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Research Article

L-cysteine ameliorated testicular toxicity induced by acrylamide in rats

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ABSTRACT

The general public is exposed to acrylamide from industrial manufacturing, laboratory work, foods rich in carbohydrates that have been cooked at high-temperature, and through cigarette smoke. The present experiment was conducted to investigate the reproductive toxicity of acrylamide exposure in male rats and the role of L-cysteine supplementation in amelioration of this toxicity. Forty eight adult male albino rats (weighing 120-140 g) were divided into four groups (16 rats/group). Group I - negative control group, drank tap water, group II - positive control, drank tap water that contains acrylamide (25 mg/kg body weight) for 28 days and group III drank tap water that contains acrylamide (25 mg/kg body weight) and L-cysteine (100 mg/kg body weight). Four rats from each group were killed at 7, 14, 21 and 28 days time intervals from the beginning of the experiment. In general, exposure to acrylamide induced a significant elevation in testes lipid peroxides and nitric oxide levels and a significant reduction in the level of glutathione and the activity of superoxide dismutase and catalase in all periods of experiment. However, plasma testosterone was significantly decreased in acrylamide treated rats with congestion and interstitial edema, necrosis, calcification and degeneration of spermatogenic cells in the seminiferous tubules and formation of spermatid gaint cells. Co-treatment of rats with L-cysteine reduced the changes in oxidative stress parameters and improved the pathological changes in testis. Therefore, supplementation of L-cysteine can be useful when there is a risk of acrlamide toxicity.

Keywords: Acrylamide, Testis, Oxidative stress, L-cysteine, Testosterone.

1. INTRODUCTION

Acrylamide (ACR) is formed through the Maillard reaction during the heating process by interactions of amino acids, especially asparagine, with reducing sugars like glucose [1, 2]. There is variance in literature about the levels of ACR in different foods and the potential risk from dietary exposure. The daily intakes of dietary ACR for the

general population are estimated to be in the range of 0.3-2.0 mg/kg body weight [3]. ACR can be reactive in three different ways, radical-mediated polymerization, and addition to thiol, hydroxyl, or amino groups result in alkylation of proteins or metabolized to an epoxide derivative, glycidamide being readily reactive toward DNA and other macromolecules [4-6]. The mechanism by which ACR exposure causes cellular dysfunction in experimental animals and humans is not completely clear. However, it is thought that oxidative stress was associated with ACR cytotoxicity. Reproductive toxicity in rodents exposed to ACR includes alterations in gonadal and pituitary hormones associated with histopathological changes that includes formation of multinucleated giant cells, vacuolation and production of high numbers of apoptotic cells in the seminiferous tubules [7-11]. Acrylamide induced oxidative stress is capable of disrupting the steroidogenic capacity of Leydig cells [12] as well as the capacity of the germinal epithelium to differentiate into normal spermatozoa [13]. Fortunately, testes contain a complicated group of antioxidants and free radical scavengers to protect the spermatogenic and steroidogenic functions of testis from oxidative stress [14].

Kurebayashi and Ohno [15] reported that glutathione (GSH) precursors such as N-acetyl-L-cysteine and L-methionine increased the protection against the cytotoxicity of ACR in isolated rat hepatocytes. Moreover, N-acetyl-L-cysteine as potent antioxidant protects tissues from ACR toxicity by inhibiting neutrophil infiltration, balancing the oxidant-antioxidant status, and regulating the generation of inflammatory mediators [8]. Also, α-lipoic acid protect cells from oxidative stress induced by ACR exposure via enhances cellular antioxidant defense capacity [9]. In this regard, the objectives of the present study were to measures the markers of oxidative stress in testis of rats exposed to ACR and to evaluate the protective role of L-cysteine as precursor of GSH against ACR toxicity.

2. MATERIALS AND METHODS

Forty eight adult male albino rats (weighing 120-140 g) were purchased from the Animal House, Faculty of Medicine, Assiut University, Assiut, Egypt. The animals were housed in cages at a controlled temperature (25±3°C) and ambient humidity (50-60%). Lights were maintained on a 12-h light-dark cycle. All animals received basal diet and water *ad-libitum* for one week as an adaptation period. Following one week of acclimatization, the rats were randomly divided into three groups (16 rats/group):

- Group I: Negative control, fed on basal diet and normal drinking water for 4 weeks.
- Group II: Positive control, fed on basal diet and drinking water that contains ACR (25 mg/kg body weight) according to Alturfan et al. [8].
- Group III: Fed on basal diet and drinking water that contains ACR (25 mg/kg body weight and L-cysteine (100 mg/kg body weight) according to Omar et al. [16].

Then, each week from the beginning of the experiment 4 rats from each group were killed under anesthesia with ether. The blood samples were collected directly from portal vein into centrifuge tubes for separation of serum by centrifugation at 3000 rpm for 15 minutes and were frozen at -20°C for subsequent biochemical analysis. Immediately after killing rats, small piece of testes were excised and fixed in formaline for histological studies, and the remnant was washed in cold saline, immersed in liquid nitrogen and stored at -20°C for biochemical assay. All animal experiments were carried out in accordance with Ethical Committee Acts.

2.1. Determination of oxidative stress biomarker

Lipid peroxidation (LPO) products as TBARS content were determined according to the method of Ohkawa et al. [17]. Nitric oxide (NO) content was measured as nitrate concentration colorimetrically using the method of Ding et al. [18]. Glutathione (GSH) content was determined using the method of Beutler et al. [19]. The activity of superoxide dismutase (SOD) was determined basing on its ability to inhibit the autoxidation of epinephrine at alkaline medium according to the method of Misra and Fridovich [20]. The activity of catalase (CAT) was determined basing on its ability to decompose H_2O_2 to H_2O and O_2 according to Gregory and Fridovich [21]. Protein content in the spleen tissues was determined by the method of Lowry et al. [22].

2.2. Estimation of testosterone

Testosterone hormone in plasma was determine by Enzyme Immunoassay Method (ELISA), Biocheck, Inc, 323 vintage Park Dr. Forster City, CA, USA, according to the kit manufacture instructions.

2.3. Statistical analysis

The data was expressed as mean ± SE. The results were analyzed statistically using column statistics and one-way analysis of variance with the Newman-Keuls multiple comparison test as a post-test. These analyses were carried out using the computer prism program for windows, version 6.0 (Graph pad software Inc., San Diego, California, USA). Differences between the groups were considered significant if $P < 0.05$, 0.01, or 0.001.

3. RESULTS

Compared to control rats, ACR treated rats exhibited a significant decrease in plasma testosterone in 2nd, 3rd, and 4th weeks and LC treatment resulted in an increase in testosterone level especially in 2nd and 4th week (Fig. 1).

In relation to control rats, ACR treated rats had greater level of LPO in testis especially in the 3rd week ($P<0.001$) and LC co-treatment failed to restore the elevation of LPO especially in 1st and 4th week (Fig. 2). Also, Fig. 2 showed that NO was significantly increased ($P<0.001$) in all periods of the experiment in comparison with control and LC treatment resulted in significant reduction in NO level ($P<0.001$).

In the ACR group, GSH level in testis was decreased significantly ($P<0.001$) in comparison to the control group. In the LC + ACR group, GSH level was resorted to the normal level in control group (Fig. 3). Moreover, SOD and CAT activities were significantly increased ($P<0.05$) in the 3rd week in ACR treated rats, while they significantly decreased ($P<0.001$) in the same week in LC and ACR treated groups (Fig. 3).

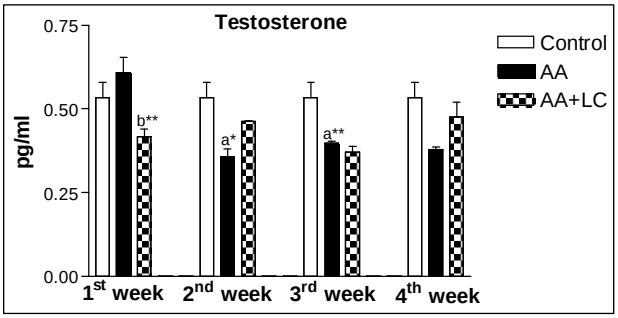
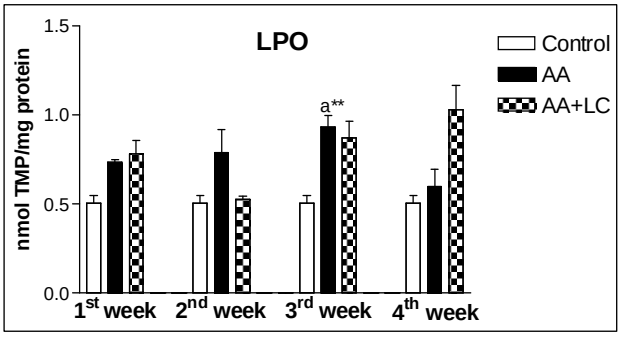


Fig. 1. Testosterone level in plasma of control and treated rats.



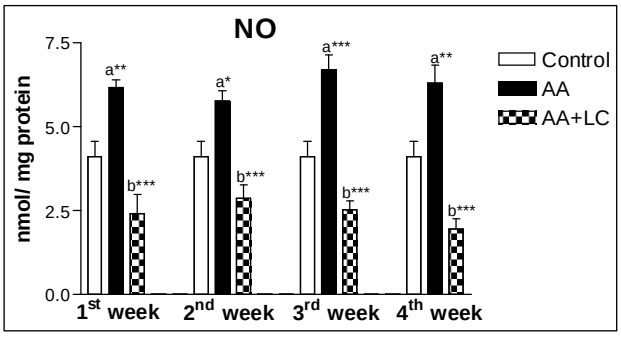


Fig. 2. Levels of LPO and NO in testis tissue of control and treated rats.

3.1. Histopathology of testes

In the present study, it was observed that testis of control rats stained with hematoxylin and eosin (H&E) was formed of seminiferous tubules and interstitial cells of Leydig. Most tubules contain normal spermatogenic cell layers and spermatozoa (Fig. 4A). After one week of treatment with ACR, testes showed mild degeneration of spermatogenic cells in the seminiferous tubules, interstitial edema and degeneration in spermatogenic cells with formation of spermatid giant cells (arrows) (Fig. 4B). After two weeks, the testes showed severe degeneration in the spermatogenic cells with the presence of spermatid giant cells, the absence of

some spermatogenic cells layers and the presence of spermatid giant cells in some cases was also noticed (Fig. 4D). After three weeks, testes showed severe coagulative necrosis of seminiferous tubules and the absence of spermatogenic cells (Fig. 4F). And after four weeks, testes showed severe necrosis with dystrophic calcification in the seminiferous tubules (Fig. 4H). In group of rats co-treated with LC for one week, testes showed congestion, interstitial edema and hyperplasia Leydig cells in the first week and mild degeneration in the spermatogenic cells (Fig. 4C). After 2 weeks of treatment with LC, testes showed mild degeneration of the spermatogenic cells with the presence of multiple spermatid giant cells (Fig. 4E). In rats co-treated with LC for 3 and 4 weeks, testes showed mild degeneration in some seminiferous tubules (Figs. 4. G and I).

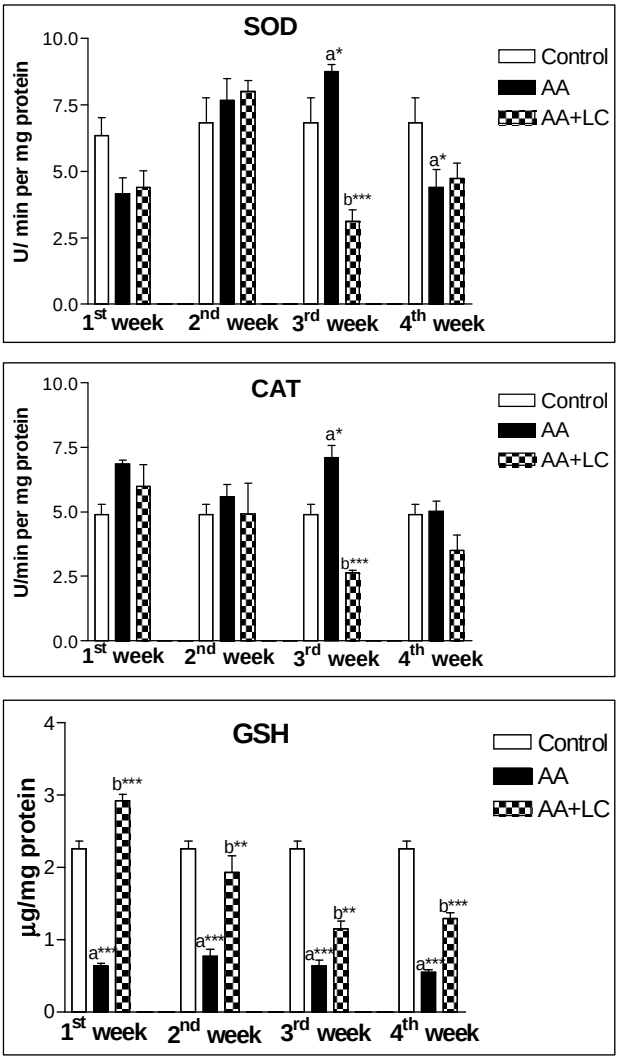


Fig. 3. Level of GSH and the activities of SOD and CAT in testis tissue of control and treated rats.

4. DISCUSSION

ACR is one of the major environmental public health problems; it induced oxidative organ damages in the brain, lung, liver, kidney, spleen, and testes tissues [8**,** 23-25]. The present result in (Fig. 2) revealed that administration of ACR in drinking water increased LPO and NO in testes and co-treatment of rats with LC decreased the level of LPO and NO in comparison with ACR treated rats. It is known that, NO may show either cytoprotective or cytotoxic effects and the elevated NO level has the ability to induce γ-glutamyl cycle [26]. In consistence with our results, Jiazhong et al. [27] and Abd El-Halim and Mohamed [23] mentioned that ACR is able to increase LPO by inducing oxidative stress with generation of free radicals. Garlic, resveratrol and NAC may be protected against ACR-induced oxidative injury by scavenging free radicals, preventing depletion of GSH and inhibiting neutrophil infiltration, and subsequent activation of inflammatory mediators that induce LPO [8, 28].

ACR is capable of interacting with vital cellular nucleophiles possessing -SH group. Therefore, it reacts with GSH in a similar manner and forms GSH S-conjugates, which is the initial step in the biotransformation of ACR [29]. Depletion of cellular GSH seems to play a pivotal role in the genotoxicity in human caused by ACR [30, 31]. In the present study, decreased GSH content in testes as presented in (Fig. 3) can be explained by the reaction of ACR with GSH, which in turn causes the depletion of GSH and the enhancement of LPO. Similarly, Abd El-Halim and Mohamed [23] found that administration of ACR caused a significant reduction in testes GSH level. However, Özturan-Özeri et al. [32] concluded that GSH is not directly capable of protecting tissues against ACR-induced oxidative stress. The present results showed that co-treatment of rats with LC attenuate GSH depletion by ACR. In consistence, treatment with garilic prior to ACR attenuated the depletion of GSH level [18]. Also, treatment of rats with lipoic acid caused an increase in GSH level with a decrease in LPO which might be attributed to the oxidative damage repairing ability of lipoic acid [33].

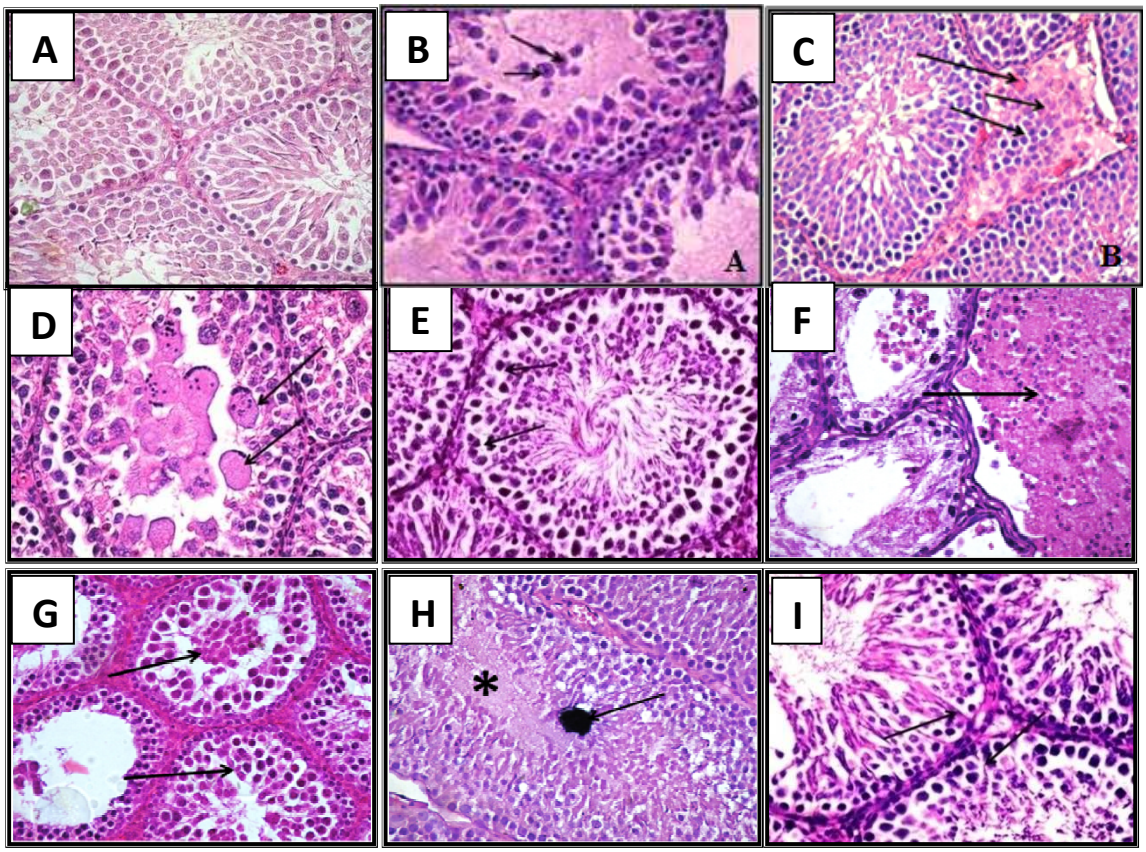


Fig. 4. Photomicrographs of control testis formed of seminiferous tubules and interstitial cells of leydig. **A.** Most tubules contain normal spermatogenic cells layers and spermatozoa in control rats. B-I testis of ACR and ACR plus L-cysteine treated groups at different weeks of treatment. **B**. mild degeneration of spermatogenic cells with formation of spermatid giant cells in the seminiferous tubules (arrows). **C.** Congestion interstitial edema and hyperplasia of Leydig cells (arrows). **D**. Severe degeneration in the spermatogenic cells with presence of spermatid giant cells (arrows). **E.** Mild degeneration in the spermatogenic cells (arrows)**. F.** Severe coagulative necrosis of seminiferous tubules and absence of spermatogenic cells (arrows). **G**. Moderate degeneration of the spermatogenic cells and presence of multiple spermatid gaint cells (arrows). **H**. Severe necrosis (star) with dystrophic calcification in the seminiferous tubules (arrows)**. I**. mild degeneration in some seminiferous tubules (arrows). H&E X400.

Superoxide radical may oxidize SH groups and undergo dismutation to form H_2O_2 and singlet oxygen [34]. This change in the redox status of the cell may modulate gene expression directly or via the transcription factors that are redox-regulated, and may lead to apoptosis, cell proliferation, or transformation (29**)**. In the current study, (Fig. 3) showed alteration in the testicular SOD and CAT activities depending on the period of treatments. Also, it showed that co-treatments of rats with LC ameliorate these changes by different levels. These results are in agreement with Abd El-Halim and Mohamed [23] who found that administration of ACR caused a significant reduction in the activity of SOD in testes tissues. The reduction in antioxidant enzyme activities was increased with increasing doses of ACR [35]. Treatment with *Curcuma longa* L. powder and garilic prior to ACR attenuated the reduction of SOD activity [23, 36], and administration of catechin and neem leaves extracts significantly enhanced the hepatic CAT activity [37].

Testosterone level in plasma of rats treated with ACR was significantly decreased, and co-treatment with LC elevates this decrease especially in the 4th week of treatment (Fig. 1). In this aspect, administration of ACR caused a significant reduction of serum testosterone level as reported by many authors [9, 38, 39]. This significant reduction of testosterone may be a result of direct damage of ACR on the Leydig cells [40]. The previous opinion was confirmed histopathologicaly in the present

study by congestion and interstitial edema, necrosis, calcification and degeneration of spermatogenic cells in the seminiferous tubules with formation of spermatid gaint cells (Fig. 4 A, C, E & G). Moreover, ACR may affect the endocrine function of the testes by altering the androgen biosynthesis of interstitial cells in the testes [41] or inducing the enzymes activity of hepatic biotransformation, which is capable of metabolically transforming androgens into products with low androgen receptor binding activity [42]. Song et al. [43] found that ACR can directly damage Leydig cells and affect the endocrine function of the testis. Moreover, Yang et al. [44] found that ACR induces histopathological lesions such as formation of multinucleated giant cells, vacuolation and production of high numbers of apoptotic cells in the seminiferous tubules of the rat. In the present study, treatment with LC along with ACR resulted in moderate attenuation of the histopathological changes in testes that were induced by ACR.

From these observations, it can be concluded that LC ameliorates the toxicity of ACR in rat testes by alleviating LPO and NO through scavenging of free radicals, and enhancing the activity of SOD and CAT and GSH level.

AUTHORS CONTRIBUTION

All authors contributed equally in planning, conduct, data analysis, and editing the work. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The author declares no conflicts of interest.

NOTES

This paper was presented in the 48th Annual Conference of Physiology and Pathology of Reproduction and simultaneously 40th Joint Congress of Veterinary and Human Medicine, Zurich. Switzerland, 11th-13th February 2015, Abstract No 74.

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Research Article

The efficiency of *Thymus laevigatus* extract on the penconazole toxicity in some rabbit tissues

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ABSTRACT

The *Thymus laevigatus* (Vahl), Lamiaceae (Labiatae), an endemic species of Yemen, is traditionally used in the treatment of various disorders including stomach and respiratory system diseases. Penconazole is a systemic triazole fungicide with preventive and curative properties for the control of powdery mildew. This study was designed to investigate the toxicity of penconazole fungicide and effect of the extract *Thymus laevigatus* on male and female rabbit tissues. The orally administered penconazole to male and female rabbits at the dose ($^1/_{10}$ LD_{50} of 380 mg/kg b.w.) for 60 days showed signs of toxicity including piloerection, subsequent fall and swollen condition some organs such as the stomach and the intestine, reduced body weight and caused death of rabbits. Histopathological changes showed in liver tissue including mild lymphocytes infiltration around bile duct in the portal area and fibrosis, and in lungs showed less inflammation and lymphoid aggregates around the bronchus, more consolidation of the alveolar space and edema. Pare with *Thymus laevigatus* extract 50 and 100 mg/kg administered orally on two different groups of animals found that the *Thymus laevigatus* possess strong antioxidant properties and therefore it inhibits penconazole toxicity effect on rabbit tissues.

Keywords: Penconazole, *Thymus laevigatus*, Rabbit, Mortality, Behavior, Histopathology.

1. INTRODUCTION

The genus *Thymus* which belongs to the family Lamiaceae (Labiatae) includes 350 species widespread all around the world. Several studies investigated earlier the chemical compositions of the essential oils of numerous species of the *Thymus* genus and focused on their antimicrobial activities [1-2]. Recently, the essential oils of various species of the Thymus genus have been screened for their traditional indigenous uses and investigated intensive as promise sources of antibacterial, antifungal, antioxidant and other natural products.

The most species of thymus contain altimol (20-55%) (thymol), a compound that most benefit in medicine, alcarvacrol (carvacrol), allinalol (linalool), cymene, and gamma-terpinene. The proportion of these components varies depending on the

soil and the time of harvest the plant and the surrounding geographical conditions [3]. In the flora of Yemen, this genus is represented by only the species *Thymus laevigatus* which is endemic to Yemen. It occurs in the higher mountains in North Yemen in Haggah (2500 m) and in Dhamar (2200 m) [4]. The Yemeni local name of the plant is Za'tar. In Yemeni folk medicine, the fresh and dried leaves of *T. laevigatus* are used as powder in warm milk, sesame oil or olive oil to treat different stomach diseases, cough, tonsillitis, pharangitis, and renal colic [5]. It also treats respiratory infections, such as chronic bronchitis, whooping cough and asthma [6] and is also working on prevention teeth from decay and treats inflammation of the tonsils, gums and teeth [7] and is thyme useful in the treatment of emphysema and inflammation of the stomach, diarrhea, headache, migraine, nocturnal enuresis in children [8- 9].

Penconazole is a systemic triazole fungicide with the preventive and curative properties for the control of powdery mildew disease of different crops. The mode of action is by stopping the development of fungi by interfering with the biosynthesis of sterols in cell membranes. It is used on fruit, especially apples and grapes and vegetables [10]. FAO and WHO [11] report that penconazole oral acute LD_{50} for rats is 2125 mg/kg, for mice 2444 mg/kg. Skin and eye acute percutaneous LD_{50} for rats is >3000 mg/kg. It is not a skin irritant; is irritating to eyes (rabbits). Not a skin sensitize inhalation LC_{50} (4 h) is >4000 mg/m^3 [12]. The penconazole is particularly effective against powdery mildew and feedbacks caused by fungi *Deuteromycetes, Basidiomycotiona* and *Ascomycetes* as *Erysiphe cichoracearum, Microspharea alin and Phyllactinia sp.* [13].

The objective of this study was to evaluate protective role of *Thymus laevigatus* leaves extract against the effect of penconazole fungicide. Studies were conducted of the behavior changes and histopathological changes such as liver and lungs of male and female rabbits.

2. MATERIALS AND METHODS

2.1. Fungicide, plant materials and test animals

Penconazole fungicide EC 10% was from Ministry of Agriculture, Department of Plant Protection (Producer Shenzhen Pisticides Ltd., China). The leaves of *Thymus laevigatus* were collected in October 2012 from Ibb Yemen. Forty local domestic rabbits have been bought from Lahej, Alseilla, Sheikh Othman markets.

2.2. Experimental animals

Forty local rabbits weighting 800-1200 grams have been used in this study. All animal experiments were carried out in accordance with local Ethical Committee Acts. All rabbits were acclimated in cages for 15 days before experiment. Each animal was weighed in the morning and fed with carrot, lettuce and cabbage. The animals were given water *ad libitum.*

Animals were divided into four groups (ten rabbits, 5 male and 5 female per group) as the following:

Group I: Control group.

Group II: Treated with $^1/_{10}$ LD_{50} of penconazole (380 mg/kg b.w.) .

Group III: Treated with $^1/_{10}$ LD_{50} of penconazole (380 mg/kg b.w.) and extract of *T. laevigatus* (50 mg/kg b.w.).

Group IV: Treated with $^1/_{10}$ LD_{50} of penconazole (380 mg/kg b.w.) and extract of *T. laevigatus* (100 mg/kg b.w.).

The administration of the all doses was achieved orally, using insulin needle, day after day.

2.3. Extraction method

Plants extracts are made through the Soxhlet apparatus methods of extraction by Petroleum ether. Only the green and healthy leaves of the plant were used, washed twice under running tap water followed by sterilized distilled water. The washed leaves were dried in good ventilated area for 5 days. Grinding the dry leaves by cafe blender. 200 grams from each plant are put in Soxhlet apparatus with 250 ml Petroleum spirit. The extraction put in a hot plate with at 90^0C for at 6 hours. The separation solvent (Petroleum spirit) from the herbal extract are put in Rotary. The dry extract where collected from the wall round bottom flask, then 2 grams of extract added to 200 ml of distilled water, this estimate the concentration of extract [14-16].

3. RESULTS

3.1. Animals mortality

There was no mortality among the control group during 60 days, four rabbits were died during the treatment with $^{1}/_{10}$ penconazole, and two rabbits were died in the group which gavaged with $^{1}/_{10}$ penconazole and extract of *Thymus laevigatus* 50 mg/kg. Simultanously, when rabbits gavaged with $^{1}/_{10}$ penconazole and extract of *Thymus laevigatus* 100 mg/kg one rabbit died. This confirms that *Thymus laevigatus* reduced the toxicity of penconazole (Table 1).

Table 1. Showing mortality rate of rabbits exposed to penconazole at $^{1}/_{10}$ LD_{50} after 60 days of exposure.

Number of group	Number of rabbits	Concentration in mg/l	Mortality %
Control	10	-	0
I	10	$^{1}/_{10}$ penconazole	4
II	10	$^{1}/_{10}$ Penconazole + extract of *Thymus laevigatus* 50 mg/kg	2
III	10	$^{1}/_{10}$ Penconazole + extract of *Thymus laevigatus* 100 mg/kg	1

3.2. Behavioral changes

Rabbits have a different behaviors when treated with penconazole such as hidden under cages, calm, piloerection of hair, loss of appetite, strong tremors. The rabbits in group II gained weight, what may be due to the swollen of some organs such as the stomach. Animals from group III showed reddish swellings around eyes, and from group IV have a very dark blood color.

3.3. Histopatological Changes

The histopathological changes in liver male were observed in Group (II) of rabbits gavaged with a dose of $^{1}/_{10}$ of penconazole (380 mg/kg b.w.) for 60 days such as mild lymphocytes, infiltration around bile duct in the portal area periporatal and fibrosis around portal area, fatty changes and hepatocyte degeneration (Figs. 1 and 2). In Figs. 3 and 4 are showed ballon cells.

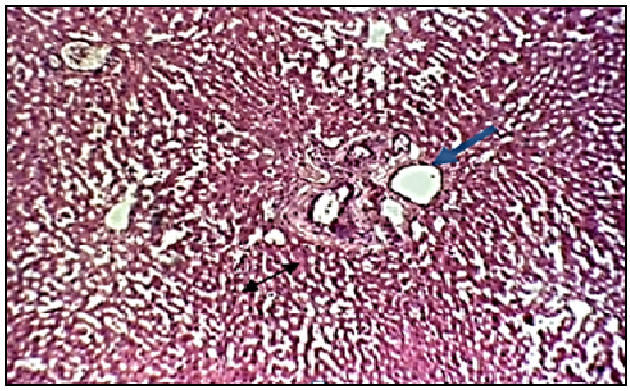

Fig. 1. Mild lymphocytes infiltration (double arrow). Fibrosis and bile duct in the portal area (blue arrow) (H&E 10x).

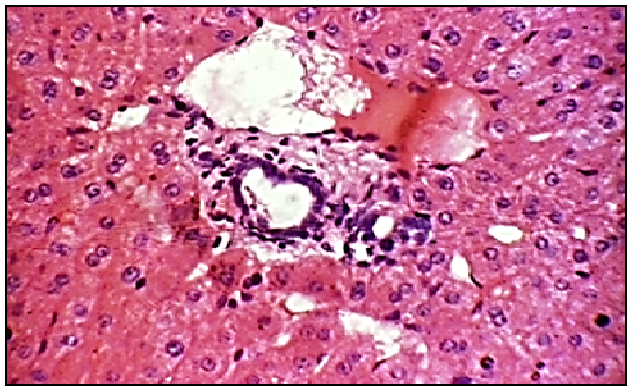

Fig. 2. Mild lymphocytes infiltration in portal area (H&E 40x).

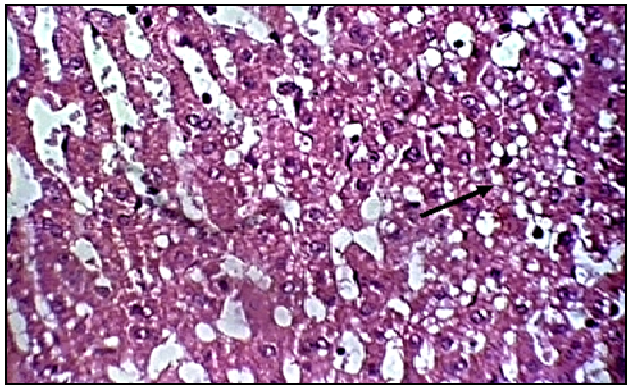

Fig. 3. Fatty changes (H&E 40x).

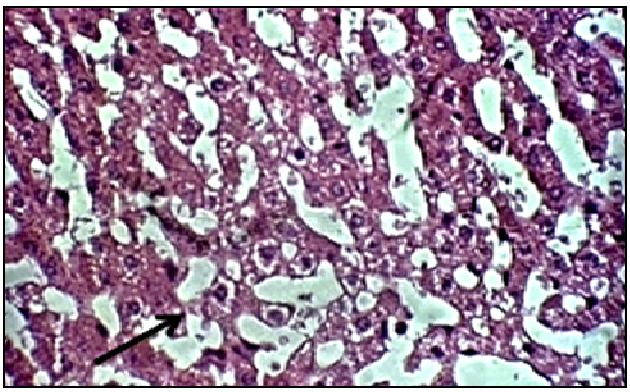

Fig. 4. Hepatocyte degeneration (ballon) (H&E 40x).

The histopathological section of Group III male rabbit liver exposed to $^{1}/_{10}$ of penconazole (380 mg/kg b.w. and extract of *Thymus laevigatus* (50 mg/kg b.w.) for 60 days showed periportal fibrosis around the bile ducts hepatocytes (Fig. 5) and cholestasis in hepatocytes (Fig. 6)

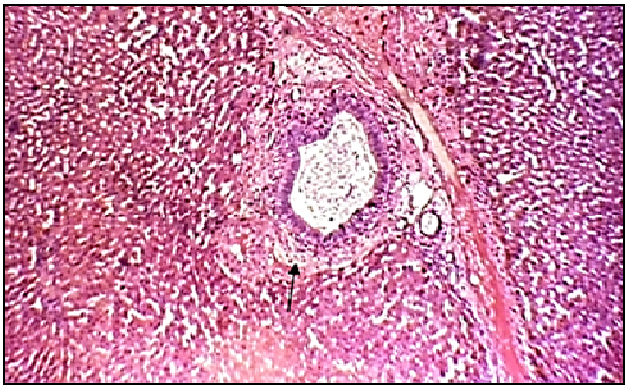

Fig. 5. Periporatal fibrosis around the bile ducts (H&E 10x).

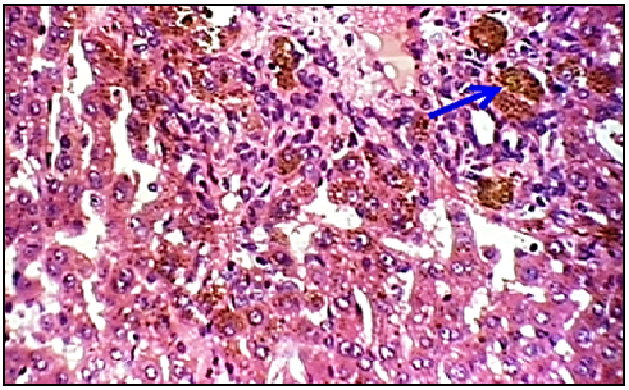

Fig. 6. Cholestasis in hepatocytes (blue arrow) (H&E 40x).

In group IV rabbits exposed to $^{1}/_{10}$ of penconazole (380 mg/kg b.w.) and extract of *Thymus laevigatus* (100 mg/kg b.w.) for 60 days a liver of the male rabbit showed no lymphocytes or fibrosis around (Fig. 7), as well as many double nuclei (Fig. 8).

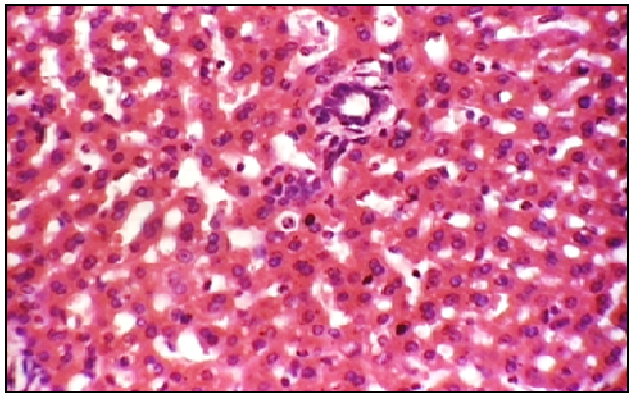

Fig. 7. No lymphocytes and no fibrosis (H&E 10x).

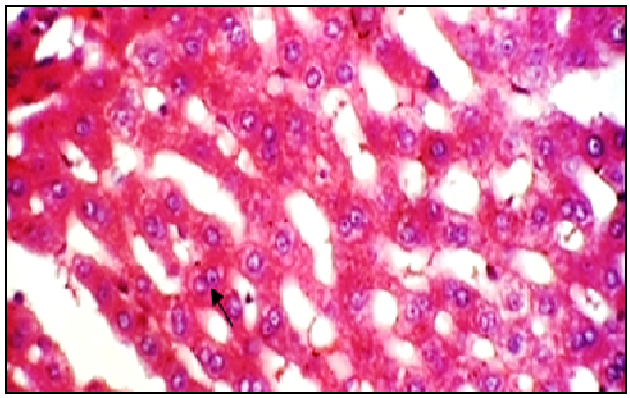

Fig. 8. Many double nuclei (arrow) (H&E 10x).

The histopathological changes in female liver (Group II), when rabbits gavaged with a dose of $^{1}/_{10}$ of penconazole (380 mg/kg b.w.) for 60 days showed balloon degeneration and fatty change (Fig. 9).

In Group III rabbits exposed to $^{1}/_{10}$ of penconazole (380 mg/kg b.w.) and extract of *Thymus laevigatus* (50 mg/kg b.w.) for 60 days, female rabbit liver showed degeneration, no fatty change or balloon hepatocytes many double nuclei (Fig. 11).

In Group IV when rabbits exposed to $^{1}/_{10}$ of penconazole (380 mg/kg b.w.) and extract of *Thymus laevigatus* (100 mg/kg b.w.) for 60 days, female rabbits liver showed mild cholestasis in the hepatocytes (Fig. 12) and increased fibrosis around bile ducts (Fig. 13).

The histopathological changes in lungs of male rabbits were observed in Group II which gava-

ged with a dose of $^{1}/_{10}$ of penconazole (380 mg/kg b.w.) for 60 days, showed thickened alveolar wall and dilation of alveolar spaces (Figs. 14 and 15).

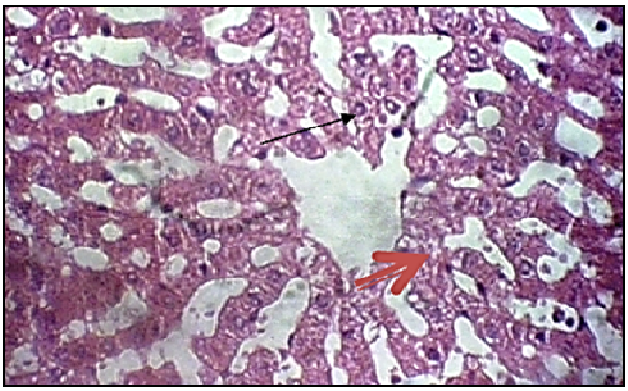

Fig. 9. Balloon degeneration (black arrow). Lymphocytes in the lobule in control and fatty change (red arrow) (H&E 40x).

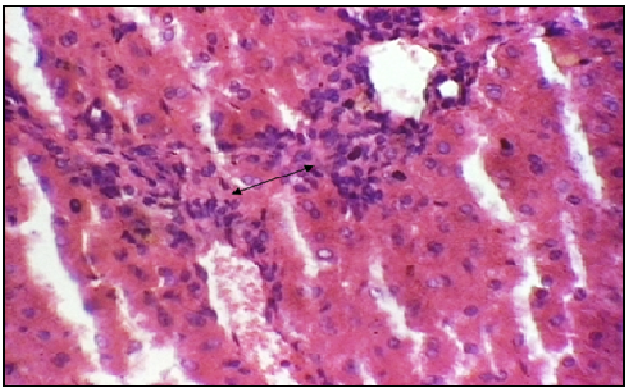

Fig. 10. Lymphocytes in the lobule in control and fatty change (arrow) (H&E 40x).

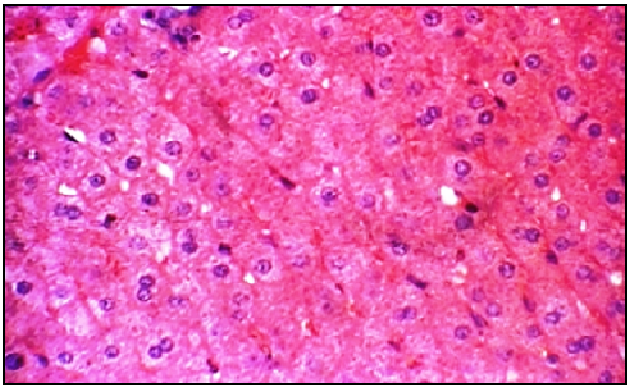

Fig. 11. Degeneration, no fatty change or balloon hepatocytes many double nuclei (H&E 40x).

The histopathological changes in Group III where female rabbits were exposed to $^{1}/_{10}$ LD_{50} of penconazole (380 mg/kg b.w.) and extract of *Thymus laevigatus* (50 mg/kg b.w.) for 60 days, lungs showed inflammation around bronchi and lymphoid aggregation (Fig. 16).

Male rabbits lungs exposed to $^{1}/_{10}$ of penconazole (380 mg/kg. b.w) and extract of *Thymus laevigatus* (100 mg/kg b.w) for 60 days at Group IV showed thickened alveolar walls, dilation alveoli space and edema (Figs. 17 and 18).

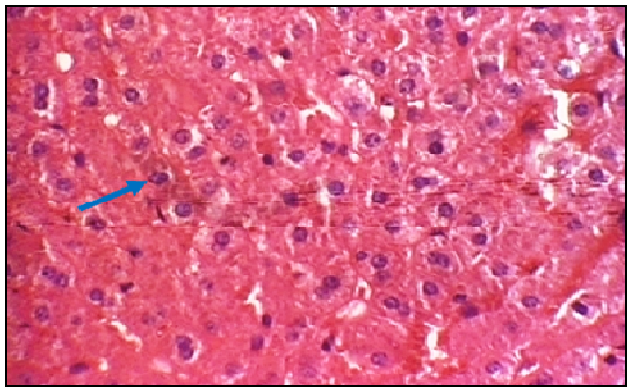

Fig. 12. Mild cholestasis in the hepatocyte (blue arrow) (H&E 10x).

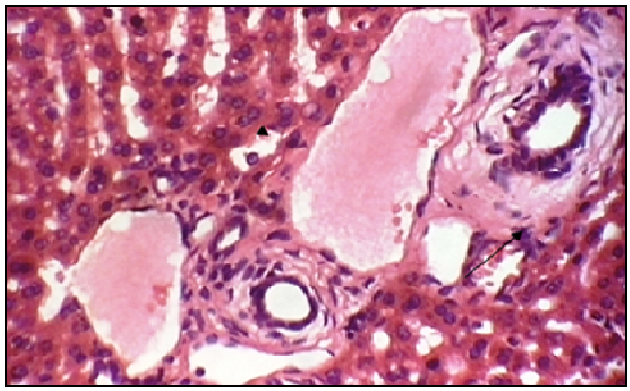

Fig. 13. Increase fibrosis around bile duct (arrow) (H&E 10x).

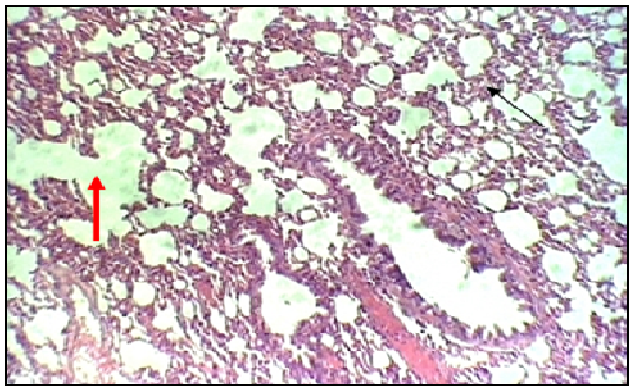

Fig. 14. Thickened alveolar wall (black arrow and dilation alveolar spaces (red arrow) (H&E 10x).

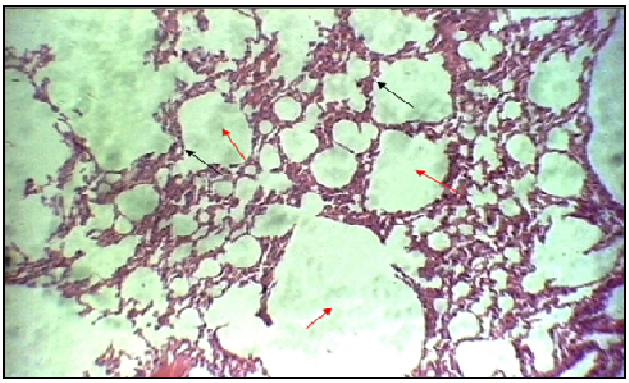

Fig. 15. Thickened alveolar walls (black arrows) and dilation alveolar spaces (red arrows) (H&E 40x).

The histopathological changes of the lungs in female rabbits in Group II which exposed of $^{1}/_{10}$ of penconazole for 60 days showed thickened alveolar walls, dilation alveolar spaces and edema (Fig. 19).

The histopathological changes of Group III which exposed to $^{1}/_{10}$ of penconazole (380mg/kg. b.w.) and extract of *Thymus laevigatus* (50 mg/kg b.w.) for 60 days, female lungs showed less edema, more alveolar space dilatation (Figs. 20 and 21).

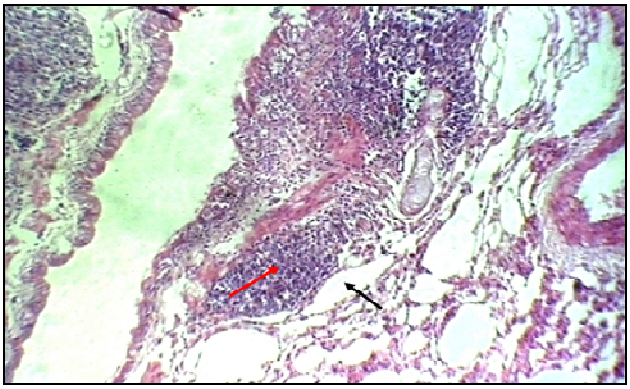

Fig. 16. Inflammation around bronchi (black arrow) and lymphoid aggregation (red arrow) (H&E 10x).

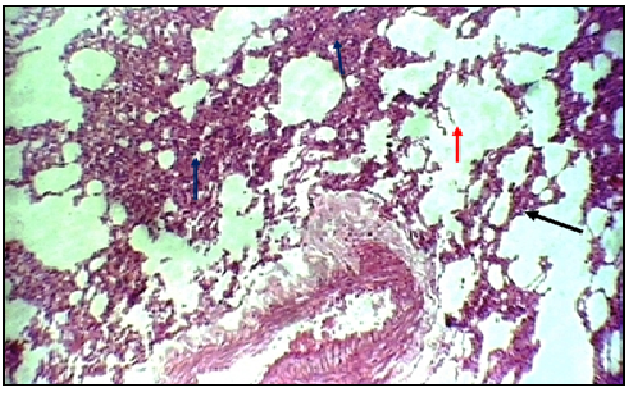

Fig. 17. Edema (black arrow) and dilation alveolar spaces (red arrow) (H&E 10x).

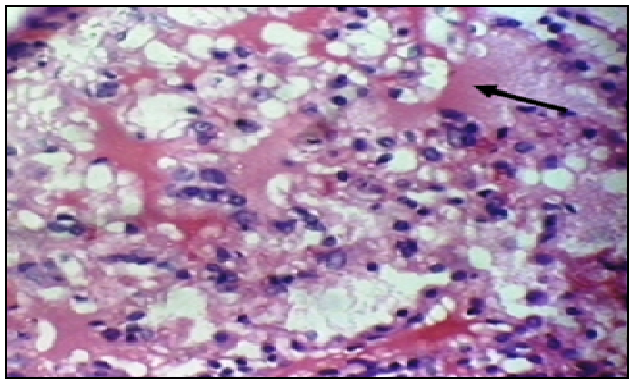

Fig. 18. Thickened alveolar walls and dilation alveolar spaces (H&E 40x).

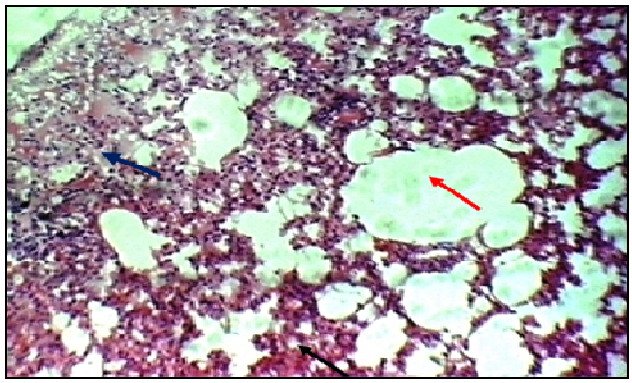

Fig. 19. Thickened alveolar wall (black arrow), dilation alveolar spaces (red arrow) and edema (blue arrow) (H&E 10x).

Female rabbits lungs of Group IV which were exposed to $^{1}/_{10}$ of penconazole (380 mg/kg b.w.) and extract of *Thymus laevigatus* (100 mg/kg b.w.) for 60 days showed dilation alveolar space (Fig. 22) and consolidation in the alveolar space and thickened alveolar wall and dilation alveolar space and no edema (Fig. 23).

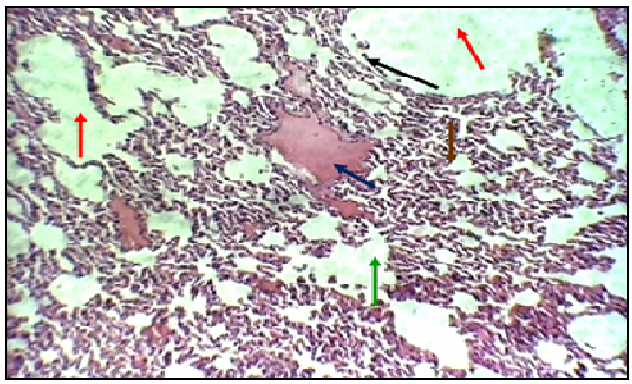

Fig. 20. Less edema (black arrow) with more alveolar space (black arrow), space dilation (brown arrow) dilation alveolar space (red arrow) and more normal area spaces (green arrow) (H&E 10x).

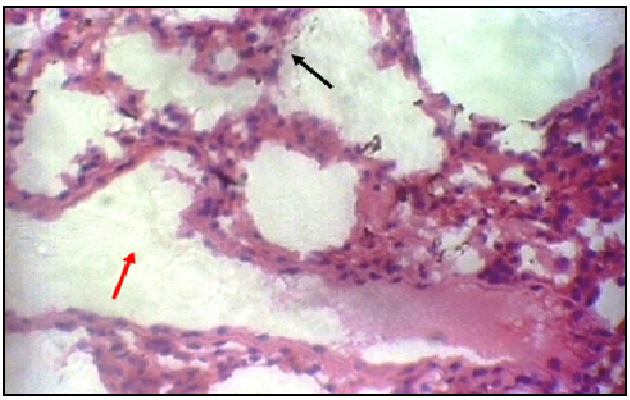

Fig. 21. Less edema (black arrow) and dilation alveolar space (red arrow) (H&E 40x).

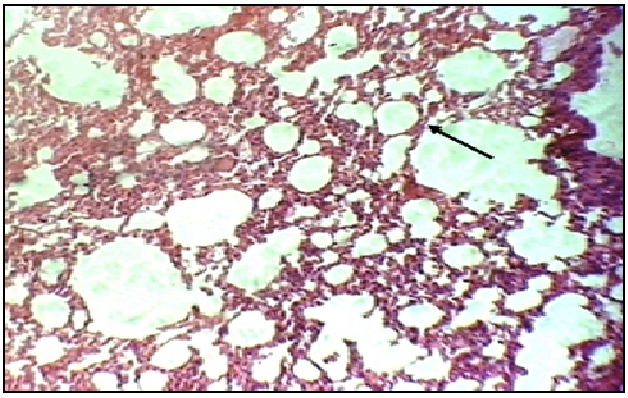

Fig. 22. Dilation alveolar space (arrow) (H&E 10x).

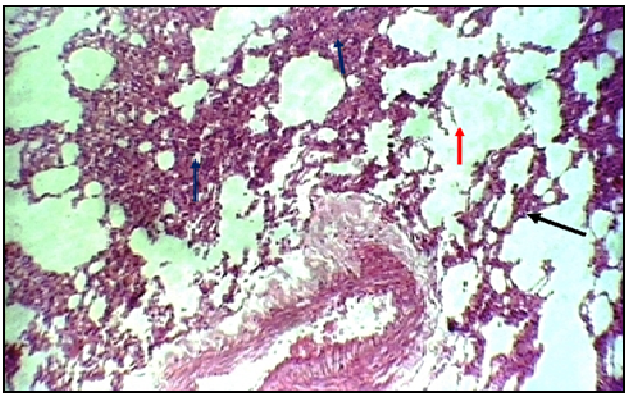

Fig. 23. Consolidation of the alveolar space and thickened alveolar wall dilated alveoli space (red) (H&E 10x).

4. DISCUSSION

The present study revealed various clinical symptoms and behavioural changes during the treatment of the experimental rabbits with penconazole fungicides, such as strong tremors which could be attributed to alternating contraction and relaxation of muscles, due to inability of the nerves to supply the muscles.

The animals also manifested piloerection followed by hair fall and reddish swellings around their eyes. These symptoms were related with skin irritation. Toxicological studies of penconazole on mice were observed by Parsons [17] and Waechter et al. [18], some treated rabbits with penconazole in the experiment lost body weight because loss of appetite according to penconazole toxicity. Schieicher and Salch [16] reported that white rabbits gavaged with 1000, 1500 or 2000 mg of penconazole/kg b.w. for 21 days caused transient signs of dyspnea, curved body position and ruffled for all dose levels.

Many histopathological changes in liver tissue of treated rabbits which were received a dose of $^{1}/_{10}$ penconazole for 60 days revealed lymphocytes infiltration around bile duct in the portal area fibrosis periporatal. These are interesting features of liver damage [19], and postulation that this damage is due to the increased level of lactic dehydrogenase in blood as well as an increased level of N-acetyl-ß-D-glucosaminidase (NAG). In groups III and IV of rabbits which were gavaged with $^{1}/_{10}$ of penconazole and extract of *Thymus laevigatus* (50 and 100 mg/kg) observed that manifested cholestasis in portal are and mild inflammation in the portal area, periporatal fibrosis and around the bile ducts, the changes was decreased as compared to group I. This is due to the active antioxidants of *Thymus* which play a major role in protecting the cells from oxidative damage, while Jürg et al. [19] observed that affect azole fungicides on the liver of rats using aromatase plant observed periporatal fibrosis and mild inflammation in the portal triad by antioxidant systems per oxidative free radicals.

Histopathological study of lung tissue in control group revealed the alveolar sac and bronchioles with normal epithelium. There are a histopathological changes in rabbits treated with penconazole such as thickened alveolar wall and dilated of alveolar spaces, more consolidation alveolar space and alveolar spaces and edema. Baciewicz et al. [20] reported vascular disruption with severe widening of the pulmonary interstitial and severe hemorrhage. Also Jürg et al. [19] recorded edema and alveolar hemorrhage in the lungs of tebuconazole in rats.

This protective effect of the extract may be mainly attributed to antioxidant for plants that offer protection against numerous diseases.

Conclusions

- Penconazole is toxic to rabbits as indicated by rabbit's behavior.
- There are histopathological changes in liver and lungs according to penconazole toxicity.
- The *Thymus laevigatus* extracts have inhibiting effects against penconazole toxicity on rabbits.

AUTHORS CONTRIBUTION

All authors contributed equally in planning, conduct, data analysis, and editing the work. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The author declares no conflicts of interest.

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Research Article

Participation of MEM+ bacteria in the bacterioplankton community in Ustka harbor, the River Słupia estuary, Southern Baltic Sea

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ABSTRACT

The number of bacteria was studied in the waters of the harbor canal in Ustka at the Baltic Sea. The studies were conducted with the use of the fluorescence microscope via live/dead method that differentiates the cells with the disintegrated membrane and live cells. The studies were conducted in a seasonal cycle at four research sites that differed in salinity and in the force of the influence of the marine environment. In all estuary zones the high percentage of dead bacteria (over 80%) was observed. The significant differences in number among particular sites were not observed. The relation between the number of live bacteria and the concentration of chlorides and organic matter was not demonstrated. It was showed that the number of bacteria falls within the greater seasonal fluctuation in the limnetic zone than in other estuary zones. Moreover, the very clear seasonal fluctuation of the studied parameters was observed. The maximum number of bacteria was stated in winter in the period of the lowest insolation.

Keywords: Estuary, Harbor, Bacteria, Live/dead, Baltic Sea.

1. INTRODUCTION

Harbors are complex economic structures fulfilling different function, apart from transport one to trade, industrial and city-forming functions [1]. Harbor waters are exposed to strong anthropogenic impact especially those ones situated in the river estuary which constitute the interesting biotope colonized by the biocenosis of organisms adopted to the existing specific environmental conditions. Heterotrophic bacteria play the significantly important role among the mentioned organisms. Both physical and chemical factors have an influence on the number and distribution [2]. The changing degree of the salinity in the harbor canal is one of the factors. The following water parameters such as organic matter content, pH, oxygen concentration have a significant influence on functioning of microorganisms [3, 4].

Harbor ports located at the estuary of rivers and seas often create unique ecosystems with a very diverse population of microorganisms due to the

transitional nature of the freshwater and seawater contact and due to the high variability of factors of physicochemical parameters of water [5]. During storms and seawater inlets, freshwaters contact masses of seawater that causes complicated processes of mixing and circulation which depends on many factors such as kinetic energy of freshwater, marine currents, tides and wind force [6]. It causes the presence of microorganisms characteristic for marine environment (halophiles) in the harbor port, while the freshwater flowing from the land introduces freshwater bacteria to the mentioned waters.

The varied physicochemical conditions of the harbor canal causes that a part of allochtonic bakteria dies in this specific ecotone due to the osmotic shock. However, the part of the microorganisms characterized by the higher tolerance to salinity changes is able to adopt and grow in a new environment [7].

The literature presents very little information on taxonomic diversification of bacteria occupying the coastal waters of the Baltic Sea, and additionally they were often performed on the basis of methods that were difficult to be mutually comparable. In Ustka region the dominant group of bacteria was *Flavobacterium-Cytophaga* with the simultaneous existence of numerous γ-proteobacteria, especially *Vibrio*. *Pseudomonas* bacteria type and cyanobacteria *Nodularia* were also numerous in the western part of the Baltic Sea [8-10]. However salinity plays the great role in the entire Baltic Sea. With the increase of the salinity level, the number of β-proteobacteria and *Actinobacteria* decreases and the number of α and γ-proteobacteria representatives and *Bacterioides* increases [11].

Harbor canals belong to the water areas which are exposed to strong anthropogenic impact that has a significant influence on the population of the existing microorganisms. Movements of ships and vessels can influence the quality of water in the harbor by resuspension of sediment deposited in the sea lane [12]. Other factors of anthropogenic origin that have an impact on the number of bacteria in the harbor canals are the discharge of the ballast water of the vessels mooring in harbors. Vessels coming from different parts of the world can introduce not only native microorganisms but also pathogens [13, 14]. Additionally, the ballast waters are polluted with huge amounts of petroleum substances.

Harbor water areas function as natural filters due to the big number of microorganisms, high bacterial production and intensively functioning of the microbial loop [15], reducing the amount of suspensions in water and the concentration of biochemical substances and petroleum pollutants flowing out from harbors.

The areas belong to the most productive water areas [16]. The flow of sea waters and the amount of biogenic soils carried by freshwaters contributes to it, especially when the discharge area of rivers is exposed to strong anthropogenic impact. As a consequence high concentration of dissolved organic matter (DOM) as well as particulate organic matter (POM) is observed [17].

2. MATERIALS AND METHODS

Ustka is a small tourist town (16.5 thousand inhabitants) located at the mouth of the River Słupia in the area of Słowińskie Shore. The town is a well-known seaside resort.

The Ustka marine harbor is the mouth of the longest river in the region - Słupia. It is 138.6 km long and it has a basin area of 1623 km^2 and the average flow at the mouth is 15.5 m^3/s. The river brings in certain amount of floating organic and non-organic matter into the Baltic Sea that originates from basin denudation and erosion. This material undergoes sedimentation to certain extend environing the mouth of the river and partially is floated as suspension with currents into the sea [18].

The marine harbor is located in the final section of the river with the length over 1100 m. It serves mainly fishing functions and passenger movement services, but the shipyard is located in the harbor as well [19].

2.1. Sample collection

Research sample collection and marking the numbers of bacteria with LIVE/DEAD method was conducted in the quarterly cycle in the years 2012-2013. In the estuary of the Ustka port, four research stations were determined (Fig. 1). The first station (st. 1) situated in the initial section of the port at a railway bridge with strongly marked impact of the river. The second station (st. 2) was located in

the middle part of the port channel. The third station (st. 3) was located in the side arm of the port, the so-called "coal basin". The fourth station (st. 4) is situated at the mouth of the port channel between breakwater heads. Samples taken from the deck of "Lucek" port tug boat belonging to the Sea Office in Słupsk. The unit owing to very low side facilitating sample collection ideally suited the research platform. Collected water samples were placed in sterile glass bottles with the capacity of 500 ml. The time between sampling and bacteriological analyzes did not usually exceed 2-3 h.

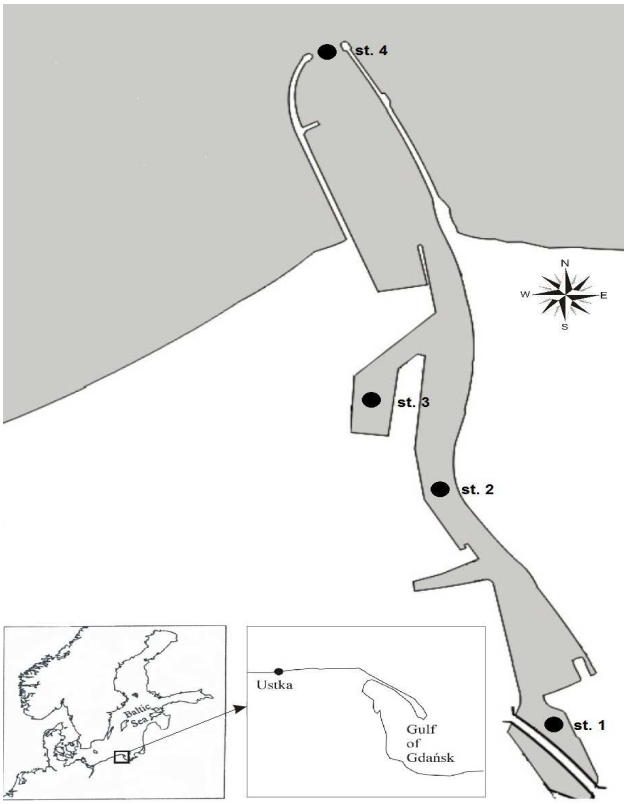

Fig. 1. Map of the study estuary with location of the sampling sites.

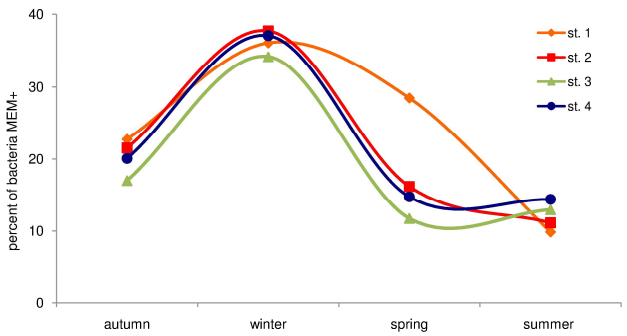

Fig. 2. Seasonal dynamics of bacteria MEM+ (live) in each areas of the estuary (in percentage).

2.2. Marking of chloride ions concentration

Samples of the examined water were analyzed chromatographically. Before injection to the chromatographic columns, samples were filtered by means of injection filters with the diameter of pores of 0.25 µm. Analysis of chloride anions in the examined waters was conducted using a ionic chromatograph by Metrohm (881 Compact IC pro), equipped with automatic sample feeder (863 Compact Autosampler). Separation of anions was carried out by means of column Metrosept A Supp 5-150/4.0 connected to protective column A Supp 4/5 Guard 4.0. The eluent was a mixture of 3.2 mM/L per$_2$ CO_3 and 1.0 mM/L of $NaHCO_3$, flowing with the intensity of 0.7 ml/min.

2.3. Marking organic matter

The organic matter was marked in terms of weight by marking out the difference between a dried sample, and a sample roasted for 4 hours in the temperature of 600°C. In order to remove carbonates the sample was previously treated with hydrochloric acid.

2.4. Marking the numbers of LIVE/DEAD bacteria

The number of "live" (MEM+) and "dead" (MEM-) bacteria was marked using the method of direct counting, under an epifluorescent microscope, of cells of bacteria stained using a commercial LIVE/DEAD test *BacLight* Bacterial Viability Kits (Invitrogen) strictly according to the instructions attached by the manufacturer. The LIVE/DEAD test consists of two agents: Syto 9 and propidium iodide. Component Syto 9 penetrates to the cells of bacteria with undamaged cell membrane and connects with DNA. After excitation with light λ=480nm connection of DNA-Syto 9 emits green light λ=500 nm. The second component of LIVE/DEAD test, propidium iodide penetrates only to the cells with disintegrated cytoplasmic membrane. After binding with DNA bacteria, excited using light λ=490 nm, propidium iodide emits red light λ=635 nm. Staining was applied to live, non-consolidated samples. Stained samples were filtered through black polycarbonate membrane filters with the diameter of pores of 0.2 µm of isopore

(Millipore) type using Millipore filtration apparatus. After drying, the filters were observed under an epifluorescent microscope Olympus BX4 using dichronous filter B-2A (Ex. λ=480/490nm, Em. λ=500/635 nm) with the magnification of 1000x ("dry" lens, ocular 10x). From each preparation, fluorescent bacteria were counted from twenty randomly selected the fields of vision. Green stained cells were classified as MEM+, while cells revealing red fluorescence were qualified as MEM-. The results obtained during microscopic observations were converted according to the formula Zimmerman, Mayer-Reil [20].

2.5. Statistical analysis

The statistical analysis of the obtained test results was done using STATISTICA 10 software. The variable distribution type was described by means of a Shapiro-Wilk normality test. If variable distribution met the condition of normality, for comparing the averages, ANOVA variance analysis was used. If variable distribution did not meet this condition, nonparametric test was used - the ANOVA Kruskal-Wallis rank test and the median test.

When testing the statistical association of two variables, the correlation coefficient was calculated. If at least one variable distribution did not meet the normality condition, Spearman's rank correlation coefficient (r) was used. In order to determine the strength of association between correlated variables, appropriate tables with critical values of the correlation coefficient were used.

3. RESULTS

Data relating to concentration of chlorides and organic matter in particular zones of the port estuary have been presented in Table 1. They imply that the highest concentration of chlorides (3237.7 mg/l) was observed in the area of port breakwaters and the lowest (328.5 mg/l) in the area of the river mouth. A very similar situation was observed in concentration of organic matter, the most of which (1.6 g/l) was observed in the mouth of the estuary to the sea and the least (0.36 g/l) in the area of the river where water was least salty.

Table 1 also presents the results of the numbers of live and dead bacteria in particular zones of the Ustka estuary. It was stated that the total number of live and dead bacteria was highest in the coal basin ($2.92 \cdot 10^5$ ml^{-1}) and in the area of breakwaters ($2.86 \cdot 10^5$ ml^{-1}) whereas the smallest number of bacteria was recorded in the limnetic zone ($2.33 \cdot 10^5$ ml^{-1}). The share of live bacteria on three research posts in the mainstream remained at the level of approximately 19% only in the blind arm of the port, (st. 3) it was lower and amounted to 16.2%.

Table 1. The number of bacteria and the concentration of chloride ions and organic matter in the different zones of the estuary.

Station	Chlorides (mg/l)	Organic matter (g/l)	Live+Dead (10^5 ml^{-1})	Live (10^5 ml^{-1})	% Live	Range (10^5 ml^{-1})
st.1	328,5	0,36	2.33	0.46	19,9	0.49 – 5.0
st.2	2204,4	1,32	2.55	0.5	19,8	1.26 – 4.7
st.3	1246,7	0,7	2.92	0.47	16,2	1.41 – 7.1
st.4	3237,7	1,6	2.86	0.54	18,9	1.1 – 5.0

Table 2 shows the dynamics of seasonal changes in the numbers of "live" bacteria on different research posts. The presented data imply that at each post, bacteria were most numerous in the winter season, whereas the smallest number of bacteria populated all zones of the estuary in autumn. In addition, it was stated that in the limnetic zone (st. 1) the number of live bacteria was characterized by the highest dynamics of seasonal changes. In the winter period, there were more than 4 times more of them on that post than in autumn. On the other hand, on other positions, only about a double increase in the numbers in winter as compared to autumn was observed.

Table 2. Seasonal dynamics of bacteria MEM+ (10^4 ml^{-1}) in different areas of the estuary.

Station	st. 1	st. 2	st. 3	st. 4
autumn	1,84	3,39	3,15	3,29
winter	7,45	6,88	5,86	7,65
spring	5,97	6,30	5,67	4,77
summer	3,27	3,59	4,23	5,92

Also, a percentage of shares of live bacteria was calculated from among all bacteria counted under the microscope. These values were presented in Fig. 2. They imply that the percentage of live bacteria at each research post was subject to similar seasonal fluctuations. In the winter season, the share of live bacteria was definitely the highest and was, depending on the position, from 34.2 to 37.7%. In spring this figure clearly decreased, to reach the minimum (9.9-14.4%) in the summer.

A statistical analysis of the correlation ratio between the number of live bacteria at specific research posts and chloride concentration and organic matter was carried out. However, no statistically significant correlation between those parameters was revealed at any of the posts. The statistical correlations between the chloride concentration in specific zones of estuary and the number of dead bacteria were analyzed. In case of those parameters it was possible to demonstrate the statistically significant correlation ($p<0.01$) only at the post located at the dead end of the harbour (post no. 3). Also, the relation between the concentration of organic matter in particular seasons and the numbers of live bacteria was tested. A statistically significant relation between these parameters ($p <0.01$) was reported only in summer. In other seasons, no significant association was stated.

Also, the statistical analysis was conducted for the significance of differences in the numbers of live and dead bacteria at particular research posts, using ANOVA test. The results of this analysis did not show the existence of significant differences between the posts.

4. DISCUSSION

Estuaries are the most dynamic water ecosystems characterized by a very large fluctuation of many abiotic parameters, including mainly salinity, reaction, temperature, oxygen concentration and alimentary substances [15, 21-24]. Therefore, they are characterized by high level of primary production and the related high accumulation of organic matter. This creates, in these water reservoirs, the optimum development conditions for bacterial microflora [25]. That is because bacteria, as a result of very high ecological flexibility, are best adapted to living in continuously changing environmental conditions, in estuary reservoirs.

Bacterial microflora plays a key role in functioning of the microbiological loop. The quantity of coal assimilated in the secondary production process by bacteria determines, to a large extent, the quantity of organic matter carried between various cells of the trophic chain [26]. One of the indicators making it possible to estimate the share of bacterial microflora in transformation of organic matter in water reservoirs is its number [27]. However, commonly used methods of direct marking of the numbers based on DAPI staining do not take into account the metabolic activity of bacteria cells [28]. For this reason, in recent years methods are used enabling differentiation between active and inactive cells. In the case of estuary reservoirs with considerable salinity gradient, the methods of this type make it possible not only to estimate the number of bacteria responsible for transformation of organic matter but also the impact environmental conditions, especially salinity, the survival rate of bacteria in particular zones of the estuary [29].

In the research conducted in the port estuary in Ustka, the number of bacteria limnetic, mesohaline and polyhaline zones was maintained at a similar level. The conducted statistical analyses did not show significant differences between particular posts nor associations between the concentration of chlorides and the number of bacteria. In estuaries, changes are observed, occurring in taxonomic composition of the population of bacteria in particular zones differing in the degree of salinity. Limnetic water is dominated by halophobic bacteria carried by the river waters. Then, with salination of water, their number decreases and in their place, saltwater bacteria carried by the sea appear. Probably this fact resulted in averaging of the numbers of bacteria throughout the Ustka estuary. This phenomenon was observed also in many other

estuary reservoirs [30] also in the area of the Polish Baltic coast [31].

Osmotic pressure changes in the environment, caused by the changing concentration of chlorides lead to the osmotic stress of cells and significant inhibition of their metabolic activity. In consequence, the osmotic shock can lead to death and then the lysis of cells. It is confirmed by the obtained results of tests proving very high numbers of dead cells or cells with disintegrated membrane. At each post of the main stream of the port channel the number dead cells was maintained at the level of more than 80%. However such high numbers of dead cells are not characteristic only for estuaries and were also observed in natural fresh waters [32]. In strongly eutrophic Mikołajskie Lake the number of metabolically active bacteria even reached 2% [33] and in Mazuria lakes it has been about 1% [34]. On the other hand, in the side arm of the coal basin it was even higher, which may have been caused by additional harmful action of anthropogenic pollution accumulated there. A similar phenomenon was observed in strongly contaminated waters in the port in Messina on Sicily [35].

In estuaries with relatively fast water flow such as port in Ustka, usually it is hard to observe a clear impact of high concentration of organic matter on the development of bacteria that would compensate the impact of the osmotic stress [36]. At fast water flow, organic matter contained there will not manage to significantly affect the development of bacteria [29]. For this reason, in such ecotones the number and the distribution of bacteria are mainly a derivative of mutual clash of sea and river waters. Probably these phenomena explain the lack of correlation between the number of bacteria and the organic matter concentration, observed in Ustka. The lack such dependence was also indicated by Freese [29] in research conducted in the mouth section of the Warnow River.

In the conditions of moderate climate, the number bacterial microflora is strongly dependent upon the season. It is confirmed by extensive research conducted both in marine waters and in lakes and rivers [37-39]. In many cases, high number of bacteria in the spring-summer period is observed. Attention is also drawn to positive correlation between the temperature of water and the number of bacterial microflora [40]. It results in a significant growth in the number of bacteria in the summer season when the water temperature is the highest. However, in the water of the Ustka estuary the highest numbers of bacteria were observed in the winter season. Also then the most live bacteria were observed. This can prove the harmful effect of sunlight on the development of bacteria. This phenomenon was described many times in numerous works [41, 42]. Light absorbed by the cells, and especially its short-wave part, lead to gradual degradation of genetic material of bacteria [43]. High-energy UV radiation contributes to passage of nitrogen alkali in nucleic acids to an excited form, prone to formation of dimers, especially between the molecules of thymine lying on neighboring threads of DNA [42, 43]. In the conducted tests, a very high percentage of dead bacteria was observed in spring and summer, when solar radiation was most intense. Decrease in numbers of live bacteria in the periods of the highest insolation were also observed by Lamy et al. [44] and Freese [29].

On the basis of the research it was also stated that at the post located at the river mouth to the estuary, the number of bacteria was subject to the highest seasonal variability. It was most likely related to the river bringing material rinsed from the drainage basin in the periods of high water level. This condition was maintained during winter and spring precipitation and thaws, when the water level in the river was the highest. In other estuary zones, the number of bacteria was subject to lower seasonal variability. This phenomenon shows that the inflow, to the estuary, of fresh water containing bacteria rinsed from the drainage basin, is noticeable only in the limnetic zone. In other estuary zones, the sea water inflow conceals changes caused by inflows, removal of bacteria from deposits or anthropopressure. In those zones the number of bacteria derives from their number in the sea. Therefore, it is subject to much smaller seasonal fluctuations. Therefore, for Ustka, the sea is a kind of a buffer with high inertia, which in a way stabilizes environmental parameters of the examined ecotone of the estuary.

AUTHORS CONTRIBUTION

All authors contributed equally in planning, conduct, data analysis, and editing the work. The final

manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The author declares no conflicts of interest.

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Research Article

Physiological effects of allelopathic activity of *Citrullus colocynthis* on *Vicia faba* and *Hordeum vulgare*

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ABSTRACT

The objective of this study was to determine the impact of allelopathic potentials of different extract of *Citrullus colocynthis* shoot system on germination and metabolite accumulation in *Vicia faba* and *Hordeum vulgare*. *Citrullus colocynthis was* collected from Al-Thomamah region, Saudi Arabia and the experimental design was a complete randomized with three replicates. Water, chloroform and methanol extracts from dried shoot system of *Citrullus colocynthis* with different concentrations (25, 50, 75 and 100%) were prepared, in addition to distilled water (as Control). The results showed that great reduce in germination of *Hordeum vulgare* but *Vicia faba* was not affected. The seedling growth was more sensitive than the seed germination. The allelopathic effects of *Citrullus colocynthis* on growth showed slightly increase in shoot and root lengths. The leaf area was decreased in *Hordeum* and *Vicia* for all concentrations. Pigments (Chlorophyll A, B and caroteins) were increased in *Vicia* for all concentrations of extracts, and showed variations in *Hordeum* compared to control. Results also showed decrease of carbohydrates and increase of proteins for the both studied plants.

Keywords: *Citrullus colocynthis*, Allelopathy, Germination, Carbohydrates, Growth, Dry weight.

1. INTRODUCTION

Plants may affect other plants growing in their vicinity in a stimulatory or inhibitory manner through released biologically active compounds often termed as allelopathics, allelocompounds or allelochemicals. This phenomenon is termed as allelopathy, receiving an increased attention recently and is considered to be applied in practice for weeds and pest managements [1]. Allelopathic effects are common to many plant species and can be observed at any level of biological organization [2-6]. Plant extract that is not decomposed was thought to contain secondary compounds with allelochemical activity or phytotoxic which cause growth inhibiton [7]. However allelopathy may alter the available resources in the environment [8]. Allelochemicals are believed to be a joint action of several secondary metabolites including phenolic compounds [9], flavonoids [10], juglone [11] and terpenoids [12]. Many researchers have found that the inhibitory substances involved in allelopathy are terpenoids and phenolic substance [13, 14].

Citrullus colocynthis (L.) is an important medicinal plant belonging to the family Cucurbitaceae. It is an annual herb widely distributed in Saudi Arabia [15]. The curative properties of medicinal plants are mainly due to the presence of various complexes chemical substances of different composition which occur as secondary metabolites [16] several active chemical constituents of *C. colocynthis* plant were recorded. They are grouped as alkaloids, flanonoids, tannins, and essential oils [17]. A number of plant secondary metabolites including flavonoids and cucurbitacins have previously been reported from *C. colocynthis* [18]. The cucurbitacins are of great interest because of the wide range of biological activities exhibited in plants and animals. Plant based natural constituents can be derived from any part of the plant like stems, leaves, flowers, roots, fruits and seeds [19]. Sunil et al. [20] studied antioxidant and free radical scavenging potential of *C. colocynthis* methanolic fruit extract. *Citrullus colocynthis* is also one of the plants belonging to family Cucurbitaceae. It has a fruit commonly known as bitter apple. It has been used in herbal treatment of diabetes [21]. The aqueous pulb extract of fruit is used for kidney, liver function treatment [22]. The phenolic compounds isolated from plants are of great interest due to their antioxidative and anticarcinogenic activity. They play a very important role in absorbing and neutralizing free radicals. They contain not only minerals and primary metabolite but also a diverse array of secondary metabolite with antioxidant potential [23].

The purpose of this study was to assess the allelopathic effects of *Citrullus colocynthis* on seed germination characteristics, primary growth and biochemical changes associated with *Vicia faba* and *Hordeum vulgare*.

2. MATERIALS AND METHODS

Samples of *Citrullus colocynthis* were collected from Al-Thomamah region, Saudi Arabia during April 2011 and identified by plant taxonomist. A voucher specimen has been deposited at the Herbarium of Botany Department, Faculty of Science, King Saud University. The seeds of studied plants were also collected as follows: seeds of bean, *Vicia faba* crop 2011, seeds of barley, *Hordeum vulgare* crop 2010.

2.1. Preparation of water extract

10 gm of powder air dried *Citrullus collocythis* in a flask and added 200 ml of distilled water. Then magnetic stirrer for 15 minutes was carried out. The samples were left for 48 hours, and then filtrate. The filtrate was centrifuged for 15 min. to get a clear solution (stock solution) [24]. Prepare of 25, 50, 75 and 100% concentrations.

2.2. Preparation of methanol extract

According to Laddy et al. [25], 10 gm of air dried plant material was extracted by 200 ml methanol in soxhlet extractor for 24 hours at 40-45˚C. The residue obtained after rotary evaporator was dissolved in 3 ml methanol and completed to 100 ml by distilled water. It were prepared the 25, 50 and 100 % concentrations.

2.3. Preparation of chloroform extract

According to Laddy et al. [25], the concentrations of 25, 50 and 100% of chloroform extract were prepared.

2.4. Germination experiment

The experimental design was randomized complete block design with three replications. Seeds of *Vicia faba* and *Hordeum vulgare* were sterilized in 5% sodium hypochlorite solution for 10 minutes, rinsed through with de ionized water several times. Their germination was conducted on water porous paper support in petri dishes (25 seed per dish) at controlled temperature of 25± 1˚C and adds 7.5-15.0 ml of extract or distilled water. Then cover the dishes with paper para film to prevent evaporation and pollution and leave for 10 days. The number of germinated seeds was recorded in each dish [26].

2.5. Determination and analysis of growth parameters

Seeds of *Vicia faba* and *Hordeum vulgare* were germinated in pots. The pots of 14 cm diameter and 18 cm in height were filled with fertile

loam up to ¾ the height of the pot. Daily supply with 15 ml of *Citrullus colocynthis* extracts (25, 50, 75 and 100% water, chloroform and methanol) and control is added to the study plants. Plant growth being conducted in controlled conditions of temperature (25± 1˚C) illumination (dark/light cycle: 14/10 h) and 80% humidity into a green house of Botany Department, Faculty of Science, King Saud University.

Extracts of *Citrullus* were added daily in different concentrations (25, 50, 75 and 100%) for each test plants. Each concentration was prepared in three replicates. After 36 days of growth, the shoot and root lengths were long enough to measure using a ruler.

Fresh and dry weights were measured, leaf surface area were measured using portable area meter Model Li – 3000. Chlorophyll A, chlorophyll B and carotenoid pigments were accomplished based on method of Stirban [27], carbohydrate content was measured according to Nelson [28] and Sonogyi [29]. Protein content was measured according to Lowry et al. [30].

2.6. Statistical analysis

Each treatment was conducted with their replicates and the results were presented as mean ± SD (standard deviation). Each of the experimental values was compared to its corresponding control. The results were analyzed by one way Anova with used statistical package for social sciences (SPSS) Version 11.5.

Table 1. Germination percentage of *Hordeum vulgare and Vicia faba.*

Treatment	*Hordeum vulgare* germination %	LSD 0.05	*Vicia faba* germination %	LSD 0.05
Control	100 ± 0		100 ± 0	
Water 100%	15 ± 1	85.00 (*)	100 ± 0	.00
Water 75%	42.33 ± 2.51	57.66 (*)	100 ± 0	.00
Water 50%	65 ± 1	35.00 (*)	90.33 ± 0.57	9.6667 (*)
Water 25%	66.16 ± 1.25	33.83 (*)	100 ± 0	.00
Chloroform 100%	-	-	100 ± 0	.00
Chloroform 50%	5 ± 1	95.00 (*)	100 ± 0	.00
Chloroform 25%	70 ± 5	30.00 (*)	100 ± 0	.00
Methanol 100%	32 ± 2	68.00 (*)	94.66 ± 0.577	5.33 (*)
Methanol 50%	46.83 ± 4.53	53.16 (*)	100 ± 0	.00
Methanol 25%	85.33 ± 1.52	14.66 (*)	100 ± 0	.00

* The mean difference is significant at the .05 level. Mean of three replications in duplicates ± Standard deviation.

3. RESULTS AND DISCUSSION

3.1. Seed germination

The effect of *Citrullus colocynthis* extracts on the seed germination of *Hordeum vulgare* and *Vicia faba* is shown in (Table 1). Water extract at low concentrations (25, 50%) have slightly inhibitory effect on the seed germination of *H. vulgare* compared to control. At high concentrations (75, 100%) it showed significance inhibition. While seed germination of *V. faba* is not affected by water extract. Chloroform and methanol extracts showed significant inhibition at high concentration, the inhibition increase by increasing the concentration. While, seed germination of *V. faba* is not affected by chloroform and methanol extracts.

This indicates that the aqueous, chloroform and methanol extracts contained growth inhibiting allelochemicals and their effects were dependent on the extract of *Citrullus* concentration. These results were in agreement with Abdel Fattah et al. [31] who

observed that allelopathic effects can cause both stimulatory and suppressive effects at lower and higher concentrations respectively.

The same results were obtained by Seyed et al. [32] who showed that in different extract concentration of *Artemisia annua*, the most germination percentage is related to control and the least was related to 100% of the extract. Also, other scientists such as Mahmood *et al*. [33] and Abhinav and Kanade [34] revealed that the inhibitory effect of the extracts increased with increasing extract concentration.

Table 2. Length of shoot at start and after 36 days of treatment for *Hordeum vulgare and Vicia faba*.

Treatment	*Hordeum vulgare*				*Vicia faba*			
	Length of shoot at the start of experiment cm^2	**LSD 0.05**	**Length of shoot after 36 days of experiment cm^2**	**LSD 0.05**	**Length of shoot at the start experiment cm^2**	**LSD 0.05**	**Length of shoot after 36 days of experiment cm^2**	**LSD 0.05**
Control	12.667 ± 0.577		18.667 ± 0.577		20 ± 1		52 ± 2	
Water 100%	12.667 ± 0.577	.000	16.333 ± 1.155	2.33	17 ± 2	3.00(*)	30 ± 3	22.00(*)
Water 75%	13 ± 1	.333	14 ± 1	4.6667(*)	17 ± 2	3.00(*)	31 ± 2	21.00(*)
Water 50%	13 ± 1	.333	20 ± 1	1.33	17 ± 2	3.00(*)	41 ± 2	11.00(*)
Water 25%	13 ± 0	.333	28 ± 2	9.33(*)	20 ± 1	.00	30 ± 3	22.00(*)
Chloroform 100%	14 ± 1	1.333(*)	17 ± 2	1.6667	20 ± 1	.00	41 ± 2	11.00(*)
Chloroform 50%	12.667 ± 0.577	.00	19 ± 1	.333	20 ± 1	.00	30 ± 3	22.00(*)
Chloroform 25%	12.667 ± 0.577	.000	16 ± 1	2.6667	20 ± 1	.00	38 ± 2	14.00(*)
Methanol 100%	12.667 ± 0.577	.000	13.333 ± 0.577	5.33(*)	20 ± 1	.00	36 ± 4	16.00(*)
Methanol 50%	13 ± 1	.333	17 ± 1.732	1.6667	20 ± 1	.00	40 ± 2	12.00(*)
Methanol 25%	13.333 ± 0.577	.6667	22.667 ± 3.786	4.00(*)	20 ± 1	.00	33 ± 3	19.00(*)

* The mean difference is significant at the .05 level. Mean of three replications in duplicates ± Standard deviation.

3.2. Shoot and root lengths

Table 2 shows that the low concentrations of aqueous extract (25, 50%) had stimulation effect on shoot lengths in *Hordeum vulgare* while higher concentrations (75, 100%) induced greater inhibition after 36 days of treatment. similar results were recorded by chloroform and methanol extract. In *Vicia faba*, various solvent extracts reduced shoot length at all different concentration after 36 days of treatment.

Table 3 shows that the aqueous extract revealed inhibition of root lengths at 100, 75 and 50% and stimulated root lengths at 25%. However, chloroform extracts stimulated root lengths at 100%, 50% and 25%. Methanol extracts stimulated root lengths at 50% only for *Hordeum vulgare.* In case of *Vicia faba* all extracts of *Citrullus colocynthis* showed significance stimulation of root lengths at all concentrations. The same results were obtained by Mahmood et al. [33], who recorded that methanolic extract significanthy inhibited root and shoot growth. The allelopathic effect of *Citrullus colocynthis* has been attributed to the production of several active chemical constituents. They are grouped as alkaloids, flavonoids [17], saponins, tannins, carbohydrates, glycosides and essential oils [19].

Table 3. Length of root after 36 days of treatment for *Hordeum vulgare and Vicia faba.*

Treatment	Length of root after 36 days of experiment cm^2	LSD 0.05	Length of root after 36 days of experiment cm^2	LSD 0.05
Control	16 ± 1		7 ± 1	
Water 100%	12.33 ± 0.58	3.6667(*)	10 ± 1	3.00(*)
Water 75 %	15 ± 1	1.00	15 ± 1	8.00(*)
Water 50%	14 ± 2	2.00	10 ± 1	3.00(*)
Water 25%	17 ± 2	1.00	12 ± 2	5.00(*)
Chloroform 100%	18 ± 1	2.00	11 ± 2	4.00(*)
Chloroform 50%	16.67 ± 0.58	.6667	19 ± 2	12.00(*)
Chloroform 25%	16.67 ± 0.58	.6667	9 ± 2	2.00
Methanol 100%	15 ± 2	1.00	11 ± 2	4.00(*)
Methanol 50%	17 ± 2	1.00	11 ± 2	4.00(*)
Methanol 25%	15 ± 2	1.00	15 ± 2	8.00(*)

* The mean difference is significant at the .05 level. Mean of three replications in duplicates ± Standard deviation.

Table 4. Fresh and dry weight of shoot after 36 days of treatment for *Hordeum vulgare and Vicia faba.*

Treatment	*Hordeum vulgare*				*Vicia faba*			
	Fresh wt. g	LSD 0.05	Dry wt. g	LSD 0.05	Fresh wt. g	LSD 0.05	Dry wt. g	LSD 0.05
Control	0.45 ± 0.01		0.11 ± 0.03		7.81 ± 0.02		0.8 ± 0.02	
Water 100%	0.26 ± 0.01	.186(*)	0.02 ± 0.01	.090(*)	3.6 ± 0.05	4.210(*)	0.35 ± 0.02	.450(*)
Water 75%	0.29 ± 0.01	.160(*)	0.03 ± 0.02	.083(*)	3.71 ± 0.02	4.100(*)	0.36 ± 0.03	.440(*)
Water 50%	0.39 ± 0.02	.060(*)	0.05 ± 0.01	.063(*)	3.85 ± 0.05	3.960(*)	0.38 ± 0.02	.320(*)
Water 25%	0.46 ± 0.02	.0100	0.05 ± 0.02	.063(*)	3.82 ± 0.02	3.990(*)	0.48 ± 0.04	.420(*)
Chloroform 100%	0.21 ± 0.01	.240(*)	0.02 ± 0.01	.096(*)	2.52 ± 0.02	2.350(*)	0.24 ± 0.04	.190(*)
Chloroform 50%	0.21 ± 0.01	.240(*)	0.02 ± 0.01	.096(*)	3.67 ± 0.03	5.290(*)	0.61 ± 0.02	.560(*)
Chloroform 25%	0.36 ± 0.01	.090(*)	0.04 ± 0.01	.073(*)	5.46 ± 0.02	4.140(*)	0.41 ± 0.02	.390(*)
Methanol 100%	0.29 ± 0.02	.070(*)	0.03 ± 0.01	.073(*)	2.44 ± 0.04	3.820(*)	0.22 ± 0.02	.350(*)
Methanol 50%	0.38 ± 0.03	.160(*)	0.04 ± 0.01	.083(*)	3.91 ± 0.03	3.900(*)	0.33 ± 0.03	.470(*)
Methanol 25%	0.77 ± 0.02	.320(*)	0.08 ± 0.02	.033(*)	3.99 ± 0.04	5.370(*)	0.45 ± 0.05	.580(*)

* The mean difference is significant at the .05 level. Mean of three replications in duplicates ± Standard deviation.

3.3. Fresh and dry weights

Chloroform extract had the highest inhibitory effect on both fresh and dry weight of *H. vulgare* (0.21 and 0.02 gm respectively) while the highest fresh and dry weights were observed at low concentrations of aqueous and methanol extracts (0.46 and 0.77 gm fresh wt.), (0.05 and 0.08 gm dry wt. respectively (Table 4).

In *Vicia faba* it was found that higher concentrations had pronounced inhibitory effect on shoot fresh and dry weights (Table 4). In general at all extracts, highest concentrations induced allelopathic effects for *H. vulgare* and *V. faba*. Maximum fresh and dry weights were observed in untreated control. These results were similar to those of Malik

[35], El Khawas and Shehata [36] and Yamagushi et al. [37] that studied allelopathic effects of *E. globulus* leaf extract on germination and seedling growth of some vegetable and crop plants. Fresh and dry weight in *H. vulgare* and *V. faba* were also reduced significantly in all extracts, these results are in agreement to those obtained by Djanaguiraman et al. [38] who found that seedling dry matter of rice, sorghum and blackgram significantly reduced by leaf leachate of *E. globulus* and highest inhibition was observed in highest concentration. Fresh and dry weights of three wheat cultivars decreased in response to aqueous eucalyptus extract [39].

3.4. Leaf surface area

The effect of aqueous extract of *C. colocynthis* on the surface area showed significant inhibition at both high and low concentration (0.17 and 0.14 cm^2 respectively) for *Hordeum vulgare*. The same results were observed for chloroform extract at high and low concentration (0.05 and 0.18 cm^2 respectively). But the methanol extract of *C. colocynthis* showed significant stimulation at both high and low concentration (0.28 and 0.92 cm^2 respectively) for *H. vulgare.* The effect of all extracts of *C. colocynthis* on *Vicia faba* showed significant inhibition at all concentration. At concentration 50% chloroform showed significant stimulation for leaf surface area (7.22 cm^2) for *V. faba* (Table 5). These results are in agreement with An et al. [17]. They showed that any secondary compound with allelochemical activity can cause both stimulatory and inhibitory effects.

3.5. Chlorophyll content

Table 6 shows that the effect of different extracts of *C. colocynthis* on chlorophyll content (Ch. A, B and carotenoids) were differ greatly on *Hordeum vulgare* and *Vicia faba*. Aqueous extract (50%) stimulated chlorophyll A, B and carotenoids (0.87, 0.34 and 0.468 mg/g respectively) for *Hordeum vulgare*, also methanol extract stimulated chlorophyll A, B and carotenoids (0.98, 0.43 and 0.546 mg/g respectively). High concentrations of aqueous and chloroform extract inhibit Ch. A, B and carotenoids for *H. vulgare*. In *Vicia faba* all extracts (Aqueous, Chloroform and Methanol) with different concentrations (100, 75, 50 and 25%) simulated chlorophyll A. B and carotenoids. The highest stimulatory was effect on Chl. A were found in 25% aqueous, 50% chloroform and 50% methanol extracts being (0.837, 1.524 and 0.737 mg/g respectively). These results are supported by the findings of Corsato et al. [40] and Gliessman [41], who stated that the allelopathic effects is a natural interference in which the plant produces substances and metabolites that may benefit or harm other plants when released.

Table 5. Leaf surface area of *Hordeum vulgare and Vicia faba.*

	Hordeum vulgare		***Vicia faba***	
Treatment	**Leaf area cm^2**	**LSD 0.05**	**Leaf area cm^2**	**LSD 0.05**
Control	0.27 ± 0.02		6.12 ± 0.217	
Water 100%	0.17 ± 1.02	3.2900(*)	4.093 ± 0.084	2.0267(*)
Water 75%	1.39 ± 2.02	.5700	5.583 ± 0.635	.5367
Water 50%	0.06 ± 3.02	.7933	6.1567 ± 0.482	.0367
Water 25%	0.14 ± 4.02	.6233	4.9 ± 0.056	1.2200(*)
Chloroform 100%	0.05 ± 5.02	.6133	4.7 ± 0.329	1.4200(*)
Chloroform 50%	0.24 ± 6.02	.6167	7.22 ± 0.437	1.1000(*)
Chloroform 25%	0.18 ± 7.02	.2900	4.6167 ± 0.499	1.5033(*)
Methanol 100%	0.28 ± 8.02	2.2567(*)	5.1167 ± 1.139	1.0033(*)
Methanol 50%	0.66 ± 9.02	.6533	4.383 ± 0.802	1.7367(*)
Methanol 25%	0.92 ± 10.02	1.2567(*)	5.133 ± 0.317	.9867(*)

* The mean difference is significant at the .05 level. Mean of three replications in duplicates ± Standard deviation.

Table 6. Chl. A, B and carotenoids of *Hordeum vulgare and Vicia faba.*

	Hordeum vulgare						***Vicia faba***					
Treatment	**Chl. A mg/g**	**LSD 0.05**	**Chl. B mg/g**	**LSD 0.05**	**Carote-noid mg/g**	**LSD 0.05**	**Chl. A mg/g**	**LSD 0.05**	**Chl. B mg/g**	**LSD 0.05**	**Carote-noid mg/g**	**LSD 0.05**
Control	0.76 ± 0.01		0.301 ± 0.003		0.424 ± 0.004		0.459 ± 0.009		0.203 ± 0.003		0.256 ± 0.006	
Water 100%	0.55 ± 0.05	.20967 (*)	0.248 ± 0.002	.05333 (*)	0.332 ± 0.002	.09200 (*)	0.769 ± 0.011	.3103 (*)	0.351 ± 0.002	.1480 (*)	0.458 ± 0.008	.2020 (*)
Water 75%	0.7 ± 0.09	.05667	0.339 ± 0.003	.03733 (*)	0.436 ± 0.004	.01200 (*)	0.563 ± 0.003	.1040 (*)	0.442 ± 0.002	.2390 (*)	0.348 ± 0.004	.0920 (*)
Water 50%	0.87 ± 0.02	.10800	0.34 ± 0.005	.03867 (*)	0.468 ± 0.003	.04400 (*)	0.664 ± 0.008	.2057 (*)	0.389 ± 0.004	.1860 (*)	0.426 ± 0.005	.1700 (*)
Water 25%	0.29 ± 0.2	.46900 (*)	0.203 ± 0.007	.09833 (*)	0.241 ± 0.003	.18300 (*)	0.837 ± 0.007	.3780 (*)	0.395 ± 0.005	.1920 (*)	0.561 ± 0.004	.3050 (*)
Chloroform %100	0.35 ± 0.05	.41000 (*)	0.262 ± 0.002	.03933 (*)	0.337 ± 0.007	.08700 (*)	0.584 ± 0.004	.1250 (*)	0.334 ± 0.004	.1310 (*)	0.343 ± 0.003	.0870 (*)
Chloroform 50%	0.48 ± 0.2	.27633 (*)	0.307 ± 0.006	.00567	0.37 ± 0.005	.05400 (*)	1.524 ± 0.004	1.0650 (*)	0.828 ± 0.008	.6250 (*)	0.577 ± 0.007	.3210 (*)
Chloroform 25%	0.79 ± 0.1	.02533	0.66 ± 0.004	.35867 (*)	0.517 ± 0.007	.09300 (*)	0.567 ± 0.009	.1080 (*)	0.323 ± 0.003	.1200 (*)	0.483 ± 0.003	.2270 (*)
Methanol 100%	0.77 ± 0.03	.00633	0.359 ± 0.007	.05767 (*)	0.475 ± 0.005	.05100 (*)	0.507 ± 0.007	.0480 (*)	0.314 ± 0.004	.1110 (*)	0.314 ± 0.004	.0580 (*)
Methanol 50%	0.98 ± 0.01	.22300 (*)	0.432 ± 0.004	.13067 (*)	0.546 ± 0.004	.12200 (*)	0.737 ± 0.007	.2780 (*)	0.496 ± 0.004	.2930 (*)	0.303 ± 0.003	.0470 (*)
Methanol 25%	0.41 ± 0.01	.35200 (*)	0.334 ± 0.004	.03267 (*)	0.429 ± 0.008	.00500	0.48 ± 0.005	.0210 (*)	0.94 ± 0.005	.7370 (*)	0.611 ± 0.01	.3550 (*)

* The mean difference is significant at the .05 level. Mean of three replications in duplicates ± Standard deviation.

3.6. Carbohydrate content

As show in Table 7 all extracts of *C. colocynthis* stimulated carbohydrates in *H. vulgare* and inhibit carbohydrates in *V. faba*. The highest stimulatory effect carbohydrates of *H. vulgare* were found at low concentrations of extract (5.5, 4.88 and 5.03 mg/g) for aqueous, chloroform and methanol respectively. While the lowest was found in *V. faba* (2.5 and 3.15 mg/g) for aqueous extract. The same findings were obtained by El-Darier [42] and Pandey and Mishra [43].

3.7. Protein Content

Table 8 shows that the protein content of *H. vulgare* and *V. faba* stimulated by all extracts of *C. colocynthis* for all concentrations compared to control. This may be due to interfering of allelochemicals with physiological and biochemical processes in tested crops. Similar results were observed by El-Khatib and Hegazy [44] and El-Khawas and Shehata [36].

Table 7. Carbohydrate content in *Hordeum vulgare and Vicia faba.*

	Hordeum vulgare		***Vicia faba***	
Treatment	**Carbohydrate mg/g**	**LSD 0.05**	**Carbohydrate mg/g**	**LSD 0.05**
Control	3.32 ± 0.02		5.15 ± 0.05	
Water 100%	4.7 ± 0.05	1.380(*)	3.15 ± 0.05	2.00(*)
Water 75%	3.92 ± 0.02	.600(*)	2.5 ± 0.05	2.650(*)
Water 50%	3.61 ± 0.02	.290(*)	5.47 ± 0.07	.320(*)
Water 25%	5.5033 ± 0.006	2.1833(*)	4.3733 ± 0.031	.7767(*)
Chloroform 100%	4.03 ± 0.03	.7100(*)	5.21 ± 0.1	.060
Chloroform 50%	2.36 ± 0.03	.9600(*)	5.86 ± 0.04	.710(*)
Chloroform 25%	4.88 ± 0.03	1.560(*)	4.72 ± 0.02	.430(*)
Methanol 100%	3.4067 ± 0.006	.0867	4.32 ± 0.02	.830(*)
Methanol 50%	4.1233 ± 0.025	.8033(*)	4.4 ± 0.06	.750(*)
Methanol 25%	5.0367 ± 0.56	1.7167(*)	5.41 ± 0.03	.260(*)

* The mean difference is significant at the .05 level. Mean of three replications in duplicates ± Standard deviation.

Table 8. Protein content in *Hordeum vulgare and Vicia faba.*

	Hordeum vulgare		***Vicia faba***	
Treatment	**Protein mg/g**	**LSD 0.05**	**Protein mg/g**	**LSD 0.05**
Control	3.2433 ± 0.006		4.77± 0.06	
Water 100%	3.9733 ± 0.574	.7300(*)	5.98 ± 0.04	1.21(*)
Water 75%	4.1533 ± 0.006	.9100(*)	5.23 ± 0.03	.46(*)
Water 50%	4.4067 ± 0.006	1.1633(*)	4.98 ± 0.04	.21(*)
Water 25%	4.77 ± 0.02	1.5267(*)	4.84 ± 0.04	.07(*)
Chloroform 100%	4.97 ± 0.02	1.7267(*)	5.97± 0.03	.2(*)
Chloroform 50%	5.25 ± 0.01	2.0067(*)	5.10 ± 0.05	.33(*)
Chloroform 25%	3.47 ± 0.03	.2267	5.10± 0.04	.33(*)
Methanol 100%	4.22 ± 0.02	.9767(*)	3.75± 0.05	.02(*)
Methanol 50%	5.61 ± 0.01	2.3667(*)	4.17± 0.02	.06(*)
Methanol 25%	4.84 ± 0.04	1.5967(*)	4.30± 0.04	.47(*)

* The mean difference is significant at the .05 level. Mean of three replications in duplicates ± Standard deviation.

From the present study, it can be concluded that various solvent and water extracts of shoot system of *C. colocynthis* had allelopathic effects on germination and growth of *Hordeum vulgare* and *Vicia faba*. The extracts reduced germination in *H. vulgare* and not affect germination of *Vicia faba*. The extracts of *C. colocynthis* reduced growth of *H. vulgare* and *Vicia faba* and this inhibitory effect increased with increasing extract concentration. Inhibitory effect of various solvent extracts was not equal and highest inhibition was observed in methanolic extract while the lowest one was observed in chloroformic and aqueous extracts. This study revealed decrease of carbohydrates and increased of proteins for the two studied plants.

AUTHORS CONTRIBUTION

HMHS: Conception and design, Acquisition of data, Writing, review and revision of the manuscript,

Administrative, technical or material support HMHS and HKAAL: Development of methodology, Analysis and interpretation of data. Both authors read and approved the final of the manuscript.

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TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Research Article

Phytochemical profile and antibacterial activity of crude extracts of the pod of *Aframomum angustifolium* (Sonn.) K. Schum.

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ABSTRACT

Aframomum angustifolium (Sonn.) K. Schum., is a perennial herb indigenous to Uganda and is widely used for medicinal and ethnodietary purposes. The ether and methanol extracts of the ripe pod of *A. angustifolium* were screened for antibacterial activity against; *Staphylococcus aureus* (ATCC 43300), *Staphylococcus aureus* **(**ATCC 25923), *Escherichia coli* (ATCC 25922) and clinical isolates of *Escherichia coli*. The plant extracts were also analysed for their phytochemical constituents. The Agar-well diffusion assay was carried out to measure the antibacterial activity. The Minimal Inhibitory Concentration (MIC) of the extracts was determined using serial dilution method. The phytochemical analysis of the extracts was done using standard procedures. The MIC of the extracts was 125 mg/ml. The methanol extract showed the strongest antibacterial activity against *S. aureus* (ATCC 25923) of 18.5 mm. Both the methanol and ether extracts tested positive for various phyto-chemicals especially flavonoids and terpenoids which are known to have antibacterial activity. The ether and methanol extracts of the ripe pod of *Aframomum angustifolium* possess antibacterial activity, justifying the use of the fruit in ethno-medicine.

Keywords: *Aframomum angustifolium*, Minimal Inhibitory Concentration, Agar-well, Phytochemical, Antibacterial, Ethnomedicine.

1. INTRODUCTION

Aframomum angustifolium is a perennial herb indigenous to Uganda [1]. The genus consists of about 50 species, with about 13 species reported to occur in Uganda [2, 3]. The ripe fruits appear above the soil surface and are often eaten as a snack [1]. *Aframomum* fruits are usually collected from the wild and often sold in markets in Uganda. However, draining of swamps and cutting of riverine forests are threatening the survival of the plant [1].

Traditionally, *Aframomum* species have been used as laxative, antiseptic, antipyretic, analgesic, antischistomosal, carminative and a sexual stimulant. They have also been used to treat dysentery,

snakebites, cataracts, abscesses, oedema, colds, migraine and toothache among others [4-6].

Since prehistoric times, humans have used indigenous plants to treat infectious diseases [7, 8]. In spite of the long history, it was not until the discovery of penicillin that large-scale screening of higher plants for antibacterial substances begun [7, 9]. It was reported that less than 5,000 plant species had been studied in depth by 2000 despite the fact that higher plants contributed about 25% of the total of all drugs in clinical use [10, 11].

Many traditional medicinal plants contain compounds with antibacterial activity that often make excellent lead compounds used in drug development [11, 12]. New antibiotics continue to be isolated from nature but most of the time; they are variants of known substances without sufficient advantage to favour their use in place of well-established compounds [13]. Synthetic compounds have also been difficult to find [13, 14]. Antimicrobial resistance threatens the effective prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses and fungi. It is now a growing global public health concern and continues to threaten the successful treatment of infectious disease [12, 15]. It is also now common to hear about methicillin-resistant *Staphylococcus aureus* (MRSA) [12, 14].

Previous studies on the seeds of *Aframomum* have indicated that they have a strong antibacterial activity as well as different active principles [6]. More recently, Ngwoke et al. [12] showed that two of the compounds isolated and purified from the rhizomes of *A. melegueta* G3 & G5b were more potent than Vancomycin, a drug of last resort used in the treatment of MRSA.

Although several authors including [2, 12, 16-19], have carried out research on the different species of *Aframomum*, especially the seeds of the West African *A. melegueta*, no studies have been condicted on *Aframomum angustifolium.* Many of the studies have concluded that although *Aframomum* is a promising therapeutic antimicrobial agent, the high toxicity of the seeds due to the presence of oxalic acid limits its wide use [5, 6, 16, 17, 19]. However, no research has been carried out on *A. angustifolium.* The appropriate utilisation of local resources to cover drug needs is dependent on preliminary scientific studies to determine their efficacy and safety [20]. This study was therefore carried out to investigate the antibacterial activity of the pod of *A. angustifolium.*

2. MATERIALS AND METHODS

2.1. Study site

The study was carried out in Microbiology laboratory, Department of Medical Microbiology and the Phytochemistry laboratory in the Department of Pharmacology and Therapeutics, College of Health Sciences in Makerere University.

2.2. Collection of the medicinal plant

The plant material was harvested from the wild in Mpigi district in Central Uganda. Voucher specimens of the plant were collected according to standard procedures described in Martin [21] and deposited in the Makerere University Herbarium for identification.

2.3. Extraction of compounds from plant material

Sequential extraction of the plant material with petroleum ether and methanol was carried out. The dried powdered plant material (250 g) was soaked in 500 ml of petroleum ether for 4 days. The mixture was decanted and filtered using Whatman filter paper and the residue was air-dried for 2 days. After drying, the same plant material was soaked in methanol for four days and the same procedure was repeated.

2.4. Phytochemical screening

Phytochemical screening of crude plant extracts was conducted following the procedures described in Sofowora [22], and Trease and Evans [6].

2.5. Preparation of plant extracts

The fruit pod was stripped off and air-dried at room temperature for two weeks. The dried material was then ground into a fine powder using a mortar and pestle to facilitate the extraction process. The dry plant extracts were obtained by recovering the solvent used for dissolving the powder using a

rotary evaporator (Büchi® rotary evaporator Model R-205). A stock solution of 250 mg/ml was prepared for the bioassays by weighing 2.5 g of the extract and dissolving it in two drops of dimethylsulphoxide (DMSO). The stock solution was kept at 4 ^{o}C and varying concentrations were made from it for the bioassay.

2.6. Bacterial selection criteria

S. aureus (ATCC 25923) and *S. aureus,* (ATCC 43300) *E. coli* (ATCC 25922) and clinical isolates of *E. coli* were used since they represent typical Gram-positive and Gram-negative organisms respectively, and are the two most common organisms used by other researchers [9, 23]. They are also implicated in food poisoning, urinary tract infections, blood stream infections and wound infections [15, 24]. *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) are known to be sensitive to antibiotics. *S. aureus* (ATCC 43300, MRSA) and clinical isolates *E. coli* that were known to be resistant were selected. It was in the interest of the study to use both known resistant and susceptible strains of the test organisms, for comparison purposes. Clinical isolates of *E. coli* which has shown resistance to different antibiotics were used. The bacteria were obtained from the freezer and incubated for 18 hours. The isolates were subcultured to obtain pure isolated colonies, which were used for the assays.

2.7. Preparation of the medium

The medium was prepared by adding 40 g of Mueller-Hinton agar powder (Sigma-Aldrich Inc.) to one liter of distilled water and then boiling the mixture. The solution was autoclaved at 121 ^{o}C at 15 psi for 15 minutes and cooled to 50 ^{o}C in a water bath. It was then transferred into sterile Petri dishes. It was allowed to cool and solidify under sterile conditions, and then incubated for 24 hours at 37 ^{o}C to ensure that there is no microbial contamination.

2.8. Agar-well diffusion assay

The Mueller-Hinton agar plates were inoculated in three planes using sterile cotton tipped swabs. Cultures of *S. aureus* (ATCC 2592), MRSA (ATCC 43300), *E. coli* (ATCC 25922), and the clinical isolates of *E. coli* were inoculated separately on the solidified agar on each Petri dish. About 250 mg/ml of the test extracts were used. Oxacillin was used as the positive control, while Dimethyl Sulphoxide (DMSO) and a blank well were used as negative controls. The extracts and controls were dispensed into the uniformly cut wells of 10 mm diameter, which were filled to about ¾ of their height. The plates were incubated at 37 ^{o}C for 24 hours without inverting them. The sensitivity of the test organisms to the extracts was determined by measuring the diameters of the zone of inhibition surrounding the wells with a metric ruler. A zone devoid of growth around the well indicated the capacity of the plant extract to inhibit growth.

2.9. Determination of Minimal Inhibitory Concentration (MIC) by serial dilution method.

The MIC values were determined by preparing two dilutions of the stock extract solution in standard nutrient broth (Sigma-Aldrich Inc). Two test tubes were arranged in a row and serial dilutions of the crude extracts were carried out with 250 mg/ml as the highest concentration in tube 1. About 0.5 ml of distilled water was poured in each test tube, and then 0.5 ml from tube 1 was poured in tube 2. It was mixed well and the process repeated to produce two dilutions. Turbidity was used as a growth indicator and growth was compared with a 0.5 McFarland inoculum.

3. RESULTS AND DISCUSSION

3.1. Qualitative phytochemical tests

Phytochemical analyses were conducted on twelve phytochemical compounds in both the ether and methanol extracts (Table 1). Triterpenoids, carotenoids, carbohydrates, flavanoids and coumarins were found in both the ether and methanol extracts. Tannins, basic alkaloids and reducing sugars were found only in the methanol extract whereas triterpenoids/steroids, unsaturated compounds and pentose sugars were limited to the ether extract.

Table 1. Phytochemical analysis of the crude extracts of the fruit pulp of *A. angustifolium.*

Compound	Methanol extract	Ether extract
1. Triterpenoids/steroids	+	++
2. Tannins	+	-
3. Basic alkaloids	+	-
4. Coumarins	+	+
5. Flavanoids	++	+
6. Saponins	-	-
7. Anthraquinones	-	-
8. Reducing sugars	+	-
9. Pentose sugars	-	+
10. Carbohydrates	+	+
11. Carotenoids	++	+
12. Unsaturated compounds	-	+

Key: ++ = Strongly present, + = Present, – = Absent

3.2. Agar-well diffusion assay

The diameters of inhibition of both the methanol and ethanol extracts show that the plant has antibacterial activity (Table 2). Both the ether extracts (14.8 mm) and methanol extracts (16.2 mm) had greater diameters of inhibition than the positive control (11.7 mm) against *S. aureus* (ATCC 43300). The ether extract showed the strongest antibacterial activity against *S. aureus* (ATCC 25922) with diameter of zone of inhibition of 18.5 mm. The methanol extract had the strongest antibacterial activity against *S. aureus* (ATCC 43300) (16.2 mm).

Diameters of the zones of inhibition include the diameters of the wells (10 mm). Both strains of *E. coli* had similar inhibition zones to the positive control. *E. coli* strains were also the most resistant strains to the extracts. Generally, the methanol extract produced larger diameters of inhibition than the ether extract. The lowest concentration that inhibited growth of the bacteria (Minimal Inhibitory Concentration) was found to be 125 mg/ml of plant extract.

Table 2. Diameters (mm) of the zones of inhibition of the extracts.

Plant Extracts	*S. aureus* (ATCC 25923)	*S. aureus* (ATCC 43300)	*E. coli* (ATCC 25922)	*E. coli* (Clinical isolates)
Ether	18.5±0.50	14.8±0.29	10.8±0.29	12.0±0.80
Methanol	14.5±0.50	16.2±0.29	12.2±0.76	12.0±0.50
Positive control	44.5±0.29	11.7±0.29	12.3±0.58	13.0±0.00

Values are means ± standard deviation (SD).

4. DISCUSSION

Although other researchers have largely studied the seeds of different *Aframomum* species, a comparison shows similar phytochemical composition and biological activity with the pod extracts of *A. angustifolium.* For example, Gröblacher [18] reported the presence of saponin, tannins, alkaloids, steroid, cardiacglycosides, flavonoids, and terpenoids in the seed extract of *Aframomum melegueta.* Similar phyochemicals have been detected in the pod extracts of *A. angustifolium* from this study.

Aframomum species are best known for the production of labdane diterpenoids and flavonoids [2, 12] isolated, purified and tested the labdane diterpenes G3 and G5 from the rhizomes of *A. melegueta* against *E. coli, L. monocytogenes* and MRSA. These compounds were shown to exhibit more potent antibacterial activity compared to the current clinically used antibiotics ampicillin, gentamicin and vancomycin and can be potential antibacterial lead compounds. Similarly, this study showed both the ether and methanol extracts of *A. angustifolium* to possess greater antibacterial activity against *S. aureus* (ATCC 43300) than the positive control Oxacillin. The greater antibacterial activity of the plant extracts against the Gram-positive *S. aureus* strains is in agreement with what is known about antibacterial diterpenoids. According to Porto et al. [25] and Fonseca et al. [26] antibacterial diterpenoids are known to have

potent antimicrobial activity against Gram-positive organisms.

Cousins and Huffman [27] found the seeds of *A. giganteum* to be rich in flavonoids, particularly quercetin and kaempferol both of which possess antibacterial and antifungal activities. Ayafor, et al. [16] also found the seed extracts of *A. danielli* and *A. aulacocarpos* to contain Aframodial [1] and other bioactive diterpenoids. In is interesting to note that both the ether and methanol pod extracts of *A. angustifolium* were rich in flavonoids. The ether extracts of the pod of *A. angustifolium* from this study also tested positive for basic alkaloids. Tane et al. [2] also reported the presence of hydroxyphenyl alkaloids in the seed extracts of *A. melegueta*.

The antibacterial activity exhibited by the pods of *A angustifolium* is in agreement with the findings of Ayafor, et al. [16] and Cousins and Huffman, [27] who reported diterpenoids from *A. aulacocarpos*, Labdane diterpenoids from *A. albo-violaceum* and flavonoids from *A. giganteum* to possess antifungal, antiviral and other bioactivity. Although the essential oil composition of the pod of *A. angustifolium* was not examined in this study, we can conjecture that it is highly likely to also contain essential oils. This can be supported by research by Eyob et al. [28] who demonstrated that the pods of *A. corrorima* had different types of essential oil components, the major one being γ-terpinene (27.1%) with a typical odour.

CONCLUSION

The extracts of the pod of *A. angustifolium* have antibacterial activity against *S. aureus* ATCC 25922, *S. aureus* ATCC 43300, clinical isolates of *E. coli* and *E. coli* ATCC 25922 *in vitro*. They also contain phytochemical compounds, some of which have proven antibacterial properties.

AUTHORS' CONTRIBUTION

GUA wrote the original concept and study design and participated in drafting the original manuscript and carrying out the phytochemical analyses. Both GUA & CK participated in carrying out the anti-bacterial laboratory analyses. Both GUA & CK participated in writing subsequent drafts on the manuscript, and have both read and approved the final version.

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TRANSPARENCY DECLARATION

The authors declare that they have no competing interests.

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