to prepare universal antivenon against scorpion venom. Furthermore, neurotoxic components of scorpion venom are least immunogenic. A recent idea for creating an universal anti-scorpion antivenom is to mix a batch of different anti-scorpion antivenin together to create a universal antivenin but this exposes patients to unnecessary antivenom from other scorpion species which are not from patient's region.

Current method for anti-scorpion antivenom production involves direct injection of crude venom into horses. Besides it, antibodies are also produced from a mixture of a number of scorpion species venoms. However, there are risks associated with injection of antibodies from other animals or passive immunization. The recipient can mount a strong immunologic response to isotype determinants to foreign antibodies. This anti-isotype response can have serious complications because some recipients will produce IgE antibody specific for injected passive antibody. Immune complexes of IgE bound to antibody can mediate systemic mast cell degranulation leading to systemic anaphylaxis. Another possibility is that the recipient will produce IgG or IgM antibodies specific for foreign antibody, which will form complement activating immune complexes. The deposition of these complexes in tissues can lead to type III hypersensitive reaction.

Another approach in neutralization of toxic effects of scorpion stings by serotherapy is possibility of raising antibodies to conserved parts of venom proteins, which could recognize several members of family. Devaux et al. have raised antibodies against an eight residue synthetic polypeptide, which represent conserved region in a set of 25 scorpion toxin sequences [177]. These peptide antibodies have been shown to cross-react with several scorpion toxins belonging to different serotype and neutralize pharmacological effects and biological activities.

Some special antivenoms are also available, which are the same horse antibodies treated with enzymes to produce $F(ab)'_2$ fragments that are used for immunotherapy [178]. Recently smaller recombinant fragments, such as classic monovalent antibody fragments (FAB, scFv and engineered variants: diabodies, triabodies, minibodies and single-domain antibodies) are now engineering as credible alternatives. These fragments retain the targeting specificity of whole antibody and can be used for therapeutic applications [179]. Single-chain Fvs are popular format in which the VH and VL domains are joined with a flexible polypeptide linker preventing dissociation. Antibody Fab and scFv fragments, comprising both VH and VL domains, usually retain the specific, monovalent, antigen binding affinity of the parent IgG, while showing improved pharmacokinetics for tissue penetration [179]. In this context, recently single chain antibodies of human origin have developed

and shown to be effective for neutralization of scorpion toxin envenomation [180-182].

9. SUMMARY

Envenomation of humans by scorpion stings is a serious health problem in some parts of the world. These venoms cause severe systemic inflammation and other complication when injected into humans. Scorpion venoms are mixture of peptides, amines, enzymes and many other bioactive compounds. The most important components, responsible for severe intoxication are short- and long-chain peptides affecting different ion channels (Na^+, K^+, Ca^{++}, Cl^-) either by blocking the channels or modifying their gating properties. They cause abnormal depolarization of the neuronal cells and if not treated on time can lead to death. Fot the neutralization of the venom's induced deleterious effects, venom itself is used for production of antivenom in experimental animals like horse and sheep. Scorpion venoms possess some peptides having antimicrobial, anticancer and insecticidal properties. These make scorpion venom make it an important pharmacological agent in future for developing antimicrobial and antitumour drugs as well as insecticides on commercial scale. Some scorpion venom components have important applications for the treatment of different diseases like autoimmune, cardiovascular and inflammatory diseases.

TRANSPARENCY DECLARATION

The author declares that there is no conflict of interest.

REFERENCES

1. Warrel DA. WHO/SEARO Guidelines for the clinical management of snake bites in the South East Asian regions. J Trop Med Pub Hlth. 1999; 30: 1-85.
2. Briggs DEG. Scorpion takes to the water. Nature. 1987; 326: 645-646.
3. Lourenco WR. Diversity and endenism in tropical versus temperate scorpion communities. Biogeographica. 1994; 70: 155-160.
4. Rochat H, Bernard P, Couraud F. Scorpion toxins: chemistry and mode of action. In: Adv. Cytopharmacol. Ceccarelli F, ed. New York: Raven, 1979: 325-334.
5. Zlotkin E, Eistan M, Bindokas VP, Adams ME, Moyer M, Burkhart W, Fowler E. Functional duality and structural uniqueness of depressant insect selective neurotoxins. Biochem. 1991; 30: 4814-4821.
6. Gordon D, Maskowitz H, Eitan M, Warner C, Catteral, WA, Zlotkin E. Localization of receptor sites for insect selective toxins on Na+ channels by site directed antibodies. Biochem. 1992; 31: 7622-7628.
7. Zlotkin E. In: Athropods venoms. Bettini S, ed. Springer, New York, 1978: 317-369.
8. Inceoglu B, Lango J, Jing J, Chen L, Doymaz F, Pessah IN, Hammock BD. One scorpion, two venoms: Prevenom of *Parabuthus transvaalis* acts as an alternative type of venom with distinct mechanism of action. Proc Natl Acad Sci. 2003; 100: 922-927.
9. Corzo G, Escoubas P, Villegas E, Barnham KJ, He W, Norton RS, Nakazima T. Characterization of unique amphipathic antimicrobial peptides from the venom of the scorpion *Pandinus imperator*. Biochem J. 2001; 359: 35-45.
10. Turkov M, Rashi S, Zilberberg N, Gordon D, Ben Khalifa R, et al. In vitro folding and functional analysis of anti-insect selective scorpion depressant neurotoxin produced in *E. coli*. Proc Express Purific. 1997; 9: 123-131.
11. Balozet L. Venomous invertebrates. In: Venomous animals and their venoms. Vol. 3, Bucherl W, Buckley EE, eds. Academic New York. 1971: 349-371.
12. Keegan HL. Scorpions of medical importance. University Press of Pississippi Jackson. 1980: 43.
13. Brownell P, Polis G. Scorpion biology and research. Oxford University Press, New York, 2001: 3-13.
14. Polis G. The biology of scorpions. Stanford University Press, Stanford, 1990: 247-193.
15. Possani LD, Becerril B, Delepierre M, Tytgat J. Scorpion toxin specific for Na^+ channel. Eur J Biochem. 1999; 264: 287-300.
16. Becceril B, Marangoni S, Possani LD. Toxin and genes isolated from the scorpion of the genus *Tityus*: a review. Toxicon. 1997; 35: 821-835.
17. Nakagawa Y, Lee YM, Lehmberg E, Herrmann R, Maskowitz H, Jones AD, Hammock BD. Antiscorpion toxin 5 (AaIT5) from *Androctonus australis*. Eur J Biochem. 1997; 246: 496-501.

18. Murugesan S, Murthy RKK, Noronha OPD, Samuel AM. Tc99m-scorpion venom: labeling, biodistribution and scintimaging. J Venom Anim Toxin. 1999; 5: 35-46.
19. Ismail M. The scorpion-envenoming syndrome. Toxicon. 1995; 33: 825-858.
20. Albert D, Rama MB, Karen W, Eddie C. Expression and secretion of a functional scorpion insecticidal toxin in cultured mouse cells. Biotechnol. 1990; 8: 339-342.
21. Stewart LMD, Hirst M, Freber ML, Merryweather AT, Clayley PJ, Posse RD. Construction of an improved baculovirus insecticide containing an insect specific toxin gene. Nature. 1991; 352: 85-88.
22. Mc Cutchen BF, Hammock BD. Recombinant baculovirus expressing an insect-selective neurotoxin. In: Natural and engineered pest management agent. Hedin PA, Menn JJ, eds. Hallingworth RM, 1994: 348-367.
23. Pelhate M, Stankiewicz M, Ben Khalifa R. Anti-insect scorpion toxins: historical account, activities and prospects. CR Seances Soc Biol Fil. 1998; 192: 463-484.
24. Karbat I, Frolow F, Froy O, Gilles N, Cohen L, Turkov M, et al. Molecular basis of the high insecticidal potency of scorpion α toxins. J Biol Chem. 2004; 279: 31679-31686.
25. Gurevitz M, Karbat I, Cohen L, Ilan N, Kahn R, Turkov M, et al. The insecticidal potential of scorpion β-toxins. Toxicon. 2007; 49: 473-489.
26. Gordon D, Karbat I, Ilan N, Cohen L, Kahn R, Gilles N, et al. The differential preference of scorpion α-toxins for insect or mammalian sodium channels: implications for improved insect control. Toxicon. 2007; 49: 452-472.
27. Pelhate M, Zlotkin E. Action of insect toxin and other toxins derived from the venom of the scorpion *Androctonus australis* on isolated giant axons of the cockroach (*Periplanata americana*). J Exp Biol. 1982; 97: 67-77.
28. Ben Khalifa R, Stankiewics M, Lapied B, Turkov M, Zilberberg N, Gurevitz M, Pelhate M. Refined electrophysiological analysis suggests that a depressant toxin is a sodium channel opener rather than a blocker. Life Sci. 1997; 61: 819-830.
29. Wang GK, Strichartz GR. Purification and physiological characterization of neurotoxins from the venoms of the scorpion *Centruroides sculpturatus* and *Leiurus quinquestriatus*. Mol Pharmacol. 1983; 23: 519-533.
30. Bontems F, Roumestand C, Gilquin B, Menez A, Toma F. Refined structure of charybdotoxin: common motifs in scorpion toxins and insect defensins. Science. 1991; 254: 1521-1523.
31. Crest M, Jacquet G, Gola M, Zerreouk H, Benslimane A, Rochat H, et al. Kaliotoxin, a novel peptidyl inhibitor of neuronal 13 K-type Ca^{++}-activated K^{+} channel characterized from *Androctonus mauretanicus mauretanicus* venom. J Biol Chem. 1992; 267: 1640-1647.
32. Zlotkin A, Fishman Y, Elazar M. AaIT: from neurotoxin to insecticide. Biochiemie. 2000; 82: 869-881.
33. Higgs S, Olson KE, Klimowski L, Powers AM, Carlson JO, Possee RD, Beaty BJ. Mosquito sensitivity to a scorpion neurotoxin expressed using an infectious Snindbis virus vector. Insect Mol Biol. 1995; 4: 97-103.
34. Loret EP, Martin-Eauclaire MF, Mansuelle P, Sampieri F, Granier C, Rochat H. An anti-insect toxin purified from the scorpion *Androctonus astralis* Hector also acts on the α and β sites of the mammalian sodium channels sequence and circular dichroism study. Biochem. 1991; 30: 633-640.
35. Eitan M, Fowler E, Herrmann R, Duval A, Pelhate M, Zlotkin E. A scorpion venom neurotoxin paralytic to insects that affects sodium current inactivation: purification, primary structure and mode of action. Biochem. 1990; 29: 5941-5947.
36. Krimm I, Gilles N, Sautire P, Stankiewicz M, Pelhate M, Gordon D, Lancelin JM. NMR structures and activity of a novel α-like toxin from the scorpion *Leiurus quinquestriatus* hebraeus. J Mol Biol. 1999; 285: 1749-1763.
37. Dhawan R, Joseph S, Sethi A, Lala AK. Purification and characterization of a short insect toxin from the venom of the scorpion *Buthus tamulus*. FEBS Lett. 2003; 528: 261-266.
38. Gawade SP. Excitatory effects of Buthus C56 *Drosophila* on larval neuromuscular junction. J Venom Anim Toxin Incl Trop Dis. 2003; 9(1): 65-75.
39. Gershburg E, Stockholm D, Froy O, Rashi S, Gurevitz M, Chejanovsky N. Baculovirus mediated expression of a scorpion depressant toxin improve the insecticidal efficacy achieved with excitatory toxin. FEBS Lett. 1998; 422: 132-136.
40. Chhatwal GS, Habbermann E. Neurotoxins, protease inhibitors and histamine releasers in the venom of the red scorpion (*Buthus tamulus*): isolation and partial characterization. Toxicon. 1981; 19: 807-823.

41. Lala K, Narayanan P. Purification, N-terminal sequence and structural characterization of a toxin protein from the Indian scorpion venom, *Buthus tamulus*. Toxicon. 1994; 32: 325-338.
42. Wudayagiri R, Inceoglu B, Herrmann R, Choudhary MD, Hammock BD. Isolation and characterization of a novel lepidopteran-selective toxin from the venom of the South Indian scorpion, *Mesobuthus tamulus*. BMC Biochem. 2001; 2: 11-16.
43. Steiner H, Hultmark D, Engstrom A, Bennich H, Boman HG. Sequence and specificity of two antimicrobial proteins involved in insect immunity. Nature. 1981; 292: 246-248.
44. Bulet P, Cociancich S, Dimarcq JL, Lambert J, Reichhart JM, Hoffmann D, et al. Insect immunity: Isolation from a coleopteran insect of novel inducible antibacterial peptide and of new members of the insect defensin family. J Biol Chem. 1991; 266: 24520-24525.
45. Nicolas P, Mor A. Peptides as weapons against microorganisms in the chemical defense system of vertebrate. Annu Rev Microbiol. 1995; 49: 277-304.
46. Hoffman JA, Kafatos FC, Janeway CA, Ezekowitz RA. Phylogenetic perspective in innate immunity. Science. 1999; 284: 1313-1318.
47. Fennel JF, Shipman WH, Cole LJ. Antibacterial action of melittin, a polypeptide from bee venom. Proc Soc Exp Biol Med. 1968; 127: 707-710.
48. Krishnakumari V, Nagaraj R. Antimicrobial and hemolytic activities of crabrolin, a 13-residue peptide from the venom of the European hornet, *Vespa crabro*, and its analogs. J Pept Res. 1997; 50: 88-93.
49. Yan L, Adams ME. Lycotoxins, antimicrobial peptides from the venom of the wolf spider *Lycosa carolinensis*. J Biol Chem. 1998; 273: 2059-2066.
50. Hwang PM, Vogel HJ. Structure-function relationship of antimicrobial peptides. Biochem Cell Biol. 1998; 76: 235-246.
51. Larrick JW, Wright SC. Cationic antimicrobial peptides. Drug Future. 1996; 21: 41-48.
52. Hancock RE, Lehrer R. Cationic peptides: a new source of antibiotics. Trends Biotechnol. 1998; 16: 82-88.
53. Zasloff M. Antimicrobial peptides of multicellular organisms. Nature. 2002; 415(6870): 389-395.
54. Hancock RE, Sahl HG. Antimicrobial and host-defense peptides as new anti infective therapeutic strategie. Nature Biotechnol. 2007; 24(12): 1551-1557.
55. Dai L, Yasuda A, Naoki H, Corzo G, Andriantsiferana M, Nakajima T. IsCT, a novel cytotoxic linear peptide from scorpion *Opisthacanthus madagascariensis*. Biochem Biophys Res Commun. 2001; 286: 820-825.
56. Arpornsuwan T, Buasakul B, Jaresitthikunchai J, Roytrakul S. Potent and rapid antigonococcal activity of the venom peptide BmKn2 and its derivatives against different Maldi biotype of multidrug-resistant *Neisseria gonorrhoeae*. Peptides. 2014; 53: 315-320.
57. Epad RM, Vogel HJ. Diversity of antimicrobial peptides and their mechanism of action. Biochim Biophys Acta. 1999; 1462: 11-28.
58. Mousli M, Bueb JL, Bronner C, Rouot B, Landry Y. G-protein activation: a receptor independent mode of action for cationic amphipathic neuropeptides and venom peptides. Trends Pharmacol Sci. 1990; 11: 358-362.
59. Bulet P, Hetru C, Dimarcq J, Hoffmann D. Antimicrobial peptides in insects: structure and function. Dev Comp Immunol. 1999; 23: 329-344.
60. Cociancich S, Goyffon M, Bontems F, Bulet P, Bouet F, Menez A, Hoffmann J. Purification and characterization of a scorpion defensin, a 4 kD antimicrobial peptide presenting structural similarities with insect defensins and scorpion toxins. Biochem Biophys Res Commun. 1993; 194: 17-22.
61. Ehret-Sabatier L, Loew D, Goyffon M, Fehlbaum P, Hoffman JA, Van Dorsselaer A, Bulet P. Characterization of novel cyteine rich antimicrobial peptides from scorpion blood. Biochem Mol Biol. 1996; 271: 29537-29544.
62. Hetru C, Letellier L, Oren Z, Hoffmann JA, Shai Y. Androctonin, a hydrophilic disulphide-bridged non-haemolytic anti-microbial peptide: a plausible mode of action. Biochem J. 2000; 345: 653-664.
63. Moerman L, Bosteels S, Noppe W, Willems J, Clynen E, Schoofs L. Antibacterial and antifungal properties of alpha-helical, cationic peptides in the venom of scorpions from southern Africa. Eur J Biochem. 2002; 269: 4799-4810.
64. Powers JP, Hancock RE. The relationship between peptide structure and antibacterial activity. Peptides. 2003; 24: 1681-1691.
65. Ramírez-Carreto S, Jiménez-Vargas JM, Rivas-Santiago B, Corzo G, Possani LD, Becerril B. Peptides from the scorpion *Vaejovis punctatus* with broad antimicrobial activity. Peptides. 2015; 73: 51-59.

66. Bao A, Zhong J, Zeng XC, Nie Y, Zhang L, Peng ZF. A novel cysteinefree venom peptide with strong antimicrobial activity against antibioticsresistant pathogens from the scorpion *Opistophthalmus glabrifrons*. J Pept Sci. 2015; 21: 758-764.
67. de Melo ET, Estrela AB, Santos EC, Machado PR, Farias KJ, Torres TM. Structural characterization of a novel peptide with antimicrobial activity from the venom gland of the scorpion *Tityus stigmurus*: stigmurin. Peptides. 2015; 68: 3-10.
68. Bandyopadhyay S, Junjie RL, Lim B, Sanjeev R, Xin WY, Yee CK. Solution structures and model membrane interactions of Ctriporin, an anti-methicillin-resistant *Staphylococcus aureus* peptide from scorpion venom. Biopolymers. 2014; 101: 1143-1153.
69. Li Z, Xu X, Meng L, Zhang Q, Cao L, Li W. Hp1404, a new antimicrobial peptide from the scorpion *Heterometrus petersii*. PLoS One. 2014; 9: 97539.
70. Conde R, Zamudio F Z, Rodriguez MH, Possani LD. Scorpine, an antimalarial and antibacterial agent purified from scorpion venom. FEBS Lett. 2000; 471: 165-168.
71. Torres-Larios A, Gurrola GB, Zamudio FZ, Possani LD. Hadrurin, a new antimicrobial peptide from the venom of the scorpion *Hadrurus aztecus*. Eur J Biochem. 2000; 267: 5023-5031.
72. Verdonck F, Bosteel S, Desmet J, Moerman L, Noppe W, Willems J, et al. A novel class of pore forming peptides in the venom of the *Parabuthus schlechteri* Purcell (Scorpions: Buthidae). Cinebasia. 2000; 16: 247-260.
73. White SH, Wimley WC, Selsted ME. Structure, function and membrane integration of defensins. Curr Opin Struct Biol. 1995; 5: 521-527.
74. Matsuzaki K. Magainins as paradigm for the mode of action of pore forming peptides. Biochim Biophys Acta. 1998; 1376: 391-400.
75. Du Q, Hou X, Wang L, Zhang Y, Xi X, Wang H, et al. AaeAP1 and AaeAP2: novel antimicrobial peptides from the venom of the scorpion, *Androctonus aeneas*: structural characterisation, molecular cloning of biosynthetic precursor-encoding cDNAs and engineering of analogues with enhanced antimicrobial and anticancer activities. Toxins. 2015; 7: 219-237
76. Al-Asmari AK, Alamri MA, Almasoudi AS, Abbasmanthiri R, Mahfoud M. Evaluation of the in vitro antimicrobial activity of selected Saudi scorpion venoms tested against multidrug-resistant microorganisms. J Global Antimicrob Resist. 2007; 10: 14-18.
77. Sikora K. Cancer survival in Britain in poorer than that of her comparable European neighbours. BMJ. 1999; 319: 461-462.
78. De Carvalho DD, Schmitmeier S, Novello JC, Markland FS. Effect of BJcuL (a lectin from the venom of the snake *Bothropos jararacussu*) on adhesion and growth of tumor and endothelial cells. Toxicon. 2001; 39: 1471-1476.
79. Chiang HS, Swaim MW, Huang TF. Characterization of platelet aggregation induced by human breast carcinoma and its inhibition by snake venom peptide, trigramin and rhodostomin. Breast Cancer Res Treat. 1995; 33: 225-235.
80. Maristela P, Daniela DC, Antonio RG, Delwood CC. The effect of lectin from the venom of the snake, *Bathrops jararacussu*, on tumor cell proliferation. Anticancer Res. 1999; 19: 4023-4026.
81. Zhou Q, Sherwin RP, Parrish C, Richters V, Groshen SG, Tsao-Wei D, Markland FS. Contortrostatin, a dimmer disintegrin from *Agkistrodon contortrix contortrix*, inhibits breast cancer progression. Breast Cancer Res Treat. 2000; 61: 249-260.
82. Yarom R, Braun K. Myocardiopathology following scorpion venom injection. Isr J Med Sci. 1969; 5: 849-852.
83. Tarasiuk A, Khvatskin S, Sofer S. Effect of antivenom serotherapy on haemodynamic patho-physiology in dogs injected with of *Leiurus quinquestriatus* scorpion venom. Toxicon. 1998; 36: 963-971.
84. Omran MAA. In vitro anticancer effect of scorpion *Leiurus quinquestriatus* and Egyptian cobra venom on human breast and prostate cancer cell lines. J Med Sci. 2003; 3: 66-86.
85. Bruses JL, Capaso J, Katz E, Pilar G. Specific in vitro biological activities of snake venom myotoxins. J Neurochem. 1993; 60: 1030-1042.
86. Deshane J, Garner CC, Sontheimer H. Chlorotoxin inhibits glioma invasion via matrix metallo-proteinase-2. J Biol Chem. 2003; 278: 4135-4144.
87. Jager H, Dreker T, Buck A, Giehl K, Gress T, Grissmer S. Blockage of intermediate-conductance Ca^{2+} activated K^+ channels inhibit human pancreatic cancer cell growth in vitro. Mol Pharmacol. 2004; 65: 630-638.
88. Gupta SD, Gomes AN, Debnath A, Saha A, Gomes AP. Apoptosis induction in human leukemic cells by a novel protein Bengalin, isolated from Indian black

scorpion venom: through mitochondrial pathway and inhibition of heat shock proteins. Chem Biol Interact. 2010; 183: 293-303.

89. Al-Asmari AK, Islam M, Al-Zahrani AM. In vitro analysis of the anticancer properties of scorpion venom in colorectal and breast cancer cell lines. Oncol Lett. 2016; 2: 1256-1262.
90. Gupta SD, Debnath A, Saha A, Giri B, Tripathi G, Vedasiromoni JR, et al. Indian black scorpion (*Heterometrus bengalensis* Koch) venom induced antiproliferative and apoptogenic activity against human leukemic cell lines U937 and K562. Leuk Res. 2007; 31: 817-825.
91. Zhang YY, Wu LC, Wang ZP, Wang ZX, Jia Q, Jiang GS, Zhang WD. Antiproliferation effect of polypeptide extracted from scorpion venom on human prostate cancer cells in vitro. J Clin Med Res. 2009; 1: 24-31.
92. Zargan J, Sajad M, Umar S, Naime M, Ali S, Khan HA. Scorpion (*Androctonus crassicauda*) venom limits growth of transformed cells (SH-SY5Y and MCF-7) by cytotoxicity and cell cycle arrest. Exp Mol Pathol. 2011; 91: 447-454.
93. Almaaytah A, Albalas Q. Scorpion venom peptides with no disulfide bridges: a review. Peptides. 2014; 51: 35-45.
94. Roger JC, Qu Y, Tanada TN, Scheur T, Catterall WA. Molecular determinants of high affinity binding of alpha-scorpion toxin and Sea anemone toxin in S3-S4 extracellular loop in domain IV of the Na+ channel alpha-subunit. J Biol Chem. 1996; 271: 15950-15962.
95. Cestele S, Caterall WA. Molecular mechanism of neurotoxic action on voltage-gated sodium channels. Biochemie (Paris). 2000; 82: 883-892.
96. Martin-Eauclaire MF, Couraud F. Scorpion neurotoxins: effects and mechanism. In: Hand book of neurotoxicology. Chang LW, Dyer RS, eds Marcel Dekker New York, 1995: 683-716.
97. Gwee MC, Nirthanan S, Khoo HE, Gopalkrishnakone P, Kini RM, Cheah LS. Autonomic effect of some scorpion venoms and toxins. Clin Exp Pharmacol Physiol. 2002; 29: 795-801.
98. Couraud F, Jover E, Dubois JM, Rochat H. Two types of scorpion toxin receptor sites, one related to the activation, the other to the inactivation of the action potential Na+ channel. Toxicon. 1982; 20: 9-16.
99. Couto AS, Moreaes-Santos T, Azevedo AD, Almeida AP, Freire-Maia L. Effect of Tsgamma, purified from *Tityus serrulatus* scorpion venom, on the isolated rat atria. Toxicon. 1992; 30: 339-343.
100. Yatani A, Kirsh GE, Possani LD, Brown AM. Effects of New World ecorpion toxins on single channel and whole cell cardiac sodium currents. Am J Physiol. 1988; 254: 443-451.
101. Romi-Labrun R, Martin-Eauclaire MF, Escoubas P, Wu FQ, Lebrun B, Hisada M, Nakazima T. Characterization of four toxins from *Buthus martensi* scorpion venom, which act on apaminesensitive Ca^{++}-activated K^{+} channels. Eur J Biochem. 1997; 245: 457-464.
102. D' Ajellow A, Zlotkin E, Miranda F, Lissitzky. The effect of the scorpion venom and pure toxin on the cockroach nervous system. Toxicon. 1972; 10: 399-404.
103. Miller C, Moczydlowski E, Latore R, Philips M. Charybdotoxin, a potent inhibitor of single Ca^{++}-activated K^{+} channels from mammalian skeleton muscle. Nature. 1985; 313: 316-318.
104. Miller C. The charybdotoxin family of K^{+} channel blocking peptides. Neurons. 1995; 15: 5-10.
105. Hoffmann JA, Hetru C. Insect defensins: inducible antimicrobial peptides. Immunol Today. 1992; 13: 411-415.
106. Bablito J, Jover E, Couraud F. Activation of the voltage sensitive sodium channel by a β-scorpion toxin in the rat brain nerve ending particles. J Neurochem. 1986; 37: 1763-1770.
107. Garcia CM, Leonard RJ, Novik J, Stevens SP, Schmalhofer W. Purification, characterization and biosynthesis of margaritatus venom that selectively inhibits voltage dependent potassium channel. J Biol Chem. 1993; 689(25): 18866-18874.
108. Lin CS, Boltz RC, Blake JT, Nguyen M, Talento A, Fischer PA, et al. Volatge-gated potassium channels regulate calcium dependent pathways involved in human T-lymphocytes activation. J Exp Med. 1993; 177: 637-645.
109. Bednarek MA, Bugianesi RM, Leonard RJ, Felix JP. Chemical synthesis and structurefunction studies of margtoxin, a potent inhibitor of voltage-dependent potassium channel in human T-lymphocytes. Biocem Biophys Res Commun. 1994; 198: 619-625.
110. Garcia ML, Garcia-Calvo M, Hidalgo P, Lee A, Mac Kinnon R. Purification and characterization of three inhibitors of voltage dependent K+ channels from *Leiurus quinquestriatus* var. hebraeus venom. Biochem. 1994; 33: 6834-6839.

111. Martins JC, Van JC, Borremanus FA. Determination of the three-dimensional solution structure of the scyllatoxin by 1H NMR. J Mol Biol. 1995; 253: 590-603.

112. Pappone PA, Chalan MD. *Pandinus imperator* scorpion venom blocks voltage-gated K+ channel in nerve fibers. J Neurosci. 1987; 7: 3300-3305.

113. Sands SB, Lewis RS, Chalan MD. Charbdotoxin blocks voltage-gated K^+ channel in human and murine T-lymphocytes. J Gen Physiol. 1989; 93: 1061-1074.

114. Valdivia H, Kirby MS, Lederer WJ, Coronado R. Scorpion toxins targated against the sarcoplasmic reticulum Ca^{++} release channel of skeletal and cardiac muscle. Proc Nat Acad Sci USA. 1992; 89: 12185-12189.

115. el Havek R, Lokuta AJ, Arevalo C, Valdivia HH. Peptide probe of ryanodine receptor function. Imperatoxin A, a peptide from the venom of the scorpion *Pandinus imperator*, selectively activates skeletaltype ryanodine receptor isoforms. J Biol Chem. 1995; 270: 28696-28704.

116. Pappone PA, Lucero MT. *Pandinus imperator* scorpion venom blocks voltage gated potassium channels in GH3 cells. J Gen Physiol. 1988; 91: 817-833.

117. Galvez A, Gimenez-Gallego G, Ruben JP, Rov-Contancin L, Feigenbaun P, Kaczorowski GJ, Garcia ML. Purification and characterization of a unique peptidyl probe for the high conductance calcium-activated potassium channel from the venom of the scorpion *Buthus tamulus*. J Biol Chem. 1990; 265: 11083-11090.

118. Marangoni S, Ghiso J, Sampaio SV, Arantes EC, Giglio JR, Oliviera B, Frangione B. The complete amino acid sequence of toxin TsTX-VI isolated from the venom of the scorpion *Tityus serrulatus*. J Prot Chem. 1990; 9: 595-601.

119. Grishin EV, Korolkova YV, Kozlov KA, Lipkin AV, Nosyreva ED, Pluzhnikov KA, et al. Structure and function of potassium channel inhibitor from black scorpion venom. Pure Appl Chem. 1996; 68: 2105-2109.

120. More SS, Mirajkar KK, Gadag JR, Menon KS, Mathew MK. A novel Kv1.1 potassium channel-blocking toxin from the venom of *Palamneus gravimanus* (Indian black scorpion). J Venom Anim Toxin Incl Trop. 2005; 11(3): 315-335.

121. De Bin JA, Maggio JE, Strichartz GR (1993) Purification and characterization of chlorotoxin, a Clchannel ligand from the venom of the scorpion. Am J Physiol Cell Physiol 264:361-369.

122. Lippens G, Najib S, Wodak J, Tartar A. Sequential assignment and solution structure of chlorotoxin, a small peptide from scorpion that blocks chloride channel. Biochem. 1995; 34: 13-21.

123. Zamudio FZ, Conde R, Arevalo C, Becerril B, Martin BM, Valdivia HH, Possani LD. The mechanism of inhibition of ryanodine receptor channel by imperotoxin I, a heterodimeric protein from the scorpion *Pandius imperator*. J Biol Chem. 1997; 272: 11886-11894.

124. Zamudio FZ, Gurrola GB, Arvalo C, Sreekumar R, Walker JW, Valdivia HH, Possani LD. Primary structure and synthesis of imperotoxin A (Iptxa), a peptide activator of Ca^{++} release channels/rynodine receptors. FEBS Lett. 1997; 405: 385-389.

125. Chuang RSI, Jaffe H, Cribbs L, Perez-Reyes EJ, Stwartz KJ. Inhibition of T-type voltagegated calcium channels by a new scorpion toxin. Natl Neuro Sci. 1998; 1: 668-674.

126. Freire-Maia L, Pinto GI, Franco I. Mechanism of the cardiovascular effects produced by purified scorpion toxin in the rat. J Pharmacol Exp Ther. 1974; 188: 207-213.

127. Ismail M. The scorpion-envenoming syndrome. Toxicon. 1995; 33: 825-858.

128. Tarasiuk A, Janco J, Sofer S. Effect of scorpion venom on central and peripheral circulatory response in an open-chest dog model. Acta Physiol Scand. 1997; 161: 141-146.

129. Ismail M, Osmon OH, Gumma KA, Karrar MA. Some pharmacological studies with scorpion (*Pandinus exitiallis*) venom. Toxicon. 1974; 2: 75-82.

130. Almeida AP, Alpoim NC, Freire-Maia L. Effects of purified scorpion toxin (*Tityus* toxin) on the isolated guinea pig heart. Toxicon. 1982; 20: 855-865.

131. Moss J, Kajik T, Henery DP, Kopin IJ. Scorpion venom induced discharge of catecholamines accompanied by hypertension. Brain Res. 1973; 54: 381-385.

132. Freire-Maia L, Campos JA. Pathophysiology and treatment of scorpion poisoning. In: Ownby LC, Odel GV, eds. Natural toxins, characterizations, pharmacology and therapeutics. Pergamon Press Oxford, 1989: 139-159.

133. Tarasiuk A, Sofer S. Effect of adrenergic blockade and ligation of spleen vessels on haemodynamics of dogs injected with scorpion venom. Crit Care Med. 1999; 27: 365-372.

134. Sofer S. Scorpion envenomation. Int Care Med. 1995; 21: 627-628.

135. Ramchandran LK, Agrawal OP, Achyutan KE, Chudhary L, Vedasiromani JR, Ganguli DK. Fractionation and biological activities of the venom of the Indian scorpions *Buthus tamulus* and *Heterometrus bengalensis*. Ind J Biochem Biophys. 1986; 23: 355-358.

136. Russel FE. Toxic effects of animal toxins. In: Idaassen CD, Amdur MO, Doull J, eds. Toxicology basic science of poisons. 3rd edn, New York: Macmillan, 1986: 706-756.

137. Henriques MC, Gassinelli G, Diniz CR, Gomez MV. Effect of the venom of the scorpion *Tityus serrulatus* on adrenal gland catecholamines. Toxicon. 1968; 5: 175-179.

138. Radha Krishna Murthy K, Vakil AE. Elevation of plasma angiotensin level in dogs by Indian red scorpion (*Buthus tamulus*) venom and its reversal by administration of insulin and tolazoline. Indian J Med Res. 1988; 88: 376-379.

139. Margulis G, Sofer S, Zalstein E, Zucker N, Ilia R, Gueron M. Abnormal coronary perfusion in experimental scorpion envenomation. Toxicon. 1994; 32: 1675-1678.

140. Wang R, Moreau P, Deschamps A, de Champlain J, Sauve R, Foucart S, et al.Cardiovascular effects of *Buthus martensi* (Karsch) scorpion venom. Toxicon. 1994; 32: 191-200.

141. Amaral CFS, Lopes JA, Magalhaes RA, de Rezende NA. Electrocardiographic, enzymatic and echocardiographic evidence of myocardial damage after *Tityus serrulatus* scorpion poisoning. Am J Cardiol. 1991; 67: 655-657.

142. Abroug E, Ayari M, Nouria S, Gamra H, Boujdaria R, Elatrons S, et al. Assessment of left ventricular function in severe scorpion envenomation: combined haemodynamic and echodopller study. Int Care Med. 1995; 21: 629-635.

143. Gueron M, Adolf RJ, Grupp IL, Gabel M, Grupp G, Fowler NO. Haemodynamic and myocardial consequences of scorpion venom. Am J Cardiol. 1980; 45: 1979-1986.

144. Couture R, Harrisson M, Vianna RM, Cloutier F. Kinin receptors in pain and inflammations. Eur J Pharmacol. 2001; 429: 161-176.

145. Campbell DJ, Dixon B, Kladis A, Kemme M, Santmaria, JD. Activation of the kallikrein-kinin system by cardiopulmonary bypass in humans. Am J Physiol. 2001; 281: 1059-1070.

146. Gwee MC, Cheah LS, Nirthanan S, Gopalkrishnakone P, Wang PT. Pre-junctional action of the venom from the Indian red scorpion *Mesobuthus tamulus* on adrenergic transmission in vitro. Toxicon. 1994; 32: 201-209.

147. Rowan EG, Vatanpour H, Furman BL, Harvey AL, Tanira MO, Gopalkrishnakone P. The effect of Indian red scorpion Buthus tamulus venom in in vivo and in vitro. Toxicon. 1992; 30: 1157-1164.

148. Murthy RKK, Medh JD, Dave BN, Vakil YE, Billimoria FR. Acute pancreatitis and reduction of H^+ ion concentration in gastric secretions in experimental acute myocarditis produced by Indian red scorpion (*Buthus tamulus*) venom. Indian J Exp Biol. 1989; 27: 242-244.

149. Murthy RKK, Hossein Z. Increased osmotic fragility of red cells of incubation at 37^0C for 20 hours in dogs with acute myocarditis produced by scorpion (*Buthus tamulus*) venom. Indian J Exp Biol. 1986; 38: 206-210.

150. Murthy RKK, Anita AG, Dave BN, Billimoria FR. Erythrocyte Na^+K^+ATPase activity inhibition and increased red cell fragility in experimental myocarditis produced by red scorpion (*Buthus tamulus*) venom. Indian J Med Res. 1988; 88: 536-540.

151. Murthy RKK, Yeolekar ME. Electrocardiographic changes in acute myocarditis produced by the scorpion (*Buthus tamulus*) venom. Indian Heart J. 1986; 38: 206-210.

152. Murthy RKK, Hossein Z, Medh JD, Kudalkar JA, Yeolekar ME, Pandit SP, et al. Disseminated intravascular coagulation and disturbances in carbohydrate and fat metabolism in acute myocarditis produced by Indian red scorpion (*Buthus tamulus*) venom. Indian J Med Res. 1988; 87: 318-325.

153. Murthy RKK, Haghanzari L. The blood level of glucagon, cortisol, and insulin following the injection of venom by the scorpion (*Mesobuthus tamulus*, Pocock) in dogs. J Venom Anim Toxin. 1999 5: 200-219.

154. Murthy RKK, Zare A. Effect of Indian red scorpion (*Mesobuthus tamulus concanesis*, Pocock) venom on thyroxine and triiodothyronine in experimental acute myocarditis and its reversible by species-specific antinenom. Indian J Exp Biol. 1998; 36: 16-21.

155. Murthy RKK, Anita AG. Reduced insulin secretion in acute myocarditis produced by scorpion (*Buthus tamulus*). Indian Heart J. 1986; 38: 467-469.

156. Gwee MC, Nirthanan S, Khoo HE, Gopalkrishnakone P, Kini RM, Cheah LS. Autonomic effect of some scorpion venoms and toxins. Clin Exp Pharmacol Physiol. 2002; 29: 795-801.

157. Murthy RKK, Zare A. The use of antivenin reverses hematological and osmotic fragility changes of erthrocytes caused by Indian red scorpion *Mesobuthus tamulus concanesis*, Pocock in experimental envenoming. J Venom Anim Toxins. 2001; 7: 113-138.
158. Freire-Maia L, Campos JA. On the treatment of the cardiovascular manifestations of scorpion envenomation. Toxicon. 1987; 25: 125-130.
159. Freire-Maia L, De Matos IM. Heparin or a PAF antagonist (BN-52021) prevents the acute pulmonary oedema induced by *Tityus serrulatus* scorpion venom on the rat. Toxicon. 1993; 31: 1207-1210.
160. Gueron M, Ovsyshcher I. What is the treatment for the cardiovascular manifestations of scorpion envenomation? Toxicon. 1987; 25: 121-124.
161. Best CH, Taylor NBA. Textbook in applied physiology of medical practice. Baltimore: Williams and Wilkins, 1967.
162. Pande SV, Mead JF. Inhibition of enzyme activities by free fatty acids. J Biol Chem. 1968; 243: 6180-6186.
163. de Rezende NA, Dias MB, Campolina D, Chavez-Olortegui C, Diniz C, Amaral CFS. Efficacy of antivenom therapy for neutralizing venom antigen in patients stung by *Tityus serrulatus* scorpion. Am J Trop Med Hyg. 1995; 52: 277-280.
164. Omran MAA, Abdel-Rahman MS. Effect of the scorpion *Leiurus quinquestriatus* (H&E) venom on the clinical chemistry parameters of the rat. Toxicol Lett. 1992; 61: 99-109.
165. Ghalim N, El-Hafny B, Sebti F, Heikel J, Lezar N, Moustanir R, Benslimane A. Scorpion venom and serotherpy in Morocco. Am J Trop Med Hyg. 2000; 62: 277-283.
166. Sofer S, Shahak E, Gueron M. Scorpion envenomation and antivenom therapy. J Pediatr. 1994; 124: 973-978.
167. Balozet L. Scorpionism in the Old World. In: Bücherl W, Buckley E, eds. Venomous animals and their venoms. New York: Academic Express, 1971: 349-371.
168. Theakston RD, Warrell DA, Grifithis E. Report of a WHO workshop on the standardization and control of antivenoms. Toxicon. 2003; 41: 541-557.
169. Demagalhaes O. Scorpionism. J Trop Med Hyg. 1938; 41: 393-399.
170. Balozet L. Scorpion venoms and anti-scorpion serum. In: Venom. Buckley EE, Porges N, eds. Washington DC. Public. No. 44. Am Adv Sci. 1956.
171. Mohammed AH, Darwish MA, Honi Ayobe M. Immunological studies on scorpions (*Leiurus quinquestriatus*) antivenin. Toxicon. 1975; 13: 67-68.
172. Kapadia ZS, Master RWP, Rao SS. Immunological studies in telson extracts of Indian and Egyptian scorpion venom. Indian J Exp Biol. 1964; 2: 75-77.
173. Kankonakar RC, Kulakarni DG, Hulikavi CB. Preparation of a potent anti-scorpion venom serum against the venom of red scorpion (*Buthus tamulus*). J Postgrad Med. 1998; 44: 85-92.
174. Amaral CFS, Dias MB, Campolina D, Proietti FA, de Rezende NA. Childrens with adrenergic manifestation of envenomation after Tityus serrulatus scorpion sting are protected from early anaphylactic antivenom reaction. Toxicon. 1994; 32: 211-215.
175. Amaral CFS, de Rezende NA. Both cardiogenic and noncardiogenic factors are involved in the pathogenesis of pulmonary oedema after scorpion envenoming. Toxicon. 1997; 35: 997-998.
176. Gueron M, Marquilis G, Sofer S. Echocardiographic and radionucleid angiographic observations following scorpion envenomation by *Leiurus quinquestriatus*. Toxicon. 1990; 28: 1005-1009.
177. Devaux C, Fourquet P, Granier C. A conserved sequence region of scorpion toxin rendered immunogenic induces broadly cross-reactive, neutralizing antibodies. Eur J Biochem. 1996; 242(3): 727-735.
178. Espino-Solis GP, Riano-Umbarila L, Becerril B, Possani LD. Antidotes against venomous animals: state of the art and prospectives. J Proteomics. 2009; 72(2): 183-199.
179. Holliger PH, Hudson JP. Engineering antibody fragments and the rise of single domains. Nat Biotechnol. 2005; 23: 1126-1136.
180. Riaño-Umbarila L, Contreras-Ferrat G, Olamendi-Portugal T, Morelos-Juárez C, Corzo G, Possani LD, Becerril B. Exploiting cross-reactivity to neutralize two different scorpion venoms with one single chain antibody fragment. J Biol Chem. 2011; 286: 6143-6151.
181. Canul-Tec J-C, Riaño-Umbarila L, Rudinño-Pinera E, Becerril B, Possani LD, Torres-Larios A. Structural basis of neutralization of the major toxic component from the scorpion *Centruroides noxius* Hoffmann by a human-derived single chain antibody fragment. J Biol Chem. 2011; 286: 20892-20900.

182. Rodríguez-Rodríguez ER, Ledezma-Candanoza LM, Contreras-Ferrat LG, Olamendi-Portugal T, Possani LD, Becerril B, Riaño-Umbarila L. A single mutation in framework 2 of the heavy variable domain improves the properties of diabody and a related single-chain antibody. J Mol Biol. 2012; 423: 337-350.

ISSN 2449-8955

European Journal of Biological Research

Research Article

Comparison of biofilm-producing *Enterococcus faecalis*, *Enterococcus faecium*, and unusual *Enterococcus* strains

Anna Sieńko*, Dominika Ojdana, Piotr Majewski, Paweł Sacha, Piotr Wieczorek, Elżbieta Tryniszewska

Department of Microbiological Diagnostics and Infectious Immunology, Faculty of Pharmacy with the Division of Laboratory Medicine, Medical University of Bialystok, 15a Waszyngtona Street, 15-269 Bialystok, Poland
*Corresponding author: Anna Sieńko; Tel.: + 48 85 746 85 71; E-mail: anna.sienko@umb.edu.pl

Received: 14 July 2017; **Revised submission:** 25 September 2017; **Accepted:** 02 October 2017

DOI: http://dx.doi.org/10.5281/zenodo.1000837

ABSTRACT

The present study focused on determining the prevalence of biofilm-forming ability in *Enterococcus faecalis*, *E. faecium*, and unusual *Enterococcus* clinical isolates, and comparison of resistance and the prevalence of selected virulence factors among biofilm-positive strains. The ability to form biofilm was detected in 13.3% of *E. faecalis*, 90% of *E. faecium*, and 57.1% of unusual *Enterococcus* strains (p=0.026). All *E. faecalis* strains were susceptible to β-lactams, while 37.5% of unusual and all *E. faecium* isolates were resistant to these antibiotics. Resistance to gentamicin was detected in 75% of *E. faecalis*, 55.5% of *E. faecium*, and 25% of other strains; resistance to streptomycin in 25%, 83.3%, and 50%, respectively. Analysis of the virulence revealed that the enterococcal surface protein (*esp*) gene was found in all *E. faecium*, 75.0% of *E. faecalis*, and 37.5% of other strains; collagen adhesin gene (*ace*) in 100%, 25.0%, and 37.5%; and hyaluronidase gene (*hyl*) in 83.3%, 0%, and 37.5%, respectively. Analysis of the resistance and virulence patterns showed that *E. faecium* isolates had the greatest variety of virulence and resistance determinants, while the lowest variety was exhibited by unusual strains. These findings indicate that unusual biofilm-producing *Enterococcus* strains have lower resistance and virulence potency than *E. faecalis* and *E. faecium*.

Keywords: *Enterococcus faecalis*; *Enterococcus faecium*; Biofilm; Resistance; Virulence.

1. INTRODUCTION

Today, *Enterococcus* spp. are the fourth most common etiological factor in nosocomial infections in Europe [1]. Although these cocci are members of the microbiota of the human gastrointestinal tract, they often infect the bloodstream, surgical sites, and urinary tract, due to their multiresistance to many antimicrobials [2, 3]. *Enterococcus* spp. have an intrinsic resistance to cephalosporins, lincosamides, and low levels of aminoglycosides, and they can easily acquire resistance, most prominently to glycopeptides and aminoglycosides (high-level resistance), by means of mutations or as a result of transfer and incorporation of genes located on mobile genetic elements, such as plasmids and transposons [1, 4]. Moreover, these bacteria have the ability to form strong biofilm structures, and to produce several virulence factors, such as enterococcal surface protein (Esp), aggregation substance

(As), collagen adhesion (Ace), hyaluronidase (Hyl), and gelatinase (GelE) [5-7]. Esp is the factor that mediates the colonization, and, together with GelE, has been suggested to be involved in biofilm formation [5-7]. Ace and EfaA are principal virulence traits associated with infective endocarditis, whereas Hyl causes tissues damages [5-7]. The majority of nosocomial enterococcal infections are caused by *E. faecalis* and *E. faecium*. However, today there is an increasing prevalence of infections caused by other rarely isolated species, for example: *E. avium, E. gallinarum, E. durans*, and *E. casseliflavus* [8-10].

Biofilm is an assemblage of microbial cells enclosed in a self-produced polysaccharide matrix and attached to a biotic or abiotic surfaces, providing an optimal microenvironment for growth, and facilitates transmission of mobile determinants between microorganisms [11, 12]. Evidence suggests that bacteria in biofilms are more resistant to antimicrobials and hosts factors than other microorganisms and are extremely difficult to eradicate [13]. Likewise, among *Enterococcus*, it is suggested that an ability to produce biofilm is a very important virulence factor which has a major impact on the course of nosocomial infections [5, 7]. Unfortunately, our knowledge about the mechanisms and determinants involved in the process of biofilm formation among enterococci is still insufficient [14]. The ability to create biofilm has been suggested to occur less frequently among *E. faecium* strains compared to *E. faecalis* strains, but, astonishingly, data about biofilm-forming ability among unusual enterococcal species are very limited and unclear [14, 15]. Furthermore, there are only a few reports about the differences in resistance and virulence of various biofilm-producing *Enterococcus* species [13, 16, 17]. This prompted us to determine the prevalence of biofilm-forming ability among *E. faecalis*, *E. faecium*, and unusual *Enterococcus* spp. clinical isolates. Then, we focused on the comparison of the antibiotic resistance, the ability to hemolyze, and the presence of selected virulence genes among these three groups of biofilm-producing *Enterococcus* spp. strains. Moreover, the next goals of this study were to determine their exact resistance profiles, and to indicate the antibiotic with the highest activity against these strains.

2. MATERIAL AND METHODS

2.1. Strains

Tests were performed on sixty-four enterococcal isolates: thirty *E. faecalis*, twenty *E. faecium*, and fourteen others (five *E. avium*, three *E. casseliflavus*, three *E. gallinarum*, three *E. durans*), isolated from clinical specimens from patients hospitalized at the University Hospital in Bialystok (Poland) from December 2013 to January 2015. Isolates were recovered from various clinical materials, mostly blood, peritoneal fluid, bronchoalveolar lavage (BAL), feces, urine, and pus. Most of the collected isolates were gathered from the intensive care unit and a hematology clinic.

2.2. Identification and susceptibility testing

The identification and susceptibility testing were conducted on the automated VITEK 2 system (bioMérieux, France) according to the manufacturer's instruction using VITEK 2 GP and AST-P516 cards, respectively. Susceptibility to ampicillin, imipenem, gentamicin, streptomycin, vancomycin, teicoplanin, linezolid, and tigecycline was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (breakpoint tables for interpretation of minimum inhibitory concentrations, MIC, and zone diameters; version 5.0, 2015; http://www.eucast.org).

2.3. Biofilm and hemolysin production

The tube method [18, 19] and Congo red agar (CRA) method [20, 21] were used to assess the ability of tested isolates to biofilm formation. Each experiment was repeated three times for each strain. Strains that demonstrated the ability to produce biofilm by both methods were considered as biofilm positive (BIO+) isolates. Hemolysin production was determined on Columbia blood agar supplemented with 5% sheep blood (OXOID, United Kingdom) [22].

2.4. DNA extraction

In the next step, genomic DNA was extracted

from overnight *E. faecium* cultures using a Genomic Mini Kit (A&A Biotechnology, Poland) according to the manufacturer's guidelines.

2.5. PCR detection of virulence genes

Then, PCR assays were performed to detect the following virulence genes: *gelE*, *ace*, *hyl*, *esp*, *as*, and *cyl*. The primers sequences are listed in Table 1. PCR amplification was performed in 25 µl mixtures using 2 µl of DNA solution, 1 µl of each primer, 8.5 µl of nuclease-free water, and 12.5 µl of PCR master mix (DNA Gdańsk, Poland). Samples were subjected to an initial denaturation at 94^{O}C for 5 min, followed by 30 cycles of denaturation at 94^{O}C for 1 min, annealing at an appropriate temperature for 1 min, and elongation at 72^{O}C for 1 min using a DNA thermocycler (SensoQuest GmbH, Germany).

PCR products were separated electrophoretically on the Sub-Cell GT apparatus (Bio-Rad, USA) at 5 V/cm for 100 min on a 1.5% agarose gel (Sigma-Aldrich, USA) containing 0.5% ethidium bromide (MP Biomedicals, USA) in Tris-borate-EDTA (ethylenediaminetetraacetic acid) buffer. Then, amplicons were visualized and photographed using the ChemiDoc XRS imaging system and Quantity One 1-D analysis software (Bio-Rad). The positions of obtained products were estimated with the molecular weight marker Perfect^{TM} 100-1000 bp DNA ladder (EURx, Poland). To confirm the presence of the above-mentioned virulence genes, DNA sequencing was carried out on selected PCR products by the GENOMED S.A. company in Poland. The sequences were aligned and compared with reference sequences achieved using GenBank with the Basic Local Alignment Search Tool (BLAST) algorithm.

Table 1. PCR primers, annealing temperatures, and product sizes for detection of virulence genes.

Virulence gene	Primers	Product size (bp)	Annealing temperature (°C)	Reference
gelE	5' AAT TGC TTT ACA CGG AAC GG 3' 5' GAG CCA TGG TTT CTG GTT GT 3'	548	52	[23]
ace	5' GGC CAG AAA CGT AAC CGA TA 3' 5' CGC TGG GGA AAT CTT GTA AA 3'	353		
hyl	5' ACA GAA GAG CTG CAG GAA ATG 3' 5' GAC TGA CGT CCA AGT TTC CAA 3'	276	55	[24]
esp	5' AGA TTT CAT CTT TGA TTC TTG G 3' 5' AAT TGA TTC TTT AGC ATC TGG 3'	510		
as	5' CACGCTATTACGAACTATGA 3' 5' TAAGAAAGAACATCACCACGA 3'	375		
cyl	5' TGG ATG ATA GTG ATA GGA AGT 3' 5' TCT TTC ATC ATC TGA TAG TA 3'	517		

2.6. Statistical analysis

STATA 13.1 (StataCorp LP, USA) was used for statistical analysis. Differences among *E. faecalis*, *E. faecium*, and unusual enterococcal strains were assessed by the Chi-square test and Fisher's exact test. Results with $p<0.05$ were considered significant.

3. RESULTS AND DISCUSSION

The present study focused on determining the prevalence of biofilm-forming ability among three enterococcal groups: *E. faecalis*, *E. faecium*, and other clinical isolates, and on comparison of the antibiotic resistance and the prevalence of selected virulence traits between BIO+ strains from these groups. Interestingly, we found that the ability to form biofilm occurred in 4/30 (13.3%) *E. faecalis*, 18/20 (90%) *E. faecium*, and 8/14 (57.1%) rarely isolated strains: 5 *E. avium*, 1 *E. durans*, 1 *E. casseliflavus*, and 1 *E. gallinarum* (statistically significant difference, $p=0.026$). Studies by other authors showed different results; in Greece, the ability to

produce biofilm was found in 60.9% of *E. faecalis* isolates [25], in Italy - in 80% of *E. faecalis* strains [26]. In the case of *E. faecium* isolates, in India, Italy, Turkey, and Spain, this ability occurred much less frequently (0%, 28.8%, 48%, and 75%, respectively) [5, 8, 27, 28]. Lleo et al. [17] described the biofilm-forming ability among four out of twelve unusual enterococcal strains (33.3%), which our study supports. However, in contrast to our findings, Dworniczek et al. [29] indicated the lack of these features in rare species. These varied results indicate that the level of the ability to produce biofilm among different *Enterococcus* species varies with geographic location.

In the next step of our research, only BIO+ strains (30/64) were chosen for further investigation. A comparison of antibiotic resistance among *E. faecalis*, *E. faecium* and other isolates showed that all *E. faecalis* strains were susceptible to tested ß-lactams, while 37.5% of other strains and all *E. faecium* isolates were resistant to these antibiotics. These results strongly overlap with results recently published by us [30] and other researchers [9, 16, 31]. Resistance to gentamicin was detected in 75% of *E. faecalis*, 55.5% of *E. faecium*, and 25% of other strains; resistance to streptomycin in 25%, 83.3%, and 50%, respectively. Findings from our previous work showed that more *E. faecium* isolates were resistant to aminoglycosides: 76% to gentamicin, and 91.4% to streptomycin [30]. Interestingly, a study by Tan et al. [9] demonstrated that all unusual enterococcal isolates from blood were susceptible to gentamicin and around 80% of them were susceptible to ß-lactams. Therefore, the authors concluded that combination therapy (penicillin with aminoglycosides) could be easily used for the treatment of serious infections caused by rare species of *Enterococcus*, such as bacteremia and sepsis. This finding is not confirmed in our survey. We revealed that resistance to glycopeptides occurred only in the case of four (22.2%) *E. faecium* isolates; two strains from the rare group, *E. gallinarum* and *E. casseliflavus*, showed intrinsic resistance to vancomycin. Similar results were obtained by other authors [9, 32]. We concluded that tigecycline and linezolid had the highest activity against all studied isolates (100% susceptibility), including those resistant to glycopeptides and aminoglycosides. Many studies confirmed that these antibiotics are a valuable therapeutic option in serious enterococcal infections [33-35]. Unfortunately, resistance to these drugs has been recently reported [34, 36, 37], indicating that resistance to newer antibiotics is also increasing, and development of new targeted enterococcal drugs is needed.

Our comparative analysis of the prevalence of virulence genes among *E. faecalis*, *E. faecium*, and other strains revealed that the *esp* gene was found in all *E. faecium*, 75% of *E. faecalis*, and 37.5% of other strains. Similar proportions were seen by other researchers [6, 11, 25, 38, 39, 40]. These findings indicate that this gene has a connection with biofilm-forming ability, especially in *E. faecium* strains. However, many authors found that there is no association between the presence of the *esp* gene and biofilm production [5, 14, 29, 41]. These conflicting results suggest that *esp* requires interactions with other virulence traits to result in biofilm enhancement.

Considering the presence of other virulence factors in our studied BIO+ groups, we found that the *ace* gene occurred in all *E. faecium*, 25% of *E. faecalis*, and 37.5% of unusual isolates; *hyl* in 83.3%, 0%, and 37.5%, respectively. The *gelE* gene was detected only in *E. faecalis* strains. According to the literature, the presence of *gelE* and *as* genes among *E. faecalis* is very common, whereas they are extremely rarely present in *E. faecium* and rare enterococcal isolates; consequently, they are not necessary in the process of biofilm formation among these species [5, 25, 28, 42, 43]. These assumptions are confirmed by our survey. However, some researchers imply that there is a strong relationship between *gelE* and the ability to form biofilm [12, 15]. Other virulence genes, *cyl* and *as*, were also found only in *E. faecalis* isolates, which is in accordance with other studies [22, 40, 44].

The exact resistance and virulence patterns among all tested BIO+ strains are shown in Table 2. No predominant profile among each group was identified, not only due to small sample size, but also because of high interindividual variability of examined traits among tested *Enterococcus* spp. groups. However, we have found that *E. faecium* isolates showed the greatest variety of virulence and resistance determinants, while the lowest variety was exhibited by the unusual strains group. Moreover, all *E. faecium* strains carried resistance to

three or more antibiotics and had the ability to hemolyse. Different results were seen in recent research by Tsikrikonis et al. [25], who detected only 1.9% of hemolysin-producing *E. faecium* clinical isolates. We also found that one *E. faecalis* isolate and three *E. avium* isolates were susceptible to all tested antibiotics.

Table 2. Characteristics of resistance and virulence patterns among BIO+ *E. faecalis*, BIO+ *E. faecium*, and other BIO+ *Enterococcus* strains. AMP, ampicillin; IMP, imipenem; CN, gentamicin; S, streptomycin; VA, vancomycin; TEI, teicoplanin; *esp*, enterococcal surface protein; *as*, aggregation substance; *gel*, gelatinase; *hyl*, hyaluronidase, *ace*, collagen adhesin; *c*, cytolysin; α, β, types of hemolysis.

No. of inactive antibiotics	Resistance pattern	No. of virulence genes	Virulence pattern	No. (%) of strains
BIO+ *E. faecalis* (n = 4)				
4	AMP IMP CN S	3	*as* *gel* *c* α	1 (25)
1	CN	4	*esp* *as* *gel* *c*	2 (50)
0		5	*esp* *as* *gel* *ace* *c*	1 (25)
BIO+ *E. faecium* (n = 18)				
5	AMP IMP S VA TEI	3	*esp* *ace* *hyl* α	2 (11.1)
	AMP IMP CN VA TEI		*esp* *ace* *hyl* α	1 (5.5)
4	AMP IMP		*esp* *ace* *hyl* α	1 (5.5)
	AMP IMP CN S		*esp* *ace* *hyl* α	5 (27.8)
	AMP IMP CN S	2	*esp* ace β	2 (11.1)
3	AMP IMP S	3	*esp* *ace* *hyl* α	5 (27.8)
	AMP IMP CN		*esp* *ace* *hyl* α	2 (11.1)
Unusual BIO+ *Enterococcus* (n = 8)				
4	AMP IMP CN VA	2	*esp* *hyl* α	1 (12.5)
	AMP IMP CN S	1	*esp* β	1 (12.5)
3	AMP IMP S	2	*esp* *hyl*	1 (12.5)
1	S		*ace* *hyl* β	1 (12.5)
	VA	0		1 (12.5)
0		1	*ace* β	2 (25)
		0	β	1 (12.5)

In conclusion, we observed that the proportion of isolates producing biofilm was the highest among *E. faecium* isolates, at the middle level among the unusual *Enterococcus* spp. group, and the lowest in *E. faecalis* isolates. Interestingly, our data demonstrated that unusual biofilm-forming *Enterococcus* strains have lower resistance to antibiotics and are characterized by possession of lower virulence capabilities than BIO+ *E. faecalis* and BIO+ *E. faecium* clinical isolates. Moreover, *E. faecium* strains showed the highest resistance and virulence levels. It is well known that *E. faecium* isolates resistant to ß-lactams, aminoglycosides, and glycopeptides are considered as multidrug resistant (MDR) bacteria, and they represent a particular threat to immunocompromised patients [9]. The problem with these strains becomes even more serious when they are also able to produce biofilm, and persist in hospital environments for a very long time. However, the high percentage of biofilm-

forming ability among unusual *Enterococcus* species, observed in this study, indicates that these isolates could also stay in the medical environment and, consequently, slowly acquire resistance and virulence traits. Therefore, the infections caused by these strains should not be underestimated, and determination of their susceptibility should always be performed. The changing epidemiology and increasing resistance to antibiotics among *Enterococcus* species stress the need to search in new directions for the treatment and new methods for preventing the spread of enterococcal nosocomial infections.

ACKNOWLEDGEMENTS

We thank Steven J. Snodgrass for editorial assistance. The results of this work were presented in part at the Biofilms 7 Conference 2016, Porto, Portugal (26-28.06.2016).

AUTHOR'S CONTRIBUTION

AS: Conception and design, Development of methodology, Acquisition of data, Analysis and interpretation of data, Writing, review and/or revision of the manuscript; DO: Acquisition of data, Analysis and interpretation of data, Administrative, technical, or material support; PM: Analysis and interpretation of data, Administrative, technical, or material support; PS and PW: Writing, review and/or revision of the manuscript; ET: Writing, review and/or revision of the manuscript, Study supervision. The final manuscript has been read and approved by all authors.

SOURCE OF FUNDING

This work was supported by funds from Medical University of Bialystok (Poland), and from the Leading National Research Centre (137/KNOW/ 2015) in Bialystok.

TRANSPARENCY DECLARATION

The authors have no conflict of interest to declare.

REFERENCES

1. Orsi GB, Ciorba V. Vancomycin resistant enterococci healthcare associated infections. Ann Ig. 2013; 25: 485-492.
2. Amyes SG. Enterococci and streptococci. Int J Antimicrob Agents. 2007; 29: 43-52.
3. Marschall J, Piccirillo ML, Fraser VJ, Doherty JA, Warren KW. Catheter removal versus retention in the management of catheter-associated enterococcal bloodstream infections. Can J Infect Dis Med. 2013; 24(3): e83-e87.
4. Fernandes SC, Dhanashree B. Drug resistance and virulence determinants in clinical isolates of *Enterococcus* species. Indian J Med Res. 2013; 137: 981-985.
5. Di Rosa R, Creti R, Venditti M, D'Amelio R, Arciola CR, Montanaro L, et al. Relationship between biofilm formation, the enterococcal surface protein (Esp) and gelatinase in clinical isolates of *Enterococcus faecalis* and *Enterococcus faecium*. FEMS Microbiol Lett. 2006; 256: 145-150.
6. Fisher K, Phillips C. The ecology, epidemiology and virulence of *Enterococcus*. Microbiology. 2009; 155: 1749-1757.
7. Sava IG, Heikens E, Huebner J. Pathogenesis and immunity in enterococcal infections. Clin Microbiol Infect. 2010; 16: 533-540.
8. Prakash VP, Rao SR, Parija SC. Emergence of unusual species of enterococci causing infections, South India. BMC Infect Dis. 2005; 5: 14.
9. Tan CK, Lai CC, Wang JY, Lin SH, Liao SH, Huang Y, et al. Bacteriemia caused by non-faecalis and non-faecium *Enterococcus* species at a medical center in Taiwan, 2000 to 2008. J Infect. 2010; 61: 34-43.
10. Kenzaka T, Takamura N, Kumabe A, Takeda K. A case of subacute infective endocarditis and blood access infection caused by *Enterococcus durans*. BMC Infect Dis. 2013; 13: 594.
11. Heikens E, Bonten MJ, Willems RJ. Enterococcal surface protein Esp is important for biofilm formation of *Enterococcus faecium* E1162. J Bacteriol. 2007; 189: 8233-8240.
12. Mohamed JA, Huang DB. Biofilm formation by enterococci. J Med Microbiol. 2007; 56: 1581-1588.
13. Paganelli FL, Willems RJ, Jansen P, Hendrickx A, Zhang X, Bonten MJ, et al. *Enterococcus faecium* biofilm formation: identification of major autolysin AtlAEfm, associated Acm surface localization, and AtlAEfm-independent extracellular DNA Release. MBio. 2013; 4(2): e00154.

14. Almohamad S, Somarajan SR, Singh KV, Nallapareddy SR, Murray BE. Influence of isolate origin and presence of various genes on biofilm formation by *Enterococcus faecium*. FEMS Microbiol Lett. 2014; 353: 151-156.
15. Kafil HS, Mobarez AM. Assessment of biofilm formation by enterococci isolates from urinary tract infections with different virulence profiles. J King Saud Univ Sci. 2015; 27: 312-317.
16. Iwen PC, Kelly DM, Linder J, Hinrichs SH, Dominguez EA, Rupp ME, et al. Change in prevalence and antibiotic resistance of *Enterococcus* species isolated from blood cultures over an 8-year period. Antimicrob Agents Chemother. 1997; 41: 494-495.
17. Lleo M, Bonato B, Tafi MC, Caburlotto G, Benedetti D, Canepari P. Adhesion to medical device materials and biofilm formation capability of some species of enterococci in different physiological states. FEMS Microbiol Lett. 2007; 274: 232-237.
18. Christensen GD, Bisno AL, Simpsom WA, Beachey EH. Adherence of slime producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect Immun. 1982; 37: 318-326.
19. Oliveira A, Cunha MD. Comparison of methods for the detection of biofilm production in coagulase-negative staphylococci. BMC Res Notes. 2010; 3: 260.
20. Freeman DJ, Falkiner FR, Keane CT. New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol. 1989; 48: 872-874.
21. Cabrera-Contreras R, Morelos-Ramirez R, Galicia-Camacho AN, Melendez-Herrada E. Antibiotic resistance biofilm production in *Staphylococcus epidermidis* strains, isolated from a Tertiary Care Hospital in Mexico City. ISRN Microbiol. 2013; 13: 1-5.
22. Vergis EN, Shankar N, Chow JW, Hayden MK, Snydman DR, Zervos MJ, et al. Association between the presence of enterococcal virulence factors gelatinase, hemolysin, and enterococcal surface protein and mortality among patients with bacteremia due to *Enterococcus faecalis*. Clin Infect Dis. 2003; 35: 570-575.
23. Camargo ILBC, Gilmore MS, Darini ALC. Multilocus sequence typing and analysis of putative virulence factors in vancomycin-resistant and vancomycin-sensitive *Enterococcus faecium* isolates from Brazil. Clin Microbiol Infect. 2006; 12: 1123-1130.
24. Zou LK, Wang HN, Zeng B, Li JN, Li XT, Zhang AY, et al. Erythromycin resistance and virulence genes in *Enterococcus faecalis* from swine in China. New Microbiol. 2011; 34: 73-80.
25. Tsikrikonis G, Maniatis AN, Labrou M, Ntokou E, Michail G, Daponte A, et al. Differences in biofilm formation and virulence factors between clinical and fecal enterococcal isolates of human and animal origin. Microb Pathog. 2012; 52: 336–343.
26. Baldassarri L, Cecchini R, Bertuccini L, Ammendolia MG, Iosi F, Arciola CR, et al. *Enterococcus* spp. produces slime and survives in rat peritoneal macrophages. Med Microbiol Immunol. 2001; 190: 113-120.
27. Latasa C, Solano C, Penadés JR, Lasa I. Biofilm-associated proteins. CR Biol. 2006; 329(11): 849-857.
28. Diani M, Esiyok OG, Ariafar MN, Yuksel FN, Altuntas EG, Akcelik N. The interactions between *esp*, *fsr*, *gelE* genes and biofilm formation and pfge analysis of clinical *Enterococcus faecium* strains. Afr J Microbiol Res. 2014; 8: 129-137.
29. Dworniczek E, Wojciech Ł, Sobieszczańska B, Seniuk A. Virulence of *Enterococcus* isolates collected in Lower Silesia (Poland). Scand J Infect Dis. 2005; 37: 630-636.
30. Sieńko A, Wieczorek P, Majewski P, Ojdana D, Wieczorek A, Olszańska D, et al. Comparison of antibiotic resistance and virulence between biofilm-producing and non-producing clinical isolates of *E. faecium*. Acta Biochim Pol. 2015; 62: 859-866.
31. Billström H, Sullivan A, Lund B. Cross-transmission of clinical *Enterococcus faecium* in relation to esp and antibiotic resistance. J Appl Microbiol. 2008; 105: 2115-2122.
32. Contreras GA, Diaz Granados CA, Cortes L, Reyes J, Vanegas S, et al. Nosocomial outbreak of *Enterococcus gallinarum*: untaming of rare species of enterococci. J Hosp Infect. 2008; 70: 346-352.
33. Franiczek R, Dolna I, Dworniczek E, Krzyżanowska B, Seniuk A, Piątkowska E. In vitro activity of tigecycline against clinical isolates of Gram-positive and Gram-negative bacteria displaying different resistance phenol-types. Adv Clin Exp Me. 2008; 17: 545-551.
34. Freitas AR, Coque TM, Novais C, Hammerum AM, Lester CH, Zervos MJ, et al. Human and swine hosts share vancomycin-resistant *Enterococcus faecium* CC17 and CC5 and *Enterococcus faecalis* CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids. J Clin Microbiol. 2011; 49: 925-931.

35. Sieńko A, Wieczorek P, Wieczorek A, Sacha P, Majewski P, Ojdana D, et al. Occurrence of high-level aminoglycoside resistance (HLAR) among *Enterococcus* species strains. Prog Health Sci. 2014; 4: 179-187.
36. Werner G, Grforer S, Fleige C, Witte W, Klare I. Tigecycline-resistant *Enterococcus faecalis* strain isolated from a German Intensive Care Unit Patient. J Antimicrob Chemother. 2008; 61: 1182-1183.
37. Baldir G, Engin DO, Kucukercan M, Inan A, Akcay S, Ozyuker S, et al. High-level resistance to aminoglycoside, vancomycin and linezolid in enterococci strains. J Microbiol Infect Dis. 2013; 3: 100-103.
38. Dupre I, Zanetti S, Schito AM, Fadda G, Sechi LA. Incidence of virulence determinants in clinical *Enterococcus faecium* and *Enterococcus faecalis* isolates collected in Sardinia (Italy). J Med Microbiol. 2003; 52: 491-498.
39. Tendolkar PM, Baghdayan AS, Gilmore MS, Shankar N. Enterococcal surface protein, Esp, enhances biofilm formation by *Enterococcus faecalis*. Infect Imm. 2004; 72: 6032-6039.
40. Hallgren A, Claesson C, Saeedi B, Monstein HJ, Hanberger H, Nilsson LE. Molecular detection of aggregation substance, enterococcal surface protein, and cytolysin genes and in vitro adhesion to urinary catheters of *E. faecalis* and *E. faecium*. Int J Med Microbiol. 2009; 299: 323-332.
41. Raad II, Hanna HA, Boktour M, Chaiban G, Hachem RY, Dvorak T, et al. Vancomycin-resistant *Enterococcus faecium*: catheter colonization, *esp* gene, and decreased susceptibility to antibiotics in biofilm. Antimicrob Agents Chemother. 2005; 49: 5046-5050.
42. Vankerckhoven V, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, et al. Development of a multiplex PCR for the detection of *asa1*, *gelE*, *cylA*, *esp*, and *hyl* genes in enterococci and survey for virulence determinants among european hospital isolates of *Enterococcus faecium*. J Clin Microbiol. 2004; 42: 4473-4479.
43. Comerlato CB, Resende MC, Caierão J, d'Azevedo PA. Presence of virulence factors in *Enterococcus faecalis* and *Enterococcus faecium* susceptible and resistant to vancomycin. Mem Inst Oswaldo Cruz. 2013; 108: 590-595.
44. Archimbaud C, Shankar N, Forestier C, Baghdayan A, Gilmore MS, Charbonne F, et al. In vitro adhesive properties and virulence factors of *Enterococcus faecalis* strains. Res Microbiol. 2002; 153: 75-80.

ISSN 2449-8955

European Journal of Biological Research

Review Article

Fungal diseases on tomato plant under greenhouse condition

Rabab Sanoubar*, Lorenzo Barbanti

Department of Agricultural Sciences, Alma Mater Studiorum - Bologna University, Viale Fanin 44, 40127 Bologna, Italy
*Corresponding author: Rabab Sanoubar; E-mail: rabab.sanoubar@unibo.it

Received: 16 January 2017; **Revised submission:** 23 September 2017; **Accepted:** 13 October 2017

DOI: http://dx.doi.org/10.5281/zenodo.1011161

ABSTRACT

The cultivation of crops in the greenhouse is the most intensive form of horticultural production. Greenhouse climatic conditions provide an ideal condition for the development of many foliar, stem and soil-borne plant diseases. Diseases are a major limiting factor for vegetable that cause serious yield reduction leading to severe economic losses. Fungi enter plants through natural openings such as stomata and through wounds caused by pruning, harvesting, hail, insects, other diseases, and mechanical damage. This chapter provides an overview of the most important diseases of tomato plants. Some of the diseases that will cover in this chapter are the follow: Early blight late, *Septoria* leaf spot, Late blight, *Fusarium* wilt, *Verticillium* wilt, Anthracnose, Buckeye rot, and Southern blight. For each disease, main symptoms and disease development are described. This review is based on combined information derived from available literature and expertise knowledge.

Keywords: Fungal diagnostic; Disease development.

1. EARLY BLIGHT

1.1. Introduction

Early blight is a common leaf-spotting fungal disease of tomato. It is also known as *Alternaria* leaf spot or target spot. Early blight, caused by the fungus *Alternaria solani* [1]. The early blight fungus can come from many sources. It can be in the soil, or on purchesed seeds or seedlings, and it can over-winter in the diseased debris of tomato plants and it can persist in the soil or debris for at least 1 year [2]. The fungus can survive winter's freezing temperatures and infect new plantings when foliage encounters contaminated dirt or dead plants. The fungus survives in the soil by forming resistant spores in association with diseased tomato debris capable of persisting for one year or more. Infection occurs rapidly under warm and humid conditions. Thousands of spores are produced in spots of infected leaves and are capable of causing more infections [3]. The fungal spores can be spread by wind and rain, irrigation, insects, workers, and on tools and equipment. Once the primary infections have occurred, they become the most important source of new spore production and are responsible for rapid disease spread. Early blight can develop quickly mid- to late season and is more severe when plants are stressed by nitrogen deficiency, drought,

or heavy fruit load. Early blight can affect almost all parts of the tomato plants, including the leaves, stems, and fruits. The plants may not die, but they will be weakened and will set fewer tomatoes than normal.

1.2. Symptoms

Premature loss of lower leaves is the most obvious symptom of the disease [4]. Infected leaves show brown to black spots (lesions) up to 1⁄2 inch diameter with dark edges and have a pattern of concentric rings resulting in the "target" appearance to the spot suggested by the common name (Figure 1) [5]. Later, spots frequently merge forming irregular blotches [6]. Defoliation progresses upward from the lower plant and infected leaves eventually turn brown and drop from the plant exposing the fruits to sun scald (Figure 2) [7]. Dark lesions on the stems start off small and slightly sunken. As they get larger, they elongate and you will start to see concentric markings like the spots on the leaves (Figure 3) [8]. Spots that form near ground level can cause some girdling of the stem or collar rot. Plants may survive, but they will not thrive or produce many tomatoes [9]. Early blight generally attacks older plants, but it can also occur on seedlings. If early blight gets on the seedlings, affected seedlings will have dark spots on their leaves and stems [10, 11].

Figure 1. Foliar symptoms of tomato early blight.

Figure 2. Defoliation caused by early blight on tomato plant.

Figure 3. Early blight of tomato stem.

They may even develop the disease on their cotyledon leaves. Basal girdling and death of seedlings may occur. This manifestation of the disease is called collar rot [12]. Early blight can infect plants at any stage during the growing season but usually progresses most rapidly after plants have set fruit (Figure 4) [13]. Green or red fruit may be infected by the fungus which invades at the point of attachment between the stem and fruit, and through growth cracks and wounds made by insects. Dark lesions enlarge in a concentric fashion and may affect large areas of the fruit. Mature lesions in fruit are typically covered by a black velvety mass of fungal spores.

Figure 4. Early blight rot symptoms on tomato fruit.

2. SEPTORIA LEAF SPOT

2.1. Introduction

Septoria leaf spot, also called *Septoria* blight, is caused by a fungus (*Septoria lycopersici*). It is one of the most common foliar disease of tomatoes. The disease is favored by warm temperatures 20-25°C and high relative humidity or the extended periods of leaf wetness caused by overhead irrigation, rain or heavy dews. Most infection probably arises from infested plant debris remaining in the soil from a previous tomato crop. *Septoria* leaf spot spreads rapidly and can quickly defoliate and weaken the plants, rendering them unable to bear fruit to maturity. The *Septoria* fungus lives on the fallen tomato plant debris and weeds that are on and in the soil. It is spread to the plants by both water and wind, usually splashing up on the plants from the soil.

2.2. Symptoms

Lower leaves are infected first, and the disease progresses upward and may also attack stems and blossoms, but rarely on fruit. Infection can occur at any stage of plant development but appears most frequently after plants have begun to set fruit. It first appears as small, water-soaked spots on the undersides of older leaves and the bottom of the plant that soon become circular spots about 1/16-1/8 inch in diameter (Figure 5) [14]. The lesions gradually develop grayish white centers with dark edges. The light-colored centers of these spots are the most distinctive symptom of *Septoria* leaf spot (Figure 6). When conditions are favorable, fungal fruiting bodies appear as tiny black specks in the centers of the spots [15]. These are fruiting structures that release spores. Spores are spread to new leaves by splashing rain. Heavily infected leaves turn yellow, wither, and eventually fall off. This will weaken the plant, send it into decline and cause sun scalding of the unprotected, exposed tomatoes. Defoliation progresses from the base of the plant upwards, and it can be severe after periods of prolonged warm and wet weather. Loss of foliage may cause fruits to become sunscalded. The *Septoria* defoliation resembles early blight disease. However, the larger dark leaf spots with concentric rings of early blight are clearly different from smaller *Septoria* leaf spots.

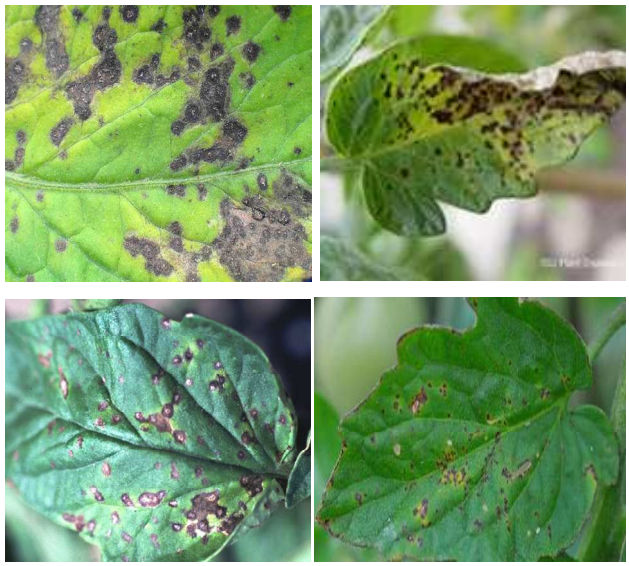

Figure 5. *Septoria* leaf spot symptoms on tomato plant.

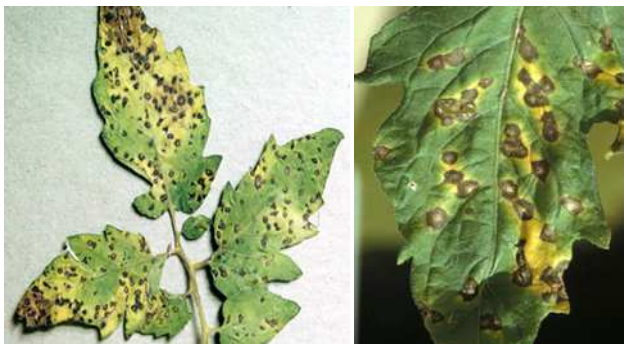

Figure 6. *Septoria* tomato leaf spot; the light-colored centers.

3. LATE BLIGHT

3.1. Introduction

Late blight is caused by the fungus *Phytophthora infestans and* usually appears in mid- or late August. The fungus is a wet weather disease favored by cool nights and warm days. Temperatures above 30°C are considered unfavorable for late blight development. The fungus survives mainly in potato seed tubers and in infected tomato transplants. Some survival may also occur in dead potato and tomato vines. The disease often begins in potato plants, from which spores of the fungus are blown by wind to infect tomatoes in favorable conditions. Disease development is rapid with extended periods of favorable conditions and ceases when weather becomes hot and dry. Usually, the warm to hot temperatures prevailing during periods of tomato production make the occurrence of this disease unlikely. However, the disease could be a potential problem during unseasonably cool and wet conditions on early-planted or fall-cropped tomatoes.

Figure 7. Late blight symptoms on tomato leaflet.

Figure 8. Brown streaks along the tomato stems caused by late blight.

3.2. Symptoms

All parts of the plant are affected and fruit decay can be severe. Late blight may infect either young (upper) or old (lower) leaves. It first appears as pale green water-soaked spots starting at leaf tips that enlarge rapidly, forming irregular, greenish black blotches (Figure 7) that expand rapidly when leaves are wet or humidity is high [13]. White mold usually develops at the margins of affected areas giving the plant a frost-damaged appearance. Entire plants may be rapidly defoliated when conditions favor the disease (Figure 7). If stems and petioles are infected, brown streaks along the stems will be presented and the areas above these infections wilt and die (Figure 8) [13]. Infection of green or ripe fruit produces large, irregularly shaped brown blotches that usually start at the stem (Figure 9). Infected fruits rapidly deteriorate into foul-smelling masses.

Figure 9. Tomato fruit rot caused by late blight.

4. FUSARIUM WILT

4.1. Introduction

Fusarium wilt, the most common tomato wilt disease, is caused by the fungus *Fusarium oxysporum* f.sp. *lycopersici*. *Fusarium* wilt is found worldwide and even resistant tomato varieties may be affected. The fungus is soil borne and makes its way into the plant through the roots. *Fusarium* wilt disease is a soil borne and can persist for many years in the soil even if no host plants are grown. It does not spread

above the ground from plant to plant. Each plant is individually infected when the organism enters the root system. This pathogen invades the vascular system (xylem tissue that moves water and some nutrients) and disrupts water flow through the plant. It clogs and blocks the xylem preventing water from traveling up the stem and out into the branches and leaves. The fungus survives and persists indefinitely in field soil. The disease is most serious in sandy soils and at temperatures between 27°C to 32°C. Soils become infested by planting infected transplants and from movement of infested soil by wind and water erosion or on farm implements.

4.2. Symptoms

Plants are susceptible at all stages of development, but symptoms are most obvious at or soon after flowering. Diseased plants first develop a yellowing of the oldest leaves (those nearing the ground). The bright yellowing which is restricted to one side of the plant or even to leaflets on one side of the petiole is considered as a recognized characteristic of tomato *Fusarium* wilt (Figure 10) [16]. The affected leaves soon wilt and dry up but they remain attached to the plant. The wilting continues successively on younger foliage and eventually results in the death of the plant. The stem remains firm and green on the outside but exhibits a narrow band of brown discoloration in the vascular tissue (Figure 11) [17]. This discoloration can be viewed easily by slicing vertically through the stem near the soil line and looking for a narrow column of browning between the central pith region (middle tissue of the stem) and the outer portion of the stem. The brown streaking in the vascular tissue of infected plants becomes plugged during the attack by the fungus, leading to wilting and yellowing of the leaves. Infected plants often die before maturing.

Figure 10. Tomato *Fusarium* wilt.

Figure 11. Vascular browning caused by *Fusarium* wilt on tomato stem.

5. VERTICILLIUM WILT

5.1. Introduction

Verticillium albo-atrum and *Verticillium dahliae,* the fungi that cause *Verticillium* wilt, can attack more than 200 plant species, including potato, pepper, eggplant, strawberry, black raspberry, watermelon, radish, and many common weeds. The fungi causing this disease overwinter in the soil as mycelium or on plant debris as microsclerotia. It invades the plant through the root system causing harms to the vascular system which results in disrupting water and mineral uptake within the plant. Infection and disease development in *Verticillium* wilt develops best at relatively cool (13-23°C) soil temperatures.

5.2. Symptoms

Wilting is the most characteristic feature of infection by *Verticillium* spp. Symptoms usually appear on the older leaves in mid-August when infected plants wilt during the warmest part of the day, and then recover at night [16]. Leaf edges and areas between the veins turn yellow and then brown. In addition, infected plants often have a characteristic V-shaped lesion at the edge of the leaf occurring in a fan pattern (Figure 12) [17]. Unlike *Fusarium* wilt, symptoms of *Verticillium* wilt do not progress along one side of a leaflet, branch, or plant. *Verticillium* wilt causes uniform yellowing and wilting of the lower leaves. As the disease progresses, younger leaves begin to wilt and die, until only a few healthy leaves remain at the top of the plant. Although diseased plants are not killed, they are stunted and weak and produce small fruit [16]. *Verticillium* wilt can be detected by presence of the internal browning streaking of vascular

system in stems (Figure 13) [18]. The discoloration is most pronounced near the soil line and does not extend quite as far up the stem. These symptoms are similar to those caused by *Fusarium* wilt, but vascular streaking caused by *Fusarium* is generally darker and progresses further up the stem than streaking caused by *Verticillium*.

Wilt caused by this disease may be differentiated from drought-stress based on the portion of the plant that is wilting and on the location of wilted plants. Diseased plants often have only a portion of the plant wilting, such as one or two stems. In addition, diseased plants usually appear in patches within the growing area. Plants suffering from drought, however, are uniformly wilted and occur throughout the growing area.

Figure 12. *Verticillium* wilt foliage symptoms on tomato plant.

Figure 13. Verticillium wilt symptoms of cut stem in tomato plant.

6. ANTHRACNOSE

6.1. Introduction

Anthracnose is a frequent problem in the latter part of the growing season on ripening tomato fruit. The disease results in a fruit rot that reduces the quality and yield of tomatoes. *Anthracnose* is caused by several fungal species in the genus *Colletotrichum*, including *C. coccodes, C. dematium, and C. gloeosporioides* [19]. The fungus can survive in infected plant debris and in the soil. During rainy weather, fungal spores are splashed onto the fruit. Most infection takes place on ripe or overripe fruit. Green fruit also can be infected, although symptoms do not develop until the tomatoes begin to mature. Disease development is favored by frequent rainfall temperatures around 26°C. The fungus survives the winter on diseased tomato vines, in the soil, and in seeds. Weeks before the fruit ripens, *anthracnose* can become established on leaf spots caused by other fungi or by insect feeding injuries. Warm, wet weather causes the disease to spread and symptoms to develop. While insect or other wounds facilitate infection, tomatoes can also become infected in the absence of wounds.

6.2. Symptoms

Although symptoms do not appear until the fruit is ripening, the infection occurs when fruits are small and green. Symptoms of *anthracnose* appear first as small, circular, slightly sunken lesions on the surface of ripening fruits (Figure 14) [20]. The spots quickly enlarge, become bruise like depressions, and develop a water-soaked appearance directly beneath the skin (epidermis) of the fruit. As these spots expand, they develop dark centers or concentric rings of dark specks. The rings consist of numerous small spore-producing bodies of the fungus (microsclerotia and acervuli) (Figure 15) [21]. In moist weather these bodies exude large numbers of spores, giving diseased areas a cream to salmon-pink color [22]. By this stage, decay has penetrated deeply into the tomato flesh. Spotted fruits often may rot completely because of attack by secondary fungi through anthracnose spots. After infection, the fungus "rests" between the cuticle and the epidermis of the fruit [23]. The fungus is activated by exposure of the fruit to low temperatures, fruit maturation or plant stress. As fruit ripens, symptoms begin to appear and susceptibilities increase. Eventually the entire fruit will rot, especially when there are several *anthracnose* spots or decay organisms enter the diseased tissue (Figure 16). Fruit nearest to the ground are most likely to be affected aand the fungus can also infect roots [24].

Despite all this apparent destruction, *Colletotrichum* is considered a weak pathogen and can often be controlled through good cultural practices [25]. The pathogen overwinters on infected plant debris, so it is very important to dispose of rotten fruit and infected plants. Because of the ability of this fungus to persist in the soil, tomatoes and other solanaceous crops, like peppers and eggplants, should be rotated on an every-other-year basis [26].

Figure 14. Circular sunken lesions on ripening tomato fruit caused by *anthracnose.*

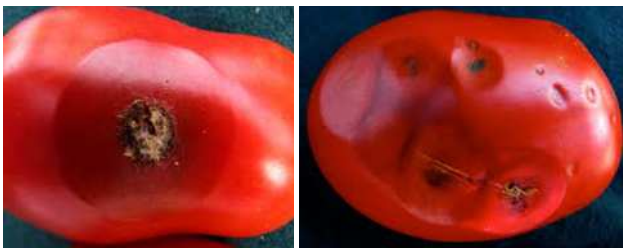

Figure 15. Fruiting bodies at the center of the lesion on ripe tomato fruit.

Figure 16. *Anthracnose* tomato fruit rot.

7. BUCKEYE ROT

7.1. Introduction

Buckeye rot caused by the soilborne fungus *Phytophthora* primarily infects fruit lying on or near moist soil. Large amounts of rainfall or frequent irrigation may result in the sudden appearance of buckeye rot. Saturation of the soil stimulates the release of zoospores (motile fungal spores) from sporangia in the soil. Buckeye fruit rot most commonly occurs under prolonged warm, wet conditions [27]. The buckeye rot fungi may be introduced through infected seeds or transplants, or through volunteer plants from the previous crop. Fungal spores are produced when the soil is wet and above 18°C. Spores are spread by surface water and splashing rain. Fruit may become infected when they come into contact with infested soil or when being splashed with mud containing fungal inoculum.

7.2. Symptoms

The initial symptom on the fruit is a grayish green or brown watersoaked spot that usually appears near the blossom end, or at the point of contact between the fruit and soil [28]. The spot further enlarges and develops into a lesion with a characteristic target-like pattern of concentric rings of narrow dark brown and wide light brown bands that resemble the markings on a buckeye (Figure 17) [29]. Fruit symptoms caused by buckeye fruit rot can be confused with symptoms of “late blight,” which is caused by the fungus *Phytophthora infestans* [30]. Buckeye fruit rot lesions are at first firm and smooth surface and lack a sharply defined margin, whereas late blight lesions are typically rough and sunken at the margins. Buckeye fruit rot may produce a white, cottony fungal growth on the lesion under moist conditions (Figure 18) [31]. The buckeye rot fungus can affect both green and ripe fruit. Diseased fruit are usually located nearest the ground in staked tomatoes.

Figure 17. Brown concentric rings cused by buckeye rot on tomato fruit.

Figure 18. Buckeye rot fungus affect both green and ripe tomato fruit.

8. SOUTHERN BLIGHT

8.1. Introduction

Southern blight, caused by the soilborne fungus *Sclerotium rolfsii*, which is nearly impossible to eradicate even though it exists in relatively low levels. The fungus infects the lower stem of the plant near the surface of the soil [32]. It is called Southern blight because it cannot survive for long stretches in frozen soil and therefore only thrives in warm climates. *Sclerotium rolfsii* can attack any parts of a plant that touch the soil, but it most commonly attacks a plant at or just below the soil line. Southern blight is favored by by high humidity and soil moisture and warm to hot temperatures (29-35°C). Disease severity increases when undecomposed organic matter is left on and in the soil. Southern blight is extremely difficult to manage because the fungus has a wide host range (more than 500 plant species in 100 plant families), which limits the use of crop rotation. Sclerotia can survive in the soil for several years, and there are few, if any, commercially available resistant varieties.

8.2. Symptoms

The initial symptom of southern blight is a rapid wilting of the entire plant (Figure 19). A water-soaked lesion on the stem near the soil line rapidly expands, turns brown, and girdles the stem (Figure 20) [33]. The fungus produces white fungal strands (mycelia or hyphae) around infected stem and can be observed on the soil surrounding the plant (Figure 20).

Figure 19. Southern blight on tomato plants.

Figure 20. White mycelia at the base of infected tomato plants, small round sclerotia of *Sclerotium rolfsii.*

Sclerotium rolfsii produces survival structures called sclerotia, which are small (0.04-0.08 inches) that enable the fungus to survive for many years in soils, even through adverse conditions [34]. Sclerotia are first white, later becoming darken to brown spherical structures or orange color. When Sclerotia is fully developed, each sclerotium is about mustard seeds size. The presence of the white mycelium and sclerotia at stem base of afected plants are very useful characteristics for identifying southern blight [35, 36]. The fungus survives in the soil as sclerotia which may build to high numbers when susceptible plants are cropped repeatedly.

After sclerotia germinate, the fungus must first colonize organic debris near the soil surface before the fungus can cause infection. Early symptoms on fruit are circular water-soaked spots followed by soft rot or decay (Figure 21).

Figure 21. Mycelia and sclerotia of *Sclerotium rolfsii* on tomato fruit.

AUTHOR'S CONTRIBUTION

RS: wrote manuscript, searched literature; LB: revised and corrected the paper. The final manuscript has been read and approved by both authors.

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interests.

REFERENCES

1. Baysal-Gurel F, Subedi N, Mera J, Miller S A. Evaluation of composted dairy manure and biorational products for the control of diseases of fresh market tomatoes in high tunnels. The sixth international IPM symposium, Portland, Oregon, 2009.
2. Kouyoumjian RE. Comparison of compost tea and biological fungicides for control of early blight in organic heirloom tomato production. MS Thesis. Clemson University, South Carolina, 2007.
3. Agricultural Marketing Service - National Organic Program. United States Department of Agriculture, 2010.
4. Tsror L. Biological control of early blight in tomatoes. Acta Horticult. 1999; 487: 271-273.
5. Vegetable MD Online. Department of Plant Pathology and Plant-Microbe Biology, Cornell University, 2010. http://vegetablemdonline.ppath.cornell.edu
6. Watson ME. Testing compost. Ohio State University Fact Sheet. ANR-15-03. Ohio State University Extension, 2003. http://ohioline.osu.edu/anr-fact/0015.html
7. Wszelaki A L, Miller S A. Determining the efficacy of disease management products in organically-produced tomatoes. Plant Health Progress, 2005. http://dx.doi.org/10.1094/PHP-2005-0713-01-RS
8. Jones JP. Early blight. In: Jones JB, Jones JP, Stall RE, Zitter TA, eds. Compendium of tomato diseases. American Phytopathological Society, St. Paul, MN, 1991: 13-14.
9. McGovern RJ, Davis TA, Seijo TE. Evaluation of fungicides for control of early blight and target spot in tomato. Fungicide Nematicide Test Reports. 1999; 55: 279.
10. McGovern RJ, Davis TA, Seijo TE. Evaluation of fungicides for control of early blight and target spot in tomato. Fungicide Nematicide Test Reports. 1999; 55: 280.
11. Sherf AF, MacNab AA, eds. Vegetable diseases and their control. John Wiley & Sons, New York, 1986.
12. Delahaut K, Stevenson W. Tomato disorders: early blight and *Septoria* leaf spot. University of Wisconsin Extension, Cooperative Extension Publishing. Disease fact sheet A2606, 2004.
13. Dixon GR. Pathogens of solanaceous crops. In: Vegetable crop diseases. AVI Publishing Company, Inc. Westport, Connecticut, 1981.
14. Hansen M A. Septoria leaf spot of tomato. Virginia Cooperative Extension Plant Disease Fact Sheet 450-711W. University of Maine Cooperative Extension, Orono, Maine, 2000.
15. Zitter TA. Septoria leaf spot of tomato (*Septoria lycopersici*). Cornell University, Department of Plant Pathology. Ithaca, NY, 1987.

16. Rowe RC, Miller SA, Riedel RM. Late blight of potato and tomato. Ohio State University Extension. Extension Fact Sheet HYG-3102-95, 1995.
17. Caldwell B, Rosen EB, Sideman E, Shelton A, Smart C. Resource guide for organic insect and disease management. New York State Agricultural Experiment Station, Geneva NY, 2005.
18. Jones JB, Jones JP, Stall RE, Zitter TA. Compendium of tomato diseases. The American Phytopathological Society, St. Paul MN, 1991.
19. Retig N, Rabinowitch HD, Cedar N. A simplified method for determining the resistance of tomato seedlings to *Fusarium* and *Verticillium* wilts. Phytoparasitica. 1973; 1(2): 111-114.
20. Fordyce C. Studies of the mechanism of variation of *Verticillium albo-atrum.* Diss Abstr. 1963; 23: 3584.
21. Caldwell B, Rosen EB, Sideman E, Shelton A, Smart C. Resource guide for organic insect and disease management. New York State Agricultural Experiment Station, Geneva NY, 2005.
22. Raudales RE, McSpadden-Gardener BB. Microbial biopesticides for the control of plant diseases in organic farming. The Ohio State University, publication HYG-3310-08, 2008.
23. Sherf AF, MacNab AA. Vegetable diseases and their control. 2nd edn. John Wiley & Sons, NY, 1986.
24. Jones JB, Jones JP, Stall RE, Brooklyn Botanic Garden. Natural disease control: A common-sense approach to plant first aid. Handbook #164. Brooklyn Botanic Garden, Inc. 1000 Washington Avenue, Brooklyn, NY, 2000.
25. CABI. Crop protection compendium. CAB International Publishing. Wallingford, UK, 2004.
26. Ploetz R. Compendium of tropical fruit diseases. APS Press, The American Phytopathological Society. Saint Paul, Minnesota, USA, 1998.
27. Thurston D. Tropical plant diseases. 2nd edn. APS Press. The American Phytopathological Society. St. Paul, Minnesota, USA, 1998.
28. Wagner G. Vegetables' pests. Personal Communication, 2004.
29. Baker RED. Notes on the diseases and fruit rots of tomatoes in the British West Indies. Trop Agr. 1939; 16: 252-257.
30. Sarejanni JA. La pourriture du collet des solanées cultivées et la classification du genre phytophthora. Beuaki Inst Phytopath. 1936: 35-52.
31. Young PA, Harrison AL, Alstatt GE. Common diseases of tomatoes. Tcx Agr Expt Sta Cir. 1940: 86-32.
32. Stevens NE, Nance NW. Spoilage of tomatoes in transit, as shown by inspection certificates. U. S. Dept. Agr. Cir. 1932: 245.
33. Poetbren van N. Verslag over de werkzaamheden van den plantenziektenkundigen dienst in het jaar 1924. Verslag. En Meded. Plantenziektenkund. Dienst Wageningen. 1924: 41-62.
34. Farr DF, Rossman AY. Fungal databases. Systematic Mycology and Microbiology Laboratory, ARS, USDA, 2016.
35. Sustainable disease management of cucurbit crops in the home garden. http://www2.ca.uky.edu/agcollege/plantpathology/ext_files/PPFShtml/PPFS-VG-19.pdf.
36. Vegetable production guide for commercial growers. http://www2.ca.uky.edu/agcomm/pubs/id/id36/id36.htm.

ISSN 2449-8955

European Journal of Biological Research

Review Article

Management of fungal plants diseases

Nadia Ghanney[1,2]

[1] National Institute of Agronomy, Tunis, Tunisia
[2] Arid Land Institute, Medenine, 4119, Tunisia
Email: nadia.ghanney@gmail.com

Received: 18 May 2017; **Revised submission:** 30 July 2017; **Accepted:** 14 October 2017

DOI: http://dx.doi.org/10.5281/zenodo.1012350

ABSTRACT

Plant diseases that can affect yield and quality of field crops everywhere around the world are numerous. Fungal parasites are by far the most prevalent plant pathogenic organisms. To develop, all components of the disease triangle must be present. These components are a susceptible host crop, a plant pathogen able to infect the host crop, and an environment that favors disease development. Management practices aiming to reduce plant diseases affect specific components of the disease triangle. They need to be combined to limit more than a single component, an approach known as integrated disease management (IDM). Integrating different tools leads to better disease reduction and decreases selection pressures. Knowing that pathogens are affected by selection pressures when certain individual management practices are over-used, and this can result in new "races" of the pathogen or fungicide-resistant strains of the pathogen being selected. The continual and indiscriminate application of chemical fungicides has caused health hazards in animals and humans due to residual toxicity. Recently, several synthetic fungicides have been banned in the western world because of their undesirable attributes such as high and acute toxicity. Nowadays, biological control is going to be the best alternative strategy for the control of plant diseases. However, other methods in IDM for crop disease control are still necessary in various environmental conditions. Consequently, for economic threshold, other control strategies of IDM besides/with biological control should be also applied to effectively reduce the disease development and the yield loss of crops in the different crop systems.

Keywords: Plant pathogenic fungi; Disease management.

1. INTRODUCTION

During their lifetime, plants are uncovered to fluctuating temperature, humidity, drought or rainfall, soils and nutrients, weeds, insects, nematodes and microorganisms. These components could be beneficial or detrimental to plant health. The disease triangle (Fig. 1), that consists of an interaction between a susceptible plant, a virulent pathogen (usually fungus) and a suitable (conductive) environment for the disease onset, is a classic concept which was formalized in the 1960s by George MacNew [1] to seek out the interrelationship of different factors in an epidemic and to understand how epidemics might be predicted, limited, or managed. It was planned as an experiment tool to presage and control diseases.

More recently, modified versions of the disease triangle concept were defined, including the disease pyramid and tetrahedron, which have 'time' and 'man' as additional factors.

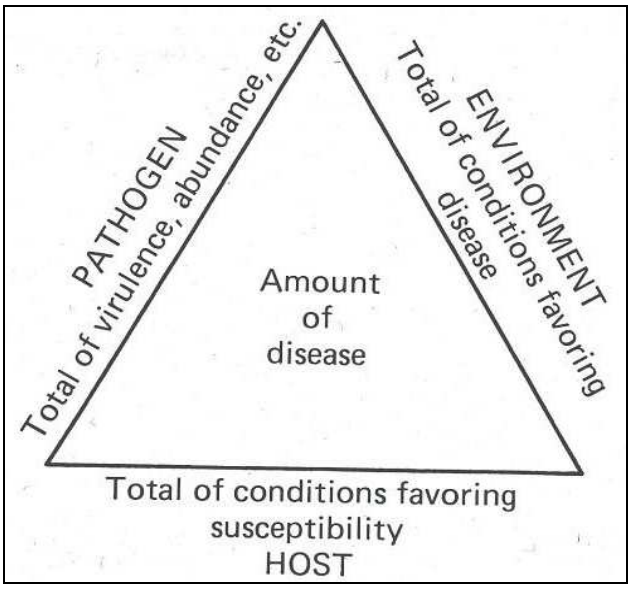

Figure 1. Disease triangle concept.

Today, theoretical and applied plant epidemiologies are advanced fields of research, incorporating the effects of climate change in the control and management of plant diseases.

Bread molds and mushrooms are examples of fungi familiar to all of us. Most of the 100,000 fungus species identified by scientists are only saprophytes and not capable of infecting plants. However, more than 8,000 plant pathogenic species have been identified making fungi the most numerous and economically important class of plant pathogens. The great diversity of fungi and the complex and intricate life cycles of some plant pathogenic species make generalizations difficult.

Plant infection by fungi occurs via a great variety of mechanisms. Some species directly penetrate plant surfaces or enter through natural openings, while others require wounds or injury for infection. During disease development, many species of fungi produces pores which are dispersed by wind, water or by other means. Each spore may cause a new infection resulting in a rapid increase in disease incidence and severity. Some fungi form special resting spores which permit survival for long periods of time (several months or years) in soil or plant debris.

2. FUNGAL DISEASES CONTROL

First of all, it should be noted that among different kinds of pathogens, the greatest losses are inflicted by fungi (42%) followed by bacteria (27%), viruses (18%), and nematodes (13%) [2, 3].

Whether the aim of disease management is to save existing plants or to prevent problems from recurring, we must know "What went wrong?" The diagnosis consists of collecting information on the problem of diseased plants and to fix the cause [4]. Once the cause is determined, it is possible to recommend a solution basing on relevant disease management. The diagnosis of plant problems can involve considerable detective work [5]. Sometimes there is not enough information and other times, the main cause of a problem is hidden by more obvious problems. Success in the diagnosis of plant problems necessarily depends on the amount of knowledge about the triangle of the disease (environment, host and pathogen).

Therefore, the environment may be altered in different ways depending on the disease to be managed. For instance, some diseases require free water for development. In this case, efficient means to reduce free water include morning irrigation, dew removal, reduction in amount and frequency of irrigation. Water manipulation might be wise tool in disease management. Improved drainage and soil conditions by aeration, straw reduction, light conditions manipulations and fertilization regulation might be relevant as steps for reducing damage from particular diseases.

On the other side, disease severity may be underplayed by suitable changes in the crop that is being grown. It is mindless practice to replant the same variety that has been killed by the same pathogen year after year, if there is another option. It is always more suitable, where possible, to use mixture or blends of various varieties, rather than seeding a single kind of crop species. Diversity in a planting almost always raises odds of survival.

The third measure of disease management is depression of the pathogen by applying chemicals which will kill the organism or keep it under threshold of harmfulness. However, most fungicides do not kill fungi, they only prevent growth.

Also, it is important to identify correctly the pathogen, so that a suitable fungicide may be selected. Random choice and application of fungicides without knowledge of the disease cause can make as much harm as good. Using

the wrong fungicide wastes money and may worsen the disease as well as causing other negative effects.

3. PREVENTIVE MEASURES

3.1. Cultural practices

Cultural practices usually affect the development of disease in plants by influencing the environment. These practices are intended to make the atmosphere, soil, or beneficial microorganisms convenient to the crop plant, inconvenient to its parasites.

For example, soil solarization process surveys the soil pathogenic organisms efficiently by trapping solar energy under cold frames subjected to direct sunlight (before planting) for sufficient periods so as to raise the temperature of the top layer of soil (to a depth of 10 cm) to 40°-60°C. The control of the soil borne pathogens, especially *Fusarium* species has changed over the last few decades [6]. Application of soil solarization for managing *Fusarium* and *Verticillium* wilt on some crops is performed generally in several countries [7].

The black root rot of tobacco seedlings caused by *Thielaviopsis basicola* were controlled by applying such treatments. Sclerotial viability of *Sclerotium rolfsii* was quickly reduced by more than 95% at 2.5 cm depth in solarized fruit orchards soil, though lowering effects were found in deeper soillayers [8]. However, the major constraints that limit the adoption of soil solarization in practice are relatively longer duration of the process and the climatic dependency. The cost of solarization is relatively low compared with other available alternative; however, it can be a limiting factor depending on the country, the crop type, the production system.

On the other side, organisms that survive in the soil can often be controlled by crop rotations with unsusceptible species, depending on the system. For example, wheat should not be monocropped or grown behind triticale, rye, or barley. Rotating to oats, annual pasture grasses, winter legumes, or a clean winter fallow for 1 to 2 years between wheat crops may be necessary in fields where serious losses to Septoria diseases have occurred [9].

Environmental factors (temperature, water, and organic and inorganic nutrients) significantly affect inoculum production. For instance, warm temperature (solarization) breaks dormancy of sclerotial structures; water may leach growth inhibitors from the soil and permit germination of resting spores; and special nutrients may stimulate the growth of sclerotes that produce inoculum.

3.2. Plant quarantine

A formal regulatory disease control is plant quarantine, the legally enforced stoppage of plant pathogens through regulations made by states concerning the movement of plant materials into them.

3.3. Sample inspection

Another preventive measure to control the diseases is the sample inspection method. Laboratory looks into of a representative sample drawn by the certification agency for the evaluation of germination, moisture content, weed seed content, purity and seedborne pathogens.

4. CONTROL MEASURES

4.1. Chemical control

Pesticides that control plant diseases can be used very differently. It depends on the pathogen to be controlled and the circumstances required for parasitic activities. For example, a water-soluble eradicative spray is applied once to dormant peach trees to remove wintering spores from the leaves, while relatively insoluble protective fungicides are repeatedly applied to the leaves of potato plants to protect them. In addition, systemic fungicides may be used curatively.

Bhuiyan et al. [10] made several studies on the effect of fungicides in inhibition of the *S. rolfsii* mycelial growth. The study used various fungicides as Ridomil, Rovral, Tilt, Dithane, Bavistin, and Provex at different concentrations. At 400 ppm, inhibition of the mycelial growth was 52.9%, 93.88%, 100%, 80.63%, 6.64% and 100%, respec-

tively. The study revealed that Provex inhibited radial mycelial growth totally even at low concentration of 100 ppm.

However, chemical control presents difficulties due to the growing resistance of the strains to the main commercial products. The ideal phytosanitary formula, as for a large number of pathogenic fungi, is far from being found and it is now only possible to limit the damage to an economically tolerable threshold.

The resistance to fungicides is a major cause of poor disease control in fungal pathogens. The development of resistance to fungicides is influenced by complex interactions of factors such as the mode of action of the fungicide, the biology of the pathogen and the crop system. Understanding the fungicide resistance, how it develops and how it can be managed is critical to ensure sustainable control of fungal diseases.

4.2. Biological control

The most logical scope for the environment to the pesticides using for the control of diseases is the use of biological approaches. Biological control is based on the phenomenon that each living entity has an adversary in nature to keep its population in check. Baker and Cook [11] defined biological control as the "reduction of inoculum density or disease producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonists, or by mass introduction of one or more antagonists".

Biological control can be fulfilled either by introducing bioinoculants or biocontrol agents (BCA) directly into a natural ecosystem or by adopting cultural practices that stimulate survival, establishment, and multiplication of the bioinoculants already existing. The first essay to control a plant disease with microorganism introduced to soil was by Hartley in 1921 [12] where introduction of isolates of saprophytic fungi and one bacterium resulted in significant reduction in severity of damping-off of pine seedlings caused by *Pythium debaryanum* [13].

Bioinoculants are primarily fungal and bacterial in origin. Bioinoculant fungi basically harness through parasitism against plant pathogenic fungi and nematodes [14]. The main genera of biocontrol fungi which have been tried on plant pathogenic fungi and nematodes including *Trichoderma*, *Aspergillus*, *Chaetomium*, *Penicillium*, *Neurospora*, *Fusarium*, *Rhizoctonia*, *Dactylella*, *Arthrobotrys*, *Catenaria*, *Paecilomyces*, *Pochonia*, and *Glomus*. Other types of BCA such as plant growth-promoting organisms have also been examined for disease management [15, 16]. A number of fungi such as *Aspergillus spp*., *Penicillium spp*., and *Trichoderma spp*. have been reported as phosphate-solubilizing microorganisms (PSM), which also suppress plant pathogens. Application of PSM can control soil-borne pathogens such as *Fusarium oxysporum*, *Macrophomina phaseolina*, *Pythium aphanidermatum*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*.

Trichoderma strains grow rapidly when inoculated in soil because they are naturally resistant to many toxic compounds such as DDT and phenolic compounds [17]. *Trichoderma* strains are efficient in controlling several fungi such as *R. solani*, *P. ultimum* and *S. rolfsii* when alternated with methylbromide, benomyl, captan, or other chemicals.

Disease suppression by bioinoculants might be performed by some mechanisms like fungistatic effects, competition for nutrients, antibiosis, myco-parasitism and stimulation of host defense response.

The practical effectiveness of biological control is clearly succeeded with relevant results *in vitro*. Thus, the need for field productivity of any biological and biotechnological approach should be addressed.

5. INTEGRATED DISEASE MANAGEMENT (IDM)

IDM is defined as: "a sustainable approach to survey diseases by combining biological, cultural, physical and chemical tools in a way that minimizes economic, health and environmental risks". This concept evolved from the original IPM (integrated pest management) [18].

The success and sustainability of IDM strategy [19], especially with resource poor farmers greatly depends on their involvement in helping

generate locally specific techniques and solutions suitable for their particular farming systems and integrating control components that are ecologically sound and readily available to them. Training and awareness raising of farmers, disease survey teams, agricultural development officers, extension agents and policy makers remains to be an important factor for the successful implementation of IDM strategies.

6. CONCLUSION

Plant fungal diseases seriously threaten crop production worldwide causing the highest yield losses among those caused by other pathogens. As a result, their management is essential to increase food production. Given the adverse effects of pesticides, bioinoculants offer a potential substitute. Many potentially useful microorganisms are available, such as *Trichoderma spp., Aspergillus niger, Penicillium digitatum, P. anatolicum, Paecilomyces lilacinus, Pochonia chlamydosporia*. These organisms can be applied directly to the soil, as a seed treatment or as a foliar spray to reduce the level of inoculum and the severity of the disease. Commercial formulations of most bioinoculants are available and offer varying degrees of disease control. The overall performance of phosphate-soluble fungi such as *A. niger*, *Trichoderma spp., Penicillium spp.,* against fungal plant diseases opens the way to commercial exploitation.

TRANSPARENCY DECLARATION

The author declares that has no conflict of interest.

REFERENCES

1. McNew, GL. The nature, origin and evolution of parasitism. In: Plant pathology: an advanced treatise. Horsfall JG, Dimond AE, eds. Academic Press, New York; 1960: 19-69.
2. Rahman Khan M, Arshad Anwer M. Fungal bioinoculants for plant disease management. Microbes and Microbial Technology. Springer New York; 2014.
3. Khan MR, Jairajpuri MS. An overview of nematode infestation in cash crops. In: Nematode infestations field crop (Part 1). Khan MR, Jairajpuri MS, eds. India: National Academy of Sciences; 2010.
4. Krishna Mohan S. Plant disease diagnosis and management; 1998.
5. Sanders PL. Turfgrass diseases: a triangular view of diagnosis and management. 1st edn. WorldCat; 1991.
6. Patel N, Desai P, Patel Ni, Jha A, Gautam HK. Agronanotechnology for plant fungal disease management. Int J Curr Microbiol App Sci. 2014; 3: 71-84.
7. Saremi H, Okhovvat SM, Ashrafi SJ. *Fusarium* diseases as the main soil borne fungal pathogen on plants and their control management with soil solarization in Iran. Afr J Biotechnol. 2011: 10: 18391-18398.
8. Shlevin E, Sagui I, Mahrer Y, Katan J. Modeling the survival of two soilborne pathogens under dry structural solarization. J Phytopathol. 2003; 93: 1247-1257.
9. Hagan AK, Mask P, Gudauskas RT, Collins D. Wheat diseases and their control. Circular ANR. 543; 1990.
10. Bhuiyan M, Rahman M, Bhuiyan K. *In vitro* screening of fungicides and antagonists against *Sclerotium rolfsii.* Afr J Biotechnol. 2012; 11: 14822-14827.
11. Baker KF, Cook RJ. Biological control of plant pathogens. San Francisco, Freeman and Co; 1974.
12. Hartley C. Damping off in forest nurseries. Washington, DC: USDA Bulletin 934; 1921.
13. Baker KF. Evolving concepts of biological control of plant pathogens. Annu Rev Phytopathol. 1987; 25: 67-85.
14. Khan MR. Biological control of Fusarial wilt an root-knot of legumes. New Delhi: Department of Biotechnology, Ministry of Science and Technology; 2005.
15. Papavizas GC. Biological control of soil borne diseases. Summa Phytopathol. 1985; 11: 173-179.
16. Nair MG, Burke BA. A few fatty acid methyl ester and other biologically active compounds from *Aspergillus niger*. Phytochemistry. 1988; 27: 3169-3173.
17. Chet I, Inbar J, Hadar I. Fungal antagonists and mycoparasites. In: The mycota IV: environmental and microbial relationships. Wicklow DT, Soderstrom B, eds. Springer: Berlin, Heidelberg, New York; 1997: 165-184.

18. Overton J. Ecologically based pest management - new solutions for a new century. National Academy Press, Washington DC, USA; 1996.
19. El Khoury W, Makkouk K. Integrated plant disease management in developing countries. J Plant Pathol. 2010: 92: S4.35-S4.42.

ISSN 2449-8955

European Journal of Biological Research

Research Article

Statistical optimization as a powerful tool for indole acetic acid production by *Fusarium oxysporum*

Ghada Abd-Elmonsef Mahmoud[1]*, Hassan H. A. Mostafa[2]

[1] Botany and Microbiology Department, Faculty of Science, Assiut University, Assiut 71516, Egypt

[2] Central Laboratory of Organic Agriculture, Agricultural Research Center, Giza 12619, Egypt

*Corresponding author: Dr. Ghada Abd-Elmonsef Mahmoud; Tel.: 20 1010711661; Fax: 20 88 2342708;

E-mail: ghada_botany@yahoo.com; ghada.moukabel@science.au.edu.eg

Received: 18 August 2017; **Revised submission:** 04 October 2017; **Accepted:** 20 October 2017

DOI: http://dx.doi.org/10.5281/zenodo.1012348

ABSTRACT

Crop production is challenged in our world by increasing food demands, decrease natural resource bases and climatic change. Nowadays plant growth regulators works like fertilizers in increasing plant growth production efficiency and needed to produce in large industrial scale. Fermentation condition and medium constituents can significantly affect on the product production and designing an acceptable fermentation medium is critical importance. In this paper *Fusarium* sp. could be considered as promising indole-3-acetic acid producers with the ability to improve the production using statistical methods. The results showed that fermentation type, incubation temperature and L-tryptophan were the most influencing parameters on the production. Maximum IAA production by *Fusarium oxysporum* was 300.4 mg/l obtained under the fermentation conditions: temperature at 25^{o}C, incubation period 5 days, pH 7, inoculums size 2%, shaking rate at 150 rpm and medium constituents: Glucose 40 g/l, yeast extract 3 g/l, L-tryptophan 1 g/l, KH_2PO_4 2 g/l, $NaNO_3$ 4 g/l, $MgSO_4 \cdot 7H_2O$ 0.1 g/l with regression analysis (R^2) 99.67% and 2.12-fold increase in comparison to the production of the original level (142 mg/l).

Keywords: Auxin; Production; Plackett-Burman; *Fusarium.*

Abbreviations: Indole-3-acetic acid (IAA), potato dextrose agar medium (PDA).

1. INTRODUCTION

In the last few years by increasing population number every year the fulfilling food requirement remains a challenging task as climate changes affected on the agricultural production systems and there has been a growing interest in increasing crop plant yield [1]. Phytohormones which could produce by microorganisms are known to play vital roles in plant growth and establishment by helping plants to acclimatize to varying environments [2].

Several phytohormones control many physiological and bio-chemical processes like abscisic acid, gibberellins, ethylene, auxins, cytokinins, and brassinosteroids [3]. Indole-3-acetic acid extensively was the first identified plant hormone and the most important member of the auxins family of phytohormones [4]. Its play a vital role in physiological processes e.g. root initiation, production of longer roots, tissue differentiation, increase number of root hairs and lateral root which are involved in nutrient uptake [5-7]. In recent paper

by Takahashi [8] revealed that indole-3-acetic acid intracellular plant concentration is controlled by the biosynthesis and degradation process.

The presence of several microorganisms synthesizes IAA as secondary metabolites through tryptophan pathway are very important factor in soil fertility [9]. Several bacterial isolates could produce IAA however; most of the previous studies do not take into account of IAA production by filamentous fungi. Indole-3-acetic acid was produced by filamentous fungi like *Colletotrichum gloeosporioides*, *Colletotrichum acutatum*, *Dibotryon morbosum*, *Fusarium*, *Rhizopus suinus*, *Phoma glomerata*, *Penicillium*, *Taphrina deformans*, *Ustilago esculenta* and *Ustilago zeae* [10-18].

A classical method of optimizing the fermentation conditions and medium constituents depends on single parameter whilst all the other factors are maintained at a fixed level [19]. However, statistically based experimental designs proved to be most popular for production optimization as it enables us to obtain the physicochemical and factors influencing on the production process with less number of planned experiments. Plackett-Burman design is practical efficient when we screening large number of factors to produce optimal response [20].

The main objective of this paper is to test the ability of different *Fusarium* isolates to produce indole-3-acetic acid on glucose medium, secondly to improve IAA production by investigating the effect of several parameters on the production process and found the optimum fermentation conditions and medium constituents for the highest IAA production using statistical approach (Plackett-Burman design).

2. MATERIALS AND METHODS

2.1. *Fusarium* sp. isolation and identification

Fusarium species, isolated from different parts of Egyptian clover, faba bean, garlic, maize and onion plants on potato dextrose agar medium (PDA). The pure cultures were maintained aerobically on the same medium and stored at 4±1°C until using [21]. *Fusarium* sp. identified based on their macroscopic and microscopic characteristics [22].

2.2. Inoculums preparation

Prior to indole-3-acetic acid production experiments, *Fusarium* sp. were grown aerobically on potato dextrose agar medium at 28±1°C for 4 days. Homogeneous spore suspension of *Fusarium* sp. was prepared by scraping fungal hyphae from culture plates and suspended in sterilized distilled water containing 0.01% (v/v) tween 80 (2×10^6 spore/ml) and stirred for 30 min. One ml of the inoculums was transferred to an Erlenmeyer flask 250 ml containing 100 ml of the production medium.

2.3. Screening for IAA production by *Fusarium* sp.

Czapek's dextrose liquid medium supplemented with 0.2 g/l L-tryptophane, was used as production medium containing (g/l): glucose, 30.0; yeast extract, 5; $NaNO_3$, 3.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5 and $FeSO_4 \cdot 7H_2O$, 0.01. These contents were dissolved in 1000 ml distilled water with initial pH adjusted to 5.5 before autoclaving. After sterilization in an autoclave at 121°C and 1.5 atm pressure for 20 min. chloramphenicol, 250 mg/ml was sterilized separately by membrane filtration, using a membrane of pore size 0.22 mm and added as bacteriostatic agent. Incubation was carried out at 28±1°C on a rotary shaking (150 rpm) for 7 days. All the experiments were carried out independently in triplicates.

2.4. Optimization using Plackett-Burman design

Plackett-Burman design was used to screen the fermentation parameters that influenced indole-3-acetic acid (IAA) production with respect to their main effect and without interaction effects between various constituents of the medium [23]. Eleven trails carried out by Plackett-Burman design for screening the fermentation parameters under investigation is shown in Table 1. Each independent variable was tested at two levels, high (+1) and low (−1). In each column and row should contain equal number of negative and positive signs. The program Sigma XL (Version 6.12) was used to analyze this experiment.

Table 1. Plackett-Burman design for different variables screening in IAA production by *Fusarium oxysporum* (I).

Variable code	Variable	Unit	Level		
			Low (-1)	(0)	High (+1)
A	Incubation temperature	C°	25	30	35
B	Incubation time	D	5	7	9
C	Fermentation type		Shaking	Shaking	Static
D	Inoculums size	%	0.5	1	2
E	Initial pH		5	6	7
F	Glucose	gl^{-1}	20	30	40
G	Yeast extract	gl^{-1}	3	5	7
H	L-tryptophan	gl^{-1}	0.1	0.5	1
J	KH_2PO_4	gl^{-1}	0.5	1	2
K	$MgSO_4 \cdot 7H_2O$	gl^{-1}	0.1	0.5	1
L	$NaNO_3$	gl^{-1}	1	2	3

Plackett-Burman design was used to screen and evaluate the important medium components that influence the response. Indole-3-acetic acid yields are explained by the following polynomial equation:

$$Y=b_o + \sum b_i X_i + \sum b_{ij} X_i X_j + E_i \quad (1)$$

Where, Y: the variable dependent response; i: the regression coefficient; X: the independent variable level and E: the experimental error. The experimental data were statistically analyzed to determine the significant difference ($p \leq 0.05$) in response under different conditions. The response surface graphs were also plotted using the same software. The quality of fit for the regression model equation was expressed as R^2.

2.5. Analytical analysis

After the incubation period, *Fusarium* mycelium was recovered by filtration through dried and weighed Whatman filter paper (No. 113), washed with distilled water three times and then dried at 70°C overnight for dry mass (DM) determination. The supernatants were centrifuged at 4,000 rpm for 15 min. and sterilized by membrane filtration, using a membrane of pore size 0.22 mm to remove any remaining spores for quantitative determination of indole-3-acetic acid (IAA).

Indole-3-acetic acid was determined spectrophotometerically (Fig. 1) using Salkowski reagent containing 1ml of 0.5 M $FeCl_3 \cdot 6H_2O$ dissolved in 50 ml of 35% $HClO_4$ [24]. Two ml of Salkowski reagent was added to one ml of culture supernatant in 10 ml test tube leaves it in room temperature and read the color after 25 min. but before 3 h at 535 nm. The developed pink color measured using T60 UV with a split beam UV visible spectrophotometer covers a wavelength range of 190-1100 nm. The amount of IAA in the supernatant was measured quantitatively at 535 nm against substrate-free blank. The standard curve was prepared using pure IAA (1-100 mg/l).

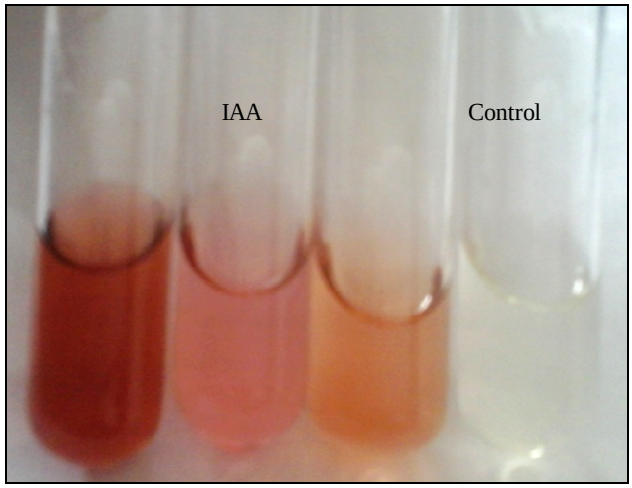

Figure 1. Different pink color degrees of IAA production after the reagent added in comparison with control (free IAA).

3. RESULTS

3.1. Isolation and identification of *Fusarium* sp.

Ten *Fusarium* isolates were isolated from different parts of Egyptian clover, faba bean, garlic, maize and onion plants on PDA medium. Based on *Fusarium* growth on the plate and the microscopic characters, *Fusarium* sp. were identified into seven species. *Fusarium solani*, *F. oxysporum*, *F. chlamydosporum*, *F. camptoceras*, *F. incarnatum*, *F. verticilloides* and *F. nygami*. The purified isolates were screened for their ability to produced indole-3-acetic acid (IAA) on fermented medium. Only one isolate was selected for further experiments based on the highest indole-3-acetic acid (mg/l) production.

3.2. Indole-3-acetic acid production by *Fusarium* sp.

All the isolates grown on the production medium and showed various degrees of dry mass and indole-3-acetic acid production. A wide variation in IAA production on the screening medium ranged from 18.37±1.04 to 142±6.46 mg/l and dry mass varied between 1.2±0.2 and 6.1±0.53 g/l. The highest fungus dry mass and indole-3-acetic acid producer was *Fusarium oxysporum* (I) isolated from onion rhizoplane giving 142±6.46 mg/l IAA (with productivity 23.14 mg/l/day) and 6.1±0.53 g/l dry mass so it was selected for the further experiments, the overall measurement results are summarized in Fig. 2.

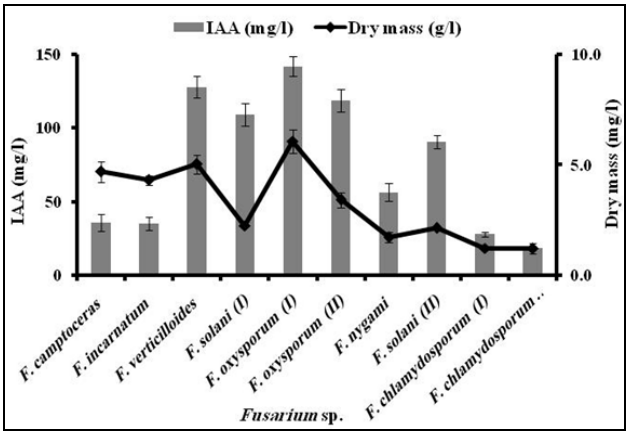

Figure 2. Screening for IAA production on glucose medium by different isolates of *Fusarium* species.

Brief description of indole-3-acetic acid highly producer *Fusarium oxysporum* (Schlechtendal) emend. Snyder & Hansen; Growth on PDA medium 50 mm in one week, Texture floccose becoming felted, Color white to pale apricot, usually with a purple tinge, Reverse purple. Conidiophores hyaline, simple, short, bearing spore masses at the apexes; two kinds of conidia: macroconidia boat-shaped, with slightly tapering apical cells and hooked basal cells, 4-celled; and microconidia ellipsoidal, 1-celled. Chlamydospores globose and usually solitary (Fig. 3).

3.3. Optimization of IAA production using Plackett-Burman design

The Plackett-Burman design was an effective way to improve IAA production. The highly IAA producer (*Fusarium oxysporum* (I)) was chosen for screening the effects of different parameters on IAA production using Plackett-Burman design. Each variable was studied at two levels (-1, 1) as declared in Table 1. Relationship between the response and the screened variables was expressed by the following polynomial equation:

IAA (mg/l) = (91.483) + (-40.02) * A: Incubation temperature + (-22.15) * B: Incubation time + (-49.82) * C: Fermentation type + (6.82) * D: Inoculum size + (2.95) * E: Initial pH + (18.35) * F: Glucose + (-1.75) * G: Yeast extract + (26.48) * H: L-tryptophane + (0.95) * J: KH_2PO_4 + (-13.78) * K: MgSO4 + (17.65) * L: $NaNO_3$ (2)

The results obtained in Table 2 indicated that there was a wide variation in IAA production from (13.6 to 300.4 mg/l) and dry mass varied between 3 and 9.1 g/l. This indicates the important effect of the medium components and environmental factors on growth and production of IAA. The ANOVA results are shown in Tables 3 showed that among the eleven variables, G (yeast extract) and J (KH_2PO_4) were found to be non-significant ($p>0.05$). Among the tested parameters, fermentation type, incubation temperature and L-tryptophan were the most effective parameters plays a crucial role in IAA production with 49.82%, 40% and 26.48% coefficient effect as shown in Pareto-Plot (Fig. 4).

All the predicted values of Plackett-Burman design were located in close proximity to the experimental values. This supports the hypothesis

that the model Eq. (2) is sufficient to describe the response of the experimental observations of IAA production (Fig. 5). Three-dimensional response surface curves were generated to study the interaction between each two variables (Fig. 6A-F). The Model F value of 324.6 ($p<0.05$) implies that the model is significant. Model F value is calculated as ratio of mean square regression and mean square residual due to the real error. The R^2 value was 99.67% indicated that the entire variation was explained by the model. The adjusted R^2 value was 99.36%.

Maximum IAA production (300.4 mg/l) by *Fusarium oxysporum* (I) obtained under the fermentation conditions: temperature at 25°C, incubation period 5 days, pH 7, inoculums size 2%, shaking rate at 150 rpm and medium constituents: Glucose 40 g/l, yeast extract 3 g/l, L-tryptophan 1 g/l, KH_2PO_4 2 g/l, $NaNO_3$ 4 g/l, $MgSO_4 \cdot 7H_2O$ 0.1 g/l.

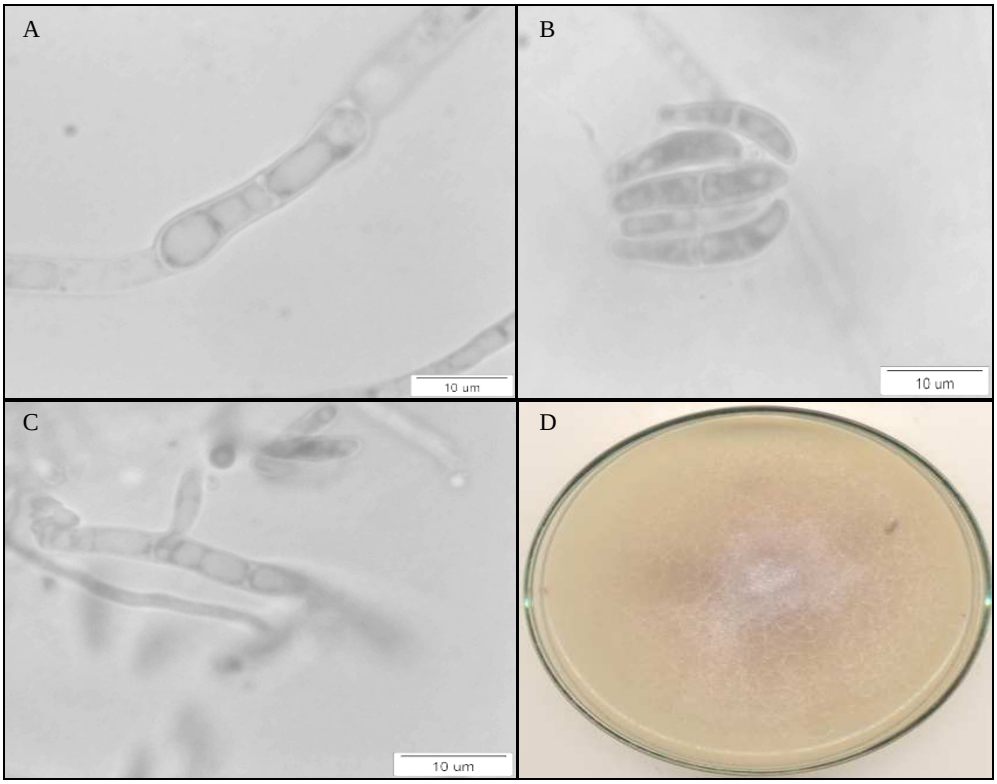

Figure 3. *Fusarium oxysporum* (I) Sch., A: Chlamysospores (Ch); B: Monophilaidic conidiogenous cell (Ph) and hypha (Hy); C: Macroconidia (Ma); Bars, 10 µm; D: Fungus growth on potato dextrose agar medium.

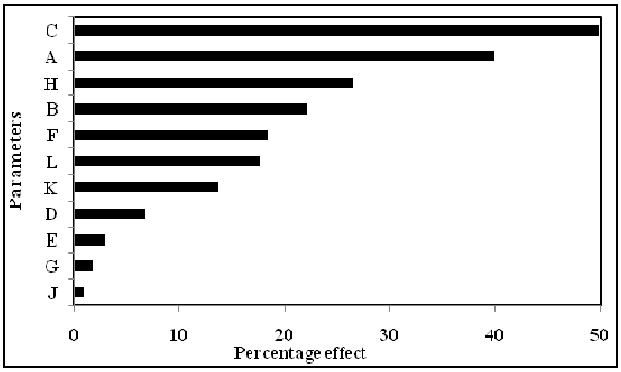

Figure 4. Pareto-Plot for Plackett-Burman parameter estimates the effect of each parameter on IAA produced by *Fusarium oxysporum* (I).

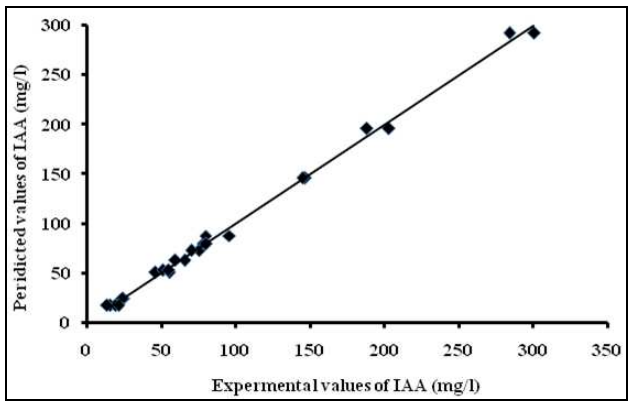

Figure 5. Comparison between IAA (mg/l) experimental and predicted values of the Plackett-Burman design.

Table 2. Plackett-Burman design variables with IAA production by *Fusarium oxysporum* (I) as response.

Trials	A	B	C	D	E	F	G	H	J	K	L	IAA (mgl^{-1})	Dry mass (gl^{-1})
1	-1	-1	1	1	1	-1	1	1	-1	1	-1	95.6	4.8
2	-1	1	1	1	-1	1	1	-1	1	-1	-1	46	6.82
3	1	1	-1	1	-1	-1	-1	1	1	1	-1	65.6	3.66
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	24.8	6
5	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	146.8	4
6	1	1	-1	1	1	-1	1	-1	-1	-1	1	75.6	9.1
7	1	1	-1	1	1	-1	1	-1	-1	-1	1	70	8.4
8	1	1	1	-1	1	1	-1	1	-1	-1	-1	15.6	3.88
9	1	-1	1	-1	-1	-1	1	1	1	-1	1	50.8	3.6
10	-1	1	-1	-1	-1	1	1	1	-1	1	1	188	7.99
11	1	1	1	-1	1	1	-1	1	-1	-1	-1	19.2	3.81
12	1	-1	1	1	-1	1	-1	-1	-1	1	1	23.6	5.4
13	1	1	-1	1	-1	-1	-1	1	1	1	-1	59.2	3.68
14	-1	1	1	1	-1	1	1	-1	1	-1	-1	55.2	8.83
15	-1	-1	-1	1	1	1	-1	1	1	-1	1	284	4.8
16	-1	1	1	-1	1	-1	-1	-1	1	1	1	13.6	5.22
17	-1	1	-1	-1	-1	1	1	1	-1	1	1	202.8	8.21
18	1	-1	-1	-1	1	1	1	-1	1	1	-1	78.4	6.8
19	-1	-1	1	1	1	-1	1	1	-1	1	-1	79.6	4.4
20	1	-1	1	-1	-1	-1	1	1	1	-1	1	54.8	4.2
21	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	144.8	3
22	1	-1	-1	-1	1	1	1	-1	1	1	-1	80	7.2
23	-1	-1	-1	1	1	1	-1	1	1	-1	1	300.4	4.6
24	-1	1	1	-1	1	-1	-1	-1	1	1	1	21.2	5.51

The sign +1 and −1 represent the two different levels (high and low) of the independent variable under investigation. A: Incubation temperature, B: Incubation time, C: Fermentation type, D: Inoculums size, E: Initial pH, F: Glucose, G: Yeast extract, H: L-tryptophan, J: KH_2PO_4, K: $MgSO_4 \cdot 7H_2O$ and L: $NaNO_3$.

Table 3. Statistical analysis of Plackett-Burman design of each variable at two levels for IAA production by *Fusarium oxysporum* (I).

Variable code	Variable	Coefficient	*t* value	*P* value
A	Incubation temperature	91.48	69.58	<0.0001*
B	Incubation time	-40.02	-30.44	<0.0001*
C	Fermentation type	-22.15	-16.85	<0.0001*
D	Inoculums size	-49.82	-37.89	0.0002*
E	Initial pH	6.82	5.19	0.0445*
F	Glucose	2.95	2.24	<0.0001*
G	Yeast extract	18.35	13.96	0.2079 N
H	L-tryptophan	-1.75	-1.33	<0.0001*
J	KH_2PO_4	26.48	20.143	0.4838 N
K	$MgSO_4.7H_2O$	0.95	0.72	<0.0001*
L	$NaNO_3$	-13.78	-10.48	<0.0001*

t – student's test, *p* – corresponding level of significance,* Significant at p ≤0.05, N, non-significant at p≥0.05.

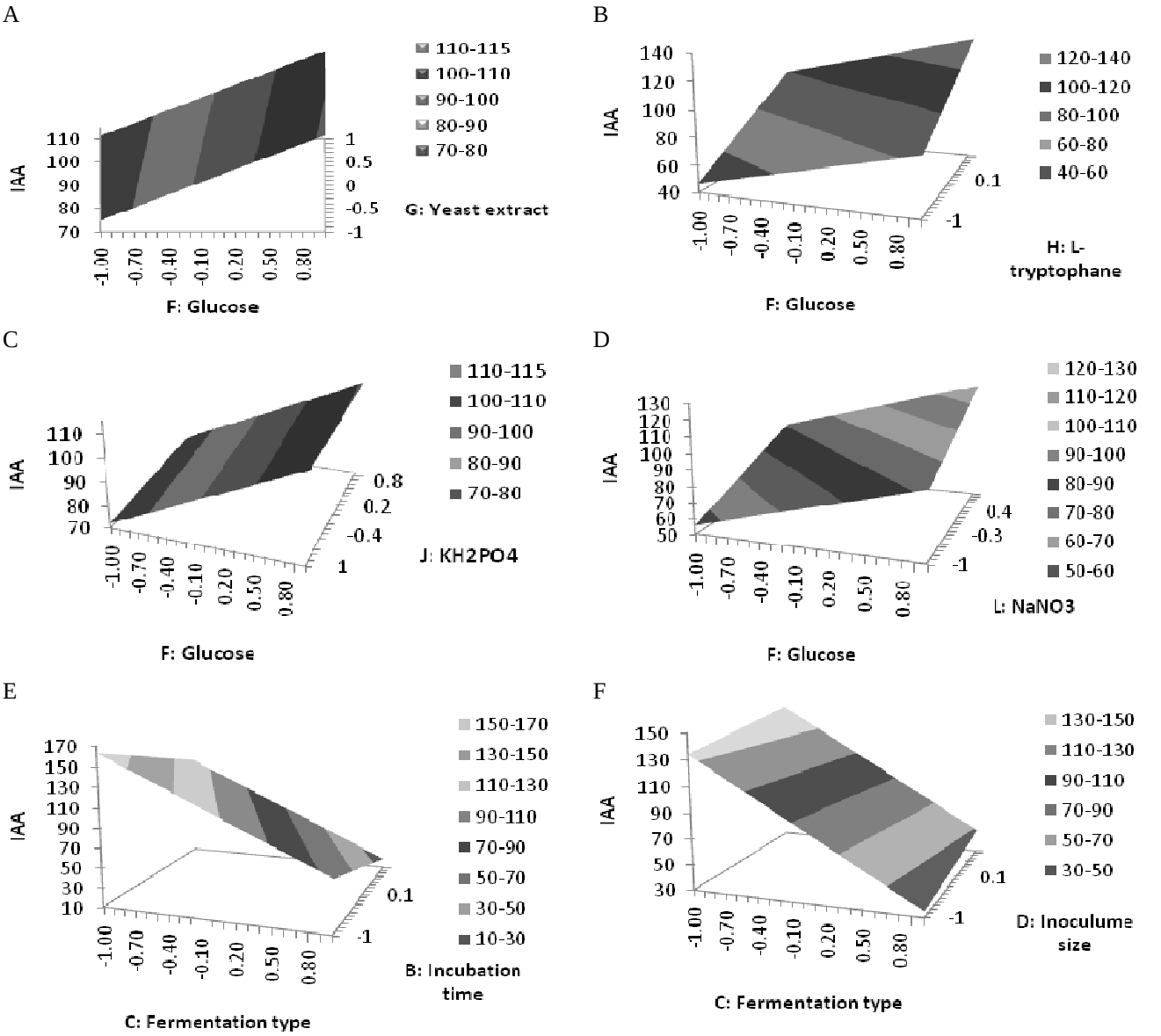

Figure 6. Response surface plots of IAA production by *Fusarium oxysporum* (I) showing the effect of two variables (other variables were kept at zero in coded unit): (A) Glucose and yeast extract, (B) Glucose and L-tryptophane, (C) Glucose and KH_2PO_4, (D) Glucose and $NaNO_3$, (E) Fermentation type and incubation time, (F) Fermentation type and inoculums size.

4. DISSCUSSION

Indole-3-acetic acid produced by all *Fusarium* isolates on the production medium with various degree of production giving maximum value by *Fusarium oxysporum.* Lynch [25] suggested that indole-3-acetic acid is a common product of L-tryptophan metabolism which produced by several microorganisms including plant growth-promoting rhizobacteria, other bacterial types and fungi. Hasan [15] found that all isolates of *Fusarium* oxysporum which isolated from different plant seeds could produce IAA (100-140 mg/l).

After screening the effect of eleven parameters on IAA we found that fermentation type, incubation temperature and L-tryptophan were the most effective parameters play a crucial role in IAA production. Thuler [26] revealed that IAA production is oxygen dependent, so agitation during production seems to be preferable when compared with a static condition. When incubation performed by agitation the production medium homogeneous better and the oxygen supplies increase, which increase both biomass and production in the medium [27]. From later researches, the optimum incubation temperature for IAA production was

range of 25-30°C by several microorganisms [15, 28, 27].

Maximum IAA production (300.4 mg/l) by *Fusarium oxysporum* (I) obtained under the fermentation conditions: temperature at 25°C, incubation period 5 days, pH 7, inoculums size 2%, shaking rate at 150 rpm and medium constituents: Glucose 40 g/l, yeast extract 3 g/l, L-tryptophan 1 g/l, KH_2PO_4 2 g/l, $NaNO_3$ 4 g/l, $MgSO4{\cdot}7H_2O$ 0.1 g/l. In agreement with our results, indole-3-acetic acid synthesis by ectomycorrhizal fungi was maximized after 30 days of incubation [29]. Indole-3-acetic acid production by *Fusarium oxysporum* maximized on 15 days and 10 for mycelium [15]. Indole-3-acetic acid production *Aspergillus niger* give maximum production after 6 days of incubation [27]. From the outcome of our investigation it is possible to conclude that genus *Fusarium* can be highly recommended in industrial production of indole-3-acetic acid. Also using statistical method in optimization for improving the production has a great potential for applications and was very effective in our study as the production of IAA (300.4 mg/l) in this paper increase with 2.12-fold in comparison to the production of original level (142 mg/l) using Plackett-Burman design.

5. CONCLUSION

From the outcome of our investigation it is possible to conclude that genus *Fusarium* can be highly recommended in industrial production of indole-3-acetic acid. Also using statistical method in optimization for improving the production has a great potential for applications and was very effective in our study as the production of IAA (300.4 mg/l) in this paper increase with 2.12-fold in comparison to the production of original level (142 mg/l) using Plackett-Burman design

AUTHORS' CONTRIBUTION

Both authors contributed in the success of this research and the final manuscript has been read and approved by both authors.

TRANSPARENCY DECLARATION

The authors declare that has no conflict of interest.

REFERENCES

1. Hussain S, Peng S, Fahad S, Khaliq A. Rice management interventions to mitigate greenhouse gas emissions: a review. Environ Sci Pollut Res Int. 2015; 22(5): 3342-3360.
2. Fahad S, Hussain S, Matloob A, Khan FA. Phytohormones and plant responses to salinity stress: a review. Plant Growth Regul. 2015; 75(2): 391-404.
3. Iqbal N, Umar S, Khan NA, Khan MIR. A new perspective of phytohormones in salinity tolerance: regulation of proline metabolism. Environ Exp Bot. 2014; 100: 34-42.
4. Fahad S, Hussain S, Bano A, Saud S. Potential role of phytohormones and plant growth-promoting rhizobacteria in a biotic stresses: consequences for changing environment. Environ Sci Pollut Res. 2015; 22(7): 4907-4921.
5. Datta C, Basu P. Indole acetic acid production by a *Rhizobium* species from root nodules of a leguminous shrub *Cajanus cojan*. Microbiol Res. 2000; 155: 123-127.
6. Teale WD, Paponov IA, Palme K. Auxin in action: signaling, transport and the control of plant growth and development. Mol Cell Biol. 2006; 7: 847-859.
7. Eyidogan F, Oz MT, Yucel M, Oktem HA. Signal transduction of phytohormones under a biotic stresses. In: Khan NA, Nazar R, Iqbal N, Anjum NA, eds. Phytohormones and a biotic stress tolerance in plants. Springer, Berlin, 2012: 1-48.
8. Takahashi H. Auxin biology in roots. Plant Root. 2013; 7: 49-64.
9. Mandal SM, Mondal KC, Dey S, Pati BR. Optimization of cultural and nutritional conditions for indole-3-acetic acid (IAA) production by a *Rhizobium* sp. isolated from root nodules of *Vigna mungo* (L.) Hepper. Res J Microbiol. 2007; 2: 239-246.
10. Thimann KV. Hydrolysis of indole acetonitrile in plants. J Biol Chem. 1953; 109: 279-291.
11. Wolf FT. The production of indole acetic acid by *Ustilago zeae* and its possible significant in tumor formation. Biochem. 1952; 38: 106-111.
12. Crady EE, Wolf FT. The production of indole acetic acid by *Dibotryon morbosum* and *Taphrina deformans.* Physiol Plant. 1959; 12: 526-533.
13. Perley JE, Stowe BB. On the ability of *Taphrina deformans* to produce indole acetic acid from tryptophan by way of tryptamine. Plant Physiol. 1966; 41: 234-237.

14. Riov RMJ, Sharon A. Indole-3-acetic acid biosynthesis in *Colletotrichum gloeosporioides* f. sp. *Aeschynomene*. Appl Environ Microbiol. 1998; 64: 5030-5032.
15. Hasan HA. Gibberellin and auxin-indole production by plant root-fungi and their biosynthesis under salinity-calcium interaction. Acta Microbiol Immunol Hung. 2002; 49: 105-118.
16. Shilts CK, Timmer EL, Ueng PP. Indole derivatives produced by the fungus *Colletotrichum acutatum* causing lime anthracnose and postbloom fruit drop of citrus. FEMS Microbiology Lett. 2003; 226: 23-30.
17. Chung KR, Tzeng DD. Biosynthesis of indole-3-acetic acid by the gall-inducing fungus *Ustilago esculenta*. J Bio Sci. 2004; 4(6): 744-750.
18. Waqas M, Khan AL, Kamran M, Hamayun M. Endophytic fungi produce gibberellins and indole acetic acid and promotes host-plant growth during stress. Molec. 2012; 17: 10754-10773.
19. Xu C, Kim S, Hwang H, Choi J. Optimization of submerged culture conditions for mycelial growth and exobiopolymer production by *Paecilomyces tenuipes* C240. Process Biochem. 2003; 38(7): 1025-1030.
20. Aravindan R, Viruthagiri T. Sequential optimization of culture medium composition for extracellular lipase production by *Bacillus sphaericus* using statistical methods. J Chem Tec Bio. 2007; 82: 460-470.
21. Booth C. The genus *Fusarium*. Commonwealth Mycological Institute, Kew, Surrey, England; 1971.
22. Leslie JF, Summerell BA. The *Fusarium* Laboratory Manual. Blackwell Publishing; 2006.
23. Plackett RL, Burman JP. The design of optimum multifactorial experiments. Biometrika. 1947; 33: 305-325.
24. Gordon SA, Weber RP. Colorimetric estimation of indole acetic acid. Plant Phys. 1951; 26: 192-195.
25. Lynch JM. Origin, nature and biological activity of aliphatic substances and growth hormones found in soil. In: Vaughan D, Malcom RE, eds. Soil organic matter and biological activity. Martinus Nijhoff /Dr. W. Junk Publishers. Dordrecht, Boston, Lancaster, 1985: 151-174.
26. Thuler DS, Floh EI, Handro W, Barbosa HR. *Beijerinckia derxii* releases plant growth regulators and amino acids in synthetic media independent of nitrogenase activity. J Appl Microbiol. 2003; 95: 799-806.
27. Bilkay IS, Karako S, Aksöz N**.** Indole-3-acetic acid and gibberellic acid production in *Aspergillus niger*. Turk J Biol. 2010; 34: 313-318.
28. Yalçınkaya Y. Effects of some physiological conditions on indole-3-acetic acid production by *Gibberella fujikuroi* G5, MSc, Hacettepe University Institute of Science and Technology; 2007.
29. Gopinathan S, Raman N. Indole-3-acetic acid production by ectomycorrhizal fungi. Indian J Exp Biol. 1992; 30: 142-143.

ISSN 2449-8955

European Journal of Biological Research

Review Article

Anthelminthic/larvicidal activity of some common medicinal plants

Kumari Sunita[1]*, Pradeep Kumar[2], Mohammad Aasif Khan[1], Sadaf[1], Syed Akhtar Husain[1], D. K. Singh[3]

[1] Human Genetics Lab, Department of Biosciences Jamia Millia Islamia (A Central University), New Delhi-110025, India
[2] Department of Zoology, S.G.N Govt. P.G College Muhammadabad, Gohana Mau 276403 (U.P), India
[3] Malacology Laboratory, Department of Zoology, DDU Gorakhpur University, Gorakhpur 273009 (U.P), India
*Corresponding author: Kumari Sunita; Mobile -+91-8756662430; E-mail: ksunita705@yahoo.com; pkumar_gpu@yahoo.co.in

Received: 16 August 2017; **Revised submission:** 25 September 2017; **Accepted:** 23 October 2017

DOI: http://dx.doi.org/10.5281/zenodo.1036819

ABSTRACT

The helminthic infection are most common disease in different animals and in human beings, which affecting a large proportion of the world population. Helminthic infection can also affect millions of livestock resulting in considerable economic loss in domestic animals. For control of helminthic disease in different part of world are uses synthetic medicines which are very effective in curing helminthiasis, but it's also causes a number of side effects. The continued uses of synthetic anthelmintic/larvicidal drugs are also causing a major drug resistance problem in several parasitic diseases. The plant derived crude products are less efficient with respect to cure of parasitic diseases but one relatively free from side effect. A large number of medicinal plants are traditionally uses to cure helminthiasis in developing countries. Thus, plant derived drugs are gaining a lot of attention for curing parasitic infection. There are several medicinal plants and their different crude products, organic extracts and active components have been scrutinized for using in various methods in helminthic/larvicidal infection control. The present reviews summarized the use of traditional medicinal plants and their different products further leads to evaluation of new researches.

Keywords: Medicinal plants; Anthelminthic activity; Larvicidal activity; Active components.

1. INTRODUCTION

Ancient man derived more than 90% of medicinal agents from higher plants. Even today, traditional system of medicine is practiced in many countries possessing ancient cultures, and major portion of their therapeutic needs are obtained from plants drugs. India with its wide eco-geographical and climatic diversity possesses a rich medicinal plant's wealth and has a very rich heritage of knowledge in the use of herbal drugs. A large part of world population depends even at the present time on the indigenous systems of medicine *Ayirveda, Unani and Sidha*, including India. Plants with anthelmintic activity have been reviewed by Akhtar et al. [1].

In many parts of the world, natural products are still in use as herbal remedies [2]. In recent

years, there has been a rapid increase in new reports of the anti-parasitic activity of natural products, both from scientific and traditional practices [1]. Thus, plant based medicines have become indispensable and are forming an integral part of the primary healthcare system over the world. The crude extracts of herbal plants have been tested for their putative anthelmintic properties. Active ingredients of these herbal products are now identified and characterized to establish their mode of action. Akhtar et al. [1] have extensively reviewed the anthelminthic activity of several herbal products. Anthelmintic activity of some plants *Alangium larmarckii* [3], *Piper betle* [4], *Piper longum* [5], *Allium sativum* [6], *Zingiber officinale* [6], *Cucurbita mexicana* and *Ficus religosa* [7], *Calotropis procera* [8], *Nicotiana tabacum* [9] and *Ferula asafetida* [10], *Dioscorea zingiberensis* [11], *Matricaria chamomillia* [12] has been reported by several workers.

In a study by Hordegen et al. [13] bromelain, the enzyme complex of the stem of *Ananas comosus* (Bromeliaceae), the ethanolic extracts of seeds of *Azadirachta indica* (Meliaceae), *Caesalpinia crista* (Caesalpiniaceae) and *Vernonia anthelmintica* (Asteraceae), and the ethanolic extracts of the whole plant of *Fumaria parviflora* (Papaveraceae) and of the fruit of *Embelia ribes* (Myrsinaceae) showed anthelmintic efficacy (up to 93%), relative to pyrantel tartrate against infective larvae of *H. contortus*. The methanol extracts of *Mentha piperita* and *Lantana camara* (leaves, stems and roots) exhibited considerable anthelmintic activity against *P. posthuma*.

Helminthic infections are among the most common infections in human beings, affecting a large proportion of the world's population. In developing countries they pose a large threat to public health and contribute to the prevalence of anaemia, malnutrition, eosinophilia and pneumonia. Although the majority of infections due to worms are generally limited to tropical countries, they can occur to travelers, who have visited those areas and some of them can be developed in temperate climates [14]. The helminthes which infect the intestine are cestodes e.g. Tapeworms (*Taenia Solium*), nematodes e.g. hookworm (*Ancylostoma duodenale*), roundworm (*Ascaris lumbricoids*) and trematodes or flukes (*Schistosoma mansoni* and *S. hematobolium*). The diseases originated from parasitic infection causing severe morbidity include lymphatic filariasis, onchocerciasis and social consequences. Helminthes infection can also affect millions of livestock resulting in considerable economic losses in domestic and farm yard animals.

2. *IN VITRO* AND *IN VIVO* ANTHELMINTIC/ LARVICIDAL ACTIVITY

In the beginning, most of the *in vitro* researches regarding anthelmintic of plants, their different extracts or oil have been based on their toxic effects on earthworm, *Pheritima posthuma* [15-22]. The essential oils of *Gardenia lucida* (Rubiaceae), *Cyperus rotendus* (Cyperaceae), *Inula racemosa* (Compositae), *Psitacia integrrima* (Anacardiaceae), *Litsea chinensis* (Lauraceae) and *Randia dumetorum* (Rubiaceae) seeds have been reported to possess good anthelmintic activity against tapeworms and earthworms [18, 19]. Most of these substances which are toxic to earthworms produce a primary irritation or agitation that results in the withdrawal of the worm from the neighborhood of the poison.

In vivo trials have also been conducted for the evaluation of anthelmintic activity of various plant materials. Githiori et al. [23] evaluated the anthelmintic properties of *Albizia anthelmintica* extracts against *H. polygrus* infections in mice. *In vivo* trials have also been carried out in domestic animals such as sheep, goats and cattle etc. for the evaluation of anthelmintic activity of various medicinal plants and its active compound. The efficacy of test substances in such studies has generally been adjudged on the basis of expulsion of worms from hosts [24-28] or reduction in the number of eggs per gram of feces (EPG) passed by the infected hosts following treatment with substances of plant origin.

By asset of this effect, anthelmintics doubtless often drive out the parasite when the concentration does not get sufficiently higher to kill the worm [29]. Some worker have also used hookworms, *Haemonchus contortus*, and tapeworms and/or *Ascaris lumbricoides* for the evaluation of *in vitro* anthelmintic activity of different plant materials [3, 4, 19-22, 30-35]. A modified egg hatch assay [36] is often used to evaluate the effect of plant products against eggs of *Haemonchus contortus*. Some other

research conducting *in vitro* studies have used an alteration of the larval development assay (LDA) or larval motility tests which are commonly used for testing of resistance of parasites to anthelmintic [37, 38].

Bany et al. [39] reported the effect of alchinal, a complex preparation of three substances *Echinacea purpurea* extract, *Allium sativum* extract and cocoa, on the development of *T. spiralis* in mice. Quinolines that exhibited good activity *in vitro* have been studied *in vivo* on *T. spiralis* in mice model [40]. The anticestodal properties of few other plants namely, *Gladiolus gandavensis, Trifolium repens*, *Strobilanthes discolor* and *Butea minor* have been well ascertained using experimentally induced *H. diminuta* in albino rats [41-43].

Extracts of *Cucurbita pepo* (Cucurbitaceaea), *Calotropis gigantean* (Asclepiadaceae), *Juglans regia* (Juglandaceae), *Momordica charantia* (Cucurbitaceae), *Musa paradisaca* (Musaceae) and *Scindapsus offcinalis* (Araceae) have been found to show profound anthelmintic activity on *Haemonchus contortus* of goat origin [30].

The cestocidal efficacy of *Acacia auriculiformis* in *H. diminuta* rat model are reported by Ghosh et al. [44]. Bogh et al. [45] reported the anthelmintic efficacy of extracts of *Embelia schimperi* against *Echinostoma caproni*, *H. polygyrus* and *H. microstoma* in mice and also against *H. diminuta* in rats. The stem bark extract of *Berlinia grandiflora* has been reported to possess anthelmintic efficacy based on its testing against *N. brasiliensis* infections in albino rats [46]. Kaushik et al. [47] evaluated extracts of 11 plants which proved lethal to *Ascaridia galli in vitro*, including those from *Amomum aromaticum* (Zingiberaceae) root and rhizome, *Ammora wallichii* stem, *Anthocephalus indicus* (Rubiaceae) stem and bark, *Calamintha umberosa* (Labiatae) plant, *Dalbergia latifolia* (Leguminosae) stem and bark, *Datura quercifolia* (Solanaceae) fruit, *Datura metal* (Solanaceae) plant, *Ficus religiosa* (Urticaceae) stem and bark, *Sentia myrtina* plant, and *Sumplocos crataegoides* (Sumplocos) leaves.

The essential oils of several plants namely, *Callistemon viminalis* (Myrtaceae), *Anacardium occidentale* (Anacardiaceae), *Buddlea asiatica* (Loganiaceae), *Chloroxylon swientenia* (Rutaceae) and oleo-gum resin of *Commiphora mukul* (Burseraceae) have been reported to possess profound anthelmintic activity against tape and hookworms and their efficacy was also noted to be comparable to that of piperazine phosphate and hexylresorcinol [48]. In other studies the essential oils of *Artemisia pallens* (Compositae), *Eupatorium triplinerve* (Compositae), *Artabotrys odoratissimus* (Annonanceae), *Capillipedium foetidum* (Poaceae) and the grass of *Cymbopogon martini* (Poaceae) have been reported to possess strong anthelmintic activity against *T. solium* and *A. lumbricoides* [22, 35, 49].

2.1. *Carica papaya*

The anthelmintic property of the aqueous extract of the seeds of *Carica papaya* (Carbicaeceae) against *Ascaris lumbricoides* and *Ascaridia galli* has been also well been established [43]. A high efficacy of *C. papaya latex* against experimental *Heligmosomoides polygyrus* infections has been reported by Satrija et al. [50]. The benzyl isothiocyanate isolated from *C. papaya* seed and use as anthelmintic activity against *Caenorhabditis elegans* [51]. Hounzangbe-Adote et al. [52] reported the anthelmintic activity of *Zanthoxylum zanthoxyloides*, *Morinda lucida* and *Newbouldia* leaf extracts and *C. papaya* seed extracts collected in Western Africa against different stages of *H. contortus*. Another study, *Z. zanthoxyloides*, *M. lucida*, *N. laevis* and *C. papaya* extracts induced a dose-dependent inhibition of egg hatching of *T. colubriformis*. These plant extracts also showed their effects against the infective larvae of *T. colubriformis*. In contrast, for adult worms, the effects were statistically significant only for *N. laevis* and *C. papaya* [53]. Okeniyi et al. [54] has been reported the seed of *C. papaya* are cheap, natural, harmless, readily available monotherapy and prevention against intestinal parasitosis.

The anthelmintic efficacy of plant cysteine proteinases of *C. papaya* have been reported in mice infected with adult *Trichuris muris*, a rodent gastrointestinal nematode [55]. In another study, Stepek et al. [56] reported the anthelmintic effects of cysteine proteinases of *C. papaya* against *Protospirura muricola* in rodent model.

2.2. *Cucurbita mexicana*

The aqueous, etheral and alcoholic extracts of *Cucurbita mexicana* (Cucurbitaceae) seeds have exhibited significant anthelmintic activity against *Moniezia expansa*, *Fasciolopsis buski*, *Ascaris lumbricoides* and *Hymenolepis diminuta*. Aqueous extract was found to possess the most significant toxicity as compared to alcoholic and etheral extracts [57]. The water and ethanol extract of *C. Mexicana* seed are effective and displayed high anthelmintic efficacy against *Aspiculuris tetraptera* in mice [58].

2.3. *Hedychium coronarium*

The rhizomes and oil of *Hedychium coronarium* (Zingiberaceae) and *H. spicatum* (Zingiberaceae) possess better anthelmintic activity than piperazine phosphate against earthworms and tapeworms [16].

2.4. *Butea monosperma*

All parts of *Butea monosperma* have been used as crude drug for the treatment of skin disease, tumors, wounds, ulcers and piles [59]. The crude seed powder of *B. monosperma* showed anthelmintic activity in sheep. The different species of *Butea* has been reported anthelmintic activity against *Ascaris lumbricoide*, *Ascaridia galli*, earthworm, *Toxocara canis*, *Dipylidium caninum* and *Taenia* [60]. Palasonin, an active principle of *Butea monosperma* (Leguminosae), has also been established to possess good anthelmintic activity against *A. lumbricoides*, using an *in vitro* assay [61].

2.5. *Azadirachta indica*

Azadirachta indica is a tree of Meliaceae family. Medicinal property of this plant is mentioned in traditional Indian Ayruvedic system of therapy [62]. All parts of the *Azadirachta indica* including the leaves, bark, fruits, seed and oil have medicinal properties and contain over ten different active components with azadirachtin as the most potent component and widely studied [63]. *Azadirachta indica* are toxic against *Salmonella* [64] *Plasmodium* and *Trypanosma* species [65, 66]. It has larvicidal activity against *L. acuminata* and larvae of *Fasciola gigantica* [6, 67]. In context of India, which is endowed with vast resources of medicinal plants, there is a strong tradition of using plant-based medicines in alternate system of medicine among native societies [1].

Phytochemical of plants and their controlled experiments associated strategies, can offer new alternatives for effective and economical control of parasite borne disease [1]. *Azadirachta indica* seeds inhibit 68.3% of larval hatching of *Haemonchus contortus* with the use of azadirachtin at 1% obtained from seeds [68]. In cattle, the consumption of dried leaves caused a reduction in the number of eggs of per gram of feces [69]. Rahman et al. [70] have evaluated the *in vitro* anthelmintic activity of Neem plant (*Azadirachta indica*) extract against third-stage *Haemonchus contortus* larvae from goats. It was recorded that 4 mg/ml methanolic extract gave 40% mortality. Aqueous leaves extract of *Azadirachta indica* leaves have significant anthelmintic activity against earthworms (*Pheretima posthuma*), tapeworms (*Raillietina spiralis*) and roundworms (*Ascaridia galli*) species [71].

2.6. *Nigella sativa*

Nigella sativa exhibits considerable anthelmintic activity against tapeworms, hookworms and nodular worms with the activity being comparable with that of hexylresorcinol against hookworms and nodular worms [72]. Mahmoud et al. [73] has been reported that the oil of *N. sativa* decreased the number of *Schistosoma mansoni* in liver and intestine of infected mice. The seed of *N. sativa* demonstrated an inhibitory effect on egg lying adult female worm and also effective against miracidium, cercaria and adult worm of *S. mansoni* [74].

2.7. *Zanthoxylum*

The anthelmintic activity of *Zanthoxylum alatum* (Rutacae) has been found to be comparable to that drug against roundworms [20], while the essential oil from the fruits of *Z. limonella* has been reported to bear better anthelmintic efficacy than that of piperazine phosphate [75].

2.8. *Punica granatum*

Inhibition of transformation of eggs to filariform larvae of *H. contortus*, Prakash et al. [76] established the dose-dependent anthelmintic activity of the alcoholic extract of *Punica granatum*. Swarnakar et al. [77] has been reported the methanolic extract of *P. granatum* shows anthelmintic activity against *Pheretima pasthuma*.

2.9. *Ocimum sanctum*

Various essential oils and eugenol isolated from *Ocimum sanctum* Linn. (Lamiaceae) have shown potent anthelmintic activity against *C. elegans*. Martinez-Ortiz-de-Montellano et al. [78] studied the effect of a tropical tannin-rich plant, *Lysiloma latisiliquum* on adult populations of *H. contortus* in sheep and suggested that a short-term consumption of *L. latisiliquum* can modulate directly the biology of adult *H. contortus* affecting the worm size and female fecundity. The essential oil of *Ocimum sanctum* and eugenol, tested *in vitro*, showed potent anthelmintic activity in the *Caenorhabditis elegans* model [79]. Singh and Nagaichi, [80] evaluated the antiparasitic effects of ethyl alcohol phytochemicals as cure of worm infections in traditional medicine systems extract of *Ocimum sanctum* against *A. galli in vitro*.

2.10. *Berlina grandiflora*

Berlina grandiflora and its active compound triterpenoid, betulinic acid shows or showed anthelmintic activity against *C. elegans* [46] in different solvent fractions. The bark and stem of *B. grandiflora* are effective anthelmintic against *N. brasiliensis* in infected albino rats [46].

2.11. *Evolvulus alsinoides*

In vitro anthelmintic activities of *Evolvulus alsinoides* extract against earthworm, *P. posthuma* and reported it to be better than piperazine citrate Dash et al. [81]. The essential oil of *Ocimum gratissimum*, a tropical plant well known for its ethnoveterinary use, showed strong anthelmintic activity *in vitro* against *H. contortus* [68].

2.12. *Melia azedarach*

The anthelmintic activity of ethanolic extract of *Melia azedarach* Linn (Meliaceae) was found to be better against *T. solium* than that of piperazine phosphate [82]. The anthelmintic activity of *M. azedarach*, *in vivo* studies have been performed with aqueous methanolic and ethanolic extracts of the fruits in chicken [83], of the seed in sheep [84] and seed, leaves in *in vitro* against *Haemonchus contortus* [85].

2.13. *Rubus fructicosus*

The woody plants, *Rubus fructicosus*, *Quercus robur* and *Corylus* showed remarkable anthelmintic activity when tested on 3rd-stage larvae (L3) and adult worms of *Teladorsagia circumcincta*, *H. contortus* and *Trichostrongylus colubriformis* [86]. The crude methanol extract of *R. fructicosus* fruits are showed anthelmintic activity against *Ascaridia galli* [87].

2.14. *Mangifera indica*

The anthelmintic properties of Vimang, an aqueous extract of *Mangifera indica* family stem bark and mangiferin, the major polyphenol present in Vimang, were investigated in the experimentally induced *T. spiralis* infections in mice [88]. Patil et al. [89] reported the methanolic extract of *M. indica* leaves were show anthelmintic activity against *Phertima posthma*.

2.15. *Punica granatum*

The fruit rind powder of *Punica granatum* tested for efficacy against gastrointestinal nematodes of sheep showed a remarkable decrease of 85% in the EPG counts in the treated groups. In a separate experiment the same fruit rind powder also showed considerable reduction in EPG in sheep naturally infected with mixed cestode species [83]. The glycosides and alkaloids of *P. granatum* have also shown good anticestodal efficacy in goats [90, 91].

2.16. *Melia azedarach*

Melia azedarach was also reported to be

capable of reducing the EPG in *A. galli* infected chickens [83]. Based on reduction in EPG, the whole plant powder of *Fumaria parviflora*, its water and ethanol extracts were also observed to be possessing significant anthelmintic efficacy against *Trichostrongylus*, *Haemonchus* and *Trichuris* infections in sheep [92].

2.17. *Saussurea lappa*

Saussurea lappa roots powder, its water and methanol extracts have also been found to possess anthelmintic effects in mixed infections of nematodes in sheep [93]. The toxicity of glycosides extracted from the roots of *S. lappa* was noted to be even better than aqueous or methanol extracts in sheep and buffalo-calves infected with mixed species of nematodes [94].

2.18. *Zingiber officinale*

Zingiber officinale is perennial plant and is considered to be the universal medicine in ayurveda. The anthelminthic activity of ethanol extracts of rhizomes of *Z. officinale* against human *Ascaris lumbricoldes* is appreciable [31, 95]. Goto et al. [96] reported the lethal effect of *Z. officinale* on *Anisakis* larvae *in vitro.* The antifilarial effect of *Z. officinale* against *Driofilaria immitis* has been reported by Datta and Sukul, [97]. Adewunmi et al. [98]; Sunita and Singh, [67] have reported the larvicidal activity of *Fasciola gigantic* larvae (sporocyst, redia and cercaria) *Z. officinale. Z. officinale* extract tested against experimentally induced *Setariacervi* infections in rats showed significant ant filarial activity [99]. Its seeds of *Carum copticum* (Umbelliferae), *Agati gratifola* (Leguminosae) and *Mangifera indica* (Anacardiaceae) have shown appreciable anthelmintic activity against human *Ascaris lumbricoides* [95]. Kalesaraj, [31] also reported that rhizomes of *Z. zerumbet* (Zingiberaceae) bear significant anthelmintic activity against human *A. lumbricoides*.

2.19. *Matricaria chamomilla*

The anthelmintic effects of *Matricaria chamomilla* L. were established in experimental *Ostertagia ostertagi* experimental infection in lambs [12].

2.20. *Dioscorea zingiberensis*

The anthelmintic activity of trillin and gracillin, the two bioactive compounds of *Dioscorea zingiberensis* C. H. Wright was investigated against *Dactylogyrus intermedius* (Monogenea) in goldfish under *in vivo* conditions. The study revealed that both trillin and gracillin are effective against *D. intermedius*, and the gracillin exhibits more interesting perspectives for the development of a candidate antiparasitic agent [11].

2.21. *Paris polyphylla*

The methanol extract of rhizomes of *Paris polyphylla* and its two steroidal saponins compounds, dioscin and polyphyllin D were established to possess a promising *in vivo* anthelmintic activity against *Dactylogyrus intermedius* [11]. The anthelmintic study of five alkaloids (sanguinarine, cryptopine, a-allocryptopine, protopine and 6-methoxyl-dihydrochelerythrine) from *Macleaya microcarpa* (Maxim) Fedde against *Dactylogyrus intermedius* in *Carassius auratus* provided evidence that the plant extract, as well as the isolated compounds, especially sanguinarine, might be the potential plant-based medicines for the treatment of *D. intermedius* infection.

2.22. *Ferula asafoetida*

Ferula asafoetida is known to possess antimicrobial, antioxidant, anti carcinogenic, antispasmodic, molluscicidal and antithelminthic activity [10, 100-104]. The alcoholic extract of *F. asafoetida* and it active component ferulic acid and umbelliferone has shown moderate anthelmintic activity against *Fasciola gigantica* larvae [6, 67]. Ferulic acid has been reported to have many physiological functions, including protection against coronary disease, lowers cholesterol and increases sperm viability [105]. Ferulic acid has been shown to potentially exert several beneficial effect on health [106], it significantly protect against UV-induced erythematic in human [107], act as a

peroxyl radical scavenger and increased the resistance of LDL to oxidation.

It also has a strong insecticidal activity and caused high percentage of mortality on eggs and larvae of insects and regarded as an ovicidal agent [108].

2.23. *Allium sativum*

Dried, powdered of *Allium sativum* contains approximately 1% allicin which is the most significant compound (S-allyl cystein sulfoxide) [109]. The most biologically active compounds, (diallyl thiosulfinat or diallyl disulfide) does not exist in *A. sativum* until it is crushed or cut; injury to the *A. sativum* bulb activates the enzyme allinase, which metabolized alliin to allicin. Allicin was first chemically isolated in the 1940, has antimicrobial effects against viruses, bacteria, fungi and parasite [110-111].

Sunita et al. [6] has been studies the larvicidal activity of allicin against *Fasciola gigantica* larvae sporocyst, redia and cercaria in different month of the year 2011-2012. However, increasing problems of development of resistance in helminthes against anthelmintic drugs [112] have led to the screening of medicinal plants for their anthelmintic activity. The alcoholic extract of bulb of *A. sativum* has also shown moderate *in vitro* anthelmintic activity against human *Ascaris lumbricoldes* [31]. *A. sativum* has been reported to be effective in dysentery and also acts as vermifuge 113, 114]. Oil of *A. sativum* has also been reported to possess anthelminthic activity [115, 116] and discards all injurious parasites in the intestine [113]. *A. sativum* has shown anthelminthic action in *in vitro* and *in vivo* condition against helminthes [31].

2.24. *Balanite*

The larvicidal activity of aqueous extracts of seed, endocarp, mesocarp and the whole fruit of *B. aegyptiaca* against adult *Biomphalaria pfeifferi* and *Lymnaea natalensis* as well as the cercariacidal activity of its seed on *Schistosoma mansoni* cercariae were investigated. With regards to the snail species, *B. pfeifferi* no mortality was observed for *B. pfeifferi* exposed to extracts' concentrations of 2, 5 and 8 ppm of all tested plant parts after 24 hours exposure. Hundred percent mortality rates were observed on *B. pfeifferi* exposed to a concentration of 100 ppm for the seeds and mesocarp, no mortality was observed at 24 hours exposure period below the concentrations of 15 ppm. From the cercariacidal investigation, the *in vitro* cercariacidal activity of the plant on *S. mansoni* cercariae showed that the mortality rates of cercariae were elevated by increasing both the concentrations of seeds and the time of exposure. The *in vivo* observation of the infectivity of *S. mansoni* cercariae was evaluated by pre-exposing the cercariae with seed extracts and then exposing to mice, it was found that infectivity of cercariae was completely inhibited at 15 ppm. And a significant reduction in tissue egg deposition occurred even at lower concentrations than 15 ppm ($p<0.05$).

2.25. *Alangium larmarckii*

The anthelmintic toxicity of the root and bark of *Alangium larmarckii* (Alangiaceaea) are use against the hookworms of dogs and poultry ascarids reported by Dubey and Gupta, [3].

2.26. *Piper betle*

The anticestodal activity of essential oil from *Piper betle* has been found to be superior to that of piperazine phosphate, and the activity against hookworms has been reported greater than that of hexylresorcinol [4]. The leaves extract of *P. betle* are potential anthelmintic [117].

2.27. *Piper longum*

The essential oil from the fruits of *Piper longum* was screened for the anthelmintic activity against *Ascaris lumbricoids*. The experiment revealed that its oil has a definite paralytic action on the nerve muscular preparation of *A. lumbricoids* [5].

2.28. *Semecarpus anacardium*

It is found throughout the hotter/warmer parts of India and its nuts are commonly known as Bhilawa. Chattopadhyaya and Khare [118] reported that anacardic acid isolated from the oil of nuts of

Semecarpus anacardium (Anacardiaceae) and its sodium salt both have good anthelmintic toxicity.

2.29. *Mimusops elengi*

The barks of *Mimusops elengi* have cardiotonic, alexipharmic, anthelmintic and astringent repoted by Kirtikar and Basu, [119]. Crude alcoholic extract and its various fractions were evaluated for their anthelmintic potential using *Pheretima posthuma* and *Ascardia galli* as testworms. The crude alcoholic extract and its ethyl acetate and n-butanol fractions significantly demonstrated paralysis and also caused death of worms especially at higher concentration of 100 mg/ml as compared to standard reference piperazine citrate (10 mg/ml).

2.30. *Cardiospermum halicacabum*

Cardiospermum halicacabum extract when tested *in vitro* for its efficacy against L3 of *Strongyloides stercoralis* showed reduction in the viability of larvae [120].

2.31. *Evolvulus alsinoides*

The ethanolic extract of *Evolvulus alsinoides* (Convolvulaceae) was observed to show more anthelmintic action as compared to piperrazine citrate Dash et al. [81].

3. CONCLUSION

The traditional use of a wide variety of common medicinal plants holds a great prominence source of easily available and effective anthelmintic/larvicidal activity in different animals. The present review of literature indicate the screening of crude products, organic extracts and different plant derived active components is need to further studies at molecular level for searching different phytochemicals which can replace the synthetic drugs in control of wide parasitic infections diseases.

AUTHOR'S CONTRIBUTION

All the authors involved in conception and design, drafting the review article. The final manuscript has been approved by all authors.

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interest regarding the publication of this article.

REFERENCES

1. Akhtar MS, Iqbal Z, Khan MN, Lateef M. Anthelmintic activity of medicinal plants with particular reference to their use in animals in Indo-Pakistan Subcontinent. Small Rumin Res. 2000; 38: 99-107.
2. Tagboto S, Townson S. Antiparasitic properties of medicinal plants and other naturally occurring products. Adv Parasitol. 2001; 50: 199-295.
3. Dubey MP, Gupta I. Studies on the anthelmintic activity of *Alangium lamarikii* Thwaites (Hindi Akol) root bark. Indian J Physiol Pharmacol. 1968; 12: 25-31.
4. Garg SC, Jain R. Biological activity of the essential oil of *Piper betle* cultivar *Sagar Bangla*. J Essent Oil Res. 1992; 4: 601-606.
5. D'Cruz JL, Nimbkark AY, Kokate CK. Evaluation of fruits of *Piper longum* Linn. And leaves of *Adhatoda vasica* seed for anthelmintic activity. Indian Drugs. 1980; 4(17): 99-101.
6. Sunita K, Kumar P, Singh VK, Singh DK. *In vitro* phytotherapy of vector snail by binary combination of larvicidal components in effective control of fasciolosis. Rev Inst Med Trop Sao Paulo. 2013; 5(55): 303-308.
7. Iqbal Z, Nadeem QK, Khan MN, Akhtar MS, Waraich FN. *In vitro* anthelmintic activity of *Allium sativum, Zingiber officinale, Cucurbita mexicana and Ficus religiosa.* Int J Agr Biol. 2001; 3: 454-457.
8. Iqbal Z, Lateef M, Jabbar A, Muhammad G, Khan MN. Anthelmintic activity of *Calotropis procera* (Ait.) Ait. F. flowers in sheep. J Ethnopharmacol. 2005; 102: 256-261.
9. Iqbal Z, Lateef M, Jabbar A, Ghayur MN, Gilani AH. *In vitro* and *in vivo* anthelmintic activity of *Nicotiana tabacum* L. leaves against gastrointestinal nematodes of sheep. Phytother Res. 2006; 20: 46-48.
10. Bakker W, Hartmans KJ, Diepenhorst P, Gorris LGM. The use of carvone in agriculture: suppression of potatoes and antifungal activity against potato tuber and other plant diseases. J Ind Crops Prod. 1995; 1(4): 3-13.

11. Wang GX, Zhou Z, Jiang DX, Han J, Wang JF, Zhao LW, Li J. *In vivo* anthelmintic activity of five alkaloids from *Macleaya microcarpa* (Maxim) Fedde against *Dactylogyrus intermedius* in *Carassius auratus*. Vet Parasitol. 2010; 171: 305-313.
12. Bahrami AM, Doosti A, Moosavi AB. Effect of *Matricaria chamomilla* L. plant extraction on experimental infected lamb with *Ostertagia ostertagi* parasites. Int J Pharmacol. 2010; 6: 712-718.
13. Hordegen P, Cabaretb J, Hertzberga H, Langhansc W, Maurera V. *In vitro* screening of six anthelmintic plant products against larval *Haemonchus contortus* with a modified methyl-thiazolyl-tetrazolium reduction assay. J Ethnopharmacol. 2006; 108: 85-89.
14. Bundy DAP. Immunoepidemiology of intestinal helmintic infection I, the global burden of intestinal nematode disease. Trans Royal Soc Trop Med Hyg. 1994; 8: 259-261.
15. Ali SM, Mehta RK. Preliminary pharmacological and anthelmintic studies of the essential oil of *Piper betle* Linn. Indian J Pharm. 1970; 32: 132-133.
16. Dixit VK, Varma KC. Anthelmintic properties of essential oils from rhizomes of *Hedychium coronarium* Koenig and *Hedychium spicatum* Koenig. Indian J Pharmacoal. 1975; 37: 143-144.
17. Girgune JB, Jain NK, Garg BD. Anthelmintic activity of some essential oils. Indian Perfumer. 1978; 22: 296-297.
18. Girgune JB, Jain NK, Garg BD. Antimicrobial and anthelmintic activity of essential oil from *Gardenia lucida* Roxb. Indian Perfumer. 1979; XXIII(3-4): 213-215.
19. Mishra SH, Gaud RS, Sharma RA, Chaturvedi SC. Anthelmintic activity of some essential oils. Indian Perfumer. 1979; XXIII(3-4): 208-209.
20. Mehta MB, Kharya MD, Srivastava R, Varma KC. Antimicrobial and anthelmintic activities of the essential oil of *Zanthoxylum alatum* Roxb. Indian Perfumer. 1981; XXV(2): 1-3.
21. Garg SC, Kasera HL. *In vitro* anthelmintic activity of the essential of *Anacardium occidentale.* Indian Perfumer. 1982; 26: 239-240.
22. Siddiqui N, Garg SC. *In vitro* anthelmintic activity of some essential oils. Pak J Sci Ind Res. 1990; 33: 536-537.
23. Githiori JB, Hoglund J, Waller PJ, Baker RL. The anthelmintic efficacy of the plant, *Albizia anthelmintica*, against the nematode parasites *Haemonchus contortus* of sheep and *Heligmosomoides polygrus* of mice. Vet Parasitol. 2003; 116: 23-24.
24. Kalesaraj, Kurup PA. Anthelmintic activity, toxicity and other pharmacological properties of palasonin, the active principle of *Butea frondosa* seeds and its piperazine salt. Ind J Med Res. 1968; 56: 1818-1825.
25. Lawrence BM. Cucurbita: a monograph. Lawrence review of natural products. Philips O. *Ficus insipida*: ethnobotany and ecology of an Amazonian anthelmintic. Econ Bot. 1990; 44: 534-536.
26. Pradhan KD, Thakur DK, Sudhan NA. Therapeutic efficacy of *P. granatum* and *C. maxima* against clinical cases of nematodiasis in calves. Ind J Indust Med. 1992; 9: 53-54.
27. Asuzu IU, Onu OU. Anthelmintic activity of the ethanolic extract of *Piliostigma thonningii* bark in *Ascaridia galli* infected chickens. Fitoterapia. 1994; 65: 291-297.
28. Desta B. Ethiopian traditional herbal drugs. Part I. Studies on the toxicity and therapeutic activity of local taenicidal medications. J Ethnopharmacol. 1995; 45: 27-33.
29. Sollman T. Anthelmintics: their efficacy as tested on earthworms. J Pharmacol. 1918; 1: 129.
30. Sharma LD, Bhaga HS, Srivastava PS. *In vitro* anthelmintic screening of indigenous medicinal plants against *Haemonchus contortus* (Rudolphi, 1803) Cobbold, 1898 of sheep and goats. Indian J Anim Res. 1971; 5: 33-38.
31. Kalesaraj R. Screening of some indigenous plants for anthelmintic action against human *Ascaris lumbricoides*. Part II. Indian J Physiol Pharmacol. 1975; 19: 47-49.
32. Shrivastava R. Anthehnintic properties of essential oil of *Cyathocline lyrata* cass. Indian J Pharm Sci. 1979; 41: 228-229.
33. Garg SC, Kasera HL. Anthelmintic activity of *Callistemon viminalis*. Fitoterapia. 1982; LIII(5-6): 179-181.
34. Kakrani HK, Kalyani GA. Anthelmintic activity of the essential oil of *Commiphora mukul.* Fitoterapia. 1984; 55: 232-234.
35. Nakhare S, Garg SC. Anthelmintic activity of some essential of *Artemisia pallens* Wall. Ancient Sci Life. 1991; 10: 185-186.
36. Coles GC, Bauer C, Borgsteede FHM, Geerts S, Klei TR, Taylor MA, Waller PJ. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P) methods for the detection of

anthelmintics resistance in nematodes of veterinary importance. Vet Parasitol. 1992; 44: 35- 44.

37. Assis LM, Bevilaqua CML, Morais SM, Vieira LS, Costa CTC, Souza JAL. Ovicidal and larvicidal activity *in vitro* of *Spigelia anthelmia* Linn. extracts on *Haemonchus contortus*. Vet Parasitol. 2003; 117: 43-49.
38. Lateef M, Iqbal Z, Khan MN, Akhtar MS, Jabbar A. Anthelmintic activity of *Adhatoda vesica* roots. Int J Agr Biol. 2003; 5: 86-90.
39. Bany J, Zdanowska D, Zdanowski R, Skopinska-Rosewska E. The effect of herbal remedy on the development of *Trichinella spiralis* infection in mice. Pol J Vet Sci. 2003; 6: 6-8.
40. Martinez-Grueiro M, Gimenez-Pardo C, Gomez-Barrio A, Franck X, Fournet A, Hocquemiller R, et al. Nematocidal and trichomonacidal activities of 2-substituted quinolines. Farmaco. 2005: 1-6.
41. Saha A, Ghosh NK, Sinhababu SP. Cestocidal activity of *Gladiolus gandavensis.* J Parasit Dis. 1999; 23: 135-136.
42. Tangpu V, Temjenmongla, Yadav AK. Anticestodal property of *Strobilanthes discolor*: an experimental study in *Hymenolepis diminuta*-rat model. J Ethnopharmacol. 2006; 105: 459-463.
43. Yadav A.K, Tangpu V. *In vitro* anticestodal evaluation of some medicinal plants used by Naga traditional healers. Pharmacologyonline. 2006; 3: 90-95.
44. Ghosh NK, Sinha Babu SP, Sukul NC, Ito A. Cestocidal activity of *Acacia auriculiformis.* J Helminthol. 1996; 70: 171-172.
45. Bogh HO, Andreassen J, Lemmich J. Anthelmintic usage of extracts of *Embelia schimperi* from Tanzania. J Ethnopharmacol. 1996; 50: 35-42.
46. Enwerem NM, Okogun JI, Wambebe CO, Okorie DA, Akah PA. Anthelmintic activity of the stem bark extracts of *Berlina grandiflora* and one of its active principles, betulinic acid. Phytomed. 2001; 8: 112-114.
47. Kaushik RK, Katiyar JC, Sen AB. A new *in vitro* screening technique for anthelmintic activity using *Ascaridia galli* as a test parasite. Indian J Anim Sci. 1981; 51: 869-872.
48. Dengre SL. Chemical and physiological examination of essential oils from Indian sources. Ph.D. Thesis, Dr. Hari Singh Gour Vishwavidyalaya, Sagar, India. 1982: 171-179.
49. Garg SC, Nakhare S. Studies on the essential oils from the flowers of *Eupatorium triplinerve*. Indian Perfumer. 1993; 37: 318-323.
50. Satrija FP, Nansen S, Murtini HS. Anthelmintic activity of papaya latex against patent *Heligmosomoides polygyrus* infections in mice. J Ethnopharmacol. 1995; 48: 161-164.
51. Kermanshai R, Brian E, Rosenfeld J, Summers SP, Sorger J. Benzyle isothiocyanate is the chief or sole anthelmintic in papaya seed extracts. Phytochem. 2001; 3(57): 427-435.
52. Hounzangbe-Adote MS, Paolini V, Fouraste I, Moutairou K, Hoste H. *In vitro* effects of four tropical plants on three stages of the parasitic nematode, *Haemonchus contortus.* Res Vet Sci. 2005; 78: 155-160.
53. Hounzangbe-Adote MS, Fouraste I, Moutairou K, Hoste H. *In vitro* effects of four tropical plants on the activity and development of the parasitic nematode, *Trichostrongylus colubriformis.* J Helminthol. 2005; 79: 29-33.
54. Okeniyi JA, Oyelami OA, Adeyemi LA. Effectiveness of dried *Carica papaya* seed against human intestinal parasitosis: a pilot. J Med Food. 2007; 1(10): 194-196.
55. Stepek G, Lowe AE, Buttle DJ, Duce IR, Behnke JM. *In vitro* and *in vivo* anthelmintic efficacy of plant cysteine proteinases against the rodent gastrointestinal nematode, *Trichuris muris.* Parasitology. 2006; 132: 681-689.
56. Stepek G, Lowe AE, Buttle DJ, Duce IR, Behnke JM. Anthelmintic action of plant cysteine proteinases against the rodent stomach nematode, *Protospirura muricola*, *in vitro* and *in vivo.* Parasitol. 2007; 134: 103-112.
57. Shrivastava MC, Singh SW. Anthelmintic activity of *Cucurbitta maxima* seeds. Ind J Med Res. 1967; 55: 629-632.
58. Erol A, Cenziz G, Hamit C, Arzu T, Seyda O, Kubra C. Evaluation of the anthelminthic activity of pumpkin seed (*Cucurbita maxima*) in mice naturally infected with *Aspiculuris tetraptrea*. J Pharmacogn Phytot. 2015; 9(7): 189-193.
59. Mengi AS, Deshpande SG. Comparative evaluation of *Butea frondosa* and flurbiprofen for ocular anti-inflammatory activity in rabbits. J Pharm Pharmacol. 1995; 47: 997-1001.
60. Iqbal Z, Lateef M, Jabbar A, Ghayur MN, Gilani AH. *In vivo* anthelmintic activity of *Butea monosperma* against *Trichostrongylid* nematodes in sheep. Fitoterapia. 2006; 77: 137-140.
61. Lal JS, Chandra, Sabir M. Modified method for isolation of *Palasonin* the anthelmintic principle of

Butea frondosa seeds. Ind J Pharmacol Sci. 1978; 40: 97-98.

62. Brototi B, Kaplay RD. *Azadirachta indica* (Neem): It's economic utility and chances for commercial planned plantation in Nanded District. Int J Pharma. 2011; 2(1): 100-104.
63. Nwosu CO. Formulation of neem-derived biopesticides as alternative to persistent organic pollutants (POPS) for the control of pest of livestock. Expert Group Meeting on Sustainable Utilization of Neem Tree in Northern Nigeria, Dust Jigawa State. 2001; 18-19th September 2001: 8.
64. ITDG, IIRR. Ethnoveterinary medicine in Kenya: a filed manual of traditional health care practices. Intermediate Technology Development Group and International Institute of Rural Reconstruction, Nairobi, Kenya. 1996.
65. Ivbijaro MF. Prospects for neem. In: Schmuttere H, Ascher KRS, eds. Natural pesticides from the neem tree (*Azadirachta indica* A Juss) and other tropical plants. German Agency for Technical Co-operation (GTZ), Nairobi, Kenya, July. 1987: 525-533.
66. Khalid SA, Duddeck H, Gouzalez-Sierra M. Isolation and characterization of antimalarial agent of the neem tree, *Azadirachta indica.* J Nat Prod. 1989; 52: 922-926.
67. Sunita K, Singh DK. Fascioliasis control: *In vivo* and *in vitro* phytotherapy of vector snail to kill *Fasciola* larva. J Parasitol Res. 2011; 2011: 1-7.
68. Pessoa LM, Morias SM, Bevilaqua CKL Luciano JHS. Anthelmintic activity of essential oil of *Ocimum gratissimum* Linn. and eugenol against *Haemonchus contortus.* Vet Parasitol. 2002; 109: 59-63.
69. Pietrosemoli S, Ovalez R, Montilla T. Empleo, de hojas de nematodes gastrointestinales de bovines a pastoreo. Rev Fac Agron. 1999; 16: 220-225.
70. Rahman WA, Lee R, Sulaiman SF. *In vitro* anthelmintic activity of neem plant (*Azadirachta indica*) extract against third-stage *Haemonchus contortus* larvae from goats. Global Veterinaria. 2011; 7: 22-26.
71. Rabiu H, Subhasish M. Investigation of *in vitro* anthelmintic activity of *Azadirachta indica* leaves. Int J Drug Dev Res. 2011; 4(3): 94-100.
72. Agarwal R, Kharya MD, Srivastava R. Antimicrobial and anthelmintic activities of the essential oil of *Nigella sativa* Linn. Indian J Exp Biol. 1979; 17: 1264.
73. Mahmoud MR, El-Abhar HS, Saleh S. The effect of *Nigella sativa* oil against the liver damage induced by *Schistosoma mansoni* infection in mice. J Ethnopharmacol. 2002; 1(79): 1-11.
74. Mohamed AM, Metwally NM, Mahmoud SS. Sativa seeds against *Schistosoma mansoni* different stages. Mem Inst Oswaldo Cruz. 2005; 2(100): 205-211.
75. Kalyani GA, Aithal KS, Srivastava KK. *In vitro* anthelmintic activity of essential oil from the fruits of *Zanthoxylum limonella.* Fitoterapia. 1989; 2(LX): 160-162.
76. Prakash V, Singhal KC, Gupta RR. Anthelmintic activity of *Punica granatum* and *Artemisia silversiana.* Indian J Pharmacol. 1980; 12: 62-65.
77. Swarnakar Y, Shroff M, Jha AK, Sahu D, Dhurandhar K. Evaluation of anthelmintic potential in fruit peel of *Punica granatum* Linn. Pomegranate. 2013; 1(2): 461-464.
78. Martínez-Ortíz-de-Montellano C, Vargas-Magana JJ, Canul-Ku HL, Miranda-Soberanis R, Capetillo-Leal C, Sandoval-Castro CA, et al. Effect of a tropical tannin-rich plant *Lysiloma latisiliquum* on adult populations of *Haemonchus contortus* in sheep. Vet Parasitol. 2010; 172: 283-290.
79. Asha MK, Prashanth D, Murali B, Padmaja R, Amit A. Anthelmintic activity of essential oil of *Ocimum sanctum* and eugenol. Fitoterapia. 2001; 72: 669-670.
80. Singh K, Nagaichi S. Anthelmintic efficacy of the alcoholic extract of *Ocimum sanctum* against common poultry worms *Ascaridia galli* and *Heterakis gallinae*. J Parasit Dis. 2002; 26: 42-45.
81. Dash GK, Suresh P, Sahu SK, Kar DM, Ganapaty S, Panda SB. Evaluation of *Evolvulus alsinoides* Linn. for anthelmintic and antimicrobial activities. J Nat Remed. 2002; 2: 182-185.
82. Szewczuk VD, Mongelli ER, Pomilio AB. Antiparasitic activity of *Melia azedarach* growing in Argentina. Mol Med Chem. 2003; 1: 54-57.
83. Akhtar MS, Riffat S. Evaluation of *Melia azedarach* Linn.seeds (Bakain) and piperazine against *Ascaridia galli* infection in chickens. Pak Vet J. 1985; 5: 34-37.
84. Pervez K, Ashraf M, Hanjira AH. Anthelmintic efficacy of *Melia azedarach* (Bakin) Linn. against gastrointestinal nematodes in sheep. Pak Vet J. 1994; 14: 135-137.
85. Maciel MV, Morais SM, Bevilaqua CML, Camurça-Vasconcelos ALF, Costa CTC, Castro CMS. Ovicidal and larvicidal activity of *Melia azedarach* extracts on *Haemonchus contortus.* Vet Parasitol. 2006; 140: 98-104.

86. Paolini V, Fouraste I, Hoste H. *In vitro* effects of three woody plant and sainfoin extracts on 3rd-stage larvae and adult worms of three gastrointestinal nematodes. Proc Nutr Soc. 2004; 63: 631-639.
87. Niaz A, Umer A, Syed WAS, Ismail S, Muhammad J, Ghayour A, et al. Acute toxicity, brine shrimp cytotoxicity, anthelmintic and relaxant potentials of fruits of *Rubus fruticosus* Agg. BMC Complem Altern Med. 2013; 13: 138.
88. Garcia D, Escalante M, Delgado R, Ubeira FM, Leiro J. Anthelmintic and antiallergic activities of *Mangifera indica* L. stem bark components Vimang and mangiferin. Phyto Res. 2003; 17: 1203-1208.
89. Patil D, Halle P, Bade A. *In-vitro* anthelminetic activity of methanoilic extract of *Mangifera indica* leaves. World J Pharm Pharmac Sci. 2014; 12(3): 771-776.
90. Akhtar MS, Aslam M. Anthelmintic efficacies of total alkaloids and glycosides isolated from *Punica granatum* fruit rinds. Pak J Agr Sci. 1988; 25: 161-168.
91. Akhtar MS, Riffat S. Efficacy of *Melia azedarach* Linn. (Bakain) and morantel against naturally acquired gastrointestinal nematodes in goats. Pak Vet J. 1984; 4: 176-179.
92. Akhtar MS, Javed I. Comparative efficacy of *Fumaria parvi*ora and morantel tartrate against gastrointestinal nematode infection in sheep. Pak J Pharmacol. 1985; 2: 31-35.
93. Akhtar MS, Hassan IJ. Evaluation of *Saussurea lappa* roots (Qust) against natural infection of gastrointestinal nematodes in sheep. Pak J Agric Sci. 1985; 22: 1-7.
94. Akhtar MS, Makhdoom S. Antinematodal efficacy of glycosides isolated from *Saussurea lappa* (Qust or Kooth) in sheep and buffalo calves. Pak J Pharmacol. 1988; 5: 59-64.
95. Kalesaraj R. Screening of some indigenous plants for anthelmintic action against human *Ascaris lumbricoides.* Indian J Physiol Pharmacol. 1974; 18: 129-131.
96. Goto C, Kasuya S, Koga K, Ohtomo H, Kaget N. Lethal efficacy of extract from *Zingiber officinale* (traditional Chinese medicine) or [6]-shogaol and [6]-gingerol in *Anisakis* larvae *in vitro*. Parasitol Res. 1990; 76: 653- 656.
97. Datta A, Sukul NC. Antifilarial effect of *Zingiber officinale* on *Dirofilaria immitis.* J Helminthol. 1987; 61: 255-258.
98. Adewunmi CO, Guntimein OBO, Furu P. Molluscicidal and antischistosomal activities of *Zingiber officinale*. Planta Med. 1990; 56: 374-376.
99. Ghosh M, Ghosh T, Sinha Babu SP, Sukul NC. Antifilarial effect of a plant, *Zingiber officinale* on *Setaria cervi* in rats. Proc Zool Soc. 1992; 45: 103-105.
100. Poolman B, Oosterhaven K, Smid EJ. S-carvone as a natural potato sprout inhibiting, fungistatic and bacteristatic compound. Indust Crop Prod. 1995; 4: 23-31.
101. Hirotaka F, Shuhei, Yumiko I, Hiroshi M, Toyokazw Y, Taru N. Ferulic acid production from clove oil by pseudomonas fluoresens E118. J Biosci Bioengin. 2003; 96: 404-405.
102. Fatehi M, Farifteh F, Hassanabad ZF. Antispasmodic and hypotensive effect *Ferula asafoetida* gum extract. J Ethnopharmacol. 2004; 91: 321-324.
103. Kumar P, Singh DK. Molluscicidal activity of *Ferula asafoetida*, *Syzygium aromaticum* and *Carum carvi* and their active components against the snail *Lymnaea acuminata*. Chemosphere. 2006; 63: 1568-1574.
104. Kumar P, Singh VK, Singh DK. Reproduction of *Lymnaea acuminata* fed to bait containing binary combination of amino acid with molluscicides. J Biol Earth Sci. 2013; 3(1): B65-B71.
105. Shiyi OU, Kin-Chor K. Ferulic acid: pharmaceutical functions, preparation and applications in foods. J Sci Food Agric. 2004; 11(84): 1261-1269.
106. Balasubashini MS, Rukkumani R, Viswanthan P, Menon VP. Ferulic acid alleviates lipid peroxidation in diabetic rats. Phytother Res. 2004; 18: 310-314.
107. Saija A, Tomaino A, Trombetta D, De Pasquale A, Uccella N, Barbuzzi T, et al. *In votro* and *in vivo* evaluation of coffeic and ferulic acid as topical phtprotective agents. Int J Pharm. 2000; 199: 39-47.
108. Razavi SM. Plant coumarins as allelopathic agents. Int J Biol Chem. 2011; 5: 86-90.
109. Dausch JG, Nixon DW. Garlic: a review of its relationship to malignant disease. Prev Med. 1990; 3(19): 346-361.
110. Bradley PR. British herbal compendium a handbook of scientific information on widely used plant drugs/published by British Herbal Medicine Association and produced by its scientific committee. Bournemouth, Dorset. 1992: 105-108.
111. Kemper KJ. Garlic (*Allium sativum*). The long wood herbal task force and the center for Holistic Pediatric Education and Research. 2000: 1-49.

112. Greek S, Dorny PP. Anthelmintic resistance in helminthes of animals of man in the tropics. Bull Sci Dutra-Mer. 1995; 3: 401-423.
113. Nadkarni KM. Indian Materia Medica. Vol. I and II Popular Prakashan, Private Limited Bombay, India. 1976.
114. Schavenberg P, Paris F. Guide to medicinal plants. Lutterworth Press, London. *Schistosomiasis*. Ann Trop Med Parasitol. 1977; 4(50): 345-349.
115. Steenis-Kruseman MJV. Select Indonesian medicinal plants organize. Sci Res Indonesia Bull. 1953; 18: 31.
116. Kirtikar KR, Basu BD. Indian medicinal plants. Part II, Indian Press, 1981.
117. Akter KN, Karmakar P, Das A, Anonna SN, Shoma SA, Sattar MM. Evaluation of antibacterial and anthelmintic activities with total phenolic contents of *Piper betel* leaves. Avicenna J Phytomed. 2014; 5(4): 320-329.
118. Chattopadhyaya MK, Khare RL. Isolation of anacardic acid from *Semecarpus anacardium* L. and study of its anthelmintic activity. Indian J Pharm. 1969; 31: 104-105.
119. Kirtikar KR, Basu BD. Indian medicinal plants. 2 edn. Vol. I Lalit Mohan Basu Allahabad, India. 1935: 785-788.
120. Boonmars T, Khunkitti W, Sithithaworn P, Fujimaki Y. *In vitro* antiparasitic activity of extracts of *Cardiospermum halicacabum* against third-stage larvae of *Strongyloides stercoralis.* Parasitol Res. 2005; 97: 417-419.

ISSN 2449-8955

European Journal of Biological Research

Research Article

Mycological and enzymatic studies on fresh beef meat sold in Taiz City, Yemen

M. A. Abdel-Sater*[1], F. A. Al-Sharjabi[2], Elham S. Al-Ashwal[2]

[1] Department of Botany & Microbiology, Faculty of Science, Assiut University, Egypt

[2] Department of Microbiology, Faculty of Applied Science, Taiz University, Yemen

*Corresponding author: M. A. Abdel-Sater; E-mail: masater59@yahoo.com

Received: 02 September 2017; **Revised submission:** 29 September 2017; **Accepted:** 25 October 2017

DOI: http://dx.doi.org/10.5281/zenodo.1037238

ABSTRACT

The mycological analysis of 30 fresh beef meat samples on Czapek's agar at 7º and 28ºC revealed that, heavily contamination with moulds was observed especially at 28ºC. A total of 234 and 400 colonies ⁄ 450 g meat were collected on both temperatures, respectively. Sixty-seven species belonging to 20 genera were identified. Members of *Aspergillus*, *Mucor*, *Penicillium* and *Trichoderma* were the most prevalent fungi. At 7°C was highly spoilage by yeasts fungi, while filamentous fungi predominated at 28°C. The ability of the common fungal isolates to produce protease and lipase enzymes revealed that most of them were positive. Among 152 isolates tested, 103 (67.8%) and 96 (63.2%) could respectively produce these enzymes. Because the deteriorative effects of the above fungi, food should be frequently and routinely analyzed. Also, it is essential to store the meat at lower temperature immediately after slaughtering and during transport and storage to reduce or prevent mould growth.

Keywords: Fresh meat; Food spoilage; Protease; Lipase.

1. INTRODUCTION

Meats still is, and will remain, part of the staple diet [1]. Meat is considered an important source of proteins, essential amino acids, B complex vitamins and minerals. Due to this rich composition, it offers a highly favorable environment for the growth of microorganisms. The microbiological contamination of meat occurs mainly during processing and manipulation, such as skinning, evisceration, storage and distribution at slaughter-houses and retail establishments [2]. Also, a variety of sources including air, water, soil, feces, feed, hides, intestines, lymph nodes, processing equip-ment, utensils and humans, contribute to the microbial contamination of the sterile muscles of healthy animals during slaughter, fabrication, and further processing and handling [3, 4]. Since it is impossible to entirely prevent contamination occurring during slaughter and dressing, some reports evaluated the microbial contamination of exposed meat surfaces at the retail level [5, 6]. The microbiology of meat spoilage has received considerable attention over the years and the characterization of the typical microflora, which develop on different types of meats during storage, has been well documented [7-19].

Enzymes have the property of causing and regulating specific chemical reactions inside or

outside living cells [20]. The major enzymes are protease, lipase, phosphatase, xanthine oxidase and lactoperoxidase [21]. Enzymatic actions are natural process in the muscle cells of the animals after they have been slaughtered and finally end up in meat self deterioration [22].

The present study was planned for the first time in Yemen to assess the fungal load in fresh beef meat, hence the purpose is to study the following: isolation and identification the moulds which contaminate the fresh beef meat in Taiz City, Yemen. The capability of the isolated moulds to produce protease and lipase enzymes was also assessed.

2. MATERIALS AND METHODS

2.1. Collection of samples

Thirty samples of fresh beef meat were collected randomly from different butchers shops and supermarkets in Taiz City. The samples were placed in sterile plastic bags and transferred in ice-cooled containers (4°C) to the laboratory for immediate fungal analysis.

2.2. Isolation and enumeration of fungi

The direct-plating technique [23] was employed. Fifteen pieces of fresh meat (1 gram each) were placed on the surface of three Czapek's agar plates. The plates were kept in a biological oxygen demand (BOD) incubator for 5-7 days at 28±2°C and 8-10 days at 7±2°C. The developing fungal colonies were isolated, identified and maintained on Czapek's agar media. Percentage incidences of fungi were calculated per 15 pieces for each sample.

2.3. Medium used for isolation of fungi

Modified Czapek′s Dox agar medium was used in which the 3% sucrose was substituted with 2% glucose. The composition of the medium (g/l) was: glucose 20; $NaNO_3$, 3; $KH2PO_4 \cdot 7H2O$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4 \cdot 7H2O$, 0.01 and agar, 15. Rose-bengal (1/15000) combined with chloramphenicol (0.5 mg/ml) were used as bacteriostatic agents [24, 25].

2.4. Identification of fungal genera and species

Fungi isolated were identified on the bases of macro- and microscopic features following the keys of Raper and Fennell [26], Booth [27], Ellis [28, 29], Pitt [30], Moubasher [31], Domsch et al. [32].

2.5. Screening for enzymatic activity of fungal isolates

The common fungal isolates recovered were tested for their abilities to produce extracellular protease and lipase on agar media as follow: three hundreds and seventeen fungal isolates belonging to eighty-three species related to twenty-three genera, commonly isolated in the current work, were tested for their abilities to produce the two enzymes. The fungal proteolytic was tested using a casin hydrolytic medium as employed by Paterson and Bridge [33]. Hydrolysis of the casein results in a clear zone around the fungal colony.

The fungi lipolytic were test using a modified medium of Ullman and Blasins [34] in which Tween 80 (poly oxy-ethylene sorbitan mono oleate) was added instead of Tween 20. The formation of crystals of calcium salt of the oleic acid liberated by the enzyme or as opaque zone surrounding the colony.

3. RESULTS AND DISCUSSION

A total of 234 and 400 colonies⁄450 g of filamentous fungi representing 67 species belonging to 20 genera were identified from 30 samples on Czapek's agar at 7 and 28±2ºC (Table 1). Member of *Mucor*, *Penicillium* and *Aspergillus* were the most common fungi. Eight species were new records in Yemen and there are: *Absidia glauca, Cochliobolus geniculata, Mucor fuscus, M. strictus, P.canescens, P. caseicolum, P. raistricki* and *Phoma exigua.* In this respect, Ismail et al. [9] examined fungal contamination of beef carcasses and could isolate 34 fungal genera, represented by 62 species and one variety of which *Aspergillus, Cladosporium* and *Penicillium* were recovered in high incidences.

Also, Farghaly et al. [35] studied the contamination of meat stored in home refrigerators and eleven mould genera could be identified and the most common genera were *Aspergillus, Penicillium*

and *Cladosporium*. Sørensen et al. [36] studied the mycobiota in the processing areas of two different meat products. The diversity of filamentous fungi in the processing areas was high. The main isolated genera were identified as *Aspergillus*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Eurotium*, *Penicillium*, *Phaeoacremonium* and *Phoma*.

Recently, Omorodion and Odu [37] analyzed three different meat samples namely; beef, chicken and pork obtained from Creek road market, Mile 3 market and Rumokoro market for their microbiological quality using differential, selective and routine media. Thirteen fungal isolates covering three genera were isolated and characterized as *Aspergillus* spp., *Mucor* spp. and *Penicillium* spp.

In the current study, *Mucor* was the first common fungus, isolated in high frequency at both incubation temperatures. It was occurred in 63% and 60% of the samples constituting 33.8% and 19.8% of total filamentous fungi, respectively. Of 5 species identified *M. circinelloides* was the most prevalent, emerging in 47% and 53% of sample having 23.5% and 18% of total fungi, respectively. *M. hiemalis* was isolated in low occurrence at 7ºC (17%) and rare at 28ºC (7% of the samples). The remaining *Mucor* species were isolated only at 7ºC in rare frequency of occurrence (Table 1). These results were greatly similar with those obtained by Mizakova et al. [13]. They studied the presence of various moulds in five kinds of fermented raw meat products and noticed that *Mucor* sp. were the most frequently isolated genus. Also, Omorodion and Odu [37] and Asefa et al. [38] reported that *Mucor* spp. were the among most prevalent genera isolated from different meat products.

Aspergillus was the second predominant genus isolated in high frequency at 28ºC and moderate occurrence at 7ºC comprising 50.5% and 13.2% of total fungi, respectively. Twenty species were identified of which *A. flavus, A. foetidus, A. fumigatus, A. niger* and *A. terreus* were the most common especially at 28ºC. They occurred in moderate or low occurrence at both temperatures. The remaining *Aspergillus* species were isolated in rare frequency of occurrence at one temperature and while missing at the other (Table 1). Pal and Bagi [39] investigated the occurrence of fungi in various lymph nodes of domestic buffaloes and isolated *A. fumigates*, *A. flavus*, *A. niger* and *A. terreus.* Ismail et al. [9] reported that *Aspergillus* was represented by 13 species and one variety of which *A. flavus* and *A. niger* were of moderate incidences on beef carcasses, while *A. alutaceus, A. fumigatus, A. sydowii, A. terreus* and *A. versicolor* were rare. Robert et al. [40] stated that the most important fungi on meat were: *A. versicolor, A. niger, A. flavus, A. restrictus* and *Eurotium* spp.

Penicillium (15 species) occupied the third common fungus isolated in high frequency at 7ºC and in moderate occurrence at 28ºC. The genus was identified from 53% and 37% of the samples contributing 26.1% and 7.3% of total fungi, respectively. However all *Penicillium* species were isolated in rare frequency except *P. chrysogenum* that was isolated in low occurrence at 7ºC. Also, counts of *Penicillium* were higher encountered at low temperature (Table 1). Robert et al. [40] noticed that the most important penicillia on meat were: *P. commune, P. crustosum, P. aurantiogriseum, P. chrysogenum. P. brevicompactum. P. nalgiovense. P. verrucosum. P. glabrum. P. variabile, P. roqueforti.* Laich et al. [12] found that some of the fungi most frequently isolated from fermented and cured meat products such as *Penicillium chrysogenum*. Some genera were isolated in low occurrence on one temperature and rare or absent on the other such as *Alternaria* (6 samples and 2 samples); *Cladosporium* (4 and 0); *Paecilomyces* (2 and 6) and *Trichoderma* (0 and 7), respectively. Iacumin et al. [18] investigated the presence of ochratoxin producing fungi on the surface of sausages from northern Italy and revealed that the most frequently species were *Penicillium nalgiovense, P. oxalicum, P. olsonii, P. chrysogenum, P. verrucosum, P. viridicatum*, *Eurotium amstelodami* and *Eupenicillium crustaceum.* Sonjak et al. [19] found that, the predominant filamentous fungal genera isolated were *Penicillium. Eurotium* spp., *Aspergillus versicolor* and *Cladosporium* spp. were isolated from meat products. Eight *Penicillium* species were identified of which *Penicillium nordicum* was recovered frequently while other penicillia were recovered less frequently.

Also, other genera were isolated in rare frequency and these were *Absidia, Cochliobolus, Emericella* (each represented by 2 spp.), *Actinomucor, Cephaliophora, Fusarium, Geotrichum, Phoma, Rhizomucor, Rhizopus, Scopulariopsis,*

Syncephalastrum, *Trichoderma*, *Tricothecsium* (1 sp. each) and sterile mycelia. Ismail and Zaky [11] found that the most frequently encountered fungi from luncheon meat were: *Aspergillus niger*, *A. flavus*, *Penicillium chrysogenum*, *Rhizopus stolonifer*, *Mucor circinelloides*, whereas *Cladosporium sphaerospermum*, *Alternaria alternata*, *Mycosphaerella tassiana*, *P. aurantiogriseum* and *P. oxalicum* were less common. Youssef et al. [41] noticed that *Aspergillus, Penicillium, Cladosporium, Mucor, Scopulariopsis, Candida* and *Rhodotorula* were the most common fungal genera contaminating ground beef. On the other hands, some species were isolated at 7ºC but not at 28ºC and vice versa (Table 1).

Table 1. Total counts (TC, calculated/450 grams in all samples), number of cases of isolation (NCI, out of 30 samples) and occurrence remarks (OR) of fungal genera and species recovered from fresh beef meat on Czapek's agar at 7 and 28±2ºC.

Genera & species	7 ± 2ºC		28 ± 2ºC	
	TC	NCI & OR	TC	NCI & OR
Absidia	**3**	**2R**	**6**	**2R**
A. corymbifera	**3**	**2R**	**1**	**1R**
A. glauca	**0**	**0**	**5**	**1R**
Actinomucor elegans	**0**	**0**	**2**	**1R**
Alternaria	**19**	**6L**	**3**	**2R**
A. alternata	**15**	**4L**	**3**	**2R**
A. chlamydospora	**4**	**2R**	**0**	**0**
Aspergillus	**31**	**9M**	**202**	**27H**
A. aculeatus	**0**	**0**	**4**	**3R**
A. awamori	**0**	**0**	**2**	**2R**
A. candidus	**3**	**3R**	**2**	**1R**
A. cervinus	**0**	**0**	**1**	**1R**
A. flavipes	**0**	**0**	**1**	**1R**
A. flavus	**5**	**3R**	**23**	**13M**
A. foetidus	**1**	**1R**	**35**	**8L**
A. fumigatus	**0**	**0**	**22**	**8L**
A. japonicas	**0**	**0**	**1**	**1R**
A. niger	**5**	**4L**	**37**	**10M**
A. ochraceus	**0**	**0**	**1**	**1R**
A. oryzae	**0**	**0**	**9**	**5L**
A. parasiticus	**0**	**0**	**7**	**3R**
A. sulphureus	**0**	**0**	**3**	**1R**
A. sydowii	**11**	**1R**	**0**	**0**
A. tamarii	**0**	**0**	**16**	**4L**
A. terreus	**0**	**0**	**27**	**9M**
A. tubingensis	**0**	**0**	**8**	**5L**
A. versicolor	**0**	**0**	**3**	**1R**
A. wentii	**6**	**1R**	**0**	**0**
Cephaliophora tropica	**0**	**0**	**1**	**1R**
Cladosporium	7	4L	0	0
C. cladosporioides	1	1R	0	0

Genera & species	7 ± 2ºC		28 ± 2ºC	
	TC	NCI & OR	TC	NCI & OR
C. herbarum	4	2R	0	0
C. macrocarpum	2	2R	0	0
Cochliobolus	6	3R	0	0
C. geniculatus	2	2R	0	0
C. ovoidea	4	1R	0	0
Emericella	0	0	4	3R
E. nidulans	0	0	3	2R
E. violacea	0	0	1	1R
Fusarium	3	2R	6	1R
F. oxysporum	2	1R	0	0
F. poae	1	1R	6	1R
Geotrichum candidum	0	0	6	2R
Mucor	79	19H	79	18H
M. circinelloides	55	14M	72	16H
M. fuscus	0	0	3	1R
M. hiemalis	15	5L	4	2R
M. strictus	7	1R	0	0
M. racemosus	2	1R	0	0
Paecilomyces	8	2R	18	6L
P. lilacinus	0	0	3	2R
P. variotii	8	2R	15	4L
Penicillium	61	16H	29	11M
P. aurantiovirens	1	1R	0	0
P. brevicompactum	11	1R	0	0
P. canescens	0	0	1	1R
P. caseicolum	1	1R	1	1R
P. chrysogenum	9	4L	1	1R
P. citrinum	10	2R	1	1R
P. corylophilum	3	2R	6	2R
P. expansum	1	1R	0	0
P. glabrum	2	1R	2	1R
P. jenseni	5	2R	5	3R
P. megasporum	0	0	1	1R
P. oxalicum	2	1R	0	0
P. raistricki	0	0	4	1R
P. purpurogenum	0	0	6	2R
P. steckii	16	3R	1	1R
Phoma	12	2R	4	1R
P. exigua	5	1R	0	0
P. glomerata	3	1R	0	0
P. herbarum	4	2R	4	1R
Rhizomucor pusillus	0	0	8	2R

Genera & species	7 ± 2ºC		28 ± 2ºC	
	TC	NCI & OR	TC	NCI & OR
Rhizopus stolonifer	1	1R	5	2R
Scopulariopsis candida	0	0	1	1R
Syncephalastrum racemosum	0	0	3	1R
Trichoderma hamatum	0	0	23	7L
Trichothecium roseum	0	0	2	1R
Sterile mycelia	4	2R	3	2R
Yeasts	297	26R	158	20H
Total count	234		400	
No. of genera = 20	11		17	
No. of species = 67	37		51	

OR = Occurrence remarks, H = High occurrence 16-30 samples, M = Moderate occurrence, 9-15 samples, L = Low occurrence, 4-8 samples, R = Rare occurrence, 1-3 samples.

The current results are greatly similar with those obtained by Tawakkol and Khafaga [42] who reported that the most commonly isolated fungi from meat were species of *Aspergillus, Penicillium, Candida* and *Rhodotorula* with *Aspergillus niger* was the most common, followed by *A. flavus, A. fumigatus* and *A. terreus. Penicillium chrysogenum, P. expansum, P. oxalicum* and *P. citrinum* were the common *Penicillium* species. Also, species of *Aspergillus, Eurotium, Penicillium, Alternaria, Emericella, Mucor, Cladosporium, Rhizopus, Botrytis, Epicoccum, Phaeacremonium* and *Phoma* were the most common in meat products such as ham [16] dry-cured mea [42], beef luncheon meat [11, 17] and fermented sausage or liver pane [36].

Battilani et al. [43] studied the pollution of dry-cured ham. They found that species from the genera *Aspergillus, Eurotium* and *Penicillium* are most frequently isolated from the surfaces of dry-cured meat products.

The experimental results showed that yeasts were isolated in high frequency of occurrence. They appeared in 87% and 67% of the samples contributing 55.9% and 28.3% of total fungi at 7ºC and 28ºC, respectively (Table 1). Nielsen et al. [44] showed the potential role of yeast in spoilage of five different processed meat products (bacon, ham, salami and two different liver patés) and found that yeasts were isolated, during storage and processing, meat products. However, with high number along the bacon production, but in low numbers during the production of Salami, cooked ham and liver pate, and in the final products, yeasts were detected in low numbers in very few samples.

3.1. Protease enzymes

The ability of common fungal isolates, recovered in the current study for protease enzyme was assessed. The results revealed that most isolates tested were able to produce protease. Among 152 isolates tested, 103 (67.8%) could produce the enzymes. From the positive isolates 1 exhibited high proteolytic, whereas 19 (18.4%) showed moderate production and 83 (80.6%) were weak producers (Table 2).

The high proteolytic isolates were related to *Alternaria alternata*, whereas the moderate isolates were related to *Aspergillus flavus, A. foetidus, A. terreus, Paecilomyces lilacinus, P. variotii, Penicillium brevicompactum, P.caseicolum, P. chrysogenum, P. citrinum, P. corylophilum, P. jenseni, P. steckii* and *Phoma herbarum*. The weak producers are related to *Absidia corymbifera, A. glauca, Alternaria chlamydospora, A. candidus, A. flavipes, A. flavus, A. foetidus, A. fumigatus, A. niger, A. oryzae, A. sulphureus, A. tamarii, A. terreus, A. tubingensis, Cladosporium cladosporioides, C. herbarum, Fusarium oxysporum, Mucor circinelloides, M. fuscus, M. hiemalis, Penicillium brevicompactum, P. caseicolum, P. corylophilum, P. expansum, P. jenseni, P. purpurogenum, P. steckii, Phoma exigua, P. herbarum, Rhizomucor pusillus, Trichoderma hamatum,* and Sterile myce-

lia. Ahmed and Abdel-Sater [45] reported that, among 73 isolates tested for proteolytic activity about 84.9% of the isolates (62 isolates) could produce protease with variable degrees. From the positive strains 30 isolates (48.4%) exhibited high protease production and these were related to *Aspergillus niger, A. flavus, A. terreus,* and *A. sydowii*. Nineteen (30.6%) isolates of the positive ones could produce enzyme moderately including *Fusarium oxysporum, A. niger, Cladosporium* and *Penicillium* species and thirteen (21%) isolates were weak producers.

El-Diasty and Salem [46] studied proteolytic fungi in some milk products and showed that most isolates of *A. flavus, A. niger, Cladosporium* spp. *Mucor* spp. and *Penicillium* have high proteolytic activity. Ghatass et al. [47] assumed that increasing permeability of the cell walls is caused by the same autolytic (enzymatic) and bacterial actions that cause deteriorations, since both give rise to the decomposition of proteins. Also, Djamel et al. [48] studied acid protease production by species of *Penicillium*. Saleem and El-Said [49] screened thirty-one fungal isolates (representing 16 genera, 28 species and 3 varieties) collected from beef luncheon meat for their abilities to produce protease and revealed that 11 isolates (35.48%) exhibited high protease production, 15 isolates (48.39%) had moderate and 5 (16.13%) were low. *Aspergillus flavus, Gibberella fujikuroi* and *Penicillium chrysogenum* were the most active producers.

3.2. Lipase enzymes

The ability of 152 isolates, to produce lipase were determined. The results revealed that most of isolates tested produced lipase enzymes. From the tested isolates 96 isolates (63.2%) could produce the enzyme. From the positive isolates, 3 (3.1%) exhibited high enzyme production, whereas 25 (26.1%) showed moderate production and 68 (70.8%) were weak producers (Table 2).

The results indicated that the high lipolytic producers were related to *Alternaria alternata, Aspergillus niger,* and *Paecilomyces variotii* whereas the moderate were related to *Alternaria alternata, Aspergillus awamori, A. flavus A. foetidus, A. niger, A. tubingensis, A. wentii, Cephaliophora tropica, Cladosporium herbarum, Mucor circinelloides, Pencillium chrysogenum, P. jenseni P. steckii, Phoma exigua, P. herbarum* and *Trichoderma hamatum,* while the remaining species (68 isolates) exhibited weak producers (Table 2). Nasser et al. [50] studied lipase production by 90 fungal isolates from keratinaceous materials and observed that 38% of the isolates produced this enzyme. Among the positive strains 14 isolates exhibited the highest lipase production and these were related to *Aspergillus versicolor, A. wentii, Geotrichum candidum, Penicillium camemberti, P. chrysogenum, P. jensenii, P. roqueforti, P. verrucosum* and *Scopulariopsis brevicaulis.* Twenty-four isolates could produce enzyme with moderate degree and 31 were weak. El-Diasty and Salem [46] studied lipolytic fungi in some milk products found that *Geotrichium* spp. and most isolates of *Candida lipolytica, C. parapasillosis* were lipolytic.

Aravindan et al. [51] reported that the main fungal producers of commercial lipases were *A. niger, A. terreus, A. carneus, C. cylindracea, Mucor miehei, Rhizopus arrhizus, R. delemar, R. japonicus, R. niveus* and *R. oryzae.* Saleem [17] isolated thirty one fungal species and 3 varieties from 30 samples of beef luncheon meat collected from different supermarkets in Qena. Screening of 31 isolates for their abilities to produce lipase showed that, ten isolates showed high production, while sixteen isolates were moderate and 5 isolates were low. They also found that *Aspergillus niger, Fusarium oxysporum* and *Nectria haematococca* were the highest lipase producers. Griebeler et al. [52] noticed that among 24 fungal isolates, 5 were good lipase producers and these were related to *Penicillium* and *Aspergillus* genera. Nwuche and Ogbonna [53] showed that the highest lipase producing strains belong to *Trichoderma* while the lowest was *Mucor* sp. The lipase activity of the *Aspergillus* species was high but varied significantly among the isolates which probably were different species of *Aspergillus.*

Rajendra [54] found that lipase production by seed-borne fungi was high in *Penicillium notatum* followed by *Fusarium equiseti* as compared to other fungi. While, *Curvularia lunata* and *C. pellescens* showed no lipase activity. Similar results were obtained by numerous workers [55, 56]. Also, similar results were observed by numerous workers [57-63].

Table 2. Protease and lipase production by fungal isolates recovered in the present investigation.

Genera & species	NIT	Protease production				Lipase production			
		NIP	High	Moderate	Weak	NIP	High	Moderate	Weak
Absidia corymbifera	2	2	—	—	2	1	—	—	1
A. glauca	2	1	—	—	1	—	—	—	—
Actinomucor elegans	1	—	—	—	—	—	—	—	—
Alternaria alternata	7	7	1	-	6	6	1	1	4
A. chlamydospora	1	1	—	—	1	1	—	—	1
Aspergillus awamori	1	—	—	—	—	1	—	1	—
A. candidus	2	1	—	—	1	2	—	—	2
A. cervinus	1	—	—	—	—	—	—	—	—
A. flavipes	1	1	-	—	1	1	-	—	1
A. flavus	12	11	-	3	8	7	-	2	5
A. foetidus	7	4	-	1	3	4	-	3	1
A. fumigatus	6	3	-	—	3	5	-	—	5
A. niger	11	1	-	—	1	6	1	4	1
A. oryzae	2	1	-	—	1	1	-	—	1
A. parasiticus	1	-	—	—	—	—	—	—	—
A. sulphureus	1	1	-	—	1	-	—	—	—
A. sydowii	1	-	—	—	—	—	—	—	—
A. tamari	1	1	—	—	1	1	—	—	1
A. terreus	14	14	—	1	13	11	—	—	11
A. tubingensis	2	2	—	—	2	2	—	1	1
A. versicolor	1	-	—	—	—	—	—	—	—
A. wentii	1	1	-	1	—	1	—	1	—
Cephaliophora tropica	1	-	—	—	—	1	—	1	—
Cladosporium cladosporioides	1	1	—	—	1	1	—	—	1
C. herbarum	2	2	-	-	2	2	-	2	-
C. macrocarpum	1	1	-	1	-	1	-	-	1
Cochliobolus geniculate	1	-	-	-	-	-	-	-	-
Emericella nidulans	1	—	—	—	—	1	—	—	1
F. oxysporum	1	1	—	—	1	1	—	—	1
Mucor circinelloides	16	11	—	—	11	7	—	2	5
M. fuscus	3	3	—	—	3	3	—		3
M. hiemalis	5	4	—	—	4	4	—	—	4
Paecilomyces lilacinus	2	2	—	2	—	2	—	—	2
P. variotii	8	1	—	1	—	2	1	—	1
Penicillium brevicompactum	1	1	—	—	1	1	—	—	1
P. caseicolum	1	1	—	—	1	1	—	—	1
P. chrysogenum	2	2	—	2	—	2	—	1	1
P. citrinum	1	1	—	1	—	1	—	—	1
P. corylophilum	2	2	—	1	1	2	—	—	2
P. expansum	1	1	—	—	1	1	—	—	1

Genera & species	NIT	Protease production				Lipase production			
		NIP	High	Moderate	Weak	NIP	High	Moderate	Weak
P. jenseni	5	4	–	3	1	3	–	2	1
P. rubrum	1	1	–	–	1	–	–	–	–
P. steckii	4	4	–	1	3	3	–	1	2
Phoma exigua	3	1	–	–	1	1	–	1	–
P. herbarum	4	4	–	1	3	4	–	1	3
Rhizomucor pusillus	1	1	–	–	1	–	–	–	–
Trichoderma hamatum	4	1	–	–	1	1	–	1	–
Trichothecium roseum	1	–	–	–	–	–	–	–	–
Sterile mycelia	1	1	–	–	1	1	–	–	1
Total isolates	152	103	1	19	83	96	3	25	68

NIT = Number of isolates tested. NIP = Number of isolates positive. H = High activity, 3-2.1 cm for proteolytic, 2.4-1.7 cm for lipolytic. M = Moderate activity, 2-1.1 cm, 1.6-0.8 cm, W = Weak activity, 1-0.1 cm, 0.7-0.1 cm.

In conclusion, because worldwide population growth and globalization of the food supply, the control of meat spoilage becomes essential in order to increase its shelf life and maintain its nutritional value, texture and flavor. Proper handling, pretreatment and preservation techniques can improve the quality of meat and meat products and increase their shelf life. For controlling enzymatic, oxidative and microbial spoilage, low temperature storage and chemical techniques are the most common in the industry today. It is essential to store the meat at lower than 4°C immediately after slaughtering and during transport and storage as it is critical for meat hygiene, safety, shelf life, appearance and eating quality. Although, microbial and enzymatic spoilage can be stopped or minimized at lower temperature.

AUTHOR'S CONTRIBUTION

All authors shared in the experimental designed and assisted in the work, formatting the tables, interpretation of data and in preparation and editing of the manuscript. The final manuscript has been approved by all authors.

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interest regarding the publication of this article.

REFERENCES

1. GIRA. World Meat Facts Book. International Meat Secretariat- GIRA, Geneva, 1997.
2. Gill CO. Microbiological contamination of meat during slaughter and butchering of cattle, sheep and pigs. In: Davies A, Board R, eds. The microbiology of meat and poultry. London: Blackie Academic and Professional, 1998; 118-157.
3. Pietzsch O, Kawerau H. Salmonellen in Schweinesch 5 lachtund Zerlegebetrieben sowie Schweinehackfleisch. Vet Med Hefte. 4. Bundesgesundheitsamt, Berlin, 1984.
4. Bell RG. Distribution and sources of microbial contamination on beef carcasses. J Appl Microobiol. 1997; 82: 292-300.
5. Farber JM, Idziak ES. Attachment of psychrotrophic meat spoilage bacteria to muscle surfaces. J Food Prot. 1984; 47: 92-95.
6. Mukhopadhyay HK, Pillai RM, Pal UK, Ajay Kumar VJ. Microbial quality of fresh chevon and beef in retail outlets of Pondicherry. Tamilnadu J Vet Animal Sci. 2009; 5 (1): 33-36.
7. Draughon FA, Melton CC, Maxedon D. Microbial profiles of country-cured hams age in Stockinettes. Barrier Bags, and Paraff, 1981.
8. Nassar AM, Ismail MA. Psychrotrophic and mesophilic fungi isolated from imported frozen lean meat in Egypt. J Food Safety. 1994; 14(4): 289-295.
9. Ismail MA, Abou-Elala AH, Nassar A, Michail DG. Fungal contamination of beef carcasses and the environment in a slaughterhouse. Food Microbiol. 1995; 12: 441-445.

10. Nunez F, Rodríguez MM, Bermudez ME, Cordoba JJ, Asensio MA. Composition and toxigenic potential of the mould population on dry-cured Iberian ham. Int J Food Microbiol. 1996; 32: 185-197.
11. Ismail MA, Zaky ZM. Evaluation of the mycological status of luncheon meat with special reference to aflatoxigenic moulds and aflatoxin residues. Mycopathol. 1999; 146: 147-154.
12. Laich F, Fierro F, Martín JF. Production of penicillin by fungi growing on food products: identification of a complete penicillin gene cluster in *Penicillium griseofulvum* and a truncated cluster in *Penicillium verrucosum*. Appl Environ Microbiol. 2001; 68(3): 1211-1219.
13. Mižãkovã A, Pipovã M, Turek P. The occurrence of moulds in fermented raw meat products. Czech J Food Sci. 2002; 20: 89-94.
14. Martín A, Juan JC, Felix N, Maria JB, Miguel AA. Contribution of a selected fungal population to proteolysis on dry-cured ham. Int J Food Microbiol. 2004; 94: 55-66.
15. Elmali M, Yaman H. Microbiological quality of raw meat balls: produced and sold in the Eastern of Turkey. Pakistan J Nut. 2005; 4(4): 197-201.
16. Wang X, Ma P, Jiang D, Peng Q, Yang H. The natural microflora of Xuanwei ham and the no-mouldy ham production. J Food Eng. 2006; 77: 103-111.
17. Saleem A. Effect of some food preservatives on the lipolytic activity of beef luncheon fungi. Mycobiol. 2008; 36(3): 167-172.
18. Iacumin L, Chiesa L, Boscolo D, Manzano M, Cantoni C, Orlic S, et al. Moulds and ochratoxin A on surfaces of artisanal and industrial dry sausages. Food Microbiol. 2009; 26: 65-70.
19. Sonjak S, Ličen M, Frisvad JC, Cimerman NG. The mycobiota of three dry-cured meat products from Slovenia. Food Microbiol. 2011; 28(3): 373-376
20. Desnuelle P. In the enzymes III. Boyer PD, ed. Academic Press, New York, 1972: 575-616.
21. Macrae AR. In microbial enzymes and technology. Fogarty WM, ed. Appl Sci London, 1983: 225-250.
22. Tauro P, Kapoor KK, Yadav KS. An introduction to microbiology. 1st edn. New Age International Publisher. New Delhi, India, 1986.
23. Pitt JI, Hocking AD. Fungi and food spoilage. 3rd edn, Springer, London, New York, 2009.
24. Smith NI, Dawson VT. The bacteriostatic action of rose bengal in media used for the plate counts of fungi. Soil Sci. 1944; 58: 467-471.
25. Al-Doory Y. Laboratory medical mycology. Lea Febiger Philadelphia Kimpton Publishers, London, 1980: 357-367.
26. Raper KB, Funnell DJ. The genus *Aspergillus.* Williams and Wilkins, Baltimore, USA, 1965.
27. Booth C. The genus *Fusarium*. Kew, UK., Commonwealth Mycological Institute, 1971.
28. Ellis MB. Dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, England, 1971.
29. Ellis MB. More dematiaceus hyphomycetes, Commonwealth Mycological institute, Kew, Surrey, England, 1976.
30. Pitt JI. The genus *Penicillium* and its telemorphic states. *Eupenicillium* and *Talaromyces*. Academic Press, London, 1979.
31. Moubasher AH. Soil fungi in Qatar and other Arab countries. Scientific and Applied Research Center. University of Qatar, Doha, Qatar, 1993.
32. Domsch KH, Gams W, Anderson T-H. Compendium of soil fungi. 2nd edn, IHW-Verlag, Eching, 2007.
33. Paterson RRM, Bridge PD. Biochemical techniques for filamentous fungi. Int. Mycol. Instit. CAB international, Surrey, 1994; 21.
34. Ullman V, Blasins G. A simple medium for the detection of different lipolytic activity of microorganisms. Zentrabl Bakteriol J Hyg. 1974; 229: 264-267.
35. Farghaly RM, Gherbawy YAMH, Yosef MS. Contamination of meat stored in home refrigerators in Qena (Egypt). Czech Mycol. 2004; 56: 53-62.
36. Sørensen LM, Jacobsen T, Nielsen PV, Frisvad JC, Koch AG. Mycobiota in the processing areas of two different meat products. Int J Food Microbiol. 2008; 124(1): 58-64.
37. Omorodion NJPN, Odu NN. Microbiological quality of meats sold in Port Harcaurt Metropolis, Nigeria. Nat Sci. 2014; 12(2): 58-62.
38. Asefa DT, Kure CF, Gjerde RO, Omer MK, Langsrud S, Nesbakken T, Skaar I. Fungal growth pattern, sources and factors of mould contamination in a dry-cured meat production facility. Int J Food Microbiol. 2010; 140: 131-135.
39. Pal M, Bagi AS. Fungi isolated from lymph nodes of buffaloes. Mycoses. 1989; 32(11): 578-580.
40. Robert AS, Ellen SH, Jens CF, Oli F. Introduction to food-borne fungi. Centraalbureau Voor Schimmel Cultures Baarn Delft, 1995.

41. Youssef BM, Mahrous SR, Aziz NH. Effect of gamma irradiation on aflatoxin B1 production by *Aspergillus flavus* in ground beef stored at 5C. J Food Safety. 1999; 19(4): 231-239.
42. Tawakkol W, Khafaga NI. Fungal contamination of meat and its environment with special reference to the strains producing aflatoxins, ochratoxins, proteinase and lipase enzymes. New Egypt J Microbiol. 2007; 17(2): 1-14.
43. Battilani P, Pietri A, Giorni P, Formenti S, Bertuzzi T, Toscani T, et al. *Penicillium* populations in dry-cured ham manufacturing plants. J Food Prot. 2007; 70: 975-980.
44. Nielsen DS, Jacbsen T, Jesperson L, Koch AG, Arneborg N. Occurrence and growth of yeasts in processed meat products-implications for potential spoilage. Meat Sci. 2008; 80(3): 919-926.
45. Ahmed KE, Abdel-Sater MA. Mycological quality of Laban Rayeb sold in Assiut City. Assiut Vet Med J. 2003; 49(99): 75-77.
46. El-Diasty EM, Salem RM. Incidence of lipolytic and proteolytic fungi in some milk products and their public health significance. J Appl Sci Res. 2007; 3(12): 1684-1688.
47. Ghatass ZF, Soliman MM, Mohamed MM. Dielectric technique for quality control of beef meat in the range 10 kHz - 1 MHz. Am Euras J Sci Res. 2008; 3(1): 62-69.
48. Djamel C, Ali T, Nelly C. Acid protease production by isolated species of *Penicillium*. Eur J Sci Res. 2009; 25(3): 469-477.
49. Saleem A, El-Said AHM. Proteolytic activity of beef luncheon fungi as affected by incorporation of some food preservatives. Acta Microbiol Immunol Hung. 2009; 56(4): 417-426.
50. Nasser AL, El-Shanawany AA, Barakat A. Ecological and physiological studies on fungi associated with camel hairs from Saudi Arabia. 8th Con. 15-17. Fac. Vet. Med. Assiut Univ. Egypt, 1998.
51. Aravindan R, Anbumathi P, Viruthagiri T. Lipase applications in food industry. Ind J Biotechnol. 2007; 6: 141-158.
52. Griebeler N, Polloni A, Remonatto D, Arbter F, Vardanega R, Cechet J, et al. Isolation and screening of lipase-producing fungi with hydrolytic activity. Food Bioprocess Technol. 2011; 4(4): 578-586.
53. Nwuche CO, Ogbonna JC. Isolation of lipase producing fungi from palm oil mill effluent (pome) dump sites at Nsukka. Braz Arch Biol Technol. 2011; 54(1): 113-116.
54. Rajendra BK. Extracellular lipase enzyme production by seed-borne fungi under the influence of physical factors. Int J Biol. 2011; 3(1): 94-100.
55. Shivkumar M, Bhajbhuje MNF. Biodiversity of seed borne microfungal flora in storage on cauliflower *(Brassica oleracea* var. botrytis) from Nagpur region. Int J Life Sci. 2014: 43-47.
56. Gayatri DA, Madhuri V. Seed mycoflora of safflower and its control by using botanicals, bio-agent and fungicides - a review. Int J Appl Biol Pharm Technol. 2014; 5(1): 208-215.
57. Ghosh PK, Saxena RK, Gupta R, Yadav RP, Davidson S. Microbial lipases: production and applications. Sci Prog. 1996; 79: 119-157.
58. Barakat A, Abdel-Sater MA. Preliminary characterization and lipolytic activity of moulds associated with raw butter. Bull Fac Sci Assiut Univ. 1999; 28(1): 112-116.
59. de Maia MMD, de Morais MMC, de Morais Jr MA, Melo EHM, de Filho JLL. Production of extracellular lipase by the phytopathogenic fungus *Fusarium solani* fs1. Revista Microbiol. 1999; 30: 304-309.
60. Colen G, Junqueira BG, Moraes-Santos T. Isolation and screening of alkaline lipase-producing fungi from Brazilian savanna soil. World J Microbiol Biotechnol. 2006; 22(8): 881-885.
61. Hasan S, Ahmad A, Purwar A, Khan N, Kundan R, Gupta G. Production of extracellular enzymes in the entomopathogenic fungus *Verticillium lecanii*. Bioinf. 2013; 9(5): 238-242.
62. Esteves AC, Saraiva M, Correia A, Alves A. Botryosphaeriales fungi produce extracellular enzymes with biotechnological potential. Can J Microbiol. 2014; 60(5): 332-342.
63. Costa-Silva TA, Souza CRF, Oliveira WP, Said S. Characterization and spray drying of lipase produced by endophytic fungus *Cercospora kikuchii*. Braz J Chem Eng. 2014; 31(4): 849-858.

ISSN 2449-8955

European Journal of Biological Research

Research Article

Immunomodulatory and hematological effects induced by diclofenac, ibuprofen or paracetamol toxicity in Swiss albino mice

Soha Gomaa

Immunology and Biotechnology Division, Zoology Department, Faculty of Science, Tanta University, Tanta 31527, Egypt
Tel.: +201091630162; Fax: +20403288501; E-mail: sohassd@science.tanta.edu.eg

Received: 25 August 2017; **Revised submission:** 20 October 2017; **Accepted:** 04 November 2017

DOI: http://dx.doi.org/10.5281/zenodo.1041933

ABSTRACT

Anti-inflammatory drugs (both COX-2 inhibitors and nonselective non-steroidal anti-inflammatory drugs = NSAIDs), paracetamol and opioid agents are associated with potentially different adverse events with varying degrees of efficacy. The present work was conducted to elucidate the haemato-immunological changes in mice when treated with diclofenac (Diclo), ibuprofen (Ibu) and paracetamol (Para). Mice were intraperitoneally administered with Diclo (7.4 mg/kg and 14.8 mg/kg), Ibu (60 mg/kg and 120 mg/kg) or Para (36.7 mg/kg and 73.4 mg/kg) daily for one month against saline-treated mice served as control. Diclo administration (14.8 mg/kg) caused decrease in RBCs count, Hb content and Hct%, depending on dose toxicity, while paracetamol and ibuprofen treatment showed increase in RBCs count, Hb content and Hct%. Additionally, all tested drugs induced activities of IgM and C-reactive protein in serum and caused perturbations in absolute and relative weight of immune related organs. Further, Diclo and Para treatments reduced levels of IgG in dose dependent manner however, Ibu administration enhanced activities of IgG that was reduced with increasing dose of Ibu. And activities of serum complement component C3 was diminished after administration of tested drugs activating alternative complement pathway. The implication of this research is that long use of diclofenac, ibuprofen or paracetamol may cause immunotoxic and hematotoxic effects in mice; and the dose plus the duration of treatment may augment their toxicity probably due to immune modulatory effects. Further studies are needed to assess the relevance between Diclo, Ibu or Para treatment and immunological and hematological perturbations.

Keywords: Immunological and hematological studies; Diclofenac; Ibuprofen; Paracetamol; Toxicity.

1. INTRODUCTION

The main analgesic agents that generally used for the most popular types of pain, include non-steroidal anti-inflammatory drugs (NSAIDs; both traditional non-selective and cyclo-oxygenase (COX)-2 selective agents), paracetamol and opioids [1]. NSAIDs have exhibited excellent efficacy in the control of acute pain producing both anti-inflammatory and analgesic effects [2, 3] by inhibition of prostaglandin synthesis via the COX enzyme that regulates normal physiological turnover of prostaglandin and maintains integrity of gastric

lining and renal homeostasis [4]. The therapeutic anti-inflammatory action of NSAIDs is produced by the inhibition of COX-2, while the potential damaging pernicious effects emerge from inhibition of physiological COX-1 activity [5].

Traditional NSAIDs such as ibuprofen or diclofenac, have been established to inhibit COX enzyme activity [6] resulting in the prevention of synthesis of prostaglandins that interfere pivotal physiological functions, including gastric cytoprotection, maintenance of renal blood flow, and platelet activation [7]. Ibuprofen (Ibu), an over-the-counter (OTC) drug, is one of the most widespread used NSAIDs as an analgesic, antipyretic, and anti-inflammatory drug globally [8, 9]. Although, NSAIDs are commonly considered to have high safety profiles, the frequent and general use of ibuprofen and other NSAIDs is likely to increase the prevalence of their adverse effects. Ibuprofen and other NSAIDs are commonly linked to gastrointestinal (GI) toxicity [10, 11] and alternation in renal function [12, 13]. So, regular toxicological evaluation of NSAIDs becomes essential.

Diclofenac (Diclo) is a widely circulated drug, used in humans and animals for the treatment and management of inflammation, fever and pain associated with disease or injury of domestic livestock and humans, regarding to its anti-inflammatory, analgesic and antipyretic properties, however it has severe pathologic conditions such as peptic ulceration, gastrointestinal bleeding, hepatotoxicity, renal papillary necrosis and renal failure on long-term of the drug administration [14-16]. Anti-inflammatory, antipyretic and analgesic action of Diclo is related to inhibition of prostaglandin synthesis from arachidonic acid by inhibition of cyclooxygenase (COX) [17]. Diclofenac was found to cause pathological changes in kidneys of the vultures leading eventually to the gout [18] and a rare but potentially fetal hepatotoxicity that may be related to reactive metabolites formation [19-21].

Alternative to NSAIDs, paracetamol (Para) is one of the most popular drugs around the world, available without a prescription, especially in childhood [22, 23]. Similar to NSAIDs, Para has a potent antipyretic and analgesic actions but without anti-inflammatory activity and a weak inhibition of prostaglandins synthesis [24, 25]. Also, it has a spectrum of action analogous to that of NSAIDs and mostly resembles the COX-2 selective inhibitors. In spite of its wide use, the mechanism of action of acetaminophen has not been fully elucidated, but it is commonly agreed that it inhibits COX-1 and COX-2 through metabolism by the peroxidase function of these isoenzymes, the possibility exists that it inhibits a so far unidentified form of COX, perhaps COX-3 [26] or there is another mechanism of action that include the effects of both the peripheral (inhibition of COX activity) and central (COX, descending serotoninergic pathways, L-arginine/NO pathway, cannabinoid system) antinociceptive processes as well as the redox mechanism [27] concluding that Para has a multifactorial mechanism of action, which may include the activation of different pain pathways hence the difficulty in clarifying the delicate mechanism of action [28]. Although Para is safe and well tolerated when taken in the usual therapeutic dose, its overdose is fairly common and often linked with hepatic and renal damage in both humans and experimental animals [29, 30].

Due to dearth of information from literature on the adverse effects of Diclofenac sodium, Ibuprofen and Paracetamol on immunological and hematological parameters in mice, therefore the present study was planned with the objective to investigate the immunomodulatory and hematological effects of repeated doses of diclofenac, ibuprofen or paracetamol in mice for one month.

2. MATERIALS AND METHODS

2.1. Experimental animals

Male Swiss albino mice were obtained from Company for Biological Products and Vaccines (VACSERA), Cairo, Egypt. Animals were 4-6 weeks old and weighed between 20-28 g at the beginning of the experiment. They were handled and kept in a specific pathogen-free facility at Faculty of Science, Tanta University in accordance with the ethical guidelines of Egyptian National Research Center, Cairo, Egypt and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All animals were housed under the same environmental conditions for 1 week before experimentation for acclimatization and to

ensure normal growth and behavior. The mice were housed under standard laboratory conditions (temperature 22°C ± 2°C; 12 h light-dark cycle) and kept in plastic cages with free access to the commercial basal food and water.

2.2. Drugs

The tested drugs: diclofenac sodium (Diclo) (each tablet contains 50 mg diclofenac sodium, Novartis Pharma, Cairo, Egypt), ibuprofen (Ibu) (each tablet contains 200 mg ibuprofen, Kahira Pharaceuticals & Chemical Industries Company, Cairo, Egypt), and paracetamol (Para) (each tablet contains 500 mg of active drug, Arab Drug Company, Cairo, Egypt) were purchased from public drug store (Tanta, Egypt). Each tablet was crushed to fine powder and dissolved in saline at appropriate concentrations.

2.3. Experimental design

Mice were divided into seven groups of ten animals each. Group1 was administrated saline (i.p.) as a control group, group 2 and group 3, i.p. inoculated with Diclo (14.8 mg/kg, 5 time less than LD50 and 7.4 mg/kg, 10 time less than LD50 respectively [31], group 4 and group 5, i.p. injected with Ibu (120 mg/kg, 5 time less than LD50 and 60 mg/kg, 10 time less than LD50 correspondingly [32], group 6 and group 7, i.p. injected with Para (73.4 mg/kg, 5 time less than LD50 and 36.7 mg/kg, 10 time less than LD50 separately) [33] daily for a period of one month. At the end of treatment, three mice from each group were euthanized by cervical dislocation at fasting state. Preceding to the scarifying, blood samples were collected from retro-orbital plexus for immunological and hematological analyses.

2.4. Hematological analysis

Blood parameters were proceeded for hematological analysis using a Nihon Kohden automated hematology analyzer (model MEK-6318K, Japan), including red blood cell count (RBC) (10^6/µl), hemoglobin concentrations (Hb g/dl), hematocrit percentage (Hct%) and platelet count (Plt) (10^3/µl).

2.5. Preparation of sera samples

At the end of experiment, three mice from each group were euthanized by cervical dislocation at fasting state. Prior to a euthanizing, blood samples were collected from retro-orbital plexus in plastic test tubes and allowed to stand for 3 h to ensure complete clotting. The clotted blood samples were centrifuged at 3000 rpm for 10 min and the clear sera samples were aspirated off and stored frozen at -80°C for immunological analyses. Evaluation of the different immunological parameters: Complement component C3, C4 and C-reactive protein (CRP). Serum levels of IgG and IgM in exposed mice were performed using enzyme linked immuno-sorbent assay (ELISA) as described by [34, 35] in triplicate for each sample. The manufacturer's instructions for each parameter were strictly followed in the course of the investigations.

2.6. Body weight gain and immune-related organs relative weight

Just before killing after 30 days, final body weight of mice in all experimental groups was recorded. Upon being killed, the spleen, lymph nodes and thymus were removed aseptically, weighed and their relative organ weights (ROW) were calculated according to Aniagu et al. [36] using the following formula:

ROW = [Absolute organ weight (g) / body weight of mice on sacrifice day (g)] × 100.

Percentage weight gains of mice (WG%) were calculated according to Tukmechi et al. [37] using the following formula: WG% = (final body weight - initial body weight) × 100/initial body weight).

2.7. Statistical analysis

The data were expressed as mean ± standard error of the mean (n = 3). Statistical comparisons among prospective groups were analyzed using a one-way analysis of variance (ANOVA) as part of an SPSS software package (v.16.0 for Windows, 2007; SPSS, Inc., Chicago, IL). Statistical significance was determined by a post hoc test followed by Dunnett's multiple comparison tests to compare treatment means versus respective controls. Significant differences are indicated as follows:

*, P < 0.05; **, P < 0.01 for significant and highly significant differences, respectively.

3. RESULTS

The current study was conducted to investigate the adverse effects of daily administration of different pain killers like Diclofenac sodium (Diclo), Ibuprofen (Ibu) and Paracetamol (Para) intraperitoneally for one month on immunological and hematological changes in mice. The present findings indicated that 1-month continuous treatment with Diclo, Ibu and Para has altered the immuno-hematological parameters in the processed mice.

The adverse effects of Diclo, Ibu and Para administration on body weight gain, absolute and relative weight of liver and kidney in the albino mice are indicated in Table 1. The obtained data revealed non-significant decrease in the monitored weight gain in all treatments used in this study, when compared to saline treated group. Further, absolute and relative weight of liver and kidney showed non-significant changes comparing to saline-treated mice.

Table 1. Changes in body weight, percentage of body weight gain, absolute and relative weight of liver and kidney after treatment with Diclo, Ibu or Para.

Treatment	Body wt			Liver		Kidney	
	Initial Body WT	Final Body WT	Body WT gain (%)	Absolute WT	Relative WT	Absolute WT	Relative WT
Control	23.47±2.39	30.11±3.12	28.29±3.79	1.68±0.17	5.63±0.58	0.5±0.06	1.64±0.05
Diclo (7.4 mg/kg)	22.00±0.61	28.47±0.81	29.44±2.61	1.89±0.16	6.33±0.26	0.45±0.02	1.51±0.01
Diclo (14 mg/kg)	20.80±0.12	25.40±0.81	22.10±3.58	1.42±0.10	5.92±0.18	0.38±0.01	1.59±0.15
Ibu (60 mg/kg)	23.23±0.66	28.37±0.55	22.21±2.75	1.40±0.08	5.36±0.21	0.45±0.05	1.73±0.14
Ibu (120 mg/kg)	24.33±1.79	29.23±0.75	21.01±5.93	1.57±0.14	5.35±0.34	0.4±0.02	1.36±0.03
Para (36.7 mg/kg)	23.80±0.81	29.51±0.7	24.42±6.73	1.71±0.08	5.79±0.24	0.41±0.01	1.40±0.02
Para (73.4 mg/kg)	24.47±2.09	30.67±2.37	26.07±7.71	1.61±0.21	5.22±0.44	0.46±0.03	1.49±0.02

Data were represented as mean ± SE (n = 3). *: Statistically significant comparison of control group and other treated groups ($p < 0.05$), **: Highly significant ($p < 0.01$).

Table 2. Changes in absolute and relative weight of spleen, thymus and lymph node of mice after treatment with Diclo, Ibu or Para.

Treatment	Spleen		Thymus		Lymph node	
	Absolute WT	Relative WT	Absolute WT	Relative WT	Absolute WT	Relative WT
Control	0.22±0.04	0.72±0.06	0.05±0.01	0.17±0.02	0.08±0.003	0.26±0.02
Diclo (7.4 mg/kg)	0.27±0.05	0.89±0.14	0.07±0.01	0.25±0.03	0.08±0.003	0.28±0.01
Diclo (14 mg/kg)	0.28±0.05	1.15±0.14**	0.04±0.01	0.17±0.03	0.06±0.006	0.25±0.02
Ibu (60 mg/kg)	0.14±0.02*	0.53±0.09*	0.04±0.01	0.15±0.02	0.06±0.010	0.24±0.04
Ibu (120 mg/kg)	0.13±0.02**	0.43±0.06**	0.04±0.00	0.15±0.01	0.05±0.006	0.17±0.02
Para (36.7 mg/kg)	0.17±0.01	0.57±0.03	0.03±0.00	0.11±0.01	0.04±0.006*	0.14±0.02*
Para (73.4 mg/kg)	0.16±0.05*	0.51±0.13*	0.07±0.02	0.22±0.06	0.05±0.010*	0.15±0.04*

Data were represented as mean ± SE (n = 3). *: Statistically significant comparison of control group and other treated groups ($p < 0.05$), **: Highly significant ($p < 0.01$).

The relative and absolute weights of immune organs (spleen, thymus and lymph node) in albino mice are illustrated in Table 2.

The present finding showed that Diclo (7.4 mg/kg and 14.8 mg/kg) treatments resulted in significant increase in spleen relative weight, whereas there was significant decrease with Ibu (60 mg/kg and 120 mg/kg) and Para (73.4 mg/kg) administration and non-significant decrease with Para (36.7 mg/kg) treatment compared to saline-treated mice. Moreover, Diclo (7.4 mg/kg and 14.8 mg/kg) and Ibu (60 mg/kg and 120 mg/kg) treatments reduced the relative weight of lymph node in dose dependent manner, and Para (36.7 mg/kg and 73.4 mg/kg) treated mice showed significant decrease relative to saline-treated mice. There were no significant differences in the relative and absolute weight of thymus in all treated mice when compared with saline-treated mice.

During the present study, the effects of Diclo, Ibu and Para on RBC and Plt in the Swiss albino mice are shown in figure 1. The results indicated that there is slight increase in RBCs count with Diclo (7.4 mg/kg), Ibu (60 mg/kg and 120 mg/kg) - treated mice and a significant increase with administration of Para (73.4 mg/kg), but no change in RBCs with Para (36.7 mg/kg) against control (Fig. 1-A). In the term of Plt, Ibu (60 mg/kg), Para-treated (36.7 mg/kg and 73.4 mg/kg) mice did not differ significantly from the saline-treated group, however Diclo-inoculated (7.4 mg/kg and 14.8 mg/kg) mice had increased Plt count and Ibu-treated (120 mg/kg) mice showed the highest significant value comparing to control mice (Fig. 1-B).

The results further revealed that significant increase in Hb content with high dose of Para and Ibu-injected mice as compared to saline-treated mice; while low dose of Diclo, Para, Ibu-treated mice indicated minor increase in Hb content. Also, there was slim decrease in Hb content of high dose of Diclo-inoculated mice (Fig. 2-A). Moreover, HCT percentage (Fig. 2-B) displayed a small elevation in Diclo (7.4 mg/kg); Ibu (60 mg/kg and 120 mg/kg) and Para-treated (36.7 mg/kg and 73.4 mg/kg) mice when compared to saline-treated group, even though Diclo-treated (14 mg/kg) mice presented minor decrease in HCT%.

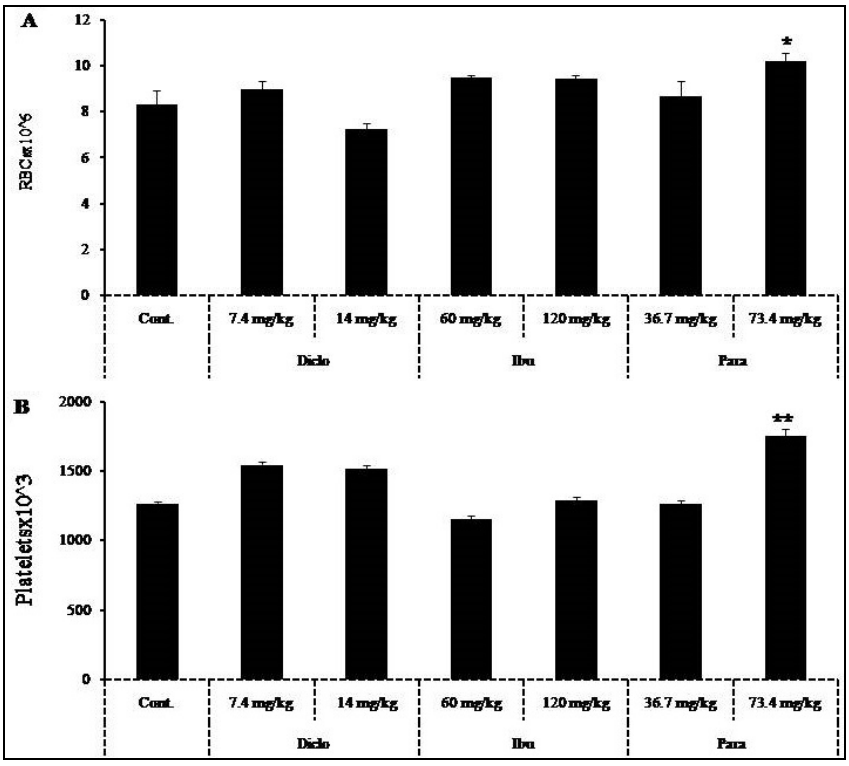

Figure 1. Effect of repeated administration of Diclo, Ibu or Para on RBCs and platelets count. Mice treated with saline (control), Diclo (7.4 mg/kg, 14.8 mg/kg), Ibu (60 mg/kg, 120 mg/kg), Para (36.7 mg/kg, 73.4 mg/kg) intraperitoneally (i.p.) daily for one month. Data were represented as mean ± SE (n = 3). *: Statistically significant comparison of control group and other treated groups ($p < 0.05$), **: Highly significant ($p < 0.01$).

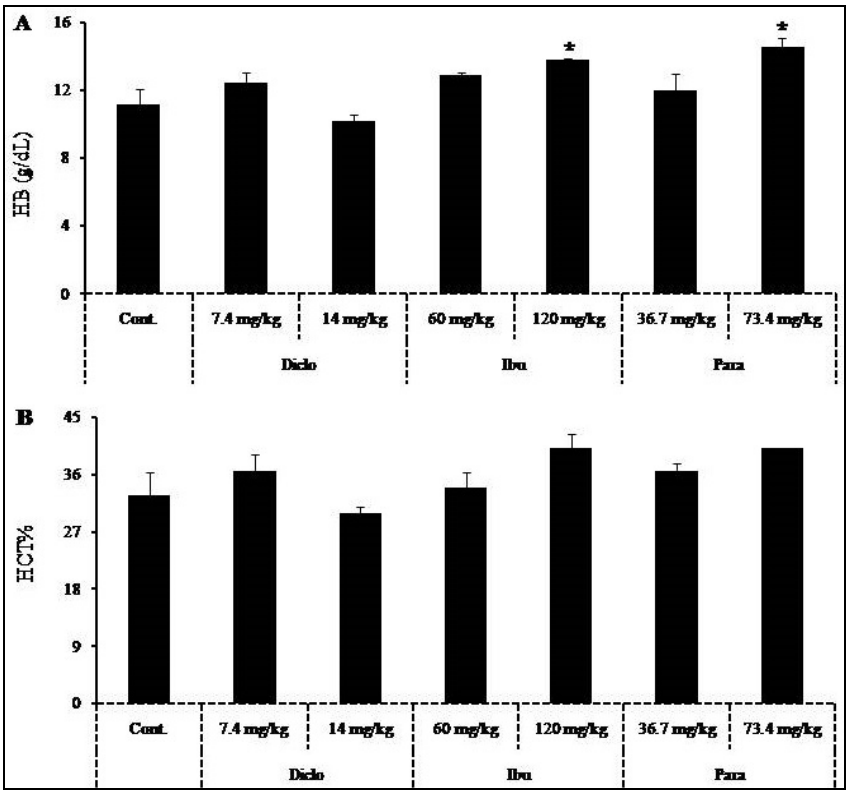

Figure 2. Changes in HB concentration and HCT% after administration of Diclo, Ibu or Para. Mice treated with saline (control), Diclo (7.4 mg/kg, 14.8 mg/kg), Ibu (60 mg/kg, 120 mg/kg), Para (36.7 mg/kg, 73.4 mg/kg) intraperitoneally (i.p.) daily for one month. Data were represented as mean ± SE (n = 3). *: Statistically significant comparison of control group and other treated groups ($p < 0.05$), **: Highly significant ($p < 0.01$).

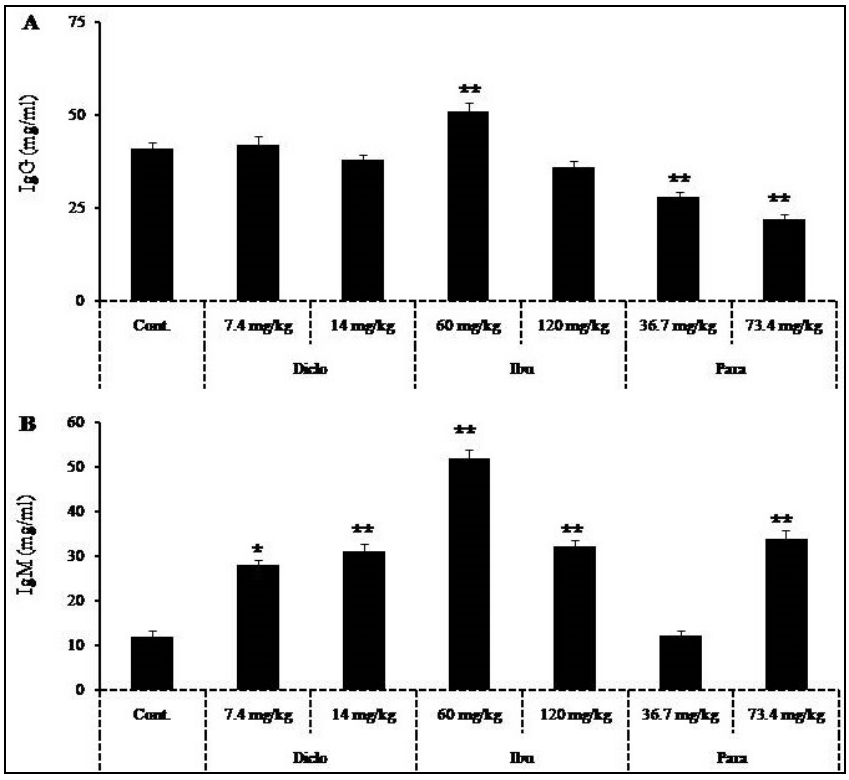

Figure 3. Changes in IgG and IgM concentration in PB after repeated administration of different pain killers. Mice treated with saline (control), Diclo 1 (7.4 mg/kg), Diclo 2 (14.8 mg/kg), Para 1 (36.7 mg/kg), Para 2 (73.4 mg/kg), Ibu 1 (60 mg/kg), Ibu 2 (120 mg/kg) intraperitoneally (i.p.) daily for one month. Data were represented as mean ± SE (n = 3). *: Statistically significant comparison of control group and other treated groups ($p < 0.05$), **: Highly significant ($p < 0.01$).

In figure 3-A, all treatments had reduced IgG concentration in dose dependent manner. Diclo treatment decreased IgG concentration from 42 to 38 (mg/ml), Para treatment from 28 to 22 (mg/ml), and Ibu treatment from 51 to 36 (mg/ml) with increasing the dose of drug compared to control (41 mg/ml). The result more revealed that there was significant increase in IgM level with all treatments compared to saline treated mice (12 mg/ml). Increase in IgM levels was depend on the dose of drug, Diclo treatment increased IgM concentration from 28 to 31 (mg/ml) and administration of Para augmented IgM level from 12.3 to 34 (mg/ml), however, Ibu treatment diminished the concentration from 52 to 32.2 (mg/ml) (Fig. 3-B).

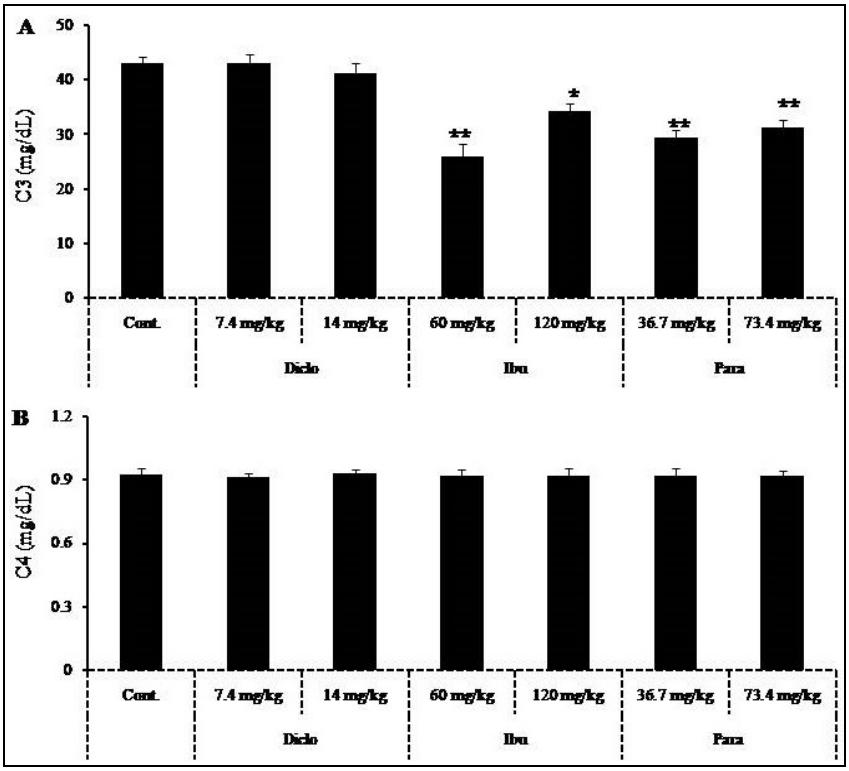

Figure 4. Changes in C3 and C4 concentration in PB after repeated administration of different pain killers. Mice treated with saline (control), Diclo 1 (7.4 mg/kg), Diclo 2 (14.8 mg/kg), Para 1 (36.7 mg/kg), Para 2 (73.4 mg/kg), Ibu 1 (60 mg/kg), Ibu 2 (120 mg/kg) intra-peritoneally (i.p.) daily for one month. Data were represented as mean ± SE (n = 3). *: Statistically significant comparison of control group and other treated groups ($p < 0.05$), **: Highly significant ($p < 0.01$).

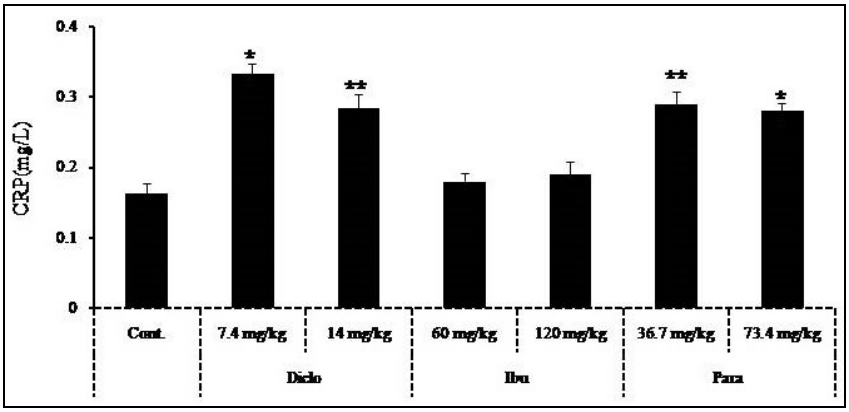

Figure 5. Changes in CRP concentration in PB after repeated administration of different pain killers. Mice treated with saline (control), Diclo 1 (7.4 mg/kg), Diclo 2 (14.8 mg/kg), Para 1 (36.7 mg/kg), Para 2 (73.4 mg/kg), Ibu 1 (60 mg/kg), Ibu 2 (120 mg/kg) intraperitoneally (i.p.) daily for one month. Data were represented as mean ± SE (n = 3). *: Statistically significant comparison of control group and other treated groups ($p < 0.05$), **: Highly significant ($p < 0.01$).

In figure 4-A, Ibu (60 mg/kg and 120 mg/kg) and Para (36.7 mg/kg and 73.4 mg/kg) treatments had significantly reduced the concentration of complement component C3; while administration of Diclo revealed a non-significant decrease in complement C3 levels depending on the dose of Diclo when compared to control mice. Further, all examined treatments did not differ significantly from the control mice in terms of complement C4 (Fig. 4-B). Moreover, Ibu (60 mg/kg and 120 mg/kg) treatments slightly elevated CRP concentration in PB, while Diclo (7.4 mg/kg and 14.8 mg/kg) and Para-treated (36.7 mg/kg and 73.4 mg/kg) mice indicated significant increase in comparison to saline-treated group (Fig. 5).

4. DISCUSSION

NSAIDs are considered as a group of the most abused drugs by mains of combining the pharmacological actions of anti-inflammatory and analgesia, so they can easily be bought over the counter [38]. Alternative to NSAIDs, Para is recommended as a first-line treatment option for mild to moderate chronic pain [39] giving analgesia by raising the pain threshold, chiefly through a central rather than peripheral mechanism [40]. Because NSAIDs and paracetamol are commonly used, we thought it is important to investigate their adverse effects on immunological and hematological parameters.

Alternations in the organ-body weight ratio may be a marker of cell constriction or inflammation and this constriction may occur as a result of lack of fluid from the organ related to damage, however an increase in organ-body weight ratio may refer to inflammation [41]. Further, numerous drugs are supposed to cause immunotoxic effects in humans and animals leading to disorders in the immune system that observed by alternations in immune related organs (spleen and thymus) weight [42]. Current results revealed all tested drugs caused non-significant change in body weight, the relative and absolute weight of liver and kidney, however there were adverse effects on the relative and absolute weight of lymphoid organs (spleen, thymus and lymph nodes). Similar results were speculated by Oyedeji et al. [43] who reported that Para caused non-significant changes in the body weight of rats post treatment for 42 days and analysis of organ weight in toxicological studies is an important endpoint for recognition of potentially deleterious effects of chemicals [44] that may occur in the absence of any morphological changes [45].

The present study showed that Diclo administration (14.8 mg/kg) caused decrease in RBCs count, Hb content and Hct%, despite there was no effect with Diclo at dose of 7.4 mg/kg concluding that it is dependent on dose toxicity. While Para and Ibu treatment showed increase in RBCs count, Hb content and Hct%. There was no change in platelets count with Para administration; however, Ibu and Diclo treatment presented elevation in their count. These results are in line with those of Thanagari et al. [46], El-Maddawy and El-Ashmawy [47] and Orinya et al. [48], who reported that Diclo induced highly significant decrease in Hb, PCV values resulting anemia that may refer to loss of blood during gastrointestinal bleeding that induced by diclofenac sodium. Moreover, chronic use of Ibu could affect hematological functions and time of exposure may promote ibuprofen toxicity depending on dose [49]. In addition, Para overdose causes liver damage based on the dose and this damage caused alterations in the red blood cell count, and packed cell volume [50, 51]. Para has the potential to inhibit erythropoietin release from the kidneys [52] resulting in the reduction in erythrocytes production, Hb concentration and Ht value and this may lead to anaemia. Further, the decrease in hematological parameters caused by Para may be attributed to the hyper-activity of bone marrow leading to the production of red blood cells with impaired integrity that are easily destroyed in the circulation [53].

NSAIDs have immunomodulatory effects by interfering with human T lymphocyte activation, proliferation and cytokine synthesis [54-56] through inhibition of Cox activity. Cox-2 is expressed in activated B lymphocytes that are required for optimal antibody production predicting that NSAID therapy can have reverberations on antibody synthesis [57, 58]. The current data revealed that by the end of treatment, there were significant down-regulated activities of IgG in response to the examined drugs; however, IgM synthesis was enhanced with all tested drugs. In agreement with the present results, Bancos et al. [59] revealed that a panel of commonly used NSAIDs dulls antibody

synthesis in human peripheral blood mononuclear cells (PBMCs) and in purified B cells. Moreover, ibuprofen's ability to diminish antibody production was dependent on concentration- and time and probably occurred via Cox-2 inhibition, as Cox-2 is responsible for ibuprofen-mediated IgG, but not IgM inhibition. In addition, Diclo forms neoantigens with RBCs that may induce the production of autoantibodies and drug-dependent antibodies [60] leading to the production of antibodies against RBCs and/or platelets [61].

Complement proteins are direct contributors in the maintenance of cellular turnover, healing, proliferation, regeneration and tissue integrity [62]. The results obtained herein revealed that Para or Ibu administration reduced levels of complement component C3 not C4 in serum, whereas Diclo treatment had a non-significant decrease in complement C3 levels in dose dependent manner and no effect on C4 level suggesting that the tested drugs activated the alternative complement pathway that relies on C3 not C4 leading to reduction of C3 level in serum. Our findings have been supported by Prohászka et al. [63] and Navratil et al. [64] who reported that hepatocytes changes or damage induced by paracetamol treatment is required for complement activation. In addition, complement components contribute in host tissue injury in several clinical conditions, and they are activated during hepatocytes regeneration for hepatoprotection through activation of C3 that is required for a normal hepatic regenerative response [65]. Further, the alternative complement pathway is activated, and may associate with deleterious reactions contributed to NSAID such as acute tubular injury induced by NSAID leading to acute kidney injury [66]. Moreover, some drugs like NSAIDs may directly stimulate effector mechanisms, such as the complement system by direct modulation of arachidonic acid pathway [67].

In the present study, a marked increase in CRP level was recorded in the sera of mice treated with Diclo, Ibu or Para for one month suggesting that continuous NSAIDs use may revert their effects on CRP levels in serum. These results were similar to those of Tarp et al. [68] who revealed the cyclooxygenase 2-selective NSAID lumiracoxib was associated with a significant increase in the CRP level and NSAIDs use for longer periods of time can lead to severe health problems like mucosal ulceration and inflammation in the lower gastrointestinal (GI) tract [69] that may be associated with elevation in CRP level [70]. Further, Para is recognized to have trifling anti-inflammatory effect and its overdose is linked with inflammation that marked by an increase in the inflammatory cytokines [71, 72].

5. CONCLUSION

From the present study, it is concluded that daily administration of Diclo, Ibu, or Para for one month caused adverse effects on hematological parameters (RBCs, HB contents, HT% and Plts counts), and they caused immunomodulatory effects on levels of IgG and IgM, in addition to perturbations in immune related organs (spleen, bone marrow and lymph node). These drugs also induced an increase in CRP level in serum and enhanced activation of alternative complement system that may contribute to deleterious reactions induced by tested drugs suggesting that continuous use of Diclo, Ibu, or Para may lead to development of haematotoxicity and immunotoxicity. So caution needs to be exercised in these drugs administration, which should be limited to the lowest therapeutic doses, to prevent its harmful effect. Further studies are needed to assess the relationships between administration of Diclo, Ibu or para and immunological and hematological perturbations.

Abbreviations

Para: Paracetamol; Diclo: Diclofenac; Ibu: Ibuprofen; COX: Cyclooxygenase; NSAIDS: non-steroidal anti-inflammatory drugs; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

ETHICS APPROVAL

All animal experimentation protocols were carried out in agreement with the Ethical Principles for Animal Research established by Egyptian National Research Center, Cairo, Egypt.

FUNDING

Not applicable.

TRANSPARENCY DECLARATION

The author declares that she has no competing interests.

REFERENCES

1. Nalamachu S. An overview of pain management: the clinical efficacy and value of treatment. Am J Managed Care. 2013; 19(14): 261-266.
2. Milsom I, Minic M, Dawood MY, Akin MD, Spann J, Niland NF, Squire RA. Comparison of the efficacy and safety of nonprescription doses of naproxen and naproxen sodium with ibuprofen, acetaminophen, and placebo in the treatment of primary dysmenorrhea: a pooled analysis of five studies. Clin Ther. 2002; 24:1384-1400.
3. Simmons DL, Botting RM, Hla T. Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. Pharmacol Rev. 2004; 56: 387-437.
4. Shafi M, Garg UK, Saqib N, Baba OK, Farid BD, Wali A. Haemato-biochemical studies on diclofenac, ibuprofen and nimesulide induced toxicity in broilers. Nat Env Poll Tech. 2012; 11(4): 649-652.
5. Zarghi A, Arfaei S. Selective COX-2 inhibitors: a review of their structure-activity relationships. Iran J Pharmac Res. 2011; 10(4): 655-683.
6. Bushra R, Aslam N. An overview of clinical pharmacology of ibuprofen. Oman Med J. 2010; 25(3): 155-161.
7. Reynolds EF. Aspirin and similar analgesic and anti-inflammatory agents. In: Martindale. The Extra Pharmacopoeia, 28th edn., Pharmaceutical Press: London, 1982: 234-282.
8. Hörl WH. Non-steroidal Antiinflammatory Drugs and the kidney. Pharmaceuticals. 2010; 3: 2291-2321.
9. Bradbury F. How important is the role of the physician in the correct use of a drug? An observational cohort study in general practice. Int J Clin Pract Suppl. 2004; 144: 27-32.
10. Capone ML, Tacconelli S, Di-Francesco L, Sacchetti A, Sciulli MG, Patrignani P. Pharmacodynamic of cyclooxygenase inhibitors in humans. Prostaglandins Other Lipid Mediat. 2007; 82(1-4): 85-94.
11. Traversa G, Walker AM, Ippolito FM, Caffari B, Capurso L, Dezi A, et al. Gastroduodenal toxicity of different nonsteroidal antiinflammatory drugs. Epidemiol. 1995; 6(1): 49-54.
12. Higuchi K, Umegaki E, Watanabe T, Yoda Y, Morita E, Murano M, et al. Present status and strategy of NSAIDs-induced small bowel injury. J Gastroenterol. 2009; 44(9): 879-888.
13. Bennett W, Henrich WL, Stoff JS. The renal effects of nonsteroidal anti-inflammatory drugs: Summary and recommendations. Am J Kidney Dis. 1996; 28: 56-62.
14. Aprioku JS and Uche FI. Renal Effects of non-steroidal antiinflammatory drugs in albino rats. Br J Pharm Res. 2013; 3(3): 314-325.
15. Fries JF. Assessing and understanding patient risk. Scand J Rheumatol. 1992; 92: 21-24.
16. Orinya OA, Adenkola AY, Ogbe RJ. Haematological and biochemical studies on the effect of diclofenac sodium on Wistar *Rattus norvegicus*. Int J Pharm Chem Biol Sci. 2016; 10(5): 2231-2242.
17. Boshra SA, Hussein MA. The protective role of colchicine on diclofenac sodium induced hepatorenal toxicity in albino rats model. Int J Pharm Sci Res. 2014; 5(12): 5136-5144.
18. Ahmad I, Qureshi TA, Khan FA, Mughal SAK, Sadique U, Shah Z, et al. Evaluation of biochemical effects of diclofenac sodium in goats. J Anim Plant Sci. 2012; 22(2): 1-4.
19. Oaks JL and Khan A. Diagnostic investigation of vulture mortality: the anti-inflammatory drug diclofenac is associated with visceral gout. J Ind Vet. 2004; 23: 152-158.
20. Bhogaraju A, Nazeer S, Al-Baghdadi Y, Rahman M, Wrestler F, Patel N. Diclofenac-associated hepatitis. J South Mediterian. 1999; 7: 711-713.
21. Aydin G, Gokcimen A, Oncu M, Clcek E, Karahan N, Golkalp O. Histopathologic changes in liver and renal tissues induced by different doses of diclofenac sodium in rats. Turk J Vet Anim Sci. 2003; 27: 1131-1140.
22. El-Maddawy ZKH, El-Ashmawy IM. Hepato-renal and haematological effects of diclofenac sodium in rats. Global J Pharmacol. 2013; 7(2): 123-132.
23. Anderson MD, Piper SE and Swan GE. Non-steroidal anti-inflammatory drug use in South Africa and possible effects on vultures. S Afr J Anim Sci. 2005; 101: 112-114.
24. Graham GG, Scott KF. Mechanism of action of paracetamol. Am J Ther. 2005; 12(1): 46-55.
25. Graham GG, Davies MJ, Day RO, Mohamudally A, Scott KF. The modern pharmacology of paracetamol: therapeutic actions, mechanism of action, metabolism, toxicity and recent

pharmacological findings. Inflammopharmacology. 2013; 21(3): 201-232.

26. Smith HS. Potential analgesic mechanisms of acetaminophen. Pain Physician. 2009; 12: 269-280
27. Jóźwiak-Bebenista M, Nowak JZ. Paracetamol: mechanism of action, applications and safety concern. Acta Pol Pharm. 2014; 71(1): 11-23.
28. Chiam E, Weinberg L, Bellomo R. Paracetamol: a review with specific focus on the haemodynamic effects of intravenous administration. Heart Lung Vessel. 2015; 7(2): 121-132.
29. McGill MR, Williams CD, Xie Y, Ramachandran A, Jaeschke H. Acetaminophen-induced liver injury in rats and mice: comparison of protein adducts mitochondrial dysfunction, and oxidative stress in the mechanism of toxicity. Toxicol Appl Pharmacol. 2012; 264(3): 387-394.
30. Seham A, Abd E, Begonia M, Morales A, Sara K, Daniel LS, Lucy FD. Localisation of cyclooxygenase-3 in rat central nervous system. Univ Cambridge J Physiol. 2004; 555P: C156.
31. European community regulations. Diclofenac sodium, Safety Data Sheet, 2008.
32. Adams SS, Bough RG, Cliffe EE, Lessel B, Mills RF. Absorption, distribution and toxicity of ibuprofen. Toxicol Appl Pharmacol. 1969; 15(2): 310-330.
33. European community regulations. Acetaminophen Safety Data Sheet, 2008.
34. Arce S, Nawar, HF, Muehlinghaus G, Russell MW, Connell TD. In vitro induction of immunoglobulin A (IgA)-and IgM-secreting plasma blasts by cholera toxin depends on T-cell help and is mediated by CD154 up-regulation and inhibition of gamma interferon synthesis. Infect Immun. 2007; 75(3): 1413-1423.
35. Keggan A, Freer H, Rollins A, Wagner B. Production of seven monoclonal equine immunoglobulins isotyped by multiplex analysis. Vet Immunol Immunopathol. 2013; 153(3): 187-193.
36. Aniagu SO, Nwinyi FC, Akumka DD, Ajoku GA, Dzarma S, Izebe KS, et al. Toxicity studies in rats fed nature cure bitters. Afr J Biotechnol. 2005; 4(1): 72-78.
37. Tukmechi A, Rezaee J, Nejati V, Sheikhzadeh N. Effect of acute and chronic toxicity of paraquat on immune system and growth performance in rainbow trout, *Oncorhynchus mykiss*. Aqua Res. 2014; 45(11): 1737-1743.
38. Gilman A, Goodman L, Gilman A. The Pharmacological basis of therapeutics. 6th edn Macmillian Pub. Co. Inc. New York. 1990.
39. Ripamonti CI, Bandieri E, Roila F. Management of cancer pain: ESMO Clinical Practice Guidelines. Ann Oncol. 2011; 22(6): 69-77.
40. Raffa RB, Stone DJ, Tallarida RJ. Unexpected and pronounced antinociceptive synergy between spinal acetaminophen (paracetamol) and phentolamine. Eur J Pharmacol. 2001; 412(2): R1-2.
41. Moore K, Dalley A, Agur, AMR. Clinically orientated anatomy. 4th edn. Lippincott Williams and Williams, Philadelphia. 1999: 263-271.
42. Descotes J. Pseudo-allergic drug reactions. Clin Res Pract Drug Reg Affairs. 1986; 4(1): 75-84.
43. Oyedeji KO, Bolarinwa AF, Jeniran SS. Effect of paracetamol (acetaminophen) on haematological and reproductive parameters in male albino rats. Res J Pharmacol. 2013; 7(2): 21-25.
44. Nirogi R, Goyal VK, Jana S, Pandey SK, Gothi A. What suits best for organ weight analysis: review of relationship between organ weight and body/brain weight for rodent toxicity studies. Int J Pharm Sci Res. 2014; 5(4): 1525-1532.
45. Bailey SA, Zidell RH, Perry RW. Relationships between organ weight and body/brain weight in the rat: What is the best analytical endpoint? Toxicol Pathol. 2004; 32(4): 448-466.
46. Thanagari BS, Fefar DT, Prajapati KS, Jivani BM, Thakor KB, Patel JH. Haemato-biochemical alterations induced by diclofenac sodium toxicity in Swiss albino mice. Vet World. 2012; 5: 417-419.
47. El-Maddawy ZKH, El-Ashmawy IM. Hepato-renal and haematological effects of diclofenac sodium in rats. Global J Pharmacol. 2013; 7(2): 123-132.
48. Orinya OA, Adenkola, AY, Ogbe RJ. Haematological and biochemical studies on the effect of diclofenac sodium on Wistar *Rattus norvegicus*. Int J Biol Chem Sci. 2016; 10(5): 2231-2242.
49. Aprioku JS, Nwidu LL, Amadi CN. Evaluation of toxicological profile of ibuprofen in Wistar albino rats. Am J Biomed Sci. 2014; 6(1): 32-40.
50. Al-Saady MAJ, Abdul-Latif A, Al-Shemmery HN. Pharmacological effects of diclofenac sodium on some haematological parameters of male rabbits. Med J Babyl. 2011; 8(3): 441-452.
51. Samuel SA, Francis AO, Ayomide O, Onyinyechi UO. Effects of paracetamol-induced liver damage on some hematological parameters: red blood cell (RBC) count, white blood cell (WBC) count, and

packed cell volume (PCV) in wistar rats of either sex. Indo Am J Pharm Res. 2015; 5(7): 2593-2599.

52. Dwivedi V, Mishra J, Shrivastava A. Efficacy study of livartho against paracetamol induced hepatotoxicity in adult Sprague Dawley rats. J Drug Metab Toxicol. 2015; 5: 175-181.
53. Adeneye AA, Ajagbonna OP, Adeleke TI, Bellow SP. Hematological evaluation of methanol seed extract of citrus. J Ethnopharmacol. 2006; 105: 374-379.
54. Inigues M, Punzon C, Fresno M. Induction of cyclooxygenase-2 on activated T lymphocytes: regulation of T cell activation by cyclooxygenase-2 inhibitors. J Immunol. 1999; 163: 111-119.
55. Paccani SR, Boncristiano M, Ulivieri C, D'Elios MM, Del Prete G, Baldari CT. Nonsteroidal anti-inflammatory drugs suppress T-cell activation by inhibiting p38 MAPK induction. J Biol Chem. 2002; 277: 1509-1513.
56. Hartel C, von Puttkamer J, Gallner F, Strunk T, Schultz C. Dose-dependent immunomodulatory effects of acetylsalicylic acid and indomethacin in human whole blood: potential role of cyclooxygenase-2 inhibition. Scand J Immunol. 2004; 60: 412-420.
57. Ryan EP, Pollock SJ, Murant TI, Bernstein SH, Felgar RE, Phipps RP. Activated human B lymphocytes express cyclooxygenase-2 and cyclooxygenase inhibitors attenuate antibody production. J Immunol. 2005; 174: 2619-2626.
58. Bernard MP, Phipps RP. CpG oligodeoxynucleotides induce cyclooxygenase-2 in human B lymphocytes: implications for adjuvant activity and antibody production. Clin Immunol. 2007; 125: 138-148.
59. Bancos S, Bernard MP, Topham, DJ, Phipps RP. Ibuprofen and other widely used non-steroidal anti-inflammatory drugs inhibit antibody production in human cells. Cell Immunol. 2009; 258(1): 18-28.
60. Salama A, Kroll H, Wittmann G, Mueller-Eckhardt C. Diclofenac-induced immune haemolytic anaemia: simultaneous occurrence of red blood cell autoantibodies and drug-dependent antibodies. Brit J Haematol. 1996; 95(4): 640-644.
61. Meyer O, Hoffmann T, Aslan T, Ahrens N, Kiesewetter H, Salama A. Diclofenac-induced antibodies against RBCs and platelets: two case reports and a concise review. Transfusion. 2003; 43(3): 345-349.
62. Rutkowski MJ, Sughrue ME, Kane AJ, Ahn BJ, Fang S, Parsa AT. The complement cascade as a mediator of tissue growth and regeneration. Inflamm Res. 2010; 59(11): 897-905.
63. Prohászka Z, Singh M, Nagy K, Kiss E, Lakos G, Duba J, Füst G. Heat shock protein 70 is a potent activator of the human complement system. Cell Stress Chaperones. 2002; 7(1): 17-22.
64. Navratil JS, Liu CC, Ahearn JM. Apoptosis and autoimmunity. Immunol Res. 2006; 36: 3-12.
65. Markiewski MM, DeAngelis RA, Strey CW, Foukas PG, Gerard C, Gerard N, Lambris JD. The regulation of liver cell survival by complement. J Immunol. 2009; 182(9): 5412-5418.
66. Clark A, Weymann A, Hartman E, Turmelle Y, Carroll M, Thurman JM, et al. Evidence for non-traditional activation of complement factor C3 during murine liver regeneration. Mol Immunol. 2008; 45(11): 3125-3132.
67. Palviainen MJ, Junnikkala S, Raekallio M, Meri S, Vainio O. Activation of complement system in kidney after ketoprofen-induced kidney injury in sheep. Acta Vet Scand. 2015; 57: 15-20.
68. Tarp S, Bartels EM, Bliddal H, Furst DE, Boers M, Danneskiold-Samsøe B, Christensen R. Effect of nonsteroidal antiinflammatory drugs on the C-reactive protein level in rheumatoid arthritis: A meta-analysis of randomized controlled trials. Arthritis Rheum. 2012; 64(11): 3511-3521.
69. Sostres C, Gargallo CJ and Lanas A: Nonsteroidal anti-inflammatory drugs and upper and lower gastrointestinal mucosal damage. Arthritis Res Ther. 2013; 15(3): 3.
70. Tomizawa M, Shinozaki F, Hasegawa R, Shirai Y, Motoyoshi Y, et al. Elevated C-reactive protein level predicts lower gastrointestinal tract bleeding. Biomed Rep. 2016; 4(6): 711-714.
71. Ghosh J, Das J, Manna P, Sil PC. Acetaminophen induced renal injury via oxidative stress and TNF-alpha production: therapeutic potential of arjunolic acid. Toxicology. 2010; 268: 8-18.
72. Jaeschke H, Williams CD, McGill MR, Xie Y, Ramachandran A. Models of drug-induced liver injury for evaluation of phytotherapeutics and other natural products. Food Chem Toxicol. 2013; 55: 279-289.

ISSN 2449-8955

European Journal of Biological Research

Research Article

Isozyme variants in two natural populations of *Lymnaea luteola*

Arvind Kumar Singh*, Naveen Yadav, Gurvachan Singh

Department of Zoology, Banaras Hindu University, Varanasi - 221 005, India
*Corresponding author: Arvind Kumar Singh; E-mail: aksbhu23@rediffmail.com

Received: 02 September 2017; **Revised submission:** 02 November 2017; **Accepted:** 10 November 2017

DOI: http://dx.doi.org/10.5281/zenodo.1045133

ABSTRACT

Lymnaea luteola is a fresh water gastropod snail, inhabiting ponds and lakes of different parts of India. Two populations of *L. luteola* were collected from fresh water ponds of district Varanasi (Uttar Pradesh) and analysed for their isozyme variants of *Xanthine dehydrogenase (Xdh)* and *Aldehyde oxidase (Ao)* enzymes loci. Both enzymes were found to be represented by two distinct loci and each locus of an enzyme showed polymorphic appearance. Based on the electrophoretic variant data, level of heterozygosity was computed for each enzyme locus. Our analysis clearly reveals that *L. luteola* inhabiting in these two ponds have undergone enough genetic differentiation.

Keywords: Isozyme polymorphism; Natural populations; *Lymnaea luteola*.

1. INTRODUCTION

Analyzing genetic polymorphisms of a species is the only way to decipher the level of genetic variation in that species. Measures which have been adopted for this purpose can be computing genetic variation at the level of phenotypic, chromosomal, protein and nucleotide [1-7]. A number of phenotypic features are well defined to be single gene inherited traits that follow Mendelian pattern of inheritance in a large number of sexually breeding organisms. Chromosomal polymorphisms have been used as a tool to measure genetic polymorphisms in Dipteran insects, particularly in *Drosophila*, due to presence of Polytene chromosomes in them [1, 2]. At molecular level, protein and nucleotide polymorphisms have been undertaken to see genetic variation among the different populations of a species [6, 9-11]. Study on isozyme polymorphisms started during 1960s [12, 13] and for the period of thirty years since then a large number of invertebrate and vertebrate species were involved for the perusal of their genetic profile based on allozyme/isozyme polymorphisms. It has been reported that invertebrates show more genetic differentiation than the vertebrates particularly, higher vertebrates [6, 14, 15]. Molluscs, both marine and fresh water have also been the focus of this kind of study [16-19]. The freshwater snails are of immense importance and have a useful status in the pond ecosystem. They are bio-indicators and being saprophytic animals help to clean water bodies as they consume algae, zooplanktons, diatoms and organic waste [20, 21]. They also form food of animals like fishes, birds and mammals even humans.

Lymnaea luteola is a fresh water gastropod mollusc. It is distributed across all the states of India. Its presence is also recorded from other neighboring countries of India [22]. This species is

often found in ponds, lakes and even in temporary water bodies, which may dry up in the summer months. It can withstand even unfavorable conditions by burying itself in the mud [23]. This species has also been reported to exist in water bodies that have a meager salinity [24, 25]. Its existence has fairly been recorded from different parts of Uttar Pradesh, one of the larger states of India. The main objective of our study was to observe allozyme/ isozyme polymorphism in two natural populations of *L. luteola*. To fulfill this aim, specimens were collected from two places of district Varanasi and in gel assay was performed to see whether the two populations differ from each other, on the basis of their enzyme variants. Results obtained in this regard are being presented in this paper.

2. MATERIALS AND METHODS

Allozyme polymorphism was studied in two natural populations of *L. luteola* which were collected from a small pond located in the close vicinity of Swtantrata Bhavan (SB), Banaras Hindu University, Varanasi and another pond situated outside the boundary wall of Diesel Locomotive Works (DLW), Varanasi. The distance between these two ponds was approximately six kilometers and the area in between is inhabited by thickly populated human population. During rains the two ponds do overflow but the organisms inhabiting them (especially molluscs) never come in contact to each other.

Genetic polymorphism in this invertebrate species was assessed by analyzing two enzyme systems i.e. Xdh (xanthine dehydrgenase) and Ao (aldehyde oxidase). For isozyme analysis, a small portion of visceral mass of the animal was homogenized in 50 µl of 20 mM Tris buffer (pH 7.4) and the homogenate was centrifuged at 12000 rpm at 4°C for 10 minutes. The supernatant was equally divided into two aliquots to scrutinize allelic arrangements of two enzyme systems at a time. Supernatant was separated and subjected to 8% native polyacrylamide gel electrophoresis in 25 mM Tris and 250 mM Glycine electrode buffer (pH 8.2) at 100V for 4 hours at 4°C. In-gel staining for a specific enzyme was made by adopting the procedure suggested by Ayala and his coworkers [26]. The locus and allele designations were decided by expression of enzyme bands. A single locus was marked by the appearance of its variants separated by meager distance, whereas, two loci of a gene were seen to be separated by marked distance.

The electrophoretic variants (alleles) of aldehyde oxidase and xanthine dehydrogenase observed in *L. luteola* are shown in Figure 1. A total of 4 enzyme loci (2 for *Xdh* and 2 for *Ao)*, corresponding to these two enzymes were ascertained. Based on the number of different genotypes of the four gene loci, frequency of allozyme variants were computed. By using Hardy-Weinberg equilibrium, the number of expected genotypes for their respective observed genotype was also computed. Chi-square analysis was performed to test the difference between observed and expected values. A significant deviation from expectation ($p<0.05$) indicated that the enzyme locus is under the influence of evolutionary force/s.

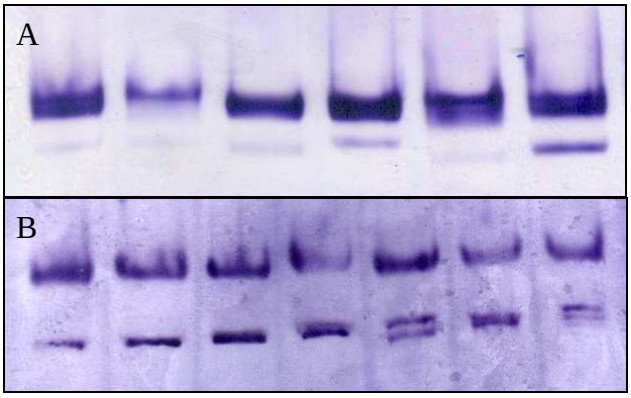

Figure 1. Electrophoretic variants (alleles) of aldehyde oxidase (A) and xanthine dehydrogenase (B) observed in *L. luteola*.

3. RESULTS

The frequency of different enzyme variants (alleles) of four gene loci of *L. luteola* is presented in Table 1. In SB population, the xanthine dehydrogenase *(Xdh)* enzyme was found to be represented by two distinct loci, *Xdh1* and *Xdh2* and each enzyme locus was expressed into two electrophoretic variants. *Xdh1* allele designated as 1.00 was in highest frequency being 0.75 whereas the same allele of *Xdh* 2 was 0.72 in this population. A measure of heterozygosity at its both loci was found to be same (0.38) in this population. Chi square analysis based on the observed and expected

numbers of genotypes of *Xdh1* and *Xdh2* revealed that the two loci are in perfect Hardy-Weinberg equilibrium. The same enzyme observed in DLW population showed the frequency of 0.61 and 0.39 for alleles 1.00 and 1.20 respectively for *Xdh1* whereas 0.45 and 0.55 for alleles 0.98 and 1.00 respectively for *Xdh2 locus*. Hardy-Weinberg equilibrium tested for these two loci revealed that they are in equilibrium.

Table 1. Frequencies of xanthine dehydrogenase (*Xdh*) and aldehyde oxidase (*Ao*) enzyme variants in two natural populations of *Lymnaea luteola.*

Enzyme locus	Alleles	Swtantrata Bhavan (SB)	Diesel Locomotive Works (DLW)
		Number 31	Number 32
Xdh1	1.00	0.75	0.61
	1.20	0.25	0.39
	$\chi 2$	0.00	0.007
Xdh2	0.98	0.28	0.45
	1.00	0.72	0.55
	$\chi 2$	0.167	3.014
		Number 31	Number 34
Ao1	1.00	0.53	0.53
	1.20	0.47	0.47
	$\chi 2$	0.057	1.106
Ao2	0.98	0.24	0.49
	1.00	0.76	0.51
	$\chi 2$	0.403	4.21*

*P<0.01

Aldehyde oxidase (*Ao*) enzyme was also studied for the same purpose and was found to be represented by two distinct polymorphic loci, i.e., *Ao1* and *Ao2* in the two natural populations. Each locus of this enzyme was expressed by two electrophoretic variants. The most common variant of each locus designated as 1.00 was 0.53 and 0.76 in their frequency in SB population. The other variant 1.20 for Ao1 and 0.98 for Ao2 were found to be 0.47 and 0.24 respectively in the same population. A study on Hardy-Weinberg equilibrium in this population for *Xdh* loci indicated that both the loci were in Hardy-Weinberg equilibrium. Aldehyde oxidase (*Ao*) enzyme considered for similar investigation in DLW population revealed that its two loci, *Ao1* and *Ao 2* were polymorphic, *Ao1* represented by variants 1.00 and 1.20 and *Ao2* by 1.00 and 0.98. The frequency of allele 1.00 and 1.20 was found to be 0.53 and 0.47 respectively. In this population another enzyme locus, *Ao2* showed frequency 0.51 and 0.49 for their respective alleles 1.00 and 0.98. Ao2 locus did not show Hardy-Weinberg equilibrium ($p<0.01$) indicating that this locus may be under the effect of some evolutionary forces.

Figure 2 is presented here to depict the frequency of heterozygotes for four gene loci studied in two different natural populations of *L. luteola.* The frequency of heterozygotes is quite high in DLW population (more than fifty percent) for Ao1 and the same enzyme was also found to be in higher heterozygosity in SB population. Overall heterozygosity was recorded to be more than thirty percent for all the loci examined. Although the two populations are completely different and exist as allopatric populations but exhibit similar pattern of evolutionary alterations depicting that similar ecological condition prevail in the area.

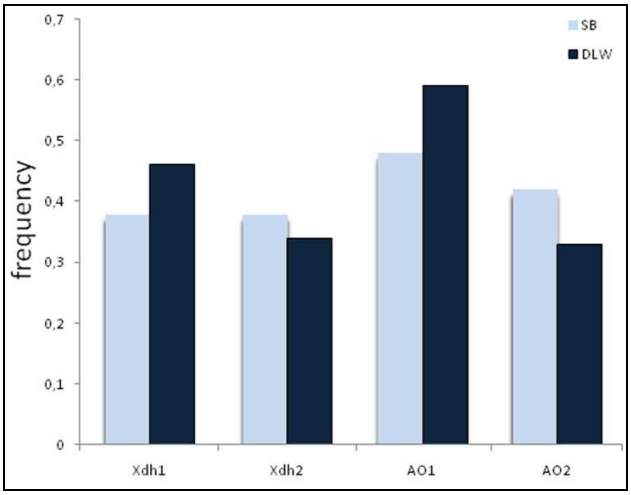

Figure 2. Bar diagram showing frequency of heterozygotes for four gene loci studied in two different natural populations of *L. luteola.*

4. DISCUSSION

The main identifying features of Lymnaeid snails are based on traits like shell morphology, structural peculiarity of radula, characteristics of renal and reproductive organs. The genus *Lymnaea*

Lamarck, includes some freshwater snails that harbours the larval stages of liver-fluke, *Fasciola hepatica*, a helminth parasite which causes fascioliasis in grazing animals and humans. Allozyme polymorphism has been studies in land snails and the significance of such studies have been used for the conservation of snails [27, 28]. Genetic variation in *Lymnaea luteola* can be studied only by following both protein or nucleotide polymorphisms and the results of such studies can be extrapolated to know genetic profile of a species. Carvalho et al. adopted polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) techniques to genetically characterize *Lymnaea columella*, *L. viatrix*, and *L. diaphana* collected from Brazil, Argentina, and Uruguay [29].

Ao and Xdh are well studied enzymes for their polymorphic status in a number of organisms particularly in different species of *Drosophila* [6, 7]. Such studies have not been undertaken in fresh water gastropods, especially in genus *Lymnaea*, from the perspective of Indian regions. We found abundant occurrence of *L. luteola* in two ponds of southern end of Varanasi City and decided to see isozyme variations in the individual of these two separate populations. Isozyme analysis clearly reveals that these two populations are genetically differentiated from each other. Since both the enzymes were represented by two loci and were polymorphic in appearance, the allelic frequencies were computed based on their genotypic frequencies and then a comparative analysis was made. A comparison made on level of heterozygosity for all the four loci studied, indicated variation between the two populations giving an idea that the two populations are genetically different from each other.

L. luteola and other species of this genus are mainly hermaphrodite mollusk species and exhibit self as well as cross fertilization [30]. Since high level of heterozygosity has been observed in both the natural populations of this species, the present study is a testimony to explain that this can happen only if individuals opt to cross fertilization. To maintain genetic heterogeneity is of prime significance to every sexually reproducing species, because species with substantial genetic variation can be better thriving in changing environmental conditions. All the four enzyme loci in the present case, in both populations show more than thirty percent heterozygosity, indicating that during breeding two individuals with varying genetic constitution get involve in reproduction.

Animal species which are migratory in nature get mixed with neighboring populations and as a result of it little genetic differences are expected to exist among the neighboring populations. Thus migration results into gene flow among the populations and consequently no substantial genetic differences can be recorded between the adjacent populations. Gastropod mollusks which remain confined in local ponds do not find it possible to get merged with other populations of neighboring water bodies until they are assisted by some other animal and therefore remain intact as a single population. Gene flow in such gastropods does not occur at all and thus their populations remain as allopatric populations. Fresh water mollusks are therefore expected to be represented by more number of species than those where substantial gene flow do occur. We could witness the existence of more than one species of snails in a single pond indicating that gastropods can be one of the best examples of sympatric speciation.

AUTHORS' CONTRIBUTION

AKS: Manuscript writing and statistical calculation; NY: Conducted experiments and literature survey; GS: Designed and conducted experiments. The final manuscript has been approved by all authors.

ACKNOWLEDGEMENT

The present work has been the part of M. Sc. dissertation of Mr. Naveen Yadav, which could be accomplished by the financial assistance provided by Centre of Advance Study, Department of Zoology, Banaras Hindu University, Varanasi.

TRANSPARENCY DECLARATION

The authors declare that they have no conflict of interest.

REFERENCES

1. Singh AK. Chromosomal polymorphism in natural populations of *Drosophila ananassae* from

Sultanpur, Uttar Pradesh. J Exp Zool. 2000; 3: 93-96.

2. Singh AK, Kumar S, Ratnam D. Genetic differentiation in natural populations and their mass culture stocks of *Drosophila ananassae*. Thai J Genetics. 2014; 7: 123-132.
3. Kumar S, Singh AK. Electrophoretic variants of xanthine dehydrogenase enzyme in natural populations of *Drosophila ananassae.* Dros Inf Serv. 2012; 95: 18-20.
4. Kumar S, Singh AK. Complete absence of linkage disequilibrium between enzyme loci in natural populations of *Drosophila ananassae.* Genetika. 2014; 46: 227-234.
5. Kumar S, Singh AK. Latitudinal clines of allozymes in Indian natural populations of *Drosophila ananassae.* Dros Inf Serv. 2014; 97: 63-67.
6. Kumar S, Singh AK. Allozyme polymorphism in *Drosophila.* Proc Zool Soc. 2016; 69: 22-31.
7. Kumar S, Singh AK. Population genetics of *Drosophhila*: genetic variation and differentiation among Indian natural populations of *Drosophila ananassae.* Zool Stud. 2017; 56: 1-10.
8. Singh G, Singh AK. Electrophoretic variants of xanthine dehydrogense enzyme in *Drosophila malerkotliana.* Dros Inf Serv. 2016; 99: 35-36.
9. Krishnamoorti K, Singh AK. Fitness differences due to allelic variation at esterase-4 locus in *Drosophila ananassae*. J Genet. 2017; 96: 625-631.
10. Presgraves DC. The molecular evolutionary basis of species formation. Nat Rev Genet. 2010; 11: 175-180.
11. Singh AK, Kumar S, Singh N. Detecting level of genetic differentiation in two closely related species of *Drosophila*: *D. bipectinata* and *D. malerkotliana*. Genetika. 2016; 48: 963-970.
12. Harris H. Enzyme polymorphism in man. Proc Roy Soc Lond B Biol Sci. 1966; 164, 298-310.
13. Lewontin, RC, Hubby JL. A molecular approach to the study of genic heterozygosity in natural populations, II. Amount of variation and degree of heterozygosity in natural populations of *Drosophila pseudoobscura.* Genetics. 1966; 54: 595-609.
14. Harris H, Hopkinson DA. Handbook of enzyme electrophoresis in human genetics. North Holland publishing Co. Amsterdam. 1976.
15. Pinsker W, Sperlich D. Allozyme variation in natural populations of *Drosophila subobscura* along a North-South gradient. Genetica. 1979; 50: 207-219.
16. Arnalud JF, Madec L, Guiller A, Bellido A. Spatial analysis of allozyme and microsatellite DNA polymorphisms in the land snail *Helix aspersa* (Gastropoda: Helicidae). Mol Ecol. 2001; 10(6): 1563-1576.
17. Davison A, Chiba S, Barton HN, Clarke B. Speciation and gene flow between snails of opposite chirality. PLOS Biol. 2005; 3: 1559-1571.
18. Janson K. Allozyme and shell variation in two marine snails *(Littorinu*, Prosobranchia) with different dispersal abilities. Biol J Linnean Soc. 1987; 11: 245-256.
19. Levan G, Fredga K. Isozyme polymorphism in three species of land snails. Hereditas. 1972; 71: 245-252.
20. Das S, Khangarot BS. Bioaccumulation of copper and toxic effects on feeding, growth, fecundity and development of pond snail *Lymnaea luteola L*. J Hazard Mater. 2011; 185: 295-303.
21. Das S, Khangarot BS. Bioaccumulation and toxic effects of cadmium on feeding and growth of an Indian pond snail *Lymnaea luteola L*. under laboratory conditions. J Hazard Mater. 2010; 182: 763-770.
22. Kakar S, Kashif K, Essote SA, Asim I, Muhammad A. Species diversity of freshwater snails (Mollusca: Gastropoda) in different sites of Balochistan province of Pakistan. Int J Biosci. 2017; 10: 251-259.
23. Gittenberger E. Sympatric speciation in snail: a largely neglected model. Evolution. 1988; 42: 826-828.
24. Subba RNV. Freshwater molluscs of India. Zoological Survey of India, Culcutta. 1989.
25. Ramakrishna, Dey A. Handbook on Indian freshwater molluscs. Zoological Survey of India, Kolkata, 2007.
26. Ayala FJ, Powell JR, Tracey ML, Mourao CA, Pérez-Salas S. Enzyme variability in *Drosophila willstoni* group IV. Genetic variation in natural population in *Drosophila willstoni*. Genetics. 1972; 70: 113-139.
27. Triggs SJ, Sherley GH. Allozyme genetic diversity in *Placostylus* land snails and implications for conservation. New Zealand J Zool. 1993; 20(1): 19-33.
28. Jordaens K, Backeljau T, Ondina P, Reise H, Verhagen R. Allozyme homozygosity and phally polymorphism in the land snail *Zonitoides nitidus* (Gastropoda, Pulmonata). J Zool Lond. 1998; 246: 95-104.

29. Carvalho OS, Cardoso PCM, Pollanah ML, Rumi A, Roche A, Berne E, et al. The use of the polymerase chain reaction and restriction fragment length polymorphism technique associated with the classical morphology for characterization of *Lymnaea columella*, *L. viatrix*, and *L. diaphana* (Mollusca: Lymnaeidae) Mem Inst Oswaldo Cruz Rio de Janeiro. 2004; 99: 503-507.
30. de Boer PACM, Jansen RF, ter Maat A. Copulation in the hermaphrodite snail *Lymnaea stagnalis*. Invertebr Reprod Dev. 1996; 30: 167-176.

ISSN 2449-8955

European Journal of Biological Research

Research Article

Virulence genes and antibiotic resistance of *Yersinia enterocolitica* strains isolated from children

Barbara Kot*, Małgorzata Piechota, Kinga Jakubiak

Department of Microbiology, Institute of Biology, Siedlce University of Natural Sciences and Humanities, 12 Bolesława Prusa Str., 08-110 Siedlce, Poland

*Corresponding author: Barbara Kot; Phone: +48 256431339, E-mail: barbara.kot@uph.edu.pl

Received: 03 October 2017; **Revised submission:** 14 November 2017; **Accepted:** 22 November 2017

DOI: http://dx.doi.org/10.5281/zenodo.1064835

ABSTRACT

Yersinia enterocolitica is a foodborne pathogen which is primarily responsible for gastrointestinal infections. The presence of the virulence genes in *Y. enterocolitica* strains isolated from children and antimicrobial resistance was studied in this work. The PCR, biotyping and disc diffusion method were used for analysis of *Y. enterocolitica* strains. Most of *Y. enterocolitica* strains belonged to biotype 4 and all carried *ail*, *myfA* and *ystaA* genes. Most of them also had the plasmid *yadA* gene. These genes were also detected in the strains of biotype 2, while in the two strains of biotype 1A only *myfA* gene was found. The *blaA* gene was present in all the strains of biotype 4 and 2, while *blaB* in the strains of biotype 2 and in some of biotype 4 strains. The presence of β-lactamase genes in *Y. enterocolitica* was not detected in biotype 1A. All strains were resistant to ampicillin, 76.2% and 47.6% were resistant to ticarcillin and piperacillin, respectively. Two strains (9.5%) were resistant to amoxicillin/clavulanic acid and aztreonam, three (14.3%) to chloramphenicol, four (19%) to amikacin and trimethoprim/sulfamethoxazole, six (28.6%) to gentamicin. A few strains of *Y. enterocolitica* were multidrug resistant. The *Y. enterocolitica* strains isolated from the faeces of children suffering from diarrhea carried virulence genes and some of them were resistant to antibiotics used in extra-intestinal yersiniosis treatment.

Keywords: *Yersinia enterocolitica*; Virulence genes; Antibiotic resistance; PCR; Yersiniosis.

1. INTRODUCTION

Yersinia enterocolitica is an important human pathogen with the global distribution and a variety of clinical disorders such as enteritidis, enterocolitis, gastroeneritidis, mesenteric lymphadenitis and others [1]. Yersiniosis is a zoonotic foodborne bacterial disease with high public health relevance. In Europe it is the third most common bacterial enteric disease after campylobacteriosis and salmonellosis [2]. Animals such as pigs, rodents, sheep, goats, cattle, horses are reservoirs of *Y. enterocolitica*. Pigs are a major reservoir for human pathogenic strains, especially for bioserotype 4/O3 [3]. This microorganism is considered an important foodborne pathogen including strains of diverse pathogenicity. Infections are most often acquired through ingestion of contaminated pork, milk, dairy foods, vegetables and contaminated drinking water or pet animal contact [4, 5]. The pathogenic *Y. enterocolitica* strains were also isolated from waste water samples in Turkey [6] or from river water in Poland [7]. *Y. enterocolitica* is rarely transmitted

through contaminated blood during transfusion [8]. The species *Y. enterocolitica* is divided into six biotypes. Strains of biotype 1A are generally regarded as nonpathogenic, whereas strains of biotypes 1B, 2, 3, 4, and 5 carry a virulence plasmid pYV. This plasmid encodes type III secretion system and the outer membrane protein YadA (*Yersinia* adhesin A). YadA was found to play multiple functions in pathogenesis because it protects bacterial cells against antibacterial activity of complement and mediates specific binding of *Y. enterocolitica* to laminin, collagen and cellular fibronectin [9]. The chromosomal *Y. enterocolitica* virulence markers are *ail*, *ystA* and *myfA* genes. The *ail* gene encodes a small outer membrane protein (Ail adhesin), which promotes adhesion of *Y. enterocolitica* and invasion of epithelial cells. The *ystA* gene encodes enterotoxin YstA, which activates the guanylate cyclase that leads to the increased cGMP level. High level of cGMP causes fluid accumulation in the intestine [10]. The major subunit of antigen Myf is encoded by the *myfA* gene. This fibrillar structure promotes the colonization of the intestine by yersiniae [11]. Biotyping is used for clinical and epidemiological classification of *Y. enterocolitica*, but the heterogenous nature of *Y. enterocolitica*, including differences in virulence, requires genotyping methods and this may be a novel way of pathogenic characterization of this microorganism.

The aim of this study was the description of *Y. enterocolitica* strains isolated from the faeces of children suffering from diarrhea by using PCR assays for the detection of some virulence genes and *in vitro* evaluation of antibiotic sensitivity of this pathogen. The presence of genes coding β-lactamases was also detected in the genome of *Y. enterocolitica* strains.

2. MATERIALS AND METHODS

2.1. Strains

Twenty one *Y. enterocolitica* strains were isolated from the faeces of children suffering from diarrhea. The strains were isolated from children treated in different hospitals and outpatients in Warsaw (Poland) over the period 2009-2015. The identification of the strains was performed with the VITEK GNI card system (VITEK 2 instrument, version 4.01, bioMérieux). Biotyping of *Y. enterocolitica* strains was performed according to Wauters et al. [12]. The strains were stored at -70°C in Brain Heart Infusion (BHI) Broth (BHI; BBL, Becton Dickinson) containing 15% glycerol.

2.2. Antibiotic susceptibility testing

The susceptibility of the strains was tested with a disc diffusion method using the following antibiotic discs (Oxoid, Basingstoke, UK): ampicillin (25 µg), amoxicillin/clavulanic acid (20/10 µg), cefepime (30 µg), cefotaxime (30 µg), cefuroxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), gentamicin (10 µg), imipenem (10 µg), norfloxacin (10 µg), piperacillin (100 µg), ticarcillin (75 µg), tobramycin (10 µg), aztreonam (30 µg), ciprofloxacin (5 µg), amikacin (30 µg), chloramphenicol (30 µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg). The results were recorded by measuring the inhibition zones and scored as susceptible, intermediately susceptible, and resistant, according to the Clinical and Laboratory Standards Institute [13].

2.3. DNA isolation

Genomic DNA was isolated from *Y. enterocolitica* strains by using the Genomic DNA PrepPlus (A&A Biotechnology, Poland), according to the manufacturer's protocol. 2.5 µl of the total extracted material from each test sample was used as a template DNA for PCR application.

2.4. Primers and PCR conditions

The primers specific for the *ail*, *ystA*, *myfA*, *yadA*, *blaA*, *blaB* and 16S rRNA genes of *Y. enterocolitica*, synthesized at DNA-Gdańsk (Gdańsk, Poland), are listed in Table 1. The duplex PCR for *ail* and *ystaA* genes was performed in a 25-µl volume containing 2.5 µl of DNA template, 1×PCR buffer, 0.2 mM each dATP, dCTP, dGTP, and dTTP (Fermentas, Lithuania), the *ail*-specific primers and *ystA*-specific primers at 50 nM, with 1 U of RedTag Genomic DNA polymerase (Sigma-Aldrich, Germany). The amplification was carried out under the following conditions: initial denaturation (94°C, 3 min), followed by 30 subsequent cycles consisting

of denaturation (94°C, 1 min), primer annealing (52°C, 1.5 min), extension (72°C, 1.5 min), and final extension (72°C, 10 min).

The duplex PCR for *blaA* and *blaB* genes was also performed in a 25 µl volume containing 2.5 µl of DNA template, 1 x PCR buffer, 200 µM of each: dATP, dCTP, dGTP, and dTTP (Fermentas, Lithuania), 100 nM of the *blaA* and the *blaB* pair of specific primers, and 1U of RedTag Genomic DNA polymerase (Sigma-Aldrich, Germany). The amplification was carried out under the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 0.5 min, primer annealing at 50°C for 0.5 min and extension at 72°C for 1 min. A 5 min extension at 72°C was performed at the end of the final cycle. The monoplex PCR for *myfA* gene and *yadA* gene as described earlier [19] and monoplex PCR for the 16S rRNA gene for species identification as described by Wannet et al. [18] were also performed.

The amplifications were carried out in the Multi Gene II thermal cycler (Labnet International, Inc., USA). The PCR products were analysed by electrophoresis in 1.5% agarose gels stained with ethidium bromide. Molecular size markers (Sigma-Aldrich) were also run for product size verification. The gel was electrophoresed in 2 × Tris-borate buffer at 70 V for 1.5 h.

Table 1. Oligonucleotide primers used in the study.

Primers	Sequence (5' → 3')	Amplicon length (bp)	References
ail-a (F)	TGGTTATGCGCAAAGCCATGT	356	[14]
ail-b (R)	TGGAAGTGGGTTGAATTGCA		
ystA-a (F)	GTCTTCATTTGGAGGATTCGGC	134	[14]
ystA-b (R)	AATCACTACTGACTTCGGCTGG		
myfA-1 (F)	CAGATA CAC CTG CCT TCC ATCT	272	[15]
myfA-2 (R)	CTCGACATATTCCTCAACACGC		
yadA-1 (F)	TAAGATCAGTGTCTCTGCGGCA	747	[16]
yadA-2 (R)	TAGTTATTTGCGATCCCTAGCAC		
blaA-1 (F)	AAATGCGCTACCGGCTTCAG	439	[17]
blaA-2 (R)	AGTGGTGGTATCACGTGGGT		
blaB-1 (F)	CCCACTTTATACCTTGGCACAAA	781	[17]
blaB-2 (R)	GAACATATCTCCTGCCTGGAAAT		
16S rRNA-Y1 (F)	AATACCGCATAACGTCTTCG	330	[18]
16S rRNA-Y2 (R)	CTTCTTCTGCGAGTAACGTC		

3. RESULTS

Biotype 4 was most numerously represented by 71.4% of *Y. enterocolitica* strains. A small group included strains of biotype 2 and biotype 1A (Table 2).

The 330 bp fragment, specific amplification product for the *Y. enterocolitica* 16S rRNA gene, was obtained in case of all the strains (Fig. 1A). A duplex PCR was used for the detection of the *ystA*-specific PCR product of 134 bp and the *ail*-specific product of 356 bp (Fig. 1B). These genes were present in all the strains of 4 and 2 biotype (Table 2). The *yadA*-specific amplification product of 747 bp was detected in all the strains of biotype 2 and the majority of strains belonging to biotype 4 (86.6%) (Fig. 1C). The *myfA*-specific PCR product of 272 bp (Fig. 1D) was detected in all the strains which belonged to different biotypes. Using multiplex PCR, 439 bp fragment for *blaA* gene in all the strains of biotype 4 and 2 was obtained (Fig. 1E). The amplification products for *blaB* (827 bp) were detected in all strains of biotype 2, and only in eight strains of biotype 4. The presence of β-lactamase genes in *Y. enterocolitica* was not detected in biotype 1A.

Table 2. Virulence genes and resistance profiles of *Y. enterocolitica* strains from the faeces of children with intestinal yersiniosis**.** Bt - biotype, AMP - ampicillin, TIC - ticarcillin, AMC - amoxicillin plus clavulanic acid, PIP - piperacillin, GM - gentamicin, AN - amicacin, C - chloramphenicol, SXT - trimethoprim/sulfamethoxazole, ATM - aztreonam, * - multidrug resistance strains, „-„ no amplification.

Strains	**Year**	**Bt**	**Results of PCR for:**						**Resistance profile**
			ail	***yadA***	***myfA***	***ystA***	***blaA***	***blaB***	
9996	2009	2	+	+	+	+	+	+	AMP/TIC/AMC
6068	2010	1A	-	-	+	-	-	-	AMP/PIP
10743	2010	4	+	+	+	+	+	+	AMP/TIC/GM
15869	2010	4	+	+	+	+	+	+	AMP/PIP/TIC
6528	2010	4	+	-	+	+	+	+	AMP/AN/C/SXT*
6701	2010	4	+	-	+	+	+	+	AMP/TIC
7217	2012	2	+	+	+	+	+	+	AMP/PIP/TIC/SXT
20179	2013	2	+	+	+	+	+	+	AMP/TIC/C/SXT*
10510	2013	1A	-	-	+	-	-	-	AMP/PIP/TIC/SXT
15395	2013	4	+	+	+	+	+	+	AMP/GM
26530	2014	4	+	+	+	+	+	+	AMP/PIP/AN/GN
13004	2015	4	+	+	+	+	+	-	AMP/PIP/TIC
2	2015	4	+	+	+	+	+	-	AMP/TIC
13571	2015	4	+	+	+	+	+	-	AMP/TIC/ATM/AMC
601	2015	4	+	+	+	+	+	-	AMP/TIC
1	2015	4	+	+	+	+	+	-	AMP, TIC
158	2015	4	+	+	+	+	+	-	AMP/TIC
450/6	2015	2	+	+	+	+	+	+	AMP/PIP/TIC/GM
448/7	2015	4	+	+	+	+	+	+	AMP/PIP/ATM/AN/GN/C*
511/8	2015	4	+	+	+	+	+	+	AMP/PIP/TIC/AN/GM
301/3	2015	4	+	+	+	+	+	-	AMP/TIC

The *Y. enterocolitica* strains showed high resistance to antibiotics belonging to penicillin group because all the strains were resistant to ampicillin, above 76% of the strains were resistant to ticarcillin and about 48% were resistant to piperacillin. Additionally, two strains (9.5%) were resistant to amoxicillin/clavulanic acid. About 29% and 19% of the strains were resistant to gentamicin and amikacin, respectively. Moreover, two strains (9.5%) were resistant to aztreonam. In case of chloramphenicol, 14.3% of the strains showed resistance and 19% of the strains were resistant to trimethoprim/sulfamethoxazole. All the strains were sensitive to cephalosporins, fluoroquinolones, imipenem and tobramycin (Fig. 2). Among the tested *Y. enterocolitica*, three strains were multidrug resistant. Two strains of biotype 4 showed resistance to antimicrobial agents from four various chemical groups and one strain of biotype 2 was resistant to antimicrobial agents belonging to three different chemical groups (Table 2).

4. DISCUSSION

Y. enterocolitica is an important foodborne pathogen which is primarily responsible for gastrointestinal infections in young children. The incidence of *Y. enterocolitica* infection is highest among children under 5 years of age [20].

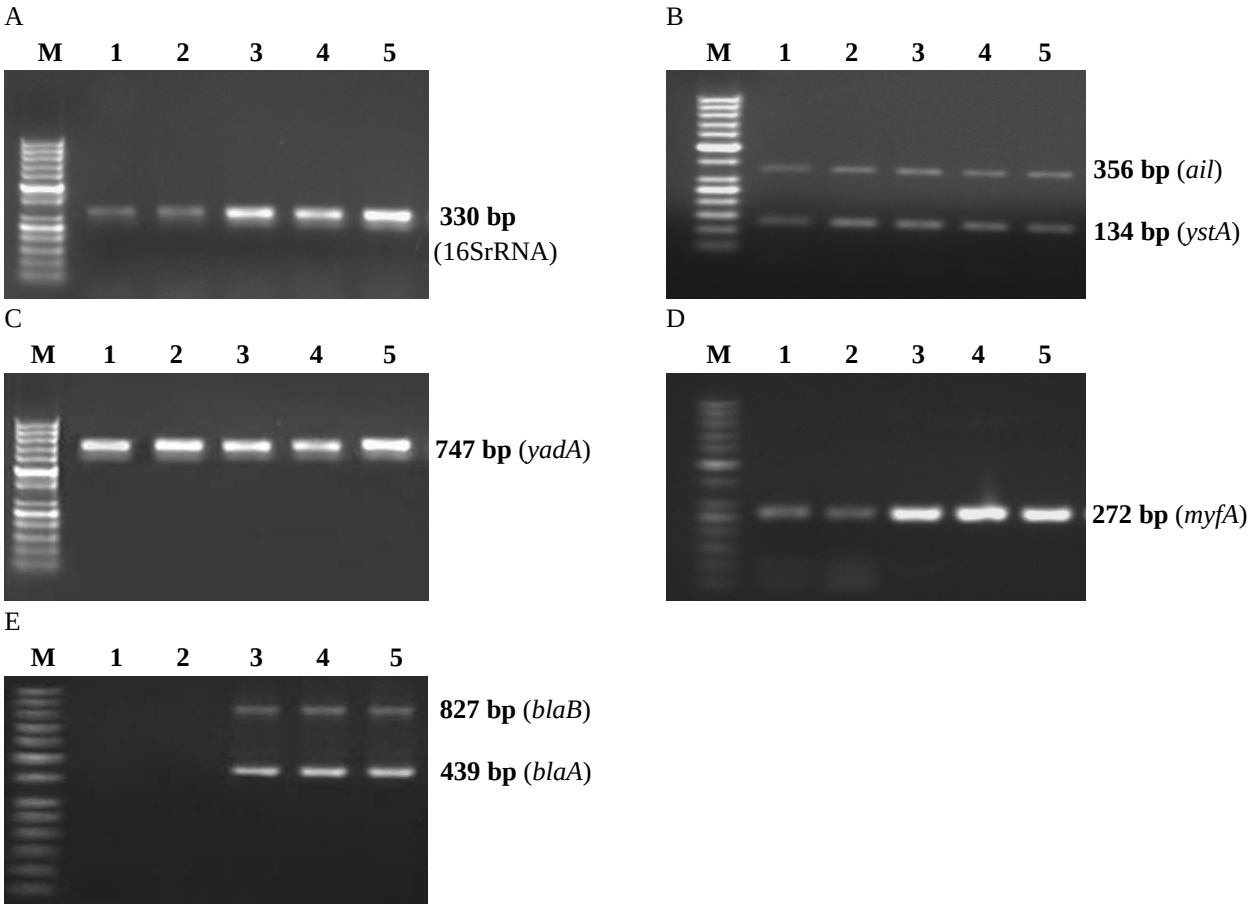

Figure 1. Electrophoresis in 1.5% agarose gel PCR products obtained by using specific primers for 16S rRNA gene (A), *ail* and *ystA* genes (B), *yadA* gene (C), *myfA* (D) and *blaA* and *blaB* genes (E).

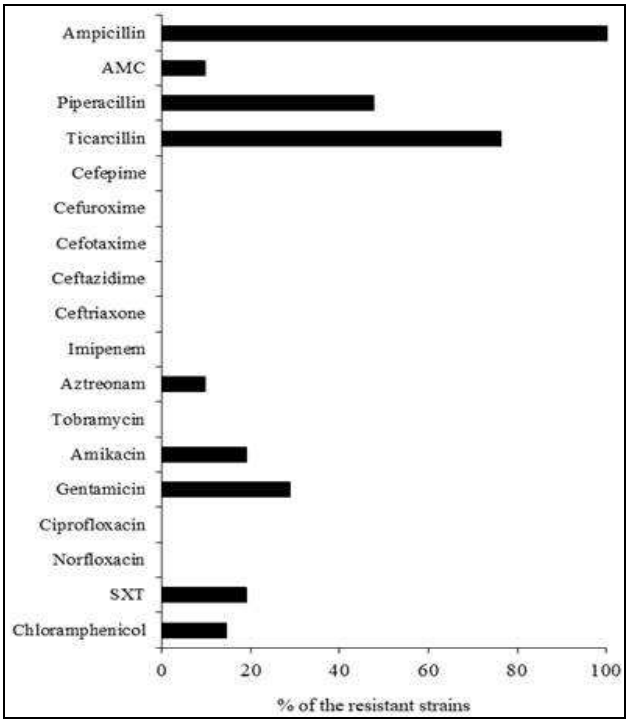

Figure 2. Antimicrobial resistance of *Y. enterocolitica* strains isolated from the faeces of humans with intestinal yersiniosis. AMC - amoxicillin/clavulanic acid, SXT - trimethoprim/sulfamethoxazole.

The high incidence of *Y. enterocolitica* infections in this age group, compared with other gastrointestinal infections, such as salmonellosis and campylobacteriosis, may result from eating food prepared from raw pork products, use of baby's dummy or contact with domestic animals, such as dogs and cats [21]. In addition, factors that may contribute to the high incidence of *Y. enterocolitica* infection in young children include an increased rate of exposure to this pathogen as a result of fecal-oral contamination, predisposition to infection due to immature immune system [22] and higher frequency of testing stool samples in case of children when affected [23]. In our research we investigated *Y. enterocolitica* strains isolated from the faeces of children suffering from diarrhea. Among them, strains belonging to biotype 4 carrying the *ail*, *myfA* and *ystaA* genes predominated. Most of them had also the plasmid gene *yadA*, confirming the presence of the plasmid pYV. These results demonstrated the pathogenic potential of the investigated strains to susceptible hosts. Our results are similar to those

obtained by other authors that also showed that strains belonging to biotype 4 are responsible for most infections caused by *Y. enterocolitca* in Europe [4, 20]. The strains of biotype 2 are rarely isolated from humans. The pathogenic potential of the biotype 2 strains examined in this study was highlighted by the occurrence of the virulence markers investigated. Similar results were obtained by Frazão and Falcão [24], who also studied strains of *Y. enterocolitica* biotype 2.

Uncomplicated course of yersiniosis usually does not require the use of antibiotics. However, some cases of yersiniosis, such as sepsis, focal extra-intestinal infection or infection in immune-compromised patients require antimicrobial treatment. *Y. enterocolitica* strains are β-lactamase producers. Most *Y. enterocolitica* strains harbored chromosomal genes *blaA* and *blaB* encoding BlaA (a non-inducible broad-spectrum carbenicillinase) and BlaB (an AmpC-type inducible cephalosporinase) [25].

In our study, the presence of *blaA* gene in all the strains of biotype 4 and 2 was detected, while *blaB* gene was carried by biotype 2 strains and over 50% of the biotype 4 strains. These genes were not detected in the strains of biotype 1A, although in previous studies, in which were used additional primers designed using the conserved regions of the *blaA* genes of *Y. enterocolitica* 8,081, biotype 1B, has been shown the presence of this gene in the majority of *Y. enterocolitica* strains of biotype 1A [26]. Heterogeneity in *blaA* gene of *Y. enterocolitica* of biotype 1A was confirmed by Sharma et al. [27]. Inability to detect *blaA* gene in these strains may result from a genetic variability in *blaA* preventing the binding of primers. The antimicrobial susceptibility test revealed high resistance of *Y. enterocolitica* to antibiotics belonging to penicillin group such as ampicillin, ticarcillin and piperacillin. This was in accordance with the results obtained by other authors [28]. Two strains (9.5%) belonging to 2 and 4 biotype were also resistant to amoxicillin with clavulanic acid, while Frazão et al. [29] showed that 19/34 of *Y. enterocolitica* strains isolated from different sources in Brazil were resistant to this combination. In our study, all the strains were sensitive to the second (cefuroxime), third (cefotaxime, ceftazidime, ceftriaxone) and fourth generation cephalosporins (cefepime), fluoroquinolones and imipenem. Fluoroquinolones and the third generation cephalosporins are the best therapeutic options to treat enterocolitis in compromised hosts and in patients with septicemia or invasive infection [30]. In case of extra-intestinal yersiniosis, also aminoglycosides in combination with other antibiotics are used for treatment. In our research, four (19%) and six (28.6%) strains were resistant to amikacin and gentamicin, respectively. Rusak et al. [28] obtained one strain (2%) resistant to amikacin, while all the strains were sensitive to gentamicin. In Switzerland during 2001-2010 also no gentamicin-resistant strains were found [4]. Trimethoprim/sulfamethoxazole are also used to treat yersiniosis. In this study, four strains (19%) were resistant to this sulfonamide. Sporadic resistance to trimethoprim/sulfamethoxazole occurred in Switzerland [4], while in Brazil trimethoprim/sulfamethoxazole resistance was found in 8.8% to 10% of the strains [28, 29]. In our study, three strains were multidrug resistant. Two strains belonging to biotype 4 showed resistance to four different classes of antimicrobial agents (penicillins, aminoglycosides, chloramphenicol, sulfonamides and penicillins, aminoglycosides, chloramphenicol, monobactams) and one strain of biotype 2 was resistant to antimicrobial agents belonging to three groups (penicillins, chloramphenicol, sulfonamides). Multiple resistance phenotypes were rarely reported in *Y. enterocolitica*. Only one out of from 60 *Y. enterocolitica* strains investigated by Rusak et al. [28] showed resistance to the three classes of antimicrobial agents (cephalosporin, sulfonamide, and tetracycline). Fredriksson-Ahomaa et al. [4] also reported that only one out of 128 *Y. enterocolitica* strains isolated from human clinical samples in Switzerland showed resistance to multiple antimicrobial agents. The multiresistance of *Y. enterocolitica* strains (19%) was found in Finland, and these strains were significantly associated with traveling abroad [31].

Our study showed that *Y. enterocolitica* strains from children in Poland belonging to biotype 4 and 2 had all investigated virulence genes, including the plasmid gene *yadA*, except the two strains of biotype 4 in which this gene was not detected. These strains showed high resistance to penicillin, although they remain susceptible to drugs used for treating gastroenteritidis, as well as extra-intestinal infections. However, it should be stressed

that some strains were resistant to antibiotics used in extra-intestinal yersiniosis treatment and few strains were multidrug resistant.

AUTHORS' CONTRIBUTION

BK: study design, laboratory investigation, data interpretation, preparation of manuscript; MP and KJ: laboratory investigation, literature analysis. The final manuscript has been approved by all authors.

TRANSPARENCY DECLARATION

The authors declare that they have no conflict of interest.

SOURCE OF FUNDING

This study was carried out with the financial support of Siedlce University of Natural Science and Humanities (Scientific Research Project no. 316/12/S).

REFERENCES

1. Bottone EJ. *Yersinia enterocolitica*: overview and epidemiologic correlates. Microb Infect. 1999; 1(4): 323-333.
2. Anonymous. The community summary report on trends and sources of zoonoses and zoonotic agents in the European Union in 2008. EFSA Journal. 2010; 10: 1496. http://www.efsa.europa.eu/fr/scdocs/doc/s1496.pdf. Accessed 19 October 2011.
3. Kot B, Woźniak-Kosek A, Kawiak J, Bukowski K. Application of the multiplex polymerase chain reaction (PCR) for identification of pathogenic plasmid markers of *Yersinia enterocolitica* strains isolated from humans and pigs [in Polish]. Med Weter. 2001; 57(10): 727-730.
4. Fredriksson-Ahomaa M, Cernela N, Hächler H, Stephan R. *Yersinia enterocolitica* strains associated with human infections in Switzerland 2001-2010. Eur J Clin Microbiol Infect Dis. 2012; 31(7): 1543-1550.
5. Saleh I, Barbour E, Shaib H, Harakeh S. Highly resistant *Yersinia enterocolitica* isolated from dairy based foods in Lebanon. IAJAA 2012; 2(1:2): 1-6.
6. Bozcal E, Uze A, Aydemir S, Skurnik M. Isolation of pathogenic *Yersinia enterocolitica* strains from different sources in Izmir region, Turkey. Folia Microbiol. 2015; 60(6): 523-529.
7. Terech-Majewska E, Pajdak J, Platt-Samoraj A, Szczerba-Turek A, Bancerz-Kisiel A, Grabowska K. Characterization of *Yersinia enterocolitica* strains potentially virulent for humans and animals in river water. J Appl Microbiol. 2016; 121(2): 554-560.
8. Milnes A, Stewart I, Clifton-Hadley FA, Davies RH, Newell DG, Sayers AR, et al. Intestinal carriage of verocytotoxigenic *Escherichia coli* O157, *Salmonella*, thermophilic *Campylobacter* and *Yersinia enterocolitica*, in cattle, sheep and pigs at slaughter in Great Britain during 2003. Epidemiol Infect. 2008; 136(6): 739-751.
9. Heesemann J, Sing A, Trulzsch K. Yersinia's stratagem: targeting innate and adaptive immune defense. Curr Opin Microbiol. 2006; 9(1): 55-61.
10. Revell PA, Miller VL. *Yersinia* virulence: more than a plasmid. FEMS Microbiol Lett. 2001; 205(2): 159-164.
11. Tennant SM, Grant TH, Robins-Browne RM. Pathogenicity of *Yersinia enterocolitica* biotype 1A. FEMS Immunol Med Microbiol. 2003; 38(2): 127-137.
12. Wauters G, Kandolo K, Janssens M. Revised biogrouping scheme of *Yersinia enterocolitica*. Contrib Microbiol Immunol. 1987; 9: 14-21.
13. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing, Twenty-Fifth Informational Supplement M100-S25, 2015.
14. Harnett N, Lin YP, Krishnan C. Detection of pathogenic *Yersinia enterocolitica* using the multiplex polymerase chain reaction. Epidemiol Infect. 1996; 117(1): 59-67.
15. Gierczyński R, Jagielski M, Rastawicki W. The evaluation of the usefulness of selected virulence markers for the identification of virulent *Yersinia enterocolitica* strains. IV. Gene *myfA* and *ureC* [in Polish]. Med Dośw Mikrobiol. 2002; 54(4): 347-355.
16. Kapperud G, Vardund T, Skjerve E, Hornes E, Michaelsen TE. Detection of pathogenic *Yersinia enterocolitica* in foods and water by immunomagnetic separation, nested polymerase chain reactions, and colorimetric detection of amplified DNA. Appl Environ Microbiol. 1993; 59(9): 2938-2944.
17. Stock I, Heisig P, Wiedemann B. Expression of β-lactamases in *Yersinia enterocolitica* strains of biovars 2, 4 and 5. J Med Microbiol. 1999; 48: 1023-1027.

18. Wannet WJB, Reessink M, Brunings HA, Maas HME. Detection of pathogenic *Yersinia enterocolitica* by a rapid and sensitive duplex PCR assay. J Clin Microbiol. 2001; 39(12): 4483-4486.
19. Kot B, Piechota M, Jakubczak A. Analysis of occurrence of virulence genes among *Yersinia enterocolitica* isolates belonging to different biotypes and serotypes. Pol J Vet Sci. 2010; 13(1): 13-19.
20. Rosner BM, Stark K, Werber D. Epidemiology of reported *Yersinia enterocolitica* infections in Germany, 2001-2008. BMC Public Health. 2010; 10: 337.
21. Boqvist S, Pettersson H, Svensson A, Andersson Y. Sources of sporadic *Yersinia enterocolitica* infection in children in Sweden, 2004: a case-control study. Epidemiol Infect. 2009; 137(6): 897-905.
22. Cohen MB. Etiology and mechanisms of acute infectious diarrhea in infants in the United States. J Pediatr. 1991; 118: S34-S39.
23. Scallan E, Jones TF, Cronquist A, Thomas S, Frenzen P, Hoefer D, et al. Factors associated with seeking medical care and submitting a stool sample in estimating the burden of foodborne illness. Foodborne Pathog Dis. 2006; 3(4): 432-438.
24. Frazão MR, Falcão JP. Genotypic diversity and pathogenic potential of *Yersinia enterocolitica* biotype 2 strains isolated in Brazil. J Appl Microbiol. 2015; 118(4): 1058-1067.
25. Liu C, Wang X, Chen Y, Hao H, Li X, Liang J, et al. Three *Yersinia enterocolitica* AmpD homologs participate in the multi-step regulation of chromosomal cephalosporinase, AmpC. Front Microbiol. 2016; 7: 1282.
26. Kot B, Rainko D. Antibiotic resistance and β-lactamases of *Yersinia enterocolitica* isolated from pigs in Poland. B Vet I Pulawy. 2009; 53: 603-607.
27. Sharma S, Mittal S, Mallik S, Virdi JS. Molecular characterization of β-lactamase genes blaA and blaB of *Yersinia enterocolitica* biovar 1A. B Vet I Pulawy. 2009; 53: 603-607.
28. Rusak LA, dos Reis CM, Barbosa AV, Santos AF, Paixão R, Hofer E, et al. Phenotypic and genotypic analysis of bio-serotypes of *Yersinia enterocolitica* from various sources in Brazil. J Infect Dev Ctries. 2014; 8(12): 1533-1540.
29. Frazão MR, Andrade LN, Darini ALC, Falcão JP. Antimicrobial resistance and plasmid replicons in *Yersinia enterocolitica* strains isolated in Brazil in 30 years. Braz J Infect Dis. 2017; 21(4): 477-480.
30. Fàbrega A, Vila J. *Yersinia enterocolitica*: pathogenesis, virulence and antimicrobial resistance. Enferm Infecc Microbiol Clin. 2012; 30(1): 24-32.
31. Sihvonen LM, Toivonen S, Haukka K, Kuusi M, Skurnik M, Siitonen A. Multilocus variable-number tandem-repeat analysis, pulsed-field gel electrophoresis, and antimicrobial susceptibility patterns in discrimination of sporadic and outbreak-related strains of *Yersinia enterocolitica*. BMC Microbiol. 2011; 11: 42.

ISSN 2449-8955

European Journal of Biological Research

Research Article

Ingredients of popular fruit teas in Poland

Artur Adamczak[1]*, Anna Forycka[1], Tomasz M. Karpiński[2]

[1] Department of Botany, Breeding and Agricultural Technology of Medicinal Plants, Institute of Natural Fibres and Medicinal Plants, Kolejowa 2, 62-064 Plewiska, Poland

[2] Department of Genetics and Pharmaceutical Microbiology, Poznań University of Medical Sciences, Święcickiego 4, 60-781 Poznań, Poland

*Corresponding author: Artur Adamczak; E-mail: artur.adamczak@iwnirz.pl

Received: 27 October 2017; **Revised submission:** 06 December 2017; **Accepted:** 18 December 2017

DOI: http://dx.doi.org/10.5281/zenodo.1175585

ABSTRACT

Fruit teas are very popular on the market of food products in many countries, due to their attractive taste and aroma as well as pro-health and medicinal properties. They are also characterized by the great wealth and diversity of composition. The purpose of this study was to analyze selected products based on the information contained on their packaging. The research included the most popular fruit teas widely available on the Polish food market, i.e. raspberry, cranberry and rosehip teas, 82 products in total. It was found that plant raw materials appearing in the tea names often constitute a small percentage of their composition, while hibiscus and apple occur very often and in the large quantities. The analysis of the content of the basic ingredient of raspberry and cranberry teas showed that they are characterized by a large diversity of quality. In addition to products with a relatively high amount of raspberry or cranberry (mean: 43.8 and 27.2%, respectively), there were teas with a very low level of these ingredients (mean: 7.5 and 1.6%). Against this background, rosehip tea has stood out positively. In this category of products, *Rosa* spp. hips, as a widely available plant raw material, most often obtained content above 30-40%.

Keywords: Composition of fruit teas; Raspberry; Cranberry; Dog rose; Foodstuffs; Plant raw material.

1. INTRODUCTION

The great popularity of fruit teas results from their attractive aroma and taste as well as health-promoting properties. Fruits, flowers, leaves and other plant raw materials being ingredients of fruit teas are an important source of phenolic compounds such as phenolic acids (hydroxybenzoic and hydroxycinnamic acids, and their derivatives), flavonols, flavanols, anthocyanins, and tannins as well as vitamins and minerals, including vitamin C. Especially berry phenolics represent a diverse group of active constituents with a high antioxidant potential [1-5].

Our earlier investigations indicated that fruit teas are characterized by a rich composition. In 187 products widely available in the retail chains in Poland, about 60 different plant raw materials were detected. The average number of ingredients in fruit teas was 7.1 (from 1 to even 12), including plant raw materials: 5.5 (1-11), and various types of additives: 1.5 (from 0 to 4). Raspberry, cranberry and rosehip teas belonged to the most numerous products in this group [6].

Fruits of red raspberry (*Rubus idaeus*), cranberry (*Oxycoccus macrocarpos* and *O. palustris*) as

well as wild rose species (*Rosa canina* and other similar species) are widely used not only in the food industry, but also in phytotherapy. Antioxidant, anti-inflammatory, antimicrobial, and anticancer properties of raspberry are associated with a high content of polyphenolic compounds, mainly anthocyanins and ellagitannins [7, 8]. Cranberry is often utilized in the treatment of urinary tract infections, but it can be used in the prevention of cardiovascular and gastric ulcer diseases [9, 10]. In turn, rose hips are mainly known as a rich source of vitamin C, lycopene, lutein, zeaxanthin, and other carotenoids. Due to the content of an anti-inflammatory galactolipid GOPO, this plant has proven useful in the treatment of osteoarthritis and rheumatoid arthritis [11, 12].

The health benefits of fruit tea drinking strongly depend on the quality and composition of the plant raw materials that were used to prepare these mixtures. The large number of products on the food market makes it difficult for consumers to choose the right ones. Unfortunately, our preliminary studies have shown that the names of many fruit teas do not describe their composition accurately [6]. The value of these products is also influenced by the presence of food additives: flavourings, acidity regulators, and sweeteners [6, 13]. Therefore, in the present work we decided to analyze this issue in detail.

The aim of the study was to describe the composition of the most popular fruit teas available on the Polish food market: raspberry, cranberry and rosehip teas. The paper presents the list of plant raw materials and food additives given by the producers as well as the percentage share of some ingredients.

2. MATERIALS AND METHODS

In the study, 82 fruit and fruit-herbal teas widely available on the Polish food market in the years 2015-2017 were used. The research included raspberry, cranberry and rosehip teas sold in the grocery stores and supermarkets, which are the most popular in this group of products. For analysis, bagged teas with raspberry, cranberry or rose on the first place of the name were selected. Products available only in pharmacies and/or herbal stores were excluded from investigations. We did not take into consideration flavoured or fruit-herbal teas containing *Camellia sinensis*, *Aspalathus linearis* (rooibos) or *Ilex paraguariensis* (yerba mate). All data about the products, in particular regarding their name and composition, came from the information on the packaging.

Incomplete data of producers concerning on the percentage share of plant raw materials and food additives in the mixtures did not allow precise description of the quantitative composition of fruit teas. Therefore, the attention was focused on the qualitative analysis of the composition of individual products and the frequency of occurrence of the different components. In the investigations, the information given on the labels on the percentage content of plant raw materials appearing in the names of fruit teas was also used. In addition, it was assumed that the order of occurrence of the individual plant materials in the list of ingredients fairly well reflects their relative quantitative contribution in a given mixtures, what was confirmed in the analysis of the collected data. Hence, the plant raw materials, which occurred from the first to the third position in the list of ingredients of fruit teas were considered as the main (dominating) components of these products [6].

Some difficulty in research resulted from the inconsistent and sometimes ambiguous way of description of the names of plant raw materials by individual producers. It was helpful comparing the composition of different teas, analysis of the pictures on the packaging, and sometimes the information from the manufacturer. In the prepared ingredient list of the fruit teas, the possibility of obtaining a plant raw material from a larger number of species was marked. The diagnosis of the plant raw materials was based on the textbooks of pharmacognosy, plant dictionaries and other similar works [14-22]. In this article, the names accepted in the herbal literature such as hibiscus flower, linden flower, raspberry fruit, rose fruit, etc. were used [23-25].

This work presents a list of plants appearing in the names of the analyzed fruit teas, giving their number of occurrences on the first, second and third place in the name, respectively and their average percentage content in the composition of these teas (Table 1). Next, the full composition of the mixtures was investigated, with division into raspberry, cranberry and rosehip teas, calculating the relative

frequency of occurrence of the individual plant raw materials (Table 2) and food additives (Table 3). For each type of tea, the bar graph showing the differentiation of the percentage content of the basic component: raspberry, cranberry or rosehip was prepared (Figs. 1, 3, 4). The statistical significan-ce of differences (Mann-Whitney U test) between product groups with the high and low amount of these ingredients was also calculated. Due to the largest number of samples (38 products), raspberry teas were analyzed in more detail. Figure 2 shows the number of occurrence of the individual plant raw materials and food additives on the first three places of the ingredient list, separately for raspberry teas with the high and low *R. idaeus* fruit content. For these groups, the statistical significance of differences (Mann-Whitney U test) regarding the number of all ingredients, plant raw materials and food additives was also assessed.

3. RESULTS

Survey of the Polish food market in the years 2015-2017 showed 38 raspberry teas, 24 cranberry teas, and 20 rosehip teas (in total: 82) produced by 20 different companies. They were described on the packaging as fruit (59.8% of cases), fruit-herbal (36.6%) or herbal-fruit (3.7%) teas. In their names, one (45.1% of cases), two (47.6%) or three (7.3%) plants occurred. In all, 23 plant species were found in the names of investigated fruit teas, but these ingredients often have a low percentage share in the mixtures (Table 1). In extreme cases, it was only 1-3% or even below 1%.

Table 1. Plants listed in the names of fruit teas and the mean content of these raw materials.

No.	Plants (raw material)	No. of occurrence in tea names and (mean content)		
		1st place	2nd place	3rd place
1	Raspberry (fruit)	38 (26.6%)	9 (9.7%)	
2	Cranberry (fruit)	24 (18.6%)	4 (6.5%)	1 (2%)
3	Dog rose (fruit, i.e. hip)	20 (46.3%)	2 (13.0%)	
4	Apple (fruit, peel)		4 (18.3%)	
5	Pomegranate (peel, juice, extract)		3 (2.0%)	1 (1%)
6	Strawberry (fruit)		3 (2.0%)	
7	Hibiscus (flower, i.e. calyx)		2 (27.5%)	
8	Linden (flower)		2 (17.5%)	
9	Quince (fruit)		2 (6.1%)	
10	Lemon (peel)		2 (5.4%)	
11	Bilberry (fruit)		2 (1.0%)	
12	Blackcurrant (fruit)		2 (0.3%)	
13	Rosebay willowherb (herb)		1 (25%)	
14	Mullein (flower)		1 (20%)	
15	Ginger (rhizome)		1 (13%)	
16	Lemongrass (herb)		1 (7.7%)	
17	Acerola (fruit)		1 (2.0%)	
18	Blackberry (fruit)		1 (0.5%)	
19	Chili pepper (fruit)		1 (0.4%)	
20	Sour cherry (juice concentrate)		1 (0.4%)	
21	Rhubarb (leaf petiole)			2 (1%)
22	Peppermint (leaf)			1 (9%)
23	Açai (juice concentrate)			1 (0.6%)

Only rosehip teas were characterized by a high mean content of the basic component (46.3%). On the other hand, ingredients rarely mentioned in the tea names such as hibiscus or apple are the permanent component of these products, and they get a share of up to 40-50% or 35%, respectively.

Depending on the type of fruit tea, hibiscus appeared in 79-95% of products and apple in 58-67% (Table 2). Cranberry teas were distinguished by a more frequent presence of chokeberry (46% of cases) and blackcurrant fruits (42%), while for raspberry teas it was elder fruits (32%). In turn, rosehip teas had the lowest total number of plant raw materials (24), but also the smallest constancy of occurrence of flavourings: 55% of cases (Tables 2-3).

Table 2. Plant raw materials of raspberry, cranberry and rosehip teas (*n*=82).

No.	Plants (raw material)	Botanical names	Frequency of occurrence in teas [%]		
			Raspberry tea (*n*=38)	**Cranberry tea (*n*=24)**	**Rosehip tea (*n*=20)**
1	Raspberry (fruit)	*Rubus idaeus* L.	100	33	25
2	Cranberry (fruit)	*Oxycoccus macrocarpos* (Aiton) Pursh, *O. palustris* Pers.	11	96	5
3	Dog rose (fruit, i.e. hip)	*Rosa canina* L. and other similar species	50	42	100
4	Hibiscus (flower, i.e. calyx)	*Hibiscus sabdariffa* L.	95	79	95
5	Apple (fruit, peel)	*Malus domestica* Borkh.	58	67	65
6	Chokeberry (fruit)	*Aronia melanocarpa* (Michx.) Elliott	32	46	25
7	Blackcurrant (fruit)	*Ribes nigrum* L.	5	42	10
8	Elder (fruit)	*Sambucus nigra* L.	32	13	15
9	Liquorice (root)	*Glycyrrhiza glabra* L.	26	17	10
10	Blackberry (leaf)	*Rubus fruticosus* L. agg.	21	17	5
11	Hawthorn (fruit)	*Crataegus monogyna* Jacq. and other similar taxa	0	13	0
12	Linden (flower)	*Tilia cordata* Mill., *T. platyphyllos* Scop.	11	4	5
13	Raspberry (leaf)	*Rubus idaeus* L.	11	0	0
14	Sweet blackberry (leaf)	*Rubus suavissimus* S. Lee	5	8	10
15	Peppermint (leaf)	*Mentha x piperita* L.	3	0	10
16	Black hollyhock (flower)	*Alcea rosea* L. var. *nigra*	0	4	10
17	Orange (peel)	*Citrus aurantium* L. ssp. *aurantium, C. sinensis* (L.) Osbeck	8	8	0
18	Bilberry (fruit)	*Vaccinium myrtillus* L.	8	4	5
19	Lemon (peel)	*Citrus limon* (L.) Osbeck	8	4	5
20	Chicory (root)	*Cichorium intybus* L.	3	8	5
21	Blackberry (fruit)	*Rubus fruticosus* L. agg.	5	4	5
22	Strawberry (fruit)	*Fragaria x ananassa* Duch.	5	4	5
23	Rowan (fruit)	*Sorbus aucuparia* L.	0	0	5
24	Blackthorn (fruit)	*Prunus spinosa* L.	0	0	5
25	Rhubarb (leaf petiole)	*Rheum rhabarbarum* L.	0	4	5
26	Nettle (leaf)	*Urtica dioica* L.	0	0	5

No.	Plants (raw material)	Botanical names	Frequency of occurrence in teas [%]		
			Raspberry tea (*n*=38)	Cranberry tea (*n*=24)	Rosehip tea (*n*=20)
27	Ginger (rhizome)	*Zingiber officinale* Roscoe	3	4	0
28	Grapefruit (peel)	*Citrus paradisi* Macfad.	0	4	0
29	Lemongrass (herb)	*Cymbopogon citratus* (DC.) Stapf.	3	4	0
30	Quince (fruit)	*Cydonia oblonga* Mill.	5	0	0
31	Pomegranate (peel)	*Punica granatum* L.	3	4	0
32	Chamomile (flower)	*Matricaria chamomilla* L.	0	4	0
33	Strawberry (leaf)	*Fragaria x ananassa* Duch.	0	4	0
34	Red currant (fruit)	*Ribes rubrum* L.	3	8	0
35	Elder (flower)	*Sambucus nigra* L.	3	0	0
36	Mullein (flower)	*Verbascum densiflorum* Bertol., *V. phlomoides* L.	3	0	0
37	Acerola (fruit)	*Malpighia glabra* L.	3	0	0
38	Cornflower (petals)	*Centaurea cyanus* L.	3	0	0
39	Rose (petals)	*Rosa* spp.	0	0	5
40	Rosebay willowherb (herb)	*Epilobium angustifolium* L.	0	4	0
41	Chili pepper (fruit)	*Capsicum annuum* L.	3	0	0
42	Ginseng (root)	*Panax ginseng* C.A. Meyer, *P. quinquefolius* L.	0	4	0
43	Sour cherry (stems)	*Prunus cerasus* L.	0	4	0

Table 3. Food additives in raspberry, cranberry and rosehip teas (*n*=82).

No.	Food additives	Frequency of occurrence in teas [%]		
		Raspberry tea (*n*=38)	Cranberry tea (*n*=24)	Rosehip tea (*n*=20)
1	Flavourings	79	71	45
2	Natural flavourings	16	8	0
3	Raspberry flavour	3	4	5
4	Strawberry flavour	0	0	5
5	Cranberry flavour	0	4	0
6	Citric acid (acidity regulator)	29	46	30
7	Malic acid (acidity regulator)	5	0	10
8	Chokeberry (juice concentrate)	5	17	10
9	Sour cherry (juice concentrate)	3	0	0
10	Açai (juice concentrate)	3	0	0
11	Raspberry (juice concentrate, dried juice)	5	0	5
12	Cranberry (juice concentrate, juice granules)	0	8	0
13	Pomegranate (extract, juice granules)	3	4	0
14	Maltodextrin	5	4	10

Detailed analysis of the composition of raspberry teas indicates two separate groups of products with different quality levels. The first group is characterized by a very low content of the basic ingredient, sometimes in the range of 0.1-0.6% and with mean value of 7.5%. In the second group, average content of raspberry was 43.8% with the highest value of 60% (Fig. 1).

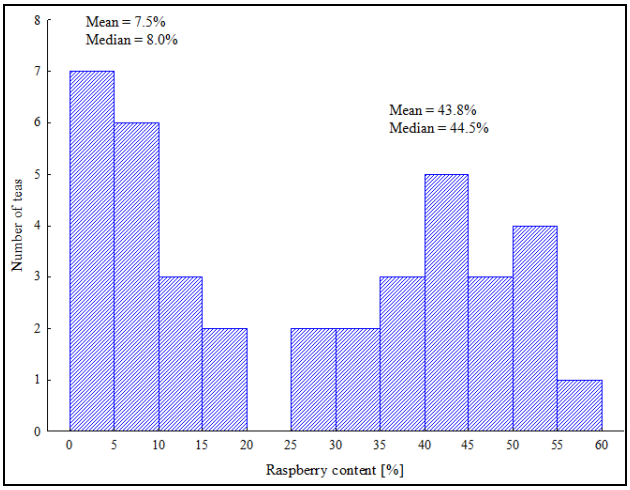

Figure 1. Content of the basic component (*Rubus idaeus* fruit) in raspberry teas (*n*=38).
Mann-Whitney U test for differences between two groups of products: with a low and high content of raspberry: $p<0.001$.

In the case of teas with a low amount of *R. idaeus* fruits, hibiscus and apple were the main component of the mixtures, and they appeared the most frequently on the first and second place in the list of ingredients, respectively. In addition, statistically significant more components, including food additives, were present in these products (Fig. 2). Similar differentiation in the product quality was also observed for the other fruit teas. The average content of the basic ingredient of cranberry tea, depending on the product group, was 1.6% and 27.2% (Fig. 3). In turn, for rosehip teas, it was 8.3% and 50.6%, respectively. However, the products with a high rosehip content were definitely dominant (Fig. 4).

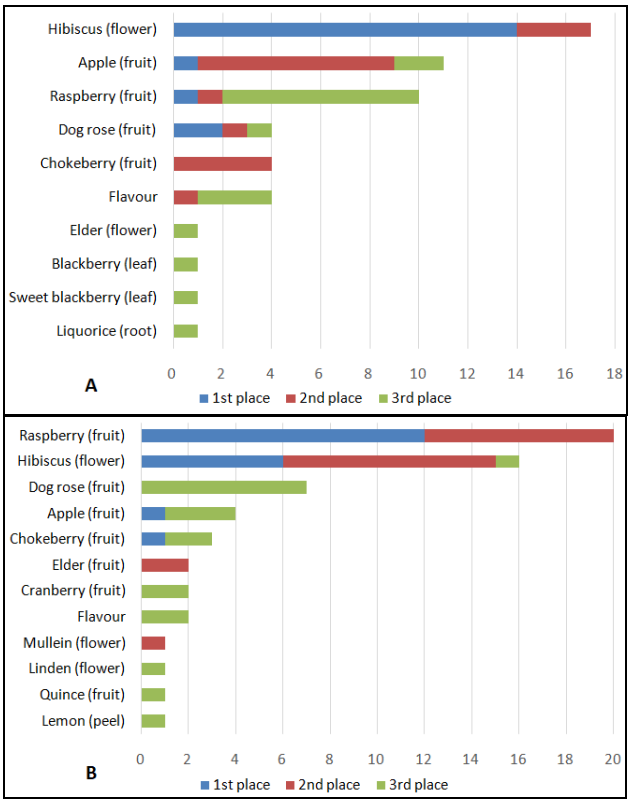

Figure 2. The number of occurrence of the individual plant raw materials and food additives on the first three places of the ingredient list of raspberry teas (*n*=38).
A) products with a low content of raspberry (0.1-20%, *n*=18); B) with a high content of raspberry (26-60%, *n*=20; compare with Fig. 1).
Mann-Whitney U test for differences between two groups of products in terms of total number of tea ingredients: $p<0.01$ (Mean=7.9 and 5.75 for A and B, respectively), food additives: $p=0.01$ (Mean=1.9 and 1.25) and plant raw materials: $p=0.015$ (Mean=6.0 and 4.5).

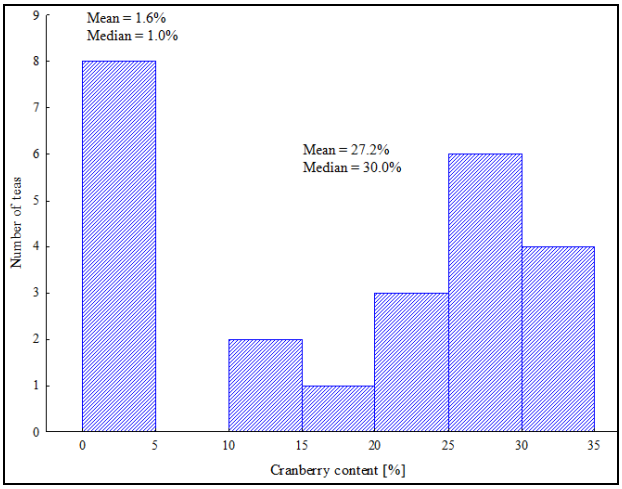

Figure 3. Content of the basic component (*Oxycoccus* spp. fruit) in cranberry teas (*n*=24).
Mann-Whitney U test for differences between two groups of products: with a low and high content of cranberry: $p<0.001$.

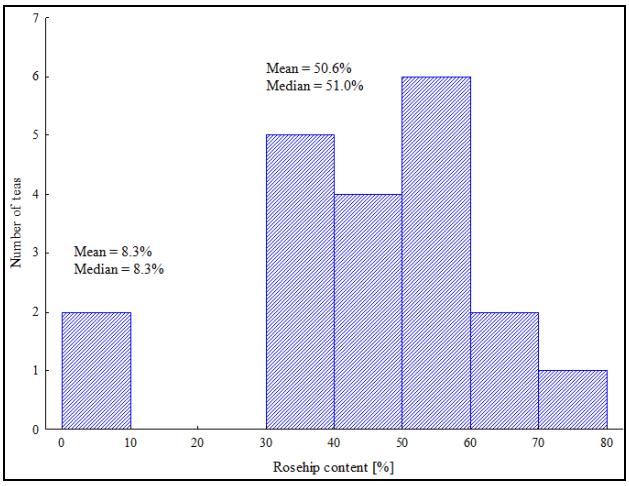

Figure 4. Content of the basic component (*Rosa* spp.) in rosehip teas (*n*=20).

4. DISCUSSION AND CONCLUSION

The survey of the Polish food market pointed to clear consumer preferences in the field of fruit teas. Considering the number of available products, it can be assumed that raspberry, cranberry and rosehip teas are most frequently chosen. This is due to the well-known pro-health and medicinal properties of these plants [7, 10, 12]. Attention was drawn to the large number of plant species occurring in the names of teas, next to raspberry, cranberry and rosehip (Table 1). Certainly, it results in increased interest in the products on the market. Importantly, the name and packaging of fruit teas are sometimes misleading, because the mentioned and illustrated plant raw materials often occur in the small quantities. On the other hand, hibiscus, apple and rosehip very often appear in large quantities and in various types of teas due to the low price of the raw material (apple and rosehip) or properties improving the taste and color of infusions (hibiscus) [6]. Unfortunately, food additives, especially flavourings, belong to the constant ingredients of fruit teas, too. Interestingly, the occurrence frequency of these components is clearly lower in the case of rosehip tea (Table 3).

Some plant raw materials present in fruit teas can be harvested from the different species [e.g. 18], what undoubtedly affects the composition and level of active compounds. Rose hips mainly collected from *Rosa canina*, but also from other wild growing species that exhibit significant phytochemical variability are a classic example of such a situation [26]. Unfortunately, there is no precise data on the labels concerning plant raw materials, especially full botanical names of taxa.

The analysis of the content of the basic ingredient of raspberry and cranberry teas showed that these products are characterized by a high variation in quality (Figs. 1, 3), which results from the high price of the discussed plant raw material. In addition to products with a relatively high amount of raspberry or cranberry fruits (mean: 43.8 and 27.2%, respectively), there were teas with a very low level of these ingredients (mean: 7.5 and 1.6%). Against this background, rosehip tea has stood out positively. In this product category, *Rosa* spp. hips, as a widely available raw material, most often obtained content above 30-40% (Fig. 4).

AUTHORS' CONTRIBUTION

AA: study design, data interpretation, preparation of manuscript; AF: preparation of tables and figures, literature analysis; TMK: preparation of manuscript, literature analysis. The final manuscript has been approved by all authors.

TRANSPARENCY DECLARATION

The authors declare that they have no conflict of interest.

REFERENCES

1. Szajdek A, Borowska EJ. Bioactive compounds and health-promoting properties of berry fruits: A review. Plant Foods Hum Nutr. 2008; 63: 147-156.
2. Belščak A, Bukovac N, Piljac-Žegarac J. The influence of ascorbic acid and honey addition on the anti-oxidant properties of fruit tea infusions: antioxidants in fruit tea infusions. J Food Biochem. 2011; 35: 195-212.
3. Şahin S. Evaluation of antioxidant properties and phenolic composition of fruit tea infusions. Antioxidants. 2013; 2: 206-215.
4. Šavikin K, Zdunić G, Janković T, Gođevac D, Stanojković T, Pljevljakušić D. Berry fruit teas: Phenolic composition and cytotoxic activity. Food Res Int. 2014; 62: 677-683.
5. Ferlemi A-V, Lamari FN. Berry leaves: An alternative source of bioactive natural products of nutritional and medicinal value. Antioxidants. 2016; 5(2): 17.

6. Adamczak A, Forycka A, Buchwald W. The composition of fruit teas available on the Polish market of foodstuffs [in Polish]. Post Fitoter. 2015; 16(4): 216-222.
7. Krauze-Baranowska M, Majdan M, Kula M. Fructus red raspberry and black raspberry as a source of biological active substances [in Polish]. Post Fitoter. 2014; 15(1): 32-39.
8. Aprea E, Biasioli F, Gasperi F. Volatile compounds of raspberry fruit: From analytical methods to biological role and sensory impact. Molecules. 2015; 20: 2445-2474.
9. Adamczak A, Buchwald W, Kozłowski J. Variation in the content of flavonols and main organic acids in the fruit of European cranberry (*Oxycoccus palustris* Pers.) growing in peatlands of North-Western Poland. Herba Pol. 2011; 57(4): 5-15.
10. Weh KM, Clarke J, Kresty LA. Cranberries and cancer: An update of preclinical studies evaluating the cancer inhibitory potential of cranberry and cranberry derived constituents. Antioxidants. 2016; 5(3): 27.
11. Willich SN, Rossnagel K, Roll S, Wagner A, Mune O, Erlendson J, et al. Rose hip herbal remedy in patients with rheumatoid arthritis – a randomised controlled trial. Phytomed. 2010; 17(2): 87-93.
12. Fan C, Pacier C, Martirosyan DM. Rose hip (*Rosa canina* L.): A functional food perspective. Functional Foods Health Disease. 2014; 4(11): 493-509.
13. Newerli-Guz J, Śmiechowska M, Piotrzkowska J. Aroma substances as ingredients of herbal-fruit teas [in Polish]. Zeszyty Nauk AM w Gdyni. 2009; 61: 19-32.
14. Muszyński J. Pharmacognosy. Outline of science about medicinal raw materials [in Polish]. PZWL, Warszawa 1957.
15. Borkowski B. Outline of the pharmacognosy [in Polish]. PZWL, Warszawa 1970.
16. Wichtl M. (ed.) Teedrogen. Ein Handbuch für Apotheker und Ärzte. Wissenschaftliche Verlagsgesellschaft mbH Stuttgart 1984.
17. Rutkowski L. The key to the determination of the vascular plants of Polish lowland [in Polish]. PWN, Warszawa 1998.
18. Kohlmünzer S. Pharmacognosy. Handbook for pharmacy students [in Polish]. PZWL, Warszawa 2000.
19. Strzelecka H, Kowalski J. (eds.) Encyclopedia of herbalism and herbal medicine [in Polish]. PWN, Warszawa 2000.
20. Anioł-Kwiatkowska J. A multilingual floristic dictionary [in Polish]. Wyd. Uniwersytetu Wrocławskiego, Wrocław 2003.
21. Podbielkowski Z, Sudnik-Wójcikowska B. Dictionary of useful plants [in Polish]. PWRiL, Warszawa 2003.
22. Lamer-Zarawska E, Kowal-Gierczak B, Niedworok J. (eds.) Phytotherapy and herbal medicines [in Polish]. PZWL, Warszawa 2007.
23. Polish Pharmacopoeia. 3rd ed. PTF, Warszawa 1954: 279.
24. Polish Pharmacopoeia. 4th ed. PTF, Warszawa 1970: 247-248.
25. Polish Pharmacopoeia. 9th ed. PTF, Warszawa 2011, 1: 1295-1296, 1405.
26. Adamczak A, Buchwald W, Zieliński J, Mielcarek S. Flavonoid and organic acid content in rose hips (*Rosa* L., section *Caninae* DC. em. Christ.). Acta Biol Cracov Ser Bot. 2012; 54(1): 105-112.