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Research Article

Enhancement of alpha amylase production by *Aspergillus flavus* AUMC 11685 on mandarin (*Citrus reticulata*) peel using submerged fermentation

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ABSTRACT

Mandarin peel as submerged fermentation (SmF) source was tested for the production of alpha amylase enzyme by strain of *Aspergillus flavus* AUMC 11685. Incubation period, concentration of substrate, temperature, pH and size of inoculum were optimized to achieve the maximum production of alpha amylase enzyme by *Aspergillus flavus* using mandarin peel. The maximum production of alpha amylase enzyme by *Aspergillus flavus* was recorded at 4-5 days of incubation, 3% substrate concentration, inoculum concentration 10%, temperature 28-40°C and pH 4-5.5.

Keywords: Mandarin; α-amylase; *Aspergillus flavus*; Submerged fermentation.

1. INTRODUCTION

Nowadays, the new potential of using micro-organism as biotechnological source of industrially relevant enzymes has stimulated interest in exploration of extracellular enzymatic activities in several microorganisms [1-3]. Enzymes have been used for thousands of years to produce food and beverages, such as cheese, yoghurt, beer and wine [4].

Enzymes are protein catalysts synthesized by living systems and are important in synthetic as well as degradative process. Alpha amylase enzyme (α-1,4 glucan-glucanohydrolase) is widely distributed in nature. This extracellular starch degrading enzyme hydrolyses α-1,4 glucosidic linkages randomly throughout the starch molecule in an endo-fashion producing oligosaccharides and monosaccharides including maltose, glucose and alpha limit dextrin [5-8]. Alpha-amylase enzymes account 65% of enzyme market in world. Amylases had numerous applications including liquefaction of starch in the traditional beverages, baking and textile industry for desizing of fabrics [9-11]. Moreover, they have been applied in paper manufacture, medical fields as digestive and as detergent additives [12, 13]. Hence, any substantial reduction in the cost of production of enzymes will be a commercial positive stimulus [4]. Fungi are particularly interesting due to their easy cultivation, and high production of extracellular enzymes of large industrial potential. These enzymes have

commercial application in various industries [14].

Many useful enzymes are produced using industrial fermentation belonging to the genus *Aspergillus* [15, 16]. In fact *Aspergillus niger* is the largest fungal source of enzymes [17, 18]. α-amylase is widespread in animals, fungi, plants, and are also found in bacteria [19, 20]. Amylases from microbial sources are generally used in industrial processes due to a number of factors including productivity, thermostability of the enzyme as well as ease of cultivating microorganisms [21]. Alpha-amylases are produced commercially in bulk from microorganisms and represent about 25-33% of the world enzyme market [22].

Many attempts have been made to optimize culture conditions and suitable strains of fungi [23]. Selection of the microbial source for α-amylase production depends on several features, such as the type of culture (solid-state or submerged fermentation), pH and genotypic characteristic of the strain [24].

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms such as bacteria and fungi. Several additional compounds also released apart from the usual products of fermentation called secondary metabolites which, range from several antibiotics to enzymes [25, 26]. The development of techniques such as Solid State Fermentation (SSF) and Submerged Fermentation (SmF) has lead to industrial-level production of useful enzymes. Submerged fermentation utilizes free flowing liquid substrates, such as broths, enzymes are secreted into the fermentation broth [27]. The purification of products is easier in SmF. More than 75% of the industrial enzymes are produced using SmF, one of the major reasons being that SmF supports the utilization of genetically modified organisms to a greater extent than SSF. Another reason why SmF is widely used is the lack of paraphernalia regarding the production of various enzymes using SSF. This is highly critical due to the fact that the metabolism exhibited by microorganisms is different in SSF and SmF [28]. Solid-state fermentation (SSF) has been defined as the fermentation process which involves solid matrix and is carried out in absence or near absence of free water. The solid matrix could be either the source of nutrients or simply a support supplemented by the suitable nutrients that allows the development of the microorganisms [29]. There are some disadvantages of SSF like difficulties on scale-up, low mix effectively, difficult control of process parameters (pH, heat, moisture, nutrient conditions), problems with heat build-up, higher impurity product and increasing recovery product costs [30]. Optimization of various parameters is one of the most important techniques used for the production of enzymes in large quantities to meet industrial demands [31]. Production of extracellular alpha-amylase in fungi is known to depend on the growth of mycelium and both morphological and metabolic state of the culture [32].

The selection of a substrate (agricultural waste) for enzyme production depends upon several factors mainly related with cost and availability of the substrate, the solid substrate not only supplies the nutrients to the microbial culture growing in it but also serves as anchorage for the cells [33]. These agriculture wastes consist of carbon and nitrogen sources necessary for the growth and metabolism of microorganisms [34, 35]. These nutrient sources included orange and mandarin wastes, rice and wheat bran, tea waste, cassava flour, oil palm waste, apple pomace and banana waste [36].

An increasing trend toward efficient utilization of natural resources has been observed around the world. The direct disposal of agro-industrial residues as a waste on the environment represents an important loss of biomass, which could be bioconverted into different metabolites, with a higher commercial value [37]. Citrus by-products are the principal solid by-product of the citrus processing industry and constitute about 50% of fresh fruit weight [38]. Mandarin considers as a source of multiple beneficial nutrients for human beings. Processing of citrus by-products potentially represents a rich source of phenolic compounds and dietary fibre. The mandarin peel wastes contribute the major industrial food waste discarded in the environment arising from juice manufacturing and home wastes [39]. Biotechnological applications of mandarin peel wastes are interesting not only from the point of view of low-cost substrate, but also in solving problems related to their disposal [40].

Although several investigations were employed on the production of enzymes by fungal strains using different agriculture wastes, only few

researches were done studying the production of enzymes by fungal strains using mandarin peel wastes. This work aims to evaluate the potentials of *Aspergillus flavus* strain AUMC 11685 isolated from accumulated rains water at Jeddah region to produce extracellular alpha amylase enzyme using mandarin peel wastes as substrate by submerged fermentation. Moreover, several factors including: pH, temperature, incubation period and concentration of each of raw material and inoculum were tested for optimization and enhancement of α-amylase enzyme production by *Aspergillus flavus* AUMC 11685 using mandarin peel wastes as a substrate in the submerged fermentation process.

2. MATERIALS AND METHODS

2.1. Microorganism

Pure culture of *Aspergillus flavus* AUMC 11685, which was isolated from accumulated rains water, Jeddah, Saudi Arabia, was grown and maintained on potato dextrose agar and it used as an inoculum during optimization steps of the study. The identification of the tested fungal species was confirmed by Assiut University Mycological Centre (AUMC) and the strain is deposited at Assiut University Mycological Centre under the code *Aspergillus flavus* AUMC 11685. The slants of the strain were grown at 28°C for seven days and stored at 4°C.

2.2. Agriculture wastes

Five grams of the agricultural waste; mandarin peel were mixed in 500 ml Erlenmeyer conical flasks containing 100 ml distilled water and sterilized in autoclave at 121°C for 20 min. Mandarin peel chosen as the sole nutrient source for submerged fermentation (SmF).

2.3. Optimization methodology of submerged fermentation (SmF)

Submerged fermentation was performed to study the effect of various physico-chemical parameters required for the optimum production of α-amylase enzyme by *A. flavus* AUMC 11685. Conidia are scrapped from mycelia of the terrestrial fungal species which are grown on slants for five days at 28°C and suspended in sterile distilled water. One ml of this suspension is used to inoculate, under aseptic conditions, Erlenmeyer flasks (500 ml capacity) each containing 100 ml of previous sterilized medium (agriculture waste medium). The inoculated flasks are incubated at 28°C on a rotary shaker at 160 rpm for 7 days (Figure 1). *Aspergillus flavus* was subjected to several optimization factors for enhancement of α-amylase enzyme production using mandarin peel wastes by SmF. Each experiment was done in thrice.

Figure 1. The inoculated flask containing the submerged fermentation medium of mandarin peel wastes.

2.3.1. Initial pH

The tested fungal strain of *Aspergillus flavus* was grown on mandarin peel medium by applying the previously mentioned fermentation process at different initial pH 2, 4, 5.5, 7 and 10. The initial pH was adjusted by 0.1 M HCl or 0.1 M NaOH. The assay of α-amylase produced was determined.

2.3.2. Incubation temperature

The tested fungal strain of *Aspergillus flavus* was grown on mandarin peel medium by applying the previously mentioned fermentation process at different incubation temperature degrees 20, 25, 28, 35, 40 and 50°C at the optimum initial pH. The assay of α-amylase produced was determined.

2.3.3. Incubation period

The tested fungal strain of *Aspergillus flavus*

was grown on mandarin peel medium by applying the previously mentioned fermentation process at several intervals of inoculation periods 2, 3, 4, 5, 6 and 7 days at both the optimum temperature and initial pH. The assay of α-amylase produced was determined.

2.3.4. Concentration of raw material

The tested fungal strain of *Aspergillus flavus* was grown on mandarin peel medium by applying the previously mentioned fermentation process at different concentration of raw material of mandarin peel waste 1, 3, 5, 7 and 9 g at the optimum temperature, initial pH and the optimal incubation period. The assay of α-amylase produced was determined.

2.3.5. Concentration of inoculum

The tested fungal strain of *Aspergillus flavus* was grown on mandarin peel medium by applying the previously mentioned fermentation process at different inoculum concentrations 0.5, 1, 2, 5 and 10 ml at the optimum temperature, initial pH, the optimal incubation period and raw material concentration. The assay of α-amylase produced was determined.

2.4. Partially purification of enzymes

Conical flasks containing the agriculture waste medium and the fungal inocula are filtered at the end of the incubation period. Then, the filtrate introduced into dialysis bag against distilled water for 24 hours. The dialyzed filtrate was centrifuged at 10,000 rpm for 20 min. The supernatant was pooled and designated as cell-free broth. The cell free broth was frozen at -20°C for further purification steps [41].

2.5. Enzyme assay

α-amylase activity was determined by measurement of glucose released from starch according to the method of Miller [42]. The reaction mixture in tubes contained 125 µl soluble potato starch 0.2%, 125 µl sodium acetate buffer, pH 5.5, 50 µl of enzyme solution and distilled water to give a final volume of 0.5 ml (test solution) and was incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.5 ml dinitrosalicylic acid reagent (DNS), followed by incubation in a boiling water bath for 10 min followed by cooling. The absorbance was recorded at 560 nm. The enzymatically liberated reducing sugar was calculated from a standard curve using glucose. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol reducing sugar as glucose per minute under the standard assay conditions.

3. RESULTS

Alpha-amylase production by *Aspergillus flavus* AUMC 11685 isolated from water habitats in Jeddah, Saudi Arabia using mandarin peel by submerged fermentation was optimized.

3.1. The effect of pH

The result of the effect of different pH values on the production of α-amylase by *Aspergillus flavus* AUMC 11685 was shown in Table 1. The lowest productivity was obtained at pH 2 (7.32 U/ml), then the α-amylase activity sharply increased at pH 4 (24.73 U/ml), and gradually increased at pH 5.5 (26.90 U/ml). At pH values higher than 5.5 the productivity sharply decreased at pH 7 (17.99 U/ml) and at alkaline pH 10 (17.03 U/ml). The highest α-amylase enzyme production was recorded at pH 5.5.

3.2. The effect of incubation temperature

The result of the effect of different incubation temperature on the production of α-amylase was shown in Table 2. *Aspergillus flavus* has ability to produce α-amylase enzyme when incubated at temperature 20°C (15.76 U/ml) and 25°C (18.24 U/ml). α-amylase productivity sharply increased and recorded the highest productivity at 28°C (26.90 U/ml), then sharply inversed at 35°C (18.36 U/ml) and then declined gradually at 40°C (18.67 U/ml) and 50°C (14.38 U/ml). There was no noticeable change in amount of produced enzyme at temperature; 25, 35 and 40°C.

Table 1. Effect of different pH values on α-amylase production (U/ml) by *Aspergillus flavus* isolated from water habitats in Saudi Arabia using mandarin peel wastes as submerged culture.

pH values	Extracellular α-amylase production (U/ml)
2	7.32
4	24.73
5.5	26.90
7	17.99
10	17.03

One unit of α-amylase enzyme activity was defined as the amount of enzyme producing 1 µmol reducing sugar as glucose per minute under the standard assay conditions.

Table 2. Effect of different incubation temperatures on α-amylase production (U/ml) by *Aspergillus flavus* isolated from water habitats in Saudi Arabia using mandarin peel wastes as submerged culture.

Incubation temperatures	Extracellular α-amylase production (U/ml)
20 ºC	15.76
25 ºC	18.24
28 ºC	26.90
35 ºC	18.36
40 ºC	18.67
50 ºC	14.38

One unit of α-amylase enzyme activity was defined as the amount of enzyme producing 1 µmol reducing sugar as glucose per minute under the standard assay conditions.

3.3. The effect of different concentrations of substrate (mandarin peel)

The result of the effect of different concentrations of mandarin peel medium on the production of α-amylase was shown in Table 3. Our results showed that *A. flavus* could produce small amount of α-amylase using mandarin peel medium at concentration 1% (g/100 ml) (12.82 U/ml), then pointedly increased to the highest yield at concentration 3% (28.28 U/ml) and slightly decreased at concentration 5% (26.90 U/ml). After this, the productivity decreased gradually at concentrations 7% (17.24 U/ml) and 9% (16.79 U/ml).

Table 3. Effect of different concentrations of mandarin peel medium on α-amylase production (U/ml) by *Aspergillus flavus* isolated from water habitats in Saudi Arabia using mandarin peel wastes as submerged culture.

Concentration of mandarin peel medium	Extracellular α-amylase production (U/ml)
1 g	12.82
3 g	28.28
5 g	26.90
7 g	17.24
9 g	16.79

One unit of α-amylase enzyme activity was defined as the amount of enzyme producing 1 µmol reducing sugar as glucose per minute under the standard assay conditions.

3.4. The effect of incubation period

Alpha-amylase production was detected at different incubation periods as shown in Table 4. *Aspergillus flavus* could start α-amylase production using mandarin peel medium after two days of incubation (13.46 U/ml) and then the productivity increased in gradual trend at three days of incubation (18.10 U/ml). α-amylase production sharply increased recording the peak rate at the fourth day of incubation (33.52 U/ml), then progressively decreased in gradual trend at five (28.93 U/ml), six (27.12 U/ml) and seven (26.90 U/ml) days of incubation. The highest α-amylase enzyme production was obtained after incubation for 4 days.

3.5. The effect of inoculum concentration

The result of the effect of different concentrations of *A. flavus* inoculum on the production of α-amylase was displayed in Table 5. Little output of α-amylase was detected by inoculum concentration 0.5% of *A. flavus* (3.04 U/ml), sharply increased by inoculum concentration of 1% *A. flavus* (26.90 U/ml). α-amylase productivity soared gradually by inoculum concentration 2% of *A. flavus* (30.04 U/ml) and by inoculum concentration 5% of *A. flavus* (35.73 U/ml), then it boosted the highest significant increment by inoculum concentration 10% of *A. flavus* (64.30 U/ml).

Table 4. Effect of different Incubation periods on α-amylase production (U/ml) by *Aspergillus flavus* isolated from water habitats in Saudi Arabia using mandarin peel wastes as submerged culture.

Incubation periods	Extracellular α-amylase production (U/ml)
2 days	13.46
3 days	18.10
4 days	33.52
5 days	28.93
6 days	27.12
7 days	26.90

One unit of α-amylase enzyme activity was defined as the amount of enzyme producing 1 µmol reducing sugar as glucose per minute under the standard assay conditions.

Table 5. Effect of different Inoculum concentrations on α-amylase production (U/ml) by *Aspergillus flavus* isolated from water habitats in Saudi Arabia using mandarin peel wastes as submerged culture.

Inoculum concentration	Extracellular α-amylase production (U/ml)
0.5 ml	3.04
1 ml	26.90
2 ml	30.04
5 ml	35.73
10 ml	64.30

One unit of α-amylase enzyme activity was defined as the amount of enzyme producing 1 µmol reducing sugar as glucose per minute under the standard assay conditions.

4. DISCUSSION

The production of α-amylase using submerged fermentation by fungi has been reported by many workers [43-46]. In the present study, the optimum conditions for α-amylase production by *Aspergillus flavus* were acidic pH range 4-5.5, a temperature of 25-40°C for a period of 4-5 days using concentration of mandarin peels medium 3-5% and the concentration of *A. flavus* microbial suspension was positively related with productivity.

From our results extracellular α-amylase could be produced by *A. flavus* using mandarin peels at all pH values used but with different amounts. Extreme pH values (highly alkaline or acidic) decreased α-amylase production. At temperature 28°C, *A. flavus* showed the maximum α-amylase production, whereas below or above this temperature α-amylase production declined gradually. Extracellular α-amylase could be produced by *A. flavus* using mandarin peels (concentration 1%) and increased at concentration 3%, above this concentration there was a negative relation between α-amylase productivity and concentration of mandarin peels medium. After 4 incubation days *A. flavus* showed the maximum α-amylase production, whereas at less than this the α-amylase production declined or more than 4 days the productivity declined gradually. There was positive relation between concentration of *A. flavus* microbial suspension and α-amylase production. Our study reported that the highest α-amylase enzyme production by *A. flavus* isolated from water habitats in Saudi Arabia using mandarin peels medium was recorded at pH 5.5, temperature 28°C and incubation period of 4 days. The maximum productivity of α-amylase was detected when using concentration 3 g/100 ml of mandarin peels medium and 10% concentration of *A. flavus* microbial suspension.

Among the physical parameters, the pH of medium plays an important role by inducing morphological changes in fungi and in enzyme secretion [47]. The synthesis of extracellular α-amylase is affected by the pH [48].

In agreement to our results, Sivaramakrishnan et al. [49] who reported that alpha amylase enzyme synthesis occurred at pH range 3-9 with an optimum at pH 5 by *Aspergillus oryzae* on wheat bran. Our results are also nearly similar to those obtained by Acourene et al. [47] who reported that a maximum biomass was produced at pH=6.0, and the lowest at pH=9.0 and pH=4.0 during their study on alpha amylase production by *Candida guilliermondii* on date wastes. Also more or less similar findings confirmed by Djekrif-Dakhmouche et al. [34], Hernandez et al. [43], Alva et al. [50] and Renato and Nelson [51] on *Aspergillus* spp., Silva et al. [52] on *Penicillium purpurogenum* and *A. niger* at pH varying between 5.0 and 6.0. Guillen-Moreira et al. [53], reported that the growth and α-amylase enzyme production by *Aspergillus tamarii* were inhibited when the initial pH of the medium was above 10.0 or below 4.0. In contrast, Pavezzi et

al. [54] reported that pH=4.0 to be the best for the production of α-amylase by *A. awamori*. With inconsistence of our results Suganyadevi et al. [55] reported that the maximum production of α-amylase by *A. niger* on tuber of *Ipomoea batatas* was attained at pH 7. Moreover, Varalakshmi et al. [56] and Arunsasi et al. [8] found that the highest production of α-amylase by *Aspergillus flavus* on wheat bran and *Cocos nucifera* meal was accomplished at pH 7.5.

Temperature is one of the important factors, which strongly affect alpha amylase production by fermentation process [19, 57, 58]. Our findings were compatible with Suganyadevi et al. [55] who observed that the maximum yield of α-amylase production by *A. niger* was possible by submerged fermentation supplied with tuber of *Ipomoea batatas* at room temperature (28°C). Our results are also similar to those obtained by Ramachandran et al. [59] who studied α-amylase enzyme synthesis by *Aspergillus oryzae* on coconut oil cake and reported that 30°C proved to be the best temperature for the enzyme synthesis. In addition, similar results were obtained by Arunsasi et al. [8] who studied α-amylase enzyme production by *Aspergillus flavus* on *Cocos nucifera* meal.

Incubation at higher temperature affected the fungus harmfully. In agreement of our output Sivaramakrishnan et al. [49] reported that alpha amylase enzyme synthesis by *Aspergillus oryzae* occurred between 20-45°C with an optimum at 30°C on wheat bran. Acourene et al. [47] reported that alpha-amylase production by *Candida guilliermondii* on date wastes was low at 20°C, and increased to a maximum at 30°C. A further increment in temperature resulted in a decrease in dry biomass and α-amylase production. At higher temperature, due to the production of large amount of metabolic heat, the fermenting substrate temperature shoots up, thereby inhibiting microbial growth and enzyme formation [60]. Temperature above 45°C results in moisture loss of the substrate, which affects metabolic activities of fungi, and results in reduced growth and α-amylase production [61]. However, Kunameni et al. [62] and Ravi et al. [63] reported that optimum temperature for amylase production by *Trichoderma lanuginosus* and *Humicola lanuginosa* is 50°C. Moreover, the optimum temperature for the maximum α-amylase activity by some *Aspergillus* spp. was 30°C [34, 45, 46, 50, 51] and also the same by *Penicillium brevicompactum* [64] and *Penicillium purpurogenum* [52].

Regarding the impact of incubation period on alpha amylase production, our findings were nearly came in agreement with Kareem et al. [36] who reported that the maximum α-amylase production by *Aspergillus oryzae* on *Cowpea* wastes was recorded after 72 hours of incubation. Sivaramakrishnan et al. [49] also reported the same during on wheat bran and Acourene et al. [47] with *Candida guilliermondii* on date wastes. In contrast to our results, Silva et al. [52] observed the highest production by *Penicillium purpurogenum* and *Penicillium brevicompactum* after 6 and 7 days of incubation and Balkan and Ertan [64] after 7 days with *Penicillium brevicompactum*.

No doubt that concentration of substrate affects α-amylase production. Similar to our findings Mohamed et al. [41] who studied the effect of mandarin peel concentration on α-amylase production by *Trichoderma harzianum* found that the highest level of enzyme activity was obtained at 5% of mandarin peel. Further concentration of mandarin peel repressed the enzyme production. Ramachandran et al. [59] reported that 0.5% concentration of starch was most suitable and higher concentrations of starch resulted in the inhibition of α-amylase enzyme synthesis by *Aspergillus oryzae* (data not shown).

The inoculum concentration has been reported as an important factor in enzymes production by fermentation. Lower inoculum concentration required longer time for the cells to multiply to sufficient number to utilize the substrate and produce enzyme. An increase in the number of spores in inoculum would ensure a rapid proliferation and biomass synthesis. Ramachandran et al. [59] reported that enzyme production increased with the increase in inoculum size from the lowest value of 0.5 ml and this in agreement of our current study, and they also reported that the maximum enzyme activity at 2 ml inoculum, further increase in the inoculum size resulted in decreased enzyme synthesis, indicating that limitation of nutrients occurred due to the increased microbial activity (results not shown) but this is not compatible with our results. Balkan and Ertan [64] reported that inoculum concentration 2.5 ml of *Penicillium brevicompactum*

gave the maximum production of alpha-amylase. Kareem et al. [36] reported that the maximum amylase production of α-amylase enzyme is attained at 4% *Aspergillus oryzae* inoculum level on *Cowpea* wastes and a further increase in the inoculums size did not increase the amylase yield. A lower level of inoculum may not be sufficient for initiating growth and enzyme synthesis.

General outlook indicates that our results are promising in enhancement of alpha-amylase production by growing strain of *Aspergillus flavus* AUMC 11685 on mandarin peel wastes in submerged culture fermentation. Based on the results obtained, mandarin peel wastes and our strain of *Apergillus flavus* were nearly more efficient in the quantity of alpha amylase production at the optimal conditions when they were compared with other wastes or substrates and microorganism in reported previous works. We have obtained 64.30 U/ml whereas Balkan and Ertan [64] detected 40 U/ml on rye straw, 50 U/ml on wheat straw, 25 U/ml on wheat branand 160 U/ml on corncob leaf by *Penicillium chrysogenum*, Farid and Shata [65] detected 1362.09 IU/g on wheat flour by *Aspergillus oryzae* LS1, Acourene et al. [47] estimated 1519.23 µmol/l/min on date wastes by *Candida guilliermondii* CGL-A10, Hang and Woodams [66] harvested 29 U/ml on baked-bean wastes and 0.06 U/ml on 2% cornmeal by *Aspergillus foetidus* NRRL 337, Suganthi et al. [67] found 43 U/mg on groundnut oil cake by *Aspergillus niger* BAN 3E, Singh et al. [27] indicated 11.0 U/ml on bacteriological peptone, $MgSO_4 \cdot 7H_2O$, KCl, starch by *Bacillus* sp., Krishna et al. [68] evaluated 23 U/ml on banana peel by *Aspergillus niger* NCIM 616 and Kumar et al. [69] produced 90 U/ml on sweet lime peel by *Aspergillus niger*.

5. CONCLUSION

The present study reveals that mandarin peel waste can be used safely as optional substrates than other agricultural/agro-industrial wastes such as wheat, corn, rice, potato and apple for the production of α-amylase enzyme. This study established the potential of the fungal strain of *Aspergillus flavus* AUMC 11685 for economic α-amylase production on mandarin peel in optimum conditions. This work gives an insight into the exploitation of a new agriculture wastes for the production of some industrial enzymes in appreciable levels.

AUTHORS' CONTRIBUTION

All the authors contributed in the success of this research article. The final manuscript has been prepared and revised by EHA and AMR. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Research Article

Influence of extracellular matrix on the proliferation and adhesion properties of stem cells derived from different sources

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ABSTRACT

One of the most important issues in regenerative medicine is the development of culture conditions mimicking the natural ones, which allows obtaining a large number of cells and their long-term maintenance in undifferentiated state. *In vivo*, cells are surrounded by a specific microenvironment called extracellular matrix (ECM), which plays an important role in the regulation of processes such as proliferation, migration, differentiation or apoptosis. In this study we assessed the influence of different extracellular matrix components (fibronectin, laminin, collagen IV, poly-D-lysine) on the *in vitro* adhesion and proliferation of stem cells isolated from bone marrow, adipose tissue and hair follicles. Our results showed that stem cells derived from different sources present various responses to ECM components. None of the tested extracellular proteins reduced the proliferation of bone marrow as well as adipose-derived mesenchymal stem cells, with the exception of laminin. This demonstrates the biocompatibility of such modified surfaces and possibility of using them for culturing these types of stem cells. Different results were obtained for hair follicle stem cells. The presented results indicate that ECM is an important component of the cellular niche in the tissue. It is also possible that ECM is required for the reconstitution of the niche of stem cells *in vitro*.

Keywords: Stem cells; Bone marrow; Adipose tissue; Hair follicles; Extracellular matrix.

1. INTRODUCTION

Tissue engineering methods offer new possibilities for the regeneration of diseased and damaged tissues and thus find an increasing attention in clinical practice. In tissue engineering, a variety of different cell types are used. However, the most attractive type are stem cells, particularly mesenchymal stem cells (MSCs).

One of the most important issues in the use of stem cells is the development of culture conditions mimicking the natural ones, which allows obtaining a large number of cells and their long-term maintenance in undifferentiated state. The proper growth and functioning of cells *in vivo* and *in vitro* depends on many factors, which result not only

from the interaction between cells (cell-cell type), but also from the interaction between cells and the extracellular environment (cell-matrix type) [1-3]. *In vivo*, cells are surrounded by a specific micro-environment called extracellular matrix (ECM), which plays an important role in the regulation of processes such as proliferation, migration, differentiation or apoptosis.

Although, fundamentally, ECM is composed of water, proteins and polysaccharides, every tissue has ECM with a unique composition and topology that is generated during tissue development through a dynamic and reciprocal biochemical and biophysical dialogue between the various cellular components and the evolving cellular and protein microenvironment. The ECM components can be divided into three major groups of molecules: insoluble (such as collagen, laminin, elastin, fibronectin), soluble (e.g. growth factors, chemokines, cytokines) and surface proteins of neighboring cells (cadherins). However, the composition and amount of all matrix molecules depends on cell type and location [2]. The selection of suitable extracellular matrix components may have a significant influence on *in vitro* cell growth. Moreover, appropriately selected ECM molecules often allow cell culturing in serum-free medium and/or without growth factors [4]. Such approach can minimize the risk of differentiation under *in vitro* conditions.

To date, both biological and synthetic materials have been used as ECM for *in vitro* cultures. However, materials derived from natural sources (e.g. collagen, laminin, fibronectin) appear to be preferable due to the presence of cell surface receptors that recognize these molecules [1].

The aim of this study was to assess the influence of different ECM components on the *in vitro* adhesion and proliferation of stem cells isolated from bone marrow, adipose tissue and hair follicles.

2. MATERIALS AND METHODS

The Local Bioethical Commitee of Nicolaus Copernicus University approved all procedures. In all studies, male Wistar rats (n=10) were used.

2.1. Isolation and culturing of bone marrow mesenchymal stem cells

Isolation of bone marrow was conducted using the Lennon and Caplan method [5]. Briefly, isolated rat femurs were washed with PBS supplemented with penicillin/streptomycin (100 μg/ml) and amphotericin B (5 μg/ml) (PAA, Austria). Distal parts of the femurs were cut off and bone marrow was flushed out using DMEM/Ham's F12 supplemented with 1% antibiotics solution. Subsesquently, the bone marrow was washed twice with PBS and centrifuged at 350 x g for 10 min. Isolated cells were cultured in the above medium containing additionally 10% FBS (PAA, Austria), 10 ng bFGF (Sigma, Germany) and L-glutamine (PAA, Austria).

2.2. Isolation and culturing of adipose mesenchymal stem cells

Adipose tissue was washed in PBS with antibiotics: penicillin/streptomycin (100 μg/ml) and amphotericin B (5 μg/ml). Subsequently, the tissue was purified from blood vessels and incubated in collagenase type I solution (1 ml/g of tissue) (Sigma, Germany) for 30 min in 37°C with shaking every 5 minutes. The digestion process was inhibited by adding an equal volume of culture medium. After that, the tissue was filtrated using a 100 μm cell strainer (BD Bioscience, USA). Thus obtained filtrate was centrifuged at 350 x g for 10 min and the cell pellet was washed twice with the culture medium. The cells were cultured in DMEM/Ham's F12 supplemented with 10% FBS (PAA, Austria), 10 ng bFGF (Sigma, Germany), amphotericin B (5 μg/ml), penicillin/streptomycin (100 μg/ml) and L-glutamine (PAA, Austria).

2.3. Isolation and culturing of follicle stem cells

Follicle stem cells were isolated from the hair follicles of rat sensory whiskers. A fragment of the skin was separated from the subcutaneous adipose and connective tissue and then washed in PBS with antibiotics: penicillin/streptomycin (100 μg/ml) and amphotericin B (5 μg/ml). Subsequently, the tissue was incubated in a dispase solution (10 mg/ml) (Gibco, USA) at 4°C for 16 h.

Hair follicles were isolated using micro-tweezers and the bulge regions were cut. Thus obtained hair follicle fragments were incubated in a solution of collagenase type P (1 mg/ml) (Roche, Switzerland) and dispase (1 mg/ml) for 0.5 h at 37ºC, followed by 0.05% trypsin solution (Biomed, Poland) for additional 1.5 h. After the incubation period, the solution was centrifuged (350 x g for 10 min). Cell culture was set up on a feeder layer (3T3 cell line) in Keratinocyte Serum-Free Medium (KSFM) (Lonza, Switzerland) supplemented with penicillin/streptomycin (100 μg/ml) and amphotericin B (5 μg/ml).

2.4. Phenotype analysis of isolated cells

Isolated stem cells were analyzed for the presence of specific surface markers by flow cytometry. Bone marrow stem cells were characterized with the use of CD90 and CD34 marker, adipose mesenchymal stem cells with the use of CD90, CD44, CD34 and CD45, while follicle stem cells with the use of cytokeratins 7, CD34 and p63. All analysis were performed according to the protocols previously described [6-8].

2.5. Evaluation of the influence of extracellular matrix proteins on the growth of stem cells

Stem cells isolated from three sources were cultured on 6-well plates coated with different ECM components such as: fibronectin, poly-D-lysine, laminin and collagen IV (BD Bioscience, USA). The number of seeded cells was 5 x 10^4/per well. Cells seeded on the polystyrene 6-well plate, not coated with any of the extracellular matrix components, served as a control. The cultures were run in media suitable for each type of stem cells at 37°C and 5% CO_2. Every 2-3 days, the medium was changed. The cells were incubated in these conditions for 7 days. Cell viability was analyzed using the MTT assay.

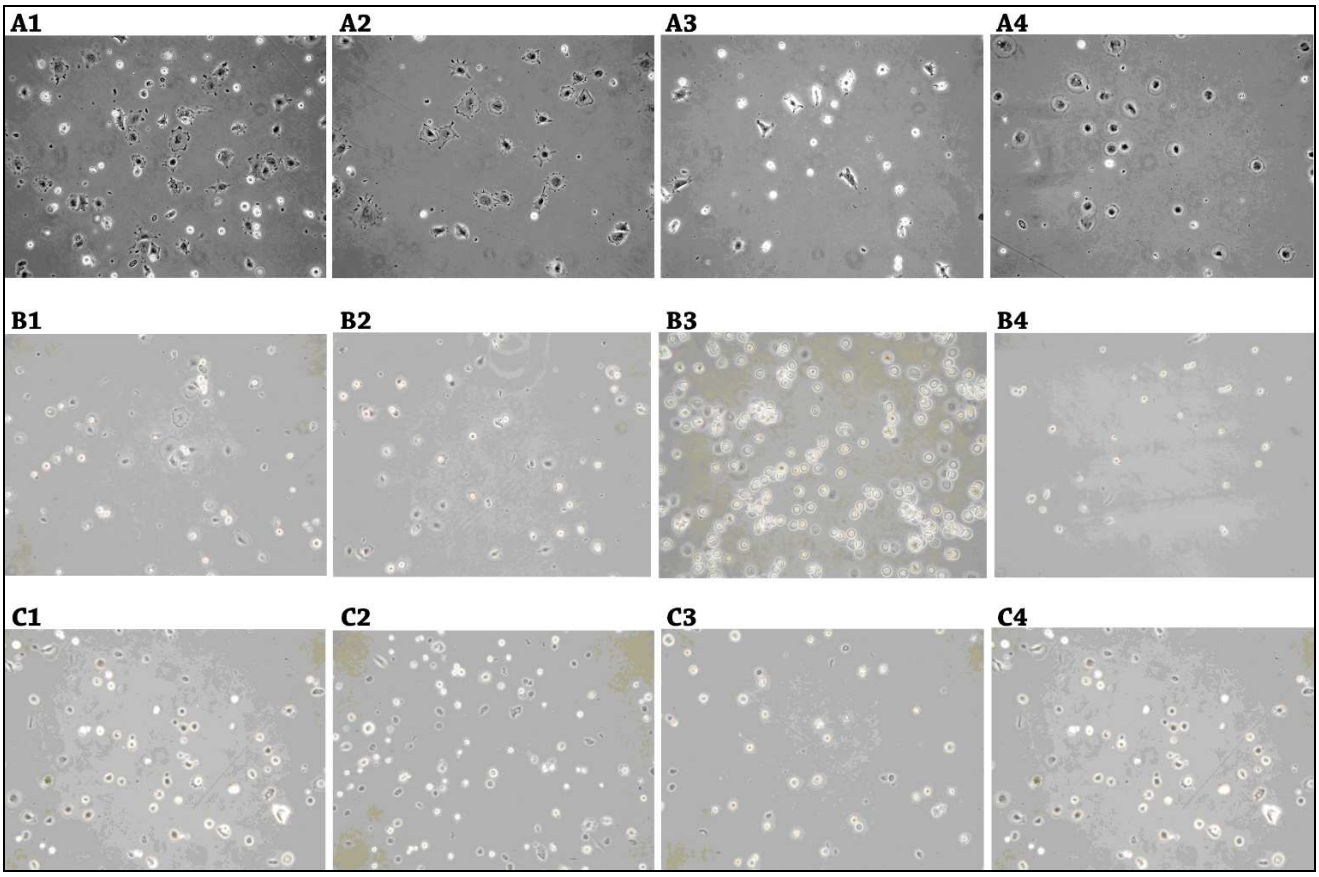

Figure 1. Stem cells isolated from bone marrow (A), adipose tissue (B), hair follicle (C) 30 minutes after seeding on plates coated with fibronectin (1), collagen IV (2), laminin (3) and poly-D-lysine (4).

3. RESULTS

For phenotypic characterization bone marrow, mesenchymal stem cells were assessed for the expression of CD90 and CD34. Expression of CD90 was at high level, while staining for CD34 was negative [6]. Adipose mesenchymal stem cells showed the high expression of CD90 and lower CD34 and CD44. The presence of CD45 was not detected [7]. Follicle stem cells expressed epithelial markers and were slighly positive for CD34 and p63 [8].

The analyzed stem cells showed significant differences in the ability of adhesion to the growth surface. The adhesion of bone marrow mesenchymal stem cells to the growth surface coated with fibronectin and collagen IV was 90% in 30 min after seeding (Fig. 1A1 and A2). Both modified surfaces supported the formation of a regular monolayer of spindle-shaped cells. However, at the same time, in the cultures on laminin- and poly-D-lysine-coated surfaces, the adhesion of cells was only 60% (Fig. 1A3 and A4).

Adipose-derived mesenchymal stem cells demonstrated a 90% adhesion during 30 min after seeding on plates coated with fibronectin, collagen IV and poly-D-lysine (Fig. 1B1, B2 and B4). However, the adhesion of the same cells cultured at the same time on laminin-coated surface was only 45% (Fig. 1B3). Hair follicle stem cells cultured on collagen IV- and laminin-coated plates showed a 50% adhesion during 3 h after seeding on the modified surfaces (Fig. 1C2 and C3). However, the adhesion of these cells to the culture plates coated with fibronectin and poly-D-lysine at the same time was only 10% (Fig. 1C1 and C4). The rate of cell proliferation was determined by MTT assay after 7 days of culture. The proliferation of bone marrow mesenchymal stem cells was the fastest on plates coated with fibronectin and collagen IV compared to the control culture. The slowest growth of these cells was observed on laminin-coated surface (Fig. 2).

The best results regarding the proliferation of adipose-derived mesenchymal stem cells were observed on the control surface, as well as the surface coated with collagen IV. The slowest growth of these cells was observed on plates coated with laminin (Fig. 3).

The proliferation of hair follicle stem cells was the fastest on the control surface that was not coated with any of analyzed extracellular matrix components. On each modified surface, cell growth was about 4 times slower (Fig. 4).

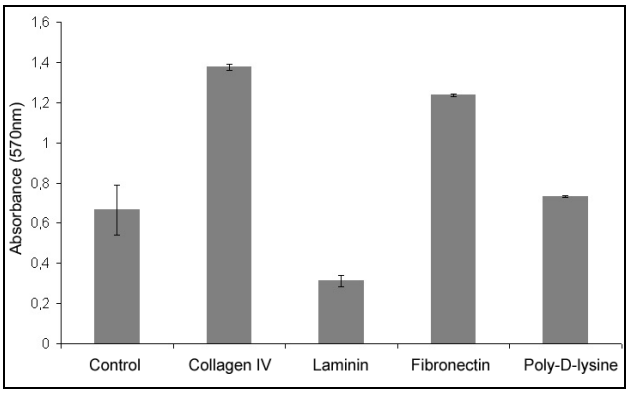

Figure 2. Proliferation rate of bone marrow mesenchymal stem cells on surfaces coated with different components of extracellular matrix.

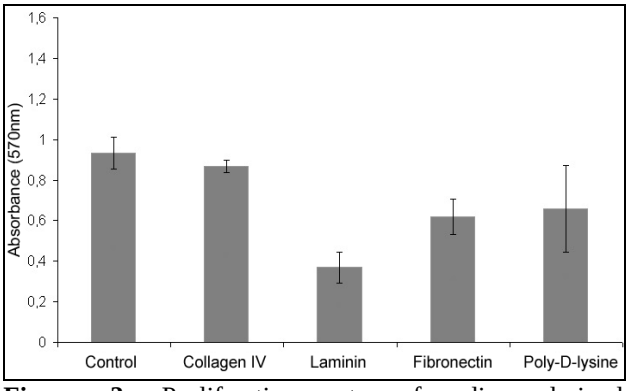

Figure 3. Proliferation rate of adipose-derived mesenchymal stem cells on plates coated with different components of extracellular matrix.

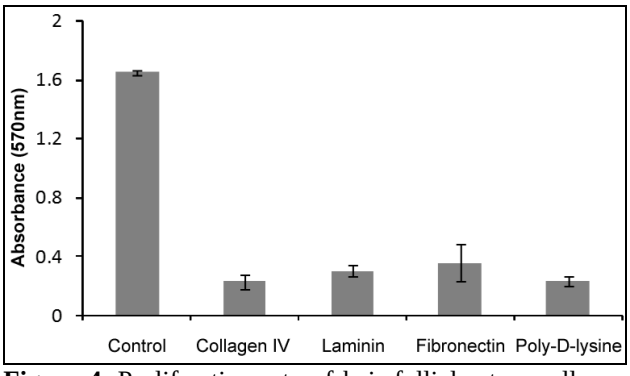

Figure 4. Proliferation rate of hair follicle stem cells on plates coated with different components of extracellular matrix.

4. DISCUSSION

The success of an *in vitro* culture often depends on the creation of environment that mimics the *in vivo* conditions. To date, a lot of biomaterials have been tested to provide a matrix for the proper cell adhesion and proliferation. Many materials such alginate, collagen or fibrin have been used. These molecules should support cell-cell and cell-matrix interactions, as well as regulate cell proliferation, migration, matrix remodeling and tissue organization - similarly to the *in vivo* conditions. Moreover, materials that are used should be biocompatible: show low antigenicity, biodegradability, non-toxicity, and should be able to maintain stem cells in their undifferentiated phenotype and promote differentiation only after induction. Due to these features of ideal micro-carriers, all attention is directed to the natural ECM components [3].

Stem cells are located in different niches that serve as their reservoir in physiological conditions. That is why it seemed very interesting to demonstrate how stem cells derived from three different sources would respond to various extracellular matrix components. In order to investigate the influence of ECM proteins on the ability of adhesion and proliferation rate of the stem cells from bone marrow, adipose tissue and hair follicles, collagen IV, fibronectin, laminin and poly-D-lysine were used.

The cell membrane of mesenchymal stem cells (MSCs), isolated inter alia from bone marrow, amniotic fluid, skin and adipose tissue, has receptors of adhesion molecules, such as ICAM-1, VCAM-1 and subunits of integrins [9, 10]. MSCs produce ECM proteins, such as collagen type I and III, laminin, vimentin and osteonectin [11]. Therefore, interaction of these cells with the matrix proteins appears to be essential for their differentiation. Lanfer et al. observed that cells grown on standard polystyrene culture vessels lose their original organization observed in physiological conditions [12].

Our results show that none of the tested substances reduced the proliferation of bone marrow, as well as adipose-derived mesenchymal stem cells, with the exception of laminin. This demonstrates the biocompatibility of such modified surfaces and possibility of using them for culturing these types of stem cells. In the case of stem cells isolated from rat bone marrow, the best proliferation rate was observed on plates coated with fibronectin and collagen IV, which also favored maintaining spindle-shaped, fibroblast-like cell morphology. Salasznyk et al. also showed that 80% of cells demonstrated adhesion during 30 min after seeding on a surface coated with fibronectin [13]. They also observed a slower proliferation of MSCs on culture dishes coated with laminin, which is consistent with the results obtained in this study. Similar findings were reported by Cool and Nurcombe [14], who demonstrated that human bone marrow mesenchymal stem cells attached to culture plates coated with fibronectin grew much better than those attached to other analyzed modified surfaces. Our studies showed that a similar effect is obtained with fibronectin, which promotes cell adhesion and proliferation.

Culture plates coated with collagen IV also showed a positive effect on the adhesion and proliferation of rat adipose-derived stem cells. However, in this case, favorable results were also observed on control plates that were not modified. This is in agreement with the suggestion by van Dijk et al. that MSCs from different sources show strong affinity to plastic culture plates [15].

We obtained different results regarding hair follicle stem cells. The adhesion of these cells to culture surfaces was much slower than in other cell types. Moreover, from all analyzed matrix proteins, the adhesion of hair follicle stem cells was the fastest on the control polystyrene surface, not coated with any of analyzed matrix components. Much poorer adhesion of these cells was observed on all analyzed modified surfaces. These results do not coincide with the previous data. Studies using mouse hair follicle stem cells, as well as limbus epithelial cells, showed that collagen IV was the best substrate for the culture of those cells [16, 17]. Adams and Watt showed a low level of adhesion of epithelial cells to culture plates coated with laminin [18]. In our studies, we noticed slow adhesion and proliferation rate not only on collagen IV and fibronectin, but also on laminin. Such weak adhesion rate of rat hair follicle stem cells to surface coated with collagen IV and fibronectin may be due to the lack of appropriate integrin receptors (α1β1,

α2, α3, α3β1, α4, α5) on the cell surface [19, 20]. It does not seem that the exposure of cells to ECM proteins could induce them to synthesize the corresponding receptors. However, the ubiquity of laminin and collagen IV in the basement membrane of hair follicles, described by Jahoda et al., may explain quite rapid adhesion of hair follicle stem cells to these substrates [21].

Extracellular matrix is a multifunctional network of fibrous, which provides structural and biochemical support to all tissues. These proteins have been implicated in many cellular processes, such as migration, proliferation, differentiation or apoptosis [21].

Regardless of the tissue type, ECM consists of different components and growth factors. More recently, it has been shown that stem cells are able to respond to the mechanical properties of the matrix. Cells can respond to microenvironment and change ECM expression, which resulting in remodeling f the matrix [22]. Besides its obvious role in determining the architecture and mechanical properties, ECM strongly influences the different cell functions [21]. However, the structure of ECM in most tissues is not well understood.

5. CONCLUSIONS

Our results showed that, depending on the origin of stem cells, their response to ECM components is different and that stem cells derived from distinct sources present differences in adhesion and proliferation rates with reference to the ECM components used. This indicates that ECM is an important component of the cellular niche in the tissue, supplying critical biochemical and physical signals to initiate or sustain cellular functions. It is also possible that ECM is required for the reconstitution of the niche of stem cells *in vitro*.

ETHICAL APPROVAL

All procedures conducted in the experiments involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

AUTHORS' CONTRIBUTION

AB - study design/planning, data collection/entry, data interpretation, literature analysis/search, wrote the initial draft of the manuscript. DP - data collection/entry, data interpretation. KR - literature analysis/search, data interpretation, statistical analysis, preparation of manuscript. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare that they have no conflict of interest.

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Research Article

Functional assessments and histopathology of hepatorenal tissues of rats treated with raw and processed herbs

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ABSTRACT

The present study ascertained the functional integrity of hepatic and renal tissues, concurrently with blood lipid patterns, of Wistar rats infused with CCl_4 and treated with raw and hydrothermal processed herbs, namely, *Monodora myristica*, *Chromolaena odorata*, *Buccholzia coriacea* and *Sphenostylis stenocarpa*. Measurement of phytochemical contents of the herbs was according to standard methods. The rats were randomly designated on the bases of diets and treatments received for 28 consecutive days. Fibrosis was induced in the Wistar rats by single dose intraperitoneal injection of CCl_4 for 2 consecutive days. Liver and kidney function tests and serum lipid profile were measured using spectrophotometric methods. Renal and hepatic tissues were subjected to histopathological examinations. The concentrations of alkaloids in the four herbal extracts were within the range of 4.83±0.03 - 31.33±0.29 mg/100 g sample, whereas the concentrations of saponins varied within a relatively narrow range: 0.33±0.09 - 4.33±0.02 mg/100 g dry sample; $p > 0.05$. The activity ratios of AST to ALT of the rat groups were generally less than 1.0 unit. Atherogenic indices of fibrotic rats were within the following ranges: TAG/HDL-C ratio (3.59±0.03 - 6.76±0.06), TC/HDL-C ratio (3.72±0.02 - 6.94±0.05) and LDL-C/HDL-C ratio (2.00±0.01 - 4.59±0.02). Losses in phytochemical contents following hydrothermal processing of the herbs did not substantially affect their overall therapeutic scores against morphological and functional impairments of hepatic and renal tissues following CCl_4 intoxication of the rats.

Keywords: Carbon tetrachloride; Histopathology; Hydrothermal; Kidney; Liver.

1. INTRODUCTION

The metabolic concerns of hepatic tissues, among several physiologic functions, ensure the detoxification of endogenously formed toxic molecules and xenobiotics through concerted processes of functional enzyme systems and facilitated by adaptable anatomical architecture [1, 2]. Additionally, administered drugs and metabolic waste products are eliminated by a combination of hepatic metabolism and renal excretion [3]. The kidneys, which are located in the posterior abdominal wall, are the principal organs for osmoregulation, electrolytes balance and regulation of blood pH levels as well as elimination of detoxified xeno-

biotics from the vascularsystem [4].

Biochemical investigations using hepatic and renal functional indices, the so-called liver function test (LFT) and kidney function test (KFT), coupled with histopathological studies have revealed that dysfunctional and morphological damage hepatic tissues predispose animal models to developing acute renal failure when exposed, in acute way, to chemical toxicants [5-9]. Carbon tetrachloride (CCl_4) is a chemical agentnoted to be toxic to hepatorenal tissues in experimental animals and human subjects [5, 6, 8, 10-13]. Reports showed that CCl_4 toxicity is elicited by microsomal *CYP2E1*-induced formation of reactive trichloromethyl ($CCl_3^{\bullet-}$) radical and the highly reactive oxygenated derivative, trichloromethylperoxyl ($Cl_3COO^{\bullet-}$) radical, which initiates lipid peroxidation and accumulation of lipid peroxidation products, engendering inflammation and fibrotic changes to hepatic and renal tissues via activation of the macrophages [1, 9, 11-14].

Herbal extracts have been reported to ameliorate hepatic and renal disorders in humans and animal models [2, 9, 13, 15]. There are vast array of Nigerian indigenous medicinal plants that are used for the treatment and management of pathologic conditions linked with overwhelming levels of reactive metabolic radicals [16, 17]. *Monodora myristica*, also called calabash Nutmeg, is an edible plant of the Annonaceae family. Phytochemical screening showed that the plant is rich in flavonoids, saponins and sterols but with minimal anti-nutrients such as cyanogenic glycosides, tannins, oxalates and phytates [18]. The medicinal usefulness of *M. myristica* has been reported elsewhere [19-22]. *Chromolaena odorata* (Linn) is commonly referred to as Siam weed, 'Elizabeth', 'Independence leaf', whereas in South-Eastern Nigeria, it is called 'Enugu plantation weed' and 'Awolowo' [23]. The proximate composition and phytochemical contents as well as the medicinal usefulness of the herb have been reported elsewhere [23-26]. *Buccholzia coriacea*, commonly called 'Wonderful Kola', is frequently use in traditional medicine practice [27]. The phytochemical contents and proximate composition as well as the medicinal usefulness of *B. coriacea* have been reported elsewhere [27-31]. *Sphenostylis stenocarpa* is commonly referred to as African yam bean. The nutraceutical usefulness of the African yam bean is associated with its antioxidant and low hepatotoxic properties [32, 33]. Furthermore, Ndidi et al. [34] reported the proximate, antinutrients and mineral composition of raw and processed African yam bean.

Studies have shown that CCl_4 infusion or inhalation elicits chemical-induced multiple organ lesions such as fibrosis, cirrhosis and hepatocarcinoma [12, 13, 35, 36], and by extension, renal dysfunction in animal models [5, 6, 12]. Organ lesion and ensuing pathology elicit wide variations in concentrations of metabolites and elevation of non-functional plasma enzymes in blood samples of experimental animals. Specifically, because plasma lipoproteins, for the most part, are biosynthesized in the hepatocytes, chemical and biotic agents that impact negatively on liver functional integrity distort plasma lipid patterns [2, 37, 38]. Serum lipid profile (SLP) describes the propensity to developing atherogenic plague [2, 39]. Accordingly, functional assessments of hepatic and renal tissues, using blood indicators in conjunction with inspection of tissue morphology and integrity tests using histopathological technique, describe the level of chemical-induced organ lesions and measure of success of therapeutic interventions.

There are growing interests in the quest for multi-dimensional applications of herbs; particularly those commonly used among traditional herbal medicine practitioners, for the alleviation of pathologic conditions [13, 17, 40]. Thus, ethno-botanical bio-prospecting offers another strategy for new herbal remedy discoveries, including those used for alleviation of co-existing liver and kidney pathologic conditions. Additionally, in ethno-medicinal practices, herbs are either administered raw or subjected to hydrothermal processing prior their application. The present study sought to ascertain the functional integrity of hepatic and renal tissues, concurrently with blood lipid patterns, of Wistar rats infused with CCl_4 and treated with raw and hydrothermal processed Nigerian indigenous herbs, namely, *M. myristica*, *C. odorata*, *B. coriacea* and *S. stenocarpa*.

2. MATERIALS AND METHODS

2.1. Collection and preparation of samples

High-grade raw seeds of *M. myristica*, *B. coriacea* and *S. stenocarpa* were purchased from Relief and Obazu-Mbieri Markets located in Owerri Capital Territory, Imo State, Nigeria. Fresh leaves of *C. odorata* were harvested from a private garden in Amakihia, Owerri-North Local Government Area, Imo State, Nigeria. The samples were transported to the laboratory, identified and authenticated by Dr. E.S. Willie at the Herbarium of the Department of Agronomy, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria. All samples were collected between the months of February and March, 2015. Voucher specimens were deposited at the Herbarium for reference purposes.

The various samples were washed separately in continues flow of distilled water for 15 min and allowed to dry at laboratory ambient temperature (T = 24 ± 5°C). The samples were divided into two portions on equal weight basis and designated as follows:

Group R: Raw samples

Group H: Hydrothermal processed samples.

Appropriate separate quantities of Group R samples were pulverized using Thomas-Willey milling machine (ASTM D-3182, India). The ground samples were transferred into corresponding vacuum desiccators and allowed to dry at laboratory ambient temperature until a constant weight was achieved. Appropriate separate quantities of Group H samples were boiled in distilled water in corresponding conical flasks (sample/water ratio = 1:4 *w/v*). According to local traditional medicine practice, hydrothermal processing of the seed samples was in the following durations: *M. myristica* = 10 min, *B. coriacea* = 1 h and *S. stenocarpa* = 1.5 h, whereas leaves of *C. odorata* were subjected to hydrothermal processing for 5 min. Next, the Group H samples were dried separately in an oven (Gallenkamp Oven 300 plus series, England) at 50°C until a constant weight was achieved. Finally, Group H samples were ground using the Thomas-Willey milling machine (ASTM D-3182; India), after which thesamples were stored in separate air-tight plastic bottles with screw caps pending extraction.

2.2. Extraction of samples

Extraction of Group R and Group H samples was according to the methods previously described [15]. Portion of 10 g each of the ground and dried Group R and Group H samples were subjected to repeated soxhlet extraction cycles for 2 h using 96% CH_3OH (BDH, U.K) as solvent to obtain final volume of 250 ml of corresponding extracts. The volumes of the extracts were concentrated and recovered in a rotary evaporator (Büch Rotavapor R-200) for 12 h at 50°C under reduced pressure. The extracts were dried invacuum desiccators for 24 h, wrapped in aluminum foil and stored in air-tight plastic bottles with screw caps at ≤ 4 °C. The yields were calculated to be as follows:

- Extract R1; Raw seeds of *M. myristica* = 8.94% (*w/w*).
- Extract R2; Raw leaves of *C. odorata* = 6.22% (*w/w*).
- Extract R3; Raw seeds of *B. coriacea* = 8.07% (*w/w*).
- Extract R4; Raw seeds of *S. stenocarpa* = 14.02% (*w/w*).
- Extract H1; Hydrothermal processed seeds of *M. myristica* = 6.41% (*w/w*).
- Extract H2; Hydrothermal processed leaves of *C. odorata* = 4.39% (*w/w*).
- Extract H3; Hydrothermal processed seeds of *B. coriacea* = 7.12% (*w/w*).
- Extract H4; Hydrothermal processed seeds of *S. stenocarpa* = 13.51% (*w/w*).

Portion of the each extract was measured for phytochemical contents. Also, the each extractwas reconstituted in phosphate buffered saline (PBS) solution that was osmotically equivalent to 100 g/l PBS (90.0 g NaCl, 17.0 $Na_2HPO_4 \cdot 2H_2O$ and 2.43 g $NaH_2PO_4 \cdot 2H_2O$) and appropriate dose was administered to corresponding experimental animals.

2.3. Phytochemicals

Quantitative compositions of some phytochemicals, namely, alkaloids, flavonoids, tannins and saponins were measured using standard methods. The concentration of alkaloids and saponins were measured using the methods of

Harborne [41]. The flavonoids content was according the methods of Boham and Kocipal [42]. The concentration of tannins was measured using the methods of Van-Burden and Robinson, [43] as reported by Belonwu et al. [44].

2.4. Experimental animals

Healthy male Wistar rats (90 days old) weighing between 150-260 g were maintained at laboratory ambient temperature of 30-55% relative humidity on a 12-h light/12-h dark cycle, with access to water and standard commercial feeds (SCF) (Ewu Feed Mill, Edo State, Nigeria) *ad libitum*, for 2 weeks acclimatization period. The Institutional Review Board of the Department of Biochemistry, Federal University of Technology, Owerri, Nigeria, granted approval for this study. The care and handling of the animals conformed to the standard principles of laboratory animal care of the United States National Institutes of Health (NIH, 1978).

2.5. Infusion of carbon tetrachloride/experimental design

A total of 72 male Wistar rats were allotted into 12 groups of 6 rats each. Fibrosis was induced in the Wistar rats by single dose intra-peritoneal (i.p) injection of CCl_4 in paraffin oil as vehicle [1:1 *v/v*; dose = 1.0 ml/kg body weight (b.wt.)] for 2 consecutive days [45]. The animals were deprived of feeds only for additional 16 h before commencement of treatment as described [1, 46]. The rats were randomly designated on the bases of diets and treatments (dose = 250 mg/kg b.wt.; i.p. of the extracts and silymarin or otherwise 1.0 ml/kg b.wt.; i.p. of PBS, paraffin oil and CCl_4/paraffin oil mixture) received for 28 consecutive days. Silymarin (Medical Union Pharmaceuticals Company) was used as the standard drug for reference treatment of fibrotic rats [1].

- Group 1: Normal rats received SCF + water *ad libitum* + PBS.
- Group 2: Normal rats received SCF + water *ad libitum* + paraffin oil.
- Group 3: Fibroticrats received SCF + water *ad libitum* + CCl_4/paraffin oil mixture.
- Group 4: Fibrotic rats received SCF + water *ad libitum* + silymarin.
- Group 5: Fibrotic rats received SCF + water *ad libitum* + Extract R1.
- Group 6: Fibrotic rats received SCF + water *ad libitum* + Extract R2.
- Group 7: Fibrotic rats received SCF + water *ad libitum* + Extract R3.
- Group 8: Fibrotic rats received SCF + water *ad libitum* + Extract R4.
- Group 9: Fibrotic rats received SCF + water *ad libitum* + Extract H1.
- Group 10: Fibrotic rats received SCF + water *ad libitum* + Extract H2.
- Group 11: Fibrotic rats received SCF + water *ad libitum* + Extract H3.
- Group 12: Fibrotic rats received SCF + water *ad libitum* + Extract H4.

At the end of the feeding and treatment period, the rats were subjected to fasting for 12 h, after which the animals were sacrificed and blood samples were drawn from the orbital sinus [47] and measured for serum biomolecules of interest. Autopsy samples of the renal and hepatic tissues were excised for histopathological examinations.

2.6. Liver function test (LFT)

2.6.1. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities

Measurement of serum AST and ALT activities were according to the methods of Reitman and Frankel [48].

2.6.2. Alkaline phosphatase (ALP) activity

Serum ALP activity was assayed by the methods described by Glogowski et al. [49], but with minor modifications according to Njoku et al. [50].

2.6.3. Bilirubin

Serum total bilirubin concentration (TBC) was measured using diazotized sulphanilic acid methods as previously described [51].

2.6.4. Total protein

Serum total protein concentration (TPC) was

measured using the biuret methods as described by Gornall et al. [52].

2.6.5. Albumin

Measurement of serum albumin concentration (AlC) was by the methods described by Doumas et al. [53].

2.7. Kidney function test (KFT)

2.7.1. Creatinine

Measurement of serum creatinine concentration (CrC) was according to the methods as described by Bonsnes and Taussky [54].

2.7.2. Urea

Estimation of serum urea concentration (UrC) was by the rapid methods as described by Fawcett and Scott [55].

2.8. Lipid profiling

2.8.1. Serum lipid profile

Blood samples were obtained from the various experimental animal groups and measured for SLP according to the methods previously described [56]. Serum total cholesterol (TC), triacylglycerol (TAG) and high-density lipoprotein cholesterol (HDL-C) concentrations were measured using commercial kits (Randox Laboratory Ltd., UK). Serum low-density lipoprotein cholesterol (LDL-C) concentration was estimated according to the formula of Friedewald et al. [57]: LDL-C = TC - (HDL-C) - (TAG/5), as reported by Shaker et al. [1]. Atherogenic index (AI) was calculated as previously reported [58].

2.9. Hepatorenal tissue morphology

2.9.1. Histopathological examinations

Organ histology was according to the methods described by Banchroft et al. [59] as previously reported [15, 60]. Autopsy samples of the renal and hepatic tissues of the different animal groups, fixed in 10% formol saline (pH = 7.2) for 24 h and washed with continuous flow of distilled water. The specimens were cleared in xylene embedded in paraffin in hot air oven at 56 ℃ for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4-mm thickness using a semi-automated rotatory microtome. The obtained tissue sections were collected on glass slides, dehydrated by immersing in serial dilutions of ethyl alcohol-water mixture, cleaned in xylene and embedded in paraffin wax. Next, the specimens were deparaffinized and stained with hematoxylin and eosin (H&E) dye for histopathological examinations. Photomicrographs of the tissue sections were captured using chare-couple device (CCD) camera under light microscope (Olympus BX51TF; Olympus Corporation, Tokyo, Japan) at × 400 magnification power.

2.10. Statistical analysis

The data collected were analyzed by the analysis of variance procedure while treatment means were separated by the least significance difference (LSD) incorporated in the statistical analysis system (SAS) package of 9.1 version, (2006).

3. RESULTS

Table 1 showed that the concentrations of alkaloids in the four herbal extracts were within the range of 4.83±0.03 - 31.33±0.29 mg/100 g sample, in which Extract R1 gave the highest concentration of alkaloids. The concentration of alkaloids in Extract R2 was approximately 1.9 fold lower than that of Extract R1; $p < 0.05$. Furthermore, the concentration of alkaloids in Extract R3 was 2.36 folds lower than that of Extract R2; $p < 0.05$. The Extract R4 gave the lowest concentration of alkaloids and was not significantly different ($p > 0.05$) from that of Extract R3.

The concentration of alkaloids in Extract H1 was significantly different ($p < 0.05$) from that of Extract H2. Conversely, the concentration of alkaloids in Extract H3 was not significantly different ($p > 0.05$) from that of Extract H4. The concentrations of alkaloids in Extract H3 and Extract H4 were significantly different ($p < 0.05$)

from those of Extract H1 and Extract H2. An overview of Table 1 showed that the concentrations of alkaloids in Extracts (R1-R4) were significantly higher ($p < 0.05$) than those of Extracts (H1-H4), except the corresponding concentrations of alkaloids between Extract R4 and Extract H4; $p > 0.05$.

Table 1. Some phytochemical contents of raw and hydrothermal processed herbal extracts.

	Concentration (mg/100 g dry sample)			
Extract	**Alkaloids**	**Flavonoids**	**Tannins**	**Saponins***
R1	31.33 ± 0.29^{a}	14.17 ± 0.10^{b}	13.28 ± 0.11^{a}	1.67 ± 0.04
R2	$16.50 \pm 0.10^{b,c}$	21.00 ± 0.18^{a}	$12.63 \pm 0.09^{a,b}$	2.23 ± 0.02
R3	$7.00 \pm 0.09^{d,e}$	$6.33 \pm 0.08^{d,e}$	$7.39 \pm 0.07^{c,d}$	2.33 ± 0.03
R4	$4.83 \pm 0.03^{d,e,f}$	$6.23 \pm 0.07^{d,e,f}$	8.10 ± 0.12^{c}	2.83 ± 0.03
H1	20.01 ± 0.21^{b}	6.50 ± 0.07^{d}	0.62 ± 0.01^{e}	1.33 ± 0.09
H2	7.33 ± 0.07^{d}	$12.33 \pm 0.09^{b,c}$	$0.42 \pm 0.01^{e,f,g,h}$	1.00 ± 0.01
H3	$1.50 \pm 0.01^{e,f,g,h}$	$4.33 \pm 0.03^{d,e,f,g}$	$0.46 \pm 0.01^{e,f,g}$	1.67 ± 0.02
H4	$2.17 \pm 0.03^{e,f,g}$	$3.17 \pm 0.03^{d,e,f,g,h}$	$0.52 \pm 0.01^{e,f}$	2.33 ± 0.02

The mean (X) ± S.D of six ($n = 6$) determinations. Means in the column with the same letter are not significantly different at $p > 0.05$ according to LSD. *Concentrations of saponins showed no significant difference $p > 0.05$ according to LSD.

Table 1 showed that Extract R2 gave the highest concentration of flavonoids, which was significantly different ($p < 0.05$) from that of Extract R1. The concentrations of flavonoids in Extract R3 and Extract R4 showed no significant difference ($p > 0.05$). In a corresponding manner, the concentration of flavonoids in Extract H2 was significantly higher ($p < 0.05$) than that of Extract H1. Likewise, the concentration of flavonoids in Extract H3 was not significantly different ($p > 0.05$) from that of Extract H4.

An overview of Table 1 showed that the concentrations of flavonoids in Extracts (R1-R4) were significantly higher ($p < 0.05$) than those of Extracts (H1-H4), except the corresponding concentrations of flavonoids in Extract R3 and Extract H3 as well as Extract R4 and Extract H4.

The concentration of tannins in Extract R1 was not significantly different ($p > 0.05$) from that of Extract R2. However, the concentration of tannins in Extract R3 was 1.79 fold lower than that of Extract R1; $p < 0.05$. The Extract R4 gave marginal higher concentration of tannins than that of Extract R3; $p > 0.05$. Table 1 showed that the concentrations of tannins in Extracts (H1-H4) were significantly lower ($p < 0.05$) than their corresponding Extracts (R1-R4). Additionally, the concentrations of tannins in Extracts (H1-H4) was within a relatively narrow range of 0.42±0.01 - 0.62±0.01 mg/100 g dry sample; $p > 0.05$.

The concentrations of saponins in the herbal extracts varied within a relatively narrow range: 0.33±0.09 - 4.33±0.02 mg/100 g dry sample; $p > 0.05$. Although the concentrations of saponins in Extracts (R1-R4) were comparatively higher than those of Extracts (H1-H4), the values showed no corresponding significant difference ($p > 0.05$). Finally, a general survey of Table 1 showed that the concentrations of the phytochemicals analyzed in the raw and hydrothermal processed herbs were in the order: alkaloids > flavonoids > tannins > saponins.

Serum ALT activity of Group 1 was not significantly different ($p > 0.05$) from that of Group 2 (Figure 1). However, Group 3 exhibited comparatively raised level of serum ALT activity, which represented 4.44 folds increase in the enzyme activity compared with that of Group 1; $p < 0.05$. Conversely, serum ALT activities of Groups (4-12) were significantly lower ($p < 0.05$) than that of Group 3, but significantly higher ($p < 0.05$) than those of Group 1 and Group 2. Specifically, serum ALT activities of Group 6, Group 10, Group 11 and Group 12 were significantly higher ($p < 0.05$) than

that of group 4, whereas serum ALT activities of Group 5, Group 7, Group 8 and Group 9 were comparable with that of Group 4; $p > 0.05$. Serum AST activities of the various experimental rat groups followed the same pattern with their serum ALT activities (Figure 1). However, the activity ratios of AST to ALT of the various experimental rat groups were generally less than 1.0 unit.

Serum ALP activity of Group 1 was not significantly different ($p > 0.05$) from that of Group 2 (Figure 1). Serum ALP from Group 3 gave the highest activity, which was significantly different ($p < 0.05$) from other experimental rat groups. Additionally, serum ALP activity of Group 3 was comparable with those of group 5, Group 6 and Group 10 ($p > 0.05$), whereas serum ALP activities of Group 7, Group 8, Group 9, Group 11 and Group 12 were significantly higher ($p < 0.05$) than that of Group 3.

Figure 2 showed that serum TBCs of Group 1 and Group 2 were less than 1.0 mg/ml and showed no significant difference ($p > 0.05$). Contrary, serum TBCs of other experimental groups, except Group 11, were above 1.0 mg/dl. Specifically, Group 3 gave the highest serum TBC, which was significantly different ($p < 0.05$) from other experimental rat groups. Furthermore, serum TBC of Group 11 was comparable with those of Group 4, Group 7, Group 9 and Group 12; $p > 0.05$.

Figure 3 showed that serum TPC of Group 1 was not significantly different ($p > 0.05$) from that of Group 2. Additionally, serum TPCs of Group 4, Group 5, Group 6, Group 7 and Group 10 were comparable; $p > 0.05$. Likewise, serum TPCs of Group 11 and Group 12 were comparable with that of Group 2; $p > 0.05$. However, serum TPC of Group 3 was significantly lower than other experimental rat groups; $p < 0.05$. A cursory look at Figure 3 showed that serum AlCs of the various experimental rat groups exhibited similar pattern with serum TPCs, exemplified by the fairly strong correlation coefficient ($r = 0.651662$) between their serum TPCs and serum AlCs.

Figure 4 showed that serum CrCs and serum UrCs of Group 1 and Group 2 did not show significant difference ($p > 0.05$). Conversely, serum CrC of Group 3 was significantly higher ($p < 0.05$) than those of Group 1 and Group 2. Additionally, serum CrC of Groups (4-12) varied within a narrow range of 0.61±0.03 - 0.66±0.05 mg/dl; $p > 0.05$.

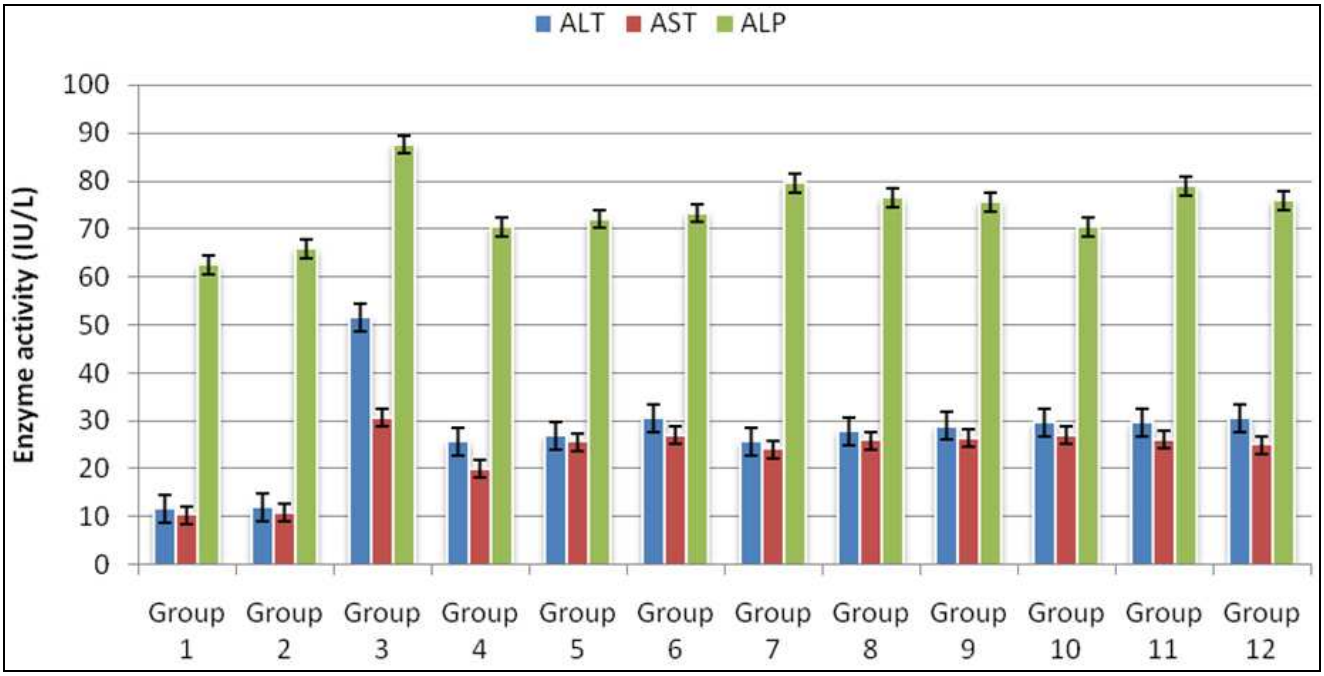

Figure 1. Serum aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities of experimental rat groups.

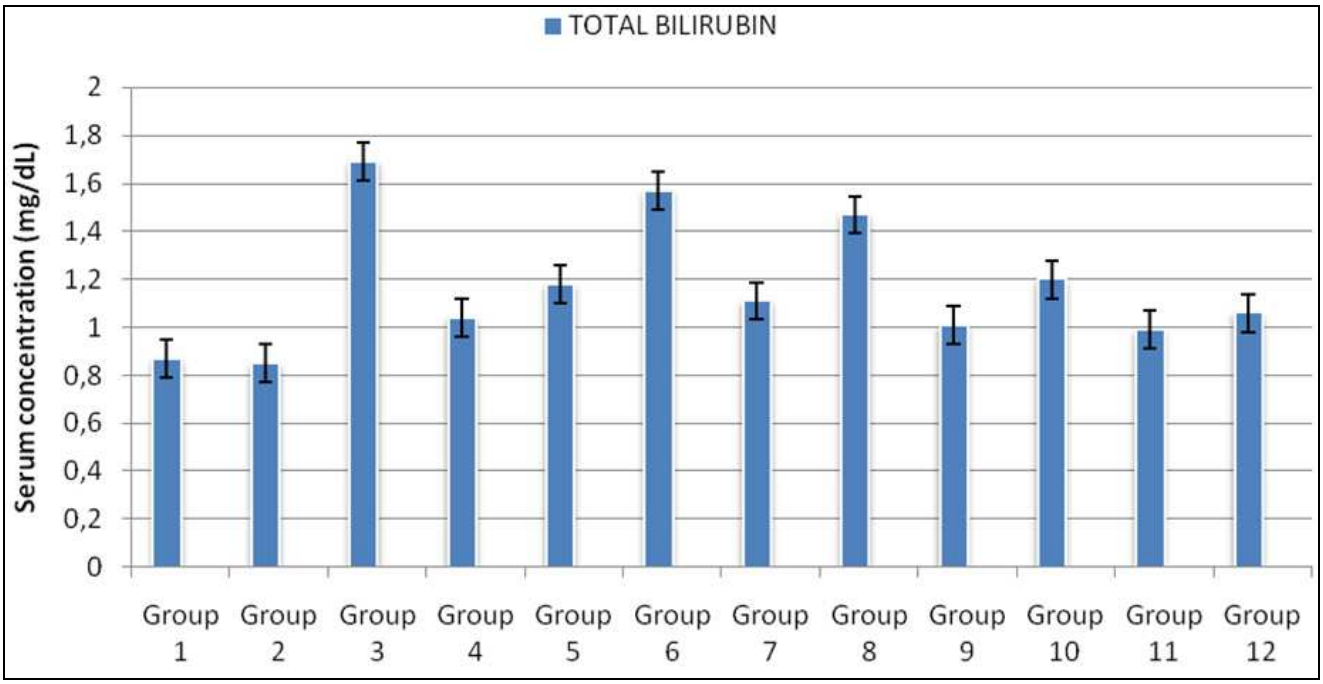

Figure 2. Serum total bilirubin concentration of experimental rat groups.

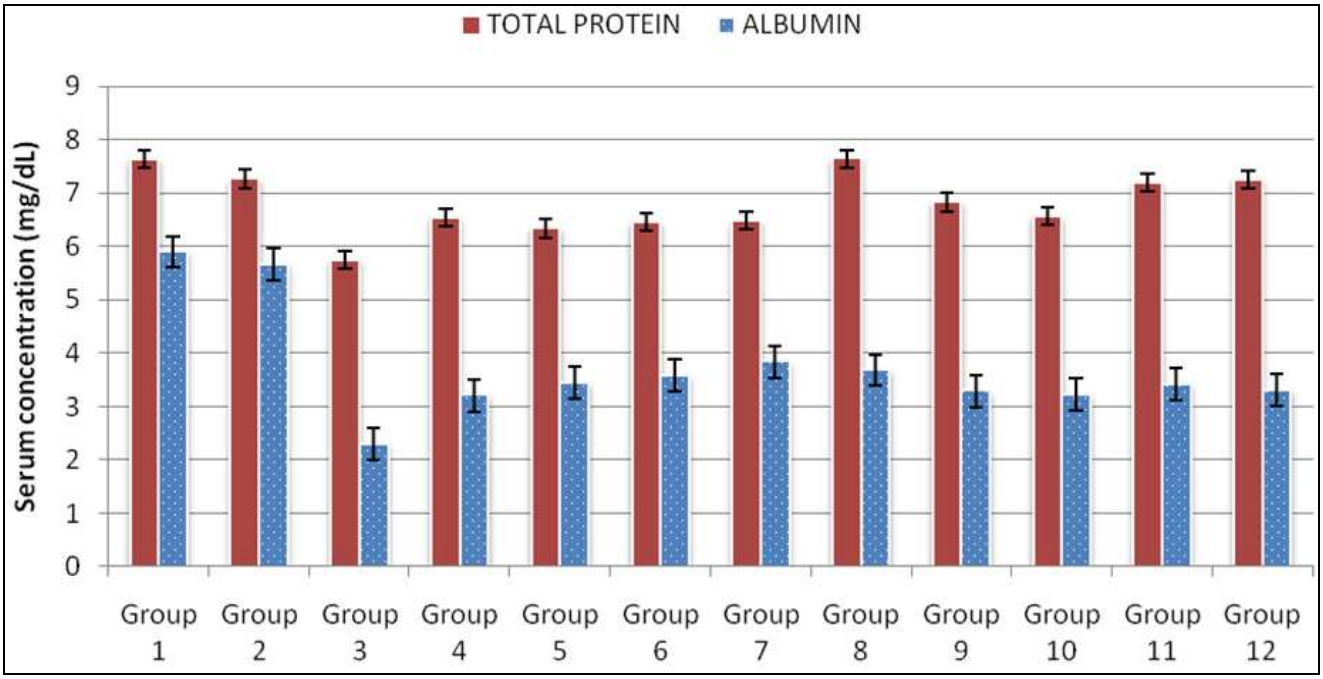

Figure 3. Serum total protein and albumin concentrations of experimental rat groups.

Serum UrC of Group 3 significantly higher ($p < 0.05$) than those of Group 1 and Group 2 as well as other experimental rat groups (5-12). The correlation coefficient ($r = 0.898134$) between serum CrC and serum UrC of the experimental rat groups indicated a strong positive correlation.

Figure 5 showed that SLP of Group 1 exhibited comparable pattern with that of Group 2 as typified by their atherogenic indices (Table 2). Specifically, serum TC, TAG and LDL-C concentrations of Group 3 were significantly raised ($p < 0.05$) compared with Group 1 and Group 2. Conversely, Group 3 exhibited the lowest serum HDL-C concentration compared with other expe-

rimental rat groups. Generally, serum TC, TAG and LDL-C concentrations of Groups (5-12) were higher than that of Group 4, and in turn, those of Group 4 was higher than those of Group 1 and Group 2; $p < 0.05$.

An overview of Table 2 showed that atherogenic indices of Group 1 and Group 2 were below the critical values, whereas those of Groups (3-12) were within the following ranges: TAG/HDL-C ratio (3.59±0.03 - 6.76±0.06), TC/HDL-C ratio (3.72±0.02 - 6.94±0.05) and LDL-C/HDL-C ratio (2.00±0.01 - 4.59±0.02).

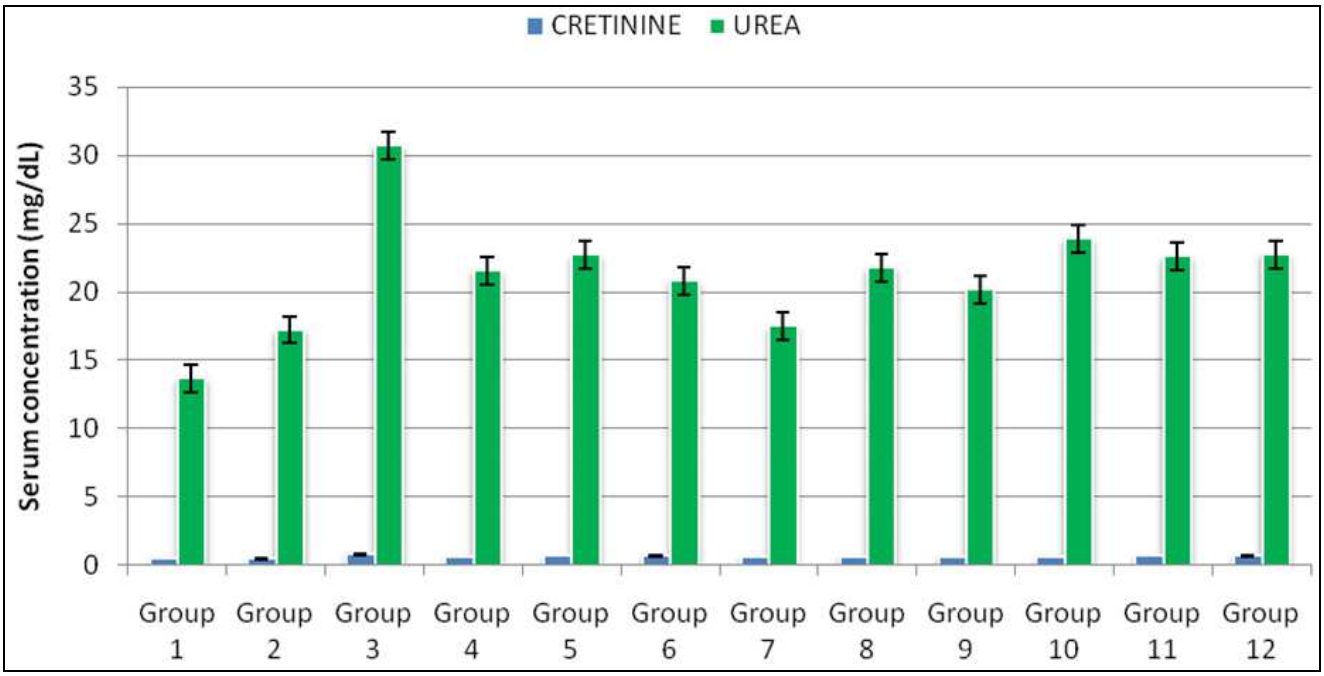

Figure 4. Serum creatinine and urea concentrations of experimental rat groups.

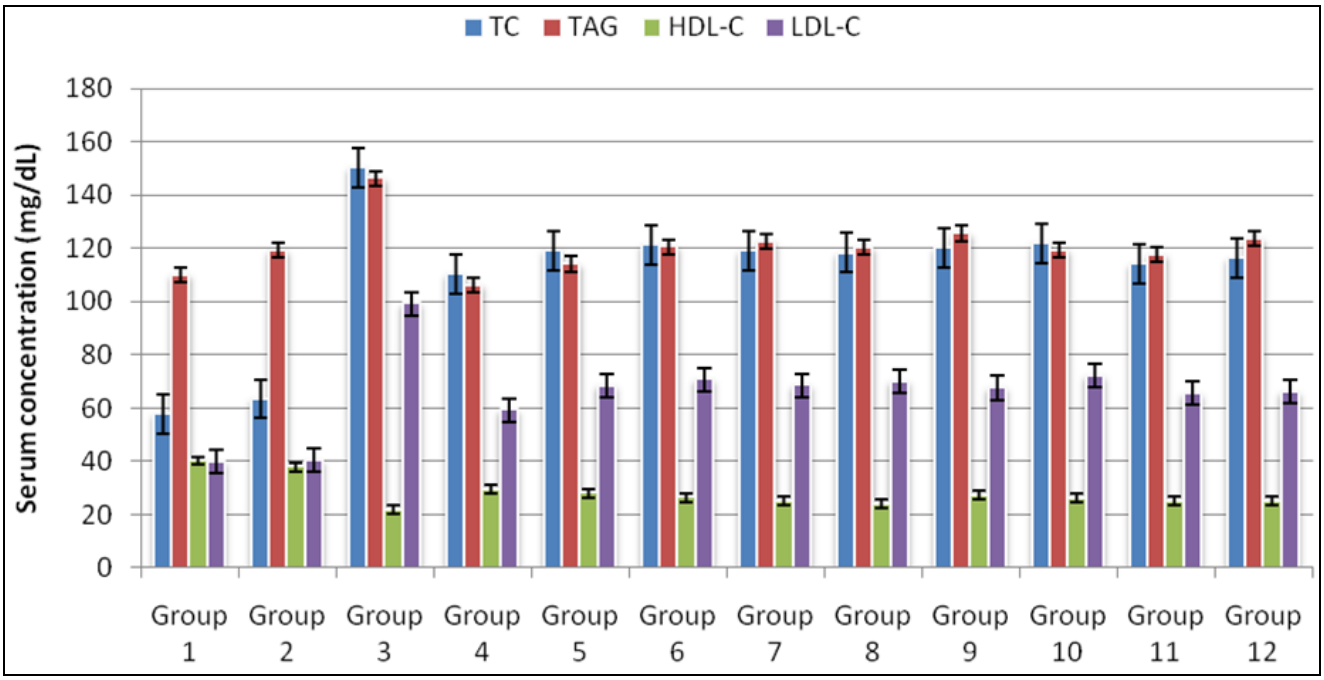

Figure 5. Serum lipid profile of experimental rat groups.

Table 2. Atherogenic indices of experimental rat groups.

Atherogenic indices			
Groups	TAG/HDL-C	TC/HDL-C	LDL-C/HDL-C
Group 1	2.74 ± 0.02	1.44 ± 0.01	0.99 ± 0.001
Group 2	2.99 ± 0.02	1.59 ± 0.01	1.01 ± 0.01
Group 3	6.76 ± 0.06	6.94 ± 0.05	4.59 ± 0.02
Group 4	3.59 ± 0.03	3.72 ± 0.02	2.00 ± 0.01
Group 5	4.07 ± 0.03	4.26 ± 0.02	2.44 ± 0.01
Group 6	4.56 ± 0.03	4.59 ± 0.02	2.68 ± 0.01
Group 7	4.91 ± 0.03	4.76 ± 0.02	2.78 ± 0.02
Group 8	5.40 ± 0.03	4.91 ± 0.02	2.91 ± 0.01
Group 9	4.59 ± 0.03	4.39 ± 0.02	2.47 ± 0.01
Group 10	4.59 ± 0.03	4.69 ± 0.02	2.77 ± 0.02
Group 11	4.69 ± 0.02	4.54 ± 0.02	2.61 ± 0.01
Group 12	4.96 ± 0.02	4.66 ± 0.02	2.65 ± 0.01

The mean (X) ± S.D of six (n = 6) determinations. Reference values: TAG/HDL-C ratio [61, 62], TC/HDL-C ratio < 1.66 and LDL-C/HDL-C ratio < 1.06 [39]. Castelli risk indices I (TC/HDL-C) and II (LDL-C/HDL-C) [63].

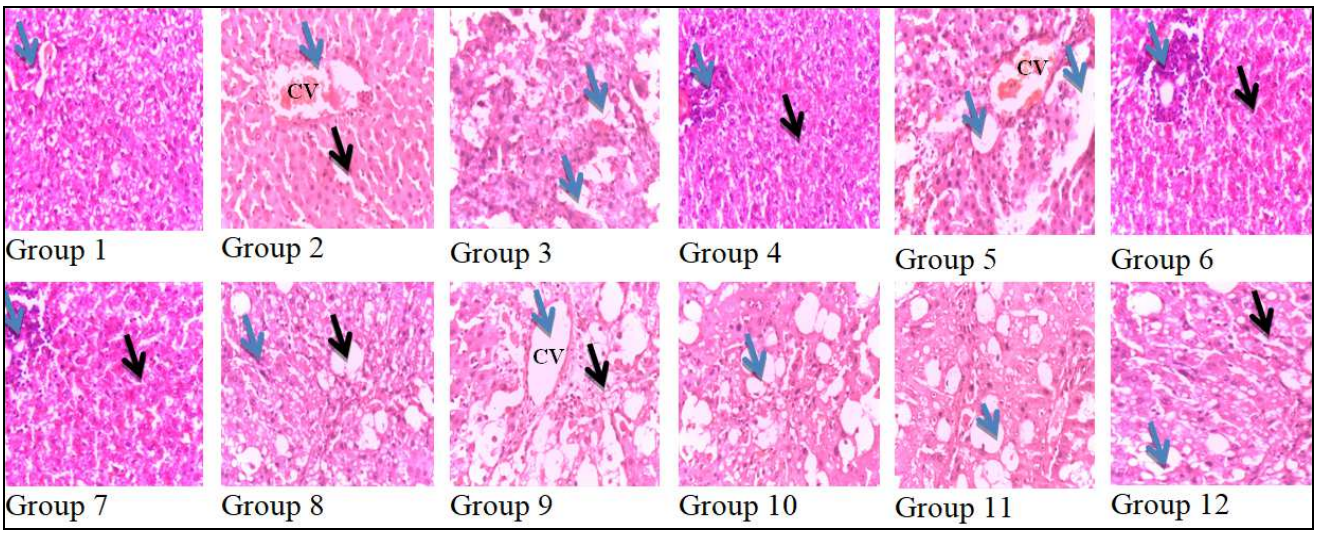

Figure 6. Photomicrograph sections of hepatic tissues (H&E x 400).

Group 1: Normal histologyshowing normal central vein (blue arrow). Group 2: Normal histology showing pronounced normal central vein (CV) (blue arrow) and Kupffer cells along the sinusoids (black arrow). Group 3: Massive tissue necrosis and fibrotic changes of hepatic parenchyma with centrilobular vacuolization and extensive fatty deposits (blue arrows). Group 4: Localized minimal distortion ofhepatic parenchyma architecture (blue arrow) with infiltration of mononuclear cells around portal area and adjoining hepatic tissues (black arrows). Group 5: Massive tissue necrosis and fibrotic changes of hepatic parenchyma with extensive fatty deposits (blue arrows). Group 6 and Group 7: Localized minimal distortion of tissue architecture (blue arrow) with massive number of apoptotic hepatocytes (black arrow). Group 8: Fibrotic changes (blue arrow) with fatty deposits (black arrow). Group 9: Fibrotic changes with fatty deposits (black arrow) as well as CV congestion (blue arrow). Group 10: Fibrotic changes with fatty deposits (blue arrow). Group 11: Fibrotic changes of hepatic parenchyma with fatty deposits (blue arrow). Group 12: Fibrotic changes with fatty deposits (blue arrow) with cytoplasmic vacuolization (black arrow).

The histopathological features of hepatic tissue sections of the experimental rat groups are presented in Figure 6. Photomicrograph section of hepatic tissues of Group 1 showed normal histology and was comparable with that of Group 4; except for slight localized distortion in hepatic parenchyma of Group 4. Additionally, hepatic section of Group 2 showed normal histology with minimal cytoplasmic vacuolization. Conversely, hepatic section of Group 3 showed evidence of massive disarrangement and distortions of tissue architecture, accompanied with infiltration of fatty deposits as well as centrilobular necrosis comparable with that of Group 5. Figure 6 showed that hepatic sections of Group 8, Group 9, Group 10, Group 11 and Group 12 showed evidence of fatty deposits and fibrotic changes of hepatic parenchyma. Specifically, hepatic tissues sections of Group 9 and Group 10 were also associated with cytoplasmic vacuolization.

The histopathological features of renal tissue sections of the experimental rat groups are presented in Figure 7. Photomicrograph section of renal tissues of Group 1 showed normal histology, which was comparable with that of Group 2. The tubular and glomeruli configurations were absent in renal tissues of Group 3, whereas those of Group 4 and Group 5 were present but distorted. Renal tissue section of Group 6 showed evidence of parenchyma necrosis with distorted glomeruli architecture. The degenerated and distorted tubular morphology of renal tissues of Group 7 was comparable with that of Group 11. There were evidence of infiltrated mononuclear cells around the glomeruli (Group 8, Group 9 and Group 11) and dispersed in renal parenchyma (Group 10).

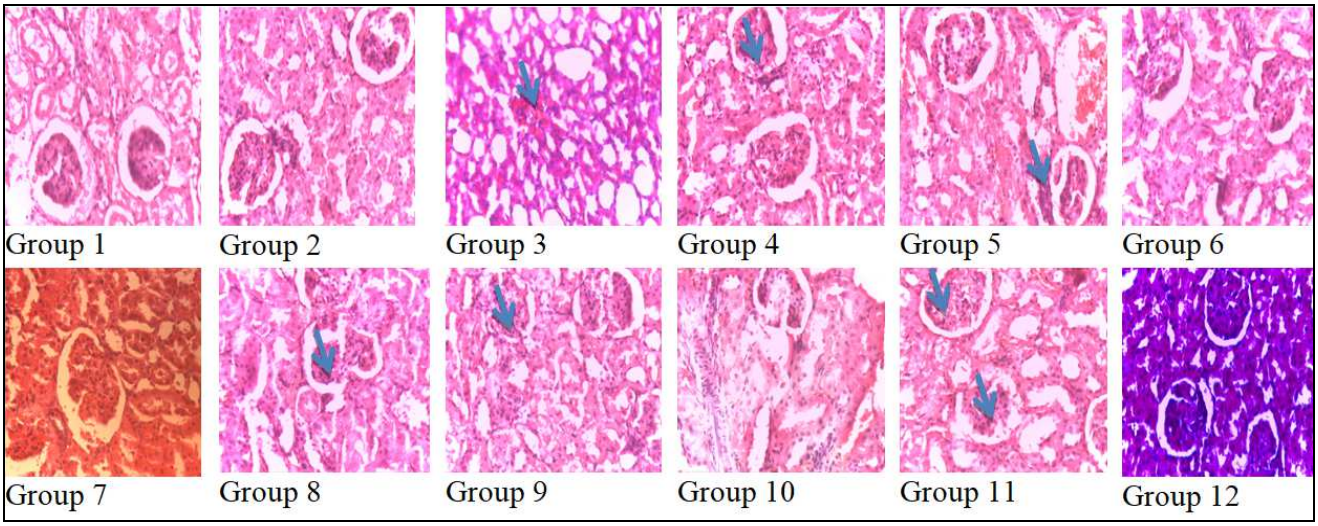

Figure 7. Photomicrograph sections of renal tissues (H&E x 400).
Group 1 and Group 2: Normal histology showing normal tubular and glomeruli architecture. Group 3: Pronounced degenerated and distorted tubular and glomeruli architecture as well as evidence of distorted renal parenchyma integrity (blue arrow). Group 4 and Group 5: Minor distorted glomeruli architecture and infiltrated mononuclear cells around the glomeruli (blue arrow). Group 6: Distorted tubular and glomeruli architecture as well as necrosis of renal parenchyma. Group 7: Minor distorted glomeruli architecture but degenerated and distorted tubular morphology. Group 8 and Group 9: Major distorted glomeruli architecture and infiltration of mononuclear cells around the glomeruli (blue arrow). Group 10: Dispersed mononuclear cells around the glomeruli and renal parenchyma. Group 11: Minor distorted glomeruli architecture and infiltrated mononuclear cells around the glomeruli (blue arrows). Group 12: Normal glomeruli architecture but degenerated and distorted tubular morphology.

4. DISCUSSION

Hydrothermal processing of herbs brings about changes in their physical characteristics and biochemical compositions [64, 65]. Expectedly, the present study showed evidence of losses in phytochemical contents following hydrothermal processing of the herbal samples. Most of these phytochemicals have been demonstrated to possessing antioxidant properties using both *in vitro* and *in vivo* evaluation models [1, 9, 66-68]. However, the effects of hydrothermal processing on the antioxidant contents and activities of herbs are still been controversially discussed [69-71]. Antioxidant phytochemicals function by modulation of nuclear factor-κB (NF-κB), p38 mitogen-activated protein kinase (MAPK), NH_2-terminal Jun kinases/stress-activated protein kinases (JNK/SAPK) and hexosamines mediated cellular stress-sensitive pathways [72-76] as well as transforming growth factor-β (TGF-β)-Smad3 signaling pathway [77]. Previous reports had associated the loss in phytochemical content of hydrothermal processed herbs to leaching and low thermal stability of most phytochemicals [78]. For instance, hydrothermal processing of broccoli, choy-sumand cabbage resulted to losses in their total phenolic contents [79]. Additionally, cooking methods and storage temperature of raw vegetables influenced their phytochemical contents and antioxidant capacities *in vitro* [64, 66]. Specifically, report showed that hydrothermal processing caused 19% loss in the phytochemical contents of cauliflower [80]. It is worthwhile to note that losses in phytochemical contents following hydrothermal processing of herbs may compromise their radical scavenging potential and the capacities of such products to exert health benefits [66, 81]. Nevertheless, hydrothermal processing serves as one of several measures for the elimination of inherent cytotoxic phytochemicals from raw medicinal herbs and vegetables [82-85].

Measurements of hepatic enzymes in serum of animal models have been used in previous studies as basis for ascertaining the capacities of herbal remedies to ameliorate chemically induced liver damage [8, 9, 86-88]. Elevated levels of serum AST, ALT and ALP activities are diagnostic of fibrotic changes of the liver, myocardial infarction, bone disorders and other related pathologic conditions associated with organ necrosis [89]. Serum ALT activity is more specific for hepatic injury than an increase in serum AST activity and may reflect fatty changes in the liver as in the case of non-alcoholic fatty liver disease (NAFLD) [90-93]. Specifically, the raised level of serum ALT activity of Group 3 (Figure 1) was obvious indication of fibrotic changes of hepatic tissues of the experimental rats as corroborated in previous findings [12, 13, 94]. However, fibrotic rats treated with raw and hydrothermal processed herbs showed evidence of amelioration of CCl_4-induced liver injury as indicated by their comparatively lower serum ALT activities, which concurred with previous findings [13, 77, 94, 95]. The present findings showed that the capacities of the raw and hydrothermal processed herbs to reverse elevated levels of serum AST and ALT activities were comparable with that of silymarin, which was used as reference treatment of fibrotic rats. Furthermore, it appeared that the raw herbs exhibited greater capacities to lower serum ALT activities of fibrotic rats than those of corresponding hydrothermal processed herbs, which was an indication of greater capacities of the raw herbs to ameliorate CCl_4-induced liver injury than those of corresponding hydrothermal processed herbs. Likewise raised level of serum ALP activity of Group 3 was a reflection of hepatobiliary disorder and fibrosis [87], which paralleled the patterns of serum AST and ALT activities of other experimental rat groups and conformed with the outcome of related investigations [1, 12, 13, 45, 96]. Overall, serum AST, ALT and ALP activities of fibrotic rats treated with raw and hydrothermal processed herbs as well as the standard herbal drug-silymarin were profoundly higher those of normal rat groups (Group 1 and Group 2), which by implication showed that the various herbal remedies did not possess the capacities to offer full therapeutic benefits within the experimental 28 days of treatment.

The factors that elicit raised level of bilirubin in serum are multifaceted and serum bilirubin concentration greater than 1.0 mg/dl is diagnostic of hyperbilirubinemia [97, 98]. Hyperbilirubinemia occurs due to rapid haemolysis such that the production of bilirubin far exceeds the capacity of normal hepatocytes to conjugate and excrete bilirubin. In the event of hepatic damage, the capacities of the hepatocytes to conjugate and excrete bilirubin

are compromised, even when bilirubin is produced in normal quantities. Furthermore, hepatobiliary obstruction prevents the excretion of bilirubin engendering the accumulation of bilirubin in the liver, which eventually diffuses into the blood system.

Previous studies have noted that reactive $CCl_3^{\bullet-}$and $Cl_3COO^{\bullet-}$ radicals generated from CCl_4 metabolism promoted membrane lipid peroxidation, in which the erythrocytes [99] and hepatorenal tissues were vulnerable [1, 9, 11-14] and consequently, prompted rapid haemolysis and hepatic necrosis with attendant hyperbilirubinemia in the experimental rats (Groups 3-12). The results of the present study (Figure 2) indicated that fibrotic rats, regardless of the type of herbal treatment administered, presented evidence of hyperbilirubinemia. However, the hydrothermal processed herbs appeared to exhibit relatively higher tendencies to lower the severity of hyperbilirubinemia than those of corresponding raw herbs. In that regard, the results appeared to suggest that hydrothermal processing of the herbs caused the elimination or reduction in the amount of cytotoxic components in the herbs that probably interfered with the capacity of the herbs to ameliorate hyperbilirubinemia.

The plasma proteins (e.g. albumin) are synthesized by the liver and therefore low circulating level of plasma proteins indicates hepatic dysfunction. Previous studies have shown that mild perturbation of hepatic tissues integrity did not profoundly affect the capacity of the liver to biosynthesize plasma proteins [58, 100]. The present study showed that serum total protein and albumin concentrations of Group 3 were lower than those of Group 1 and Group 2 (Figure 3), which confirmed compromised hepatic function. However, the administration of raw and hydrothermal processed herbs caused limited improvement inthe capacity of hepatocytes of fibrotic rats to biosynthesize plasma proteins.

The renal tissues are primarily concerned with the clearance of nitrogenous waste products and other blood low threshold substances. Creatinine is mainly the catabolic waste product from tissue protein turnover, whereas urea is derived from oxidative deamination of dietary amino acids [1, 101, 102]. Elevated blood creatinine and urea concentrations are diagnostic of impaired renal function. The present study showed evidence of renal dysfunction following infusion of the experimental rats with CCl_4, exemplified by comparatively raised serum creatinine and urea concentrations of Group 3 (Figure 4). The present findings confirmed previous research outcomes, which noted that acute chemical intoxication that caused morphological and functional damages to hepatic tissues predisposed animal models to developing acute renal dysfunction [1, 5, 6, 8, 9]. Fibrotic rats treated with raw and hydrothermal processed herbs showed evidence of limited amelioration of impaired renal function, typified by their lowered serum creatinine and urea concentrations compared to that of Group 3. In a related study, Elgazar and AboRaya [103], using serum urea and creatinine concentrations as biomarkers, noted that single and combinatorial formulations of *Petroselinum sativum*, *Eruca sativa* and *Curcuma longa* ameliorated gentamicin-induced renal tubular necrosis in adult male Sprague Dawley rats.

The metabolic concerns of the liver, amongst other functions, are to regulate the mobilization, biosynthesis and catabolism of lipoproteins in vertebrates. Previous reports showed that CCl_4 interferes with the metabolism of lipoproteins in hepatic smooth endoplasmic reticulum, engendering alterations in SLP patterns and associated dyslipidemia [12, 104, 105], whereby blood lipid concentrations are elevated as typified by raised levels of serum LDL-C and TC of Group 3 compared to that of Group 1 (Figure 5). Additionally, the SLP patterns of fibrotic rats administered with raw and hydrothermal processed herbs were identical and therapeutic scores of the herbs were not profoundly different from that of the standard herbal drug - silymarin. Overall, the present study showed that the herbs did not offer full therapeutic benefits to the fibrotic rats, in terms of their capacities to ameliorate dyslipidemia. The predisposition of the experimental rat groups to arteriosclerosis and associated cardiovascular morbidity and mortality were defined by their atherogenic indices (Table 2). According to the TAG/HDL-C and LDL-C/HDL-C ratios of the present study, adjustments of SLP patterns in the fibrotic rats, irrespective of the type of herbal treatment they received, indicated incidences of atherogenicity as defined elsewhere [39, 58, 61-63].

The massive disarrangement of hepatic tissues architecture of untreated fibrotic rats (Group 3) correlated with the levels of alterations of serum indicators, namely, serum ALT, AST and ALP activities as well as serum TBC as previously described [12, 13, 98, 99]. Additionally, photomicrograph section of hepatic tissues of fibrotic rats (Figure 6) revealed and confirmed CCl_4-induced necrosis and steatosis as previously described [12, 13, 88, 106]. The localized distortions in hepatic architecture, persistence of hepatic steatosis and hydropic degenerations in fibrotic rats following treatment with raw and hydrothermal processed herbs were indications, which confirmed limited capacities of the herbs to ameliorate CCl_4-induced morphological and functional impairments of hepatic tissues within the experimental period of 28 days. According to Sokol et al. [107], manifestation of hepatic steatosis was as a result of low availability of tissue α-tocopherol and ascorbic acid, rather than glutathione (GSH), which also predisposed the liver to oxidative injuries as exemplified by the presence of hepatic necrosis in tissue sections of the present report.

Rincón et al. [5] suggested that the effect of CCl_4 on kidney tissue morphology and function depended on the functional state of the liver. Similar to the histopathological status of the liver, photomicrograph sections of renal tissues (Figure 7) showed that the distortions in renal tissue architecture correlated with the levels of alterations in their blood indicators; serum creatinine and urea concentrations as previously described [60]. Furthermore, the tissue architecture of fibrotic rats administered with raw and hydrothermal processed herbs confirmed the limited capacities of the herbs to ameliorate renal dysfunction within the experimental period of 28 days.

5. CONCLUSION

The losses in phytochemical contents following hydrothermal processing of the herbs did not substantially affect their overall therapeutic scores against morphological and functional impairments of hepatic and renal tissues following CCl_4 intoxication of the experimental rats. However, herbal intervention against CCl_4-induced hyperbilirubinemia showed that hydrothermal processed herbs possessed greater capacity to lower the severity of hyperbilirubinemia than their corresponding raw herbs. Nevertheless, a more rewarding bio-prospecting exercisefor the alleviation of CCl_4-induced hepatorenal impairment could be achieved by subjecting the herbs to sequential multi-solvent extraction process, which perhaps, will provide improved and better therapeutic benefits than the present outcomes.

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AUTHORS' CONTRIBUTIONS

OAO: conceptualized and supervised the study, edited the manuscript. PCC: wrote the manuscript and analyzed the experimental data. DIU: performed the experiments andanalyzed the experimental data. COI: supervised the study, edited the manuscript. RNN: supervised the study, edited the manuscript. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

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

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Research Article

In vitro regeneration of plantlets from nodal explants of *Aristolochia saccata* and *Aristolochia cathcartii*

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ABSTRACT

In vitro propagation of *Aristolochia saccata* and *A. cathcartii* were carried out using nodal explant. In both the plants, nodal explants showed direct somatic embryogenesis when cultured on MS medium using various concentrations of BAP (1.0-4.0) and 2iP (1.0-4.0) separately or in combination with low concentration (0.5 and 1.0 mg l^{-1}) of auxin (NAA). It was observed that BAP in combination with NAA was more effective for shoot induction than the hormones used separately. Among different combinations of NAA and BAP, 3.0 mg/l BAP + 1.0 mg/l NAA showed better response in case of *A. saccata* of about 96%, whereas, in *A. cathcartii*, the best response was achieved in 4.0 mg/l BAP + 0.5 mg/l NAA after 28 day of culture and 88.3% explants showed proliferation in this combination. The auxins NAA and IBA were used singly to induce rooting from in vitro raised shoot lets. A range of concentration was tested (0.1, 0.5, 0.8 and 1.0 mg/l) for rooting. In the present study 1/2 strength MS basal medium and the two different auxins (NAA and IBA) were tried, the maximum results on rooting were obtained on half strength with IBA (0.5 mg/l) then NAA.

Keywords: *Aristolochia saccata*; *A. cathcartii*; *In vitro* regeneration; Nodal culture; MS medium.

1. INTRODUCTION

From the time immemorial, plants have been widely used as curative agents in traditional medicines for variety of ailments. In India about 2,500 plants species belongs to more than 1000 genera are being used in the indigenous system of medicine [1]. Northeast India including Assam is represented by about 130 different tribes out of total 427 of India having their own traditional practices. Many herbal remedies individually or in combination have been recommended for the cure of different diseases in traditional medicinal practices by the ethnic communities of Northeast India.

*Aristolochia (*Aristolochiaceae) is an important genus widely used in traditional medicine [2]. During the past two decades, this genus has attracted much interest and has been the subject of numerous chemical and pharmacological studies. It is a rich source of aristolochic acids, which are unique to this genus, and of terpenoids [3].

The family Aristolochiaceae comprises of 8 genera and 450-600 species. The members of the genus *Aristolochia* are mostly distributed in tropical, subtropical, and Mediterranean regions of the world [4-7]. The species of *Aristolochia* are shrubby or perennial herbs, usually climbing.

The genus *Aristolochia* consists of a large number of species, cultivated as ornamentals [8] and popularly used as sources of abortifacient, emmena-

gogue, sedative, analgesic, anticancer, anti-inflammatory, anti-feedant, muscle relaxant, antihistaminic, and antiallergic drugs, for intestinal worms, in the treatment of cholera, stomach ache, abdominal pain, rheumatism, malaria, wounds and skin diseases, and also useful in treatment of different types of poisonous bites and stings [2-3].

The genetic diversity of medicinal plants in the world is becoming endangered at an alarming rate because of ruinous harvesting practices and over-harvesting for production of medicines. Further, extensive destruction of the plant rich habitat as a result of forest degradation, agricultural encroachment, urbanization, etc., are also some important factors [4]. Hence, there is a strong need for proactive understanding in the conservation, cultivation and sustainable usage of important medicinal plant species for future use. Hence the present study was aimed to develop an effective, reproducible, and simple and improved protocol for in vitro propagation by using nodal explants to make two species of *Aristolochia* viz. *A. saccata* Wall. and *A. cathcartii* Hook.f. & Thomson, throughout the year for pharmaceutical use and also for conservation.

2. MATERIALS AND METHODS

2.1. Plant material and explants sterilization

Two species of *Aristolochia viz., Aristolochia saccata* and *Aristolochia cathcartii* were collected from Karbi Anlong district (25°54′20.22″ N, 93°39′41.16″ E) of Assam, India. The collected experimental plants were grown and maintained in the experimental garden of Botany Department, Gauhati University. Nodal segments from the source plants were used as explants. The explants were coarsely trimmed to a size of 3 cm and washed thoroughly under running tap water for 10 min and then treated with liquid detergent [5% (v/v) Tween-20] for 15 min. Later these explants were washed with double-distilled water for 10 min. The explants were then sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5 min and washed several times with sterile H_2O to remove all traces of $HgCl_2$. After a final wash, the explants were spread on the pre-sterilized petridishes lined with sterile blotting paper inside a laminar airflow chamber. They were then trimmed finely to the appropriate size (1-1.5 cm). The sterilized explants were inoculated onto solid basal MS medium [9], with different concentrations and combinations of 2iP, BAP and NAA for in vitro regeneration of plants.

2.2. Culture media and growth condition

The stock solution in the required quantity was pipetted out into a standard flask containing distilled water. Sucrose 3% along with 100 mg/l myoinositol were added and dissolved in the media. All the plant growth regulators; additives for the different combinations were added before making up the media to the required volume. pH was adjusted at 5.8 using 0.1 N NaOH or 0.1 N HCl. For solidification, 0.8% w/v agar (HI-MEDIA Lab. India) was then added to the medium and mixed well**.** The sterilization of the culture medium was carried out in an autoclave for 20 min at 121°C and 15 psi pressure. The medium was then poured into pre-sterilized culture vessels, 15 ml was taken in culture tubes (150 × 25 mm), 50 ml was taken in culture bottles and 100 ml was taken in petri plates (150 x 20 mm) under aseptic conditions in a laminar air-flow cabinet.

Nodal segments (1-1.5 cm) were dissected out and all the inoculation operations were carried out under strict aseptic condition inside a Laminar Air Flow chamber, which was made sterile by the incessant exposure of germicidal UV rays for half an hour before use. All operations were carried out using pre-sterilized instruments and glassware. Explants were then aseptically introduced into culture vessels. The culture tubes were then plugged tightly with non-absorbent cotton plugs and the culture bottles and petriplates were sealed tight with sealing film. All cultures were incubated under irradiance of 70 µmol m^{-2} s^{-1} for 16 hour photo-period and temperature of 25±1°C and with a relative humidity of 55-60%.

2.3. Induction of callus and regeneration of plantlets

Basal medium supplemented with different concentrations of 2-isopentenyl-adenine (2-iP) (1.0, 2.0, 3.0, 4.0 mg/l) and benzylaminopurine (BAP) (1.0, 2.0, 3.0, 4.0 mg/l) individually and in combi-

nations with naphthaleneacetic acid (NAA) (0.5, 1.0 mg/l) were tested for the induction of callus and regeneration of shoot and root from nodal explants.

Sub-culturing was done at 14 day intervals onto fresh medium for 6 weeks to induce in vitro regeneration of shoot. Shoot buds were further cultured for elongation in the same medium supplemented with low concentration of cytokinins. The responses of each explant with regard to the induction of shoots, the length of shoot and the percentage of response were recorded after 6 weeks in culture.

2.4. In vitro rooting

In vitro regenerated shoots were rooted on half strength medium supplemented with different concentrations of auxins (NAA and IBA) alone. The response of each explant with regard to the number of roots induced and root lengths per shoot after 2 weeks in culture were recorded.

2.5. Hardening and acclimatization

In vitro grown plantlets were gently removed from culture tubes and washed with slightly warm ($37^{\circ}C$) sterile double distilled H_2O to remove all traces of nutrient medium. After removing media, plants were dipped in 1% w/v solution of bavistine to prevent any fungal infection to newly developed plants. After bavistine treatment the plantlets were carefully planted in plastic pots containing soilrite. The plantlets were irrigated by sprinkling with 0.5 x MS inorganic salts for three to four times per day for seven days. Plantlets were acclimatized for two weeks in an aseptic culture room under (16 h photoperiod at 28 ± $2^{\circ}C$; 8 h in dark at 25 ± $2^{\circ}C$) conditions. Further, the plantlets were exposed gradually to sunlight for acclimatization and were maintained in a garden.

2.6. Data collection and statistical analysis

Data for the percentage of response per explants with different concentrations and combinations of cytokinins and auxins with basal MS medium (shoot regeneration, shoot lengths, number of roots and root lengths) were recorded. Thus obtained data were analyzed statistically using SPSS 16.0 software (IBM Corporation SPSS, North America).

3. RESULTS

3.1. Direct somatic embryogenesis from nodal explants

Regeneration potential of nodal segments was explored on MS medium supplemented with various plant growth regulators and results are summarized in Tables 1 and 2. Nodal segment explants remained green and fresh but failed to develop multiple shoots in growth regulators free MS medium (control). All nodal explants cultured on MS medium supplemented with various concentrations of 2iP and BAP individually and in combination with NAA have developed healthy shoots. Nodal explants cultured on MS medium fortified with cytokinins alone induced multiple shoots at a lesser frequency compared to the media supplemented with combination of cytokinin and auxin (Fig. 1 and Fig. 2).

All the concentrations of BAP and 2iP facilitated shoot bud differentiation but BAP being more efficient than 2iP in terms of percent regeneration, number of shoots and shoot length. Among the various concentrations of BAP and 2iP tested, 3.0 mg/l BAP showed the highest shoot regeneration frequency of 86.6 ± 2.8%, the highest number of shoot were recorded as 1.8 ± 0.34 in *A. saccata*, but the highest shoot length (4.34 ± 0.07 cm) was observed at reduced concentration of BAP (1.0 mg/l).

In case of *A. cathcartii*, 4.0 mg/l BAP showed the highest shoot regeneration frequency of 73.3 ± 2.8% and shoot number (3.8 ± 0.45), but the highest shoot length (4.02 ± 0.1 cm) was observed at 2.0 mg/l concentration of BAP.

The synergistic influences of auxins with cytokinins was evident when combination of optimal concentration of each cytokinins with different concentrations of NAA (0.5 and 1.0 mg/l) were tested (Tables 1 and 2). Addition of NAA markedly enhanced the percent regeneration and number of shoots for both the *Aristolochia* sp. used for *in vitro* propagation. Among all the cytokinin and auxin combinations, the maximum percent regeneration in

A. saccata was found as 96.6 ± 2.8% and number of shoots (3.4 ± 0.55) per explants were obtained at 3.0 mg/l BAP + 1.0 mg/l NAA. But the highest shoot length (4.02 ± 0.08 cm) was recorded at the combination of 1.0 mg/l BAP + 0.5 mg/l NAA.

In case of *A. cathcartii*, the maximum percent of regeneration was recorded as 88.3 ± 2.8% and number of shoots (6.2 ± 0.44) at 4.0 mg/l BAP + 0.5 mg/l NAA. Here, the highest shoot length (3.82 ± 0.1 cm) was recorded at the combination of 2.0 mg/l BAP + 1 mg/l NAA.

3.2. *In-vitro* rooting

The *in vitro* raised shootlets were sub cultured on ½ strength MS medium augmented with 0.1-1.0 mg/l either NAA or IBA for both *A. saccata* and *A. cathcartii* for root formation. At 14th day, the *in vitro* raised shootlets were produced *in vitro* rootlets without any callus proliferation. Medium containing 0.5 mg/l of IBA was proved to be the most effective for rooting of micro shoots than that containing any other concentrations of NAA in case of both the plants evaluated (Tables 3 and 4). Here, NAA did not significantly improve the parameters evaluated. Highest percentage (83.3 ± 2.8%), maximum number of rootlets/shootlet (2.8 ± 0.44) and mean length of rootlets (3.22 ± 0.16 cm) were observed in in *A. saccata.* In *A. cathcartii*, the medium containing 0.5 mg/l of IBA, highest rooting was observed (78.3 ± 2.8%) percent shoots induced rooting within 14 days of culture and the mean number of root per culture and root length was recorded as 3.8 ± 0.44 and 3.04 ± 0.08 cm respectively.

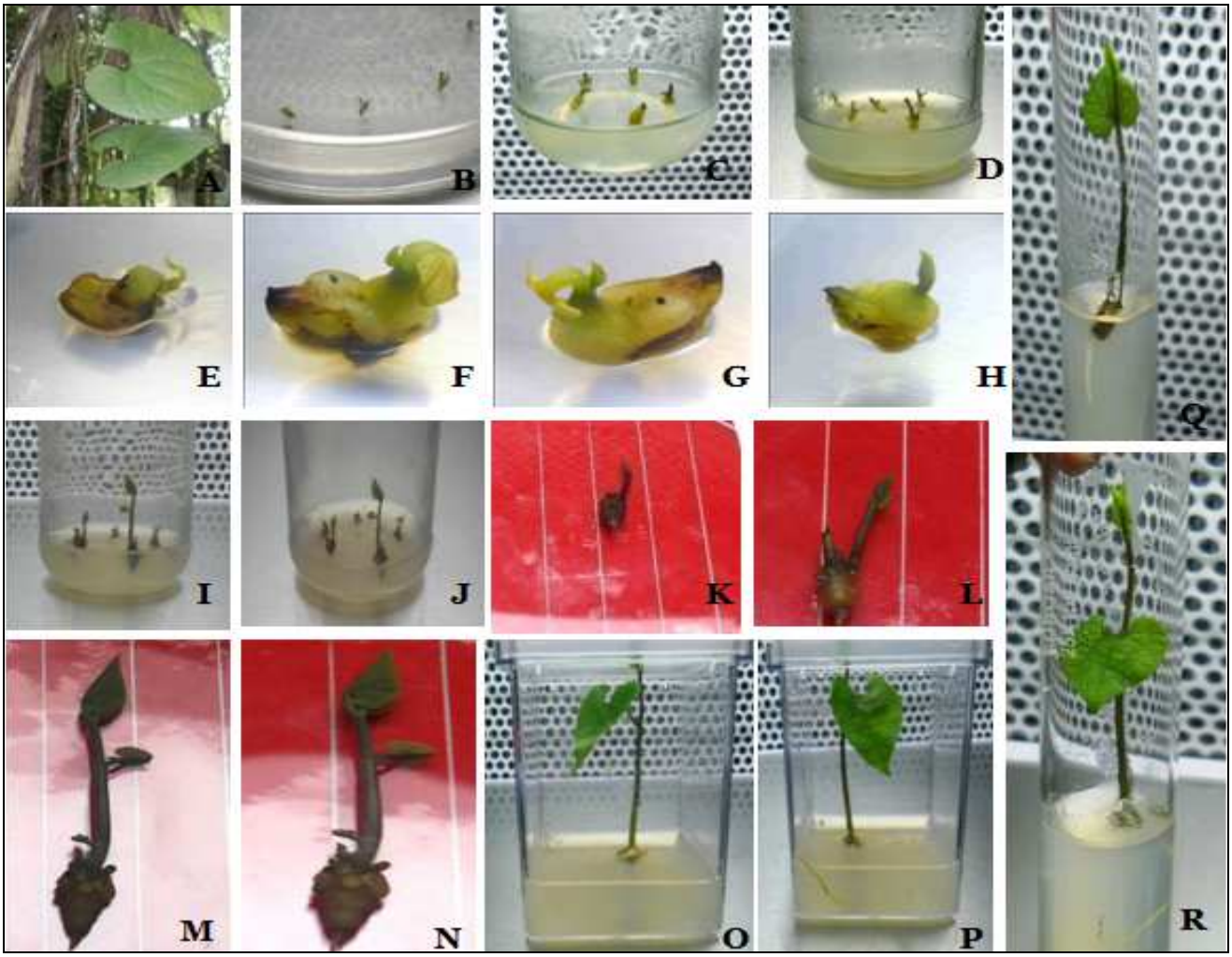

Figure 1. Different stages of *in-vitro* regeneration of *A. saccata* from nodal explant. **A** = plant in wild condition, **B** = inoculation of nodal explants in MS medium, **C-D** = initial days after inoculation in MS medium, **E-H** = direct organogenesis from explant in 3:1 mg/l of BAP and NAA in MS medium, **I-L** = multiple shoot regeneration in 3:1 mg/l of BAP and NAA, **M-N** = shoot elongation in 1 mg/l BAP, **O-R** = stages of rooting in 0.5 mg/l IBA in ½ MS medium.

Table 1. Effect of cytokinins and auxins individually and in combinations for organogenesis from nodal explants of *A. saccata* (after 6 weeks).

Plant growth regulators (mg l^{-1})			Response of nodal explants (%)	Number of shoots/ explant (Mean ± SD)	Shoot length/explant (cm) (Mean ± SD)
2iP	BAP	NAA			
Control (PGR free)			-	-	-
1			0	0	0
2			71.6±2.8	1.2±0.44	3.70±0.08
3			76.6±2.8	1.6±0.45	3.21±0.04
4			61.6±2.8	1.2±0.45	2.48±0.05
1		0.5	0	0	0
2		1	75.0	1.6±0.34	3.26±0.10
3		1	88.3±2.8	2.2±0.34	2.80±0.04
4		0.5	66.6±2.8	1.4±0.24	2.34±0.07
	1		80.0	1.6+±0.54	4.34±0.07
	2		73.3±2.8	1.4 ± 0.53	3.88±0.05
	3		86.6±2.8	1.8±0.34	3.38±0.10
	4		70.0	1.2±0.44	2.90±0.04
	1	0.5	88.3±2.8	2.2±0.44	4.02±0.08
	2	1	81.6±2.8	2.6±0.54	3.68±0.10
	3	1	96.6±2.8	3.4±0.55	3.38±0.11
	4	0.5	71.6±2.8	2.8±0.44	2.82±0.04

Data mean of 3 replicates ± S.D.

Table 2. Effect of cytokinins and auxins individually and in combinations for organogenesis from nodal explants of *A. cathcartii* (after 6 weeks).

Plant growth regulators (mg l^{-1})			Response of nodal explants (%)	Number of shoots/ explant (Mean ± SD)	Shoot length/explant (cm) (Mean ± SD)
2iP	BAP	NAA			
1			0	0	o
2			31.6± 2.8	3±0.82	3.6 ± 0.2
3			33.3± 2.8	3.25±0.50	3.24 ± 0.1
4			36.6± 2.8	3.5±0.57	2.98 ± 0.14
1		0.5	0	0	0
2		1	33.3± 2.8	3.2±0.45	3.11 ± 0.2
3		1	41.6± 2.8	3.6±0.50	3.06 ± 0.2
4		0.5	43.3± 2.8	3.8±0.42	3.02 ± 0.21
	1		0	0	0
	2		41.6± 2.8	3.4±0.54	4.02 ± 0.1
	3		43.3± 2.8	3.6±0.57	3.72 ± 0.08
	4		73.3± 2.8	3.8±0.45	3.42 ± 0.25
	1	0.5	0	0	0
	2	1	73.3± 2.8	4±0.70	3.82 ± 0.1
	3	1	81.6± 2.8	4.2 ±0.44	3.54 ± 0.25
	4	0.5	88.3± 2.8	6.2±0.44	3.26 ± 0.2

Data mean of 3 replicates ± S.D.

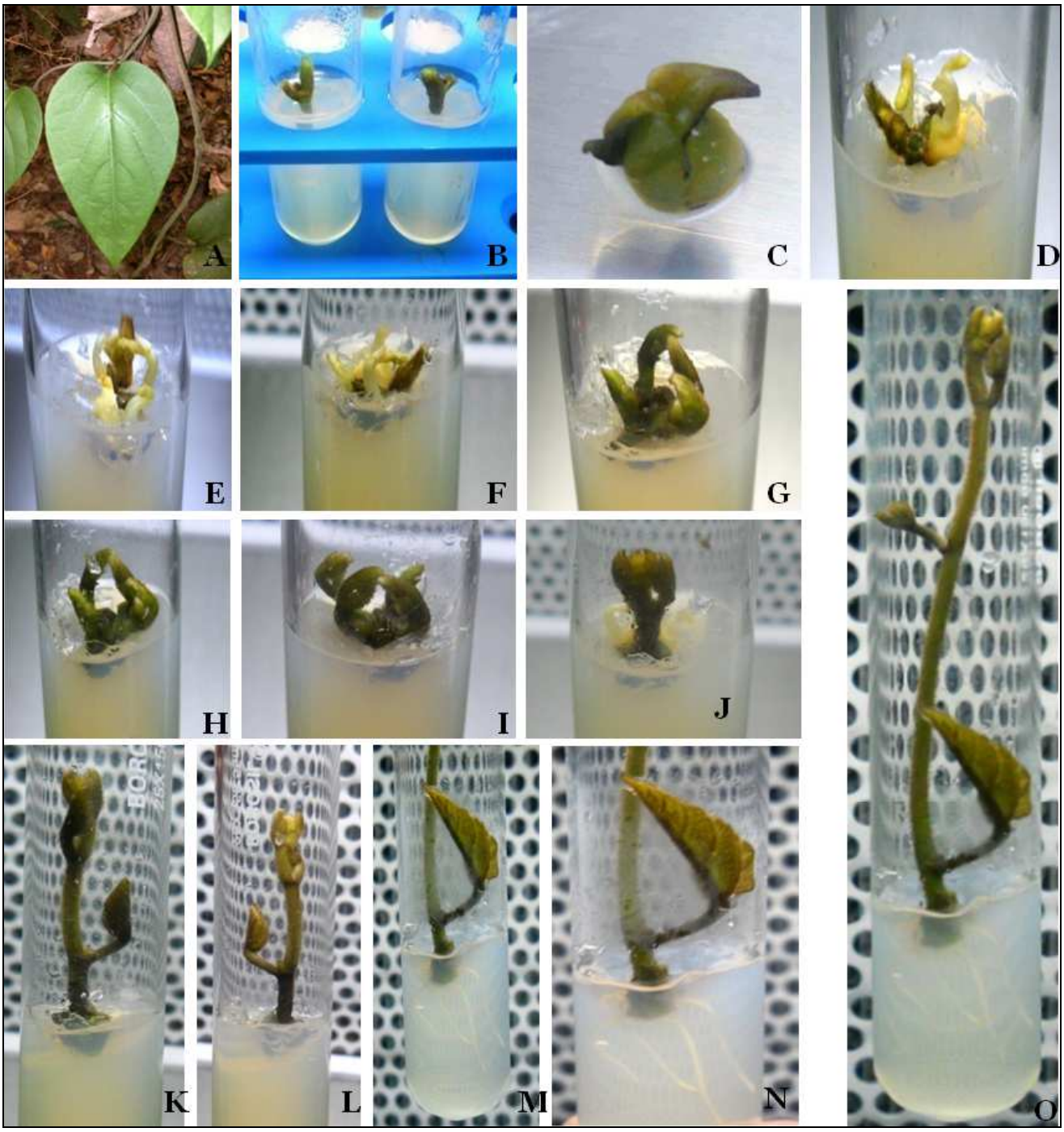

Figure 2. Different stages of *in-vitro* regeneration of *A. cathcartii* from nodal explant. **A** = plant in wild condition, **B** = inoculation of nodal explants in MS medium containing 4.0 mg/l BAP and 0.5 mg/l NAA, **C** = shoot bud initiation from an explant, **D-I** = direct organogenesis and multiple shoot regeneration in 4:0.5 mg/l of BAP and NAA, **J** = individual shoot bud transferred to shoot elongation medium containing 2 mg/l BAP, **K-L** = shoot elongation in 2 mg/l BAP, **M-O** = stages of rooting in 0.5 mg/l IBA in ½ MS medium.

3.3. Acclimatization and hardening

The rooted plantlet were successfully hardened off inside the growth room in sterile soilrite for 2 weeks and eventually established in natural soil. There was no detectable variation among the potted plants with respect to morphological and growth characteristics (Fig. 3).

After 15 days, *in vitro* raised plantlets were hardened in polycups with soilrite, irrigated with 0.5× MS liquid medium. The plants were kept in a culture room for 14 days. Approximately,

75% of plants were successfully established in polycups for both the experimental plants. After 14 days the polycups hardened plants were transferred to pots placed in and kept in poly house. Sixty five percentages of plantlets were well established for *A. saccata* and sixty percentages for *A. cathcartii* in the poly house condition. After one month, regenerated plants were successfully transferred to the field.

The protocol optimized here is efficient, reproducible and provide a rapid technique for mass propagation and multiplication of this two potential medicinal plants and could further be used in their improvement programme.

Figure 3. Growth of plantlets of two species of *Aristolochia* in Poly-house condition. **A** = *A. saccata*; **B-C** = *A. cathcartii.*

Table 3. Effect of auxins for root induction of *A. saccata* (after 2 weeks in root induction medium).

Auxin concentration (mg l^{-1})		Response (%)	Numbers of roots/shoot	Root length/culture
NAA	**IBA**			
0.1		28.3 ± 2.8	1.8±0.34	2.74±0.07
0.5		63.3 ± 2.8	1.8±0.24	3.08±0.13
0.8		51.6 ± 2.8	2.2±0.44	3.02±0.07
1		53.3 ± 2.8	1.2±0.45	2.80±0.04
	0.1	41.6 ± 2.8	1.4±0.55	2.84±0.04
	0.5	83.3 ± 2.8	2.8±0.44	3.22±0.16
	0.8	51.6 ± 2.8	2.4±0.44	3.18±0.10
	1	43.3 ± 2.8	1.6±0.55	2.98±0.11

Data mean of 3 replicates ± S.D.

Table 4. Effect of auxins for root induction of *A. cathcartii* (after 2 weeks in root induction medium)

Auxin concentration (mg l^{-1})		Response (%)	Numbers of roots/shoot	Root length/culture
NAA	**IBA**			
0.1		31.6 ± 2.8	1.8±0.45	2.36±0.11
0.5		56.6 ± 2.8	2.4±0.54	2.42±0.13
0.8		46.6 ± 2.8	2.6±0.55	2.80±0.12
1		43.3 ± 2.8	2.2±0.45	2.82±0.10
	0.1	63.3 ± 2.8	2.8±0.44	2.86±0.08
	0.5	78.3 ± 2.8	3.8±0.44	3.04±0.08
	0.8	66.6 ± 2.8	3.6±0.54	3.02±0.10
	1	56.6 ± 2.8	3.2±0.44	2.92±0.10

Data mean of 3 replicates ± S.D.

4. DISCUSSION

Clonal propagation through tissue culture can be achieved in a short time and space. Thus, it is possible to produce plants in large numbers starting from a single individual protoplast to different plant parts as an explant. Micro propagation has, wide commercial application, starting from conservation of genetic stock of threatened species to secondary metabolite production in important plant taxa and year round supply of disease free quality planting material for commercial cultivation. Since then, several crop species have been micropropagated and recipes are now available which can be adopted by growers trained in aseptic manipulations in a new era of plant husbandry. The results obtained in our experiment suggested that *in vitro* plantlet regeneration using nodal explant may be used for direct clonal propagation and conservation with a low risk of generating disease free quality planting material in large scale for *Aristolochia saccata* and *Aristolochia cathcartii*.

In this study, an *in-vitro* propagation protocol has been developed for *A. saccata* and *A. cathcartii* using nodal explant. In both the plants nodal explants showed direct somatic embryogenesis when cultured on MS medium using various concentrations of BAP (1.0-4.0) and 2iP (1.0-4.0) separately or in combination with low concentration (0.5 and 1.0 mg l^{-1}) of auxin (NAA). It was observed that BAP in combination with NAA was more effective for shoot induction than the hormones used separately.

Among the different treatments of BAP and NAA, 3.0 mg/l BAP + 1.0 mg/l NAA showed better response in case of *A. saccata*. In this concentration, 96.6% explants induced to develop shoots. The number of shoot as well as length of shoot per explant was recorded as 3.4 ± 0.55 and 3.38 ± 0.11 cm respectively. BAP is considered one of the most useful cytokinins for the multiplication of axillary buds reported by many authors [10-12]. In the present investigation, combination of BAP with NAA was found more suitable than BAP and 2iP alone. But, highest shoot length was observed in low concentration of BAP i.e., 4.34 ± 0.07 cm.

Induction of callus and multiple shoots from *A. bracteolate* using various PGRs was also reported previously [13]. Among the BAP - NAA supplemented media for *A. cathcartii*, the best response was achieved in 4.0 mg/l BAP + 0.5 mg/l NAA after 30-day of culture and 88.3% explants showed proliferation in this combination. The highest mean number of shoot per culture were 6.2 ± 0.44 in combination of BAP and NAA, but the highest shoot length was found 4.02 ± 0.1 in 2.0 mg/l BAP. These results are in agreement with the results of Sultana and Handique, Chandramu et al., Sudha et al. and Chen et al. [14-17]. In the present study, it was found that the number of shoot per culture was increased with the number of subculture. Rout et al. (2000) reported that, a rapid rate of propagation depends on the sub-culturing of proliferating shoots [18].

The MS medium augmented with auxin or cytokinin alone or in combinations induced highest percentage of shoot proliferation and maximum number of shoots from the inter-nodal segments of *A. bracteata* [19]. The results of the present study were directly coincided with previous observations [20-25]. In *A. tagala*, multiple shoot buds are produced directly from nodal explants cultured on basal medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA [26] and adventitious shoots at 1.0 mg/l of BAP [27]. *A. bracteolate* cultured on MS medium fortified with 4.0 mg/l of BAP combined with 0.5 mg/l of NAA produce maximum number of shoots (8.9) in nodal explants [28]. In the present study we optimized a protocol for large scale multiplication of *A. saccata* and *A. cathcartii* using nodal segments as explants.

In the present study, nodal explants of *A. saccata* and *A. cathcartii* showed significantly higher response in the medium with the combination of BAP + NAA. The quality of shoots and the overall growth response in terms of average shoot length was better in this growth regulator combination. A comparatively lower response was recorded when BAP or 2iP was added alone in the medium. Review of literature indicates that the addition of either IAA or NAA in the culture medium improved the response in a number of species in terms of shoot growth. Shin et al. [29] reported that the combination and interaction of BAP and NAA plays important role for *in vitro* propagation of nodal explant for multiple shoot induction.

In this study, two cytokinins were taken for higher shoot multiplication. Some authors also suggested that the combination of two cytokinins were needed for producing multiple shoots on *Aristolochia bracteolate* [13], but here higher response was observed in combination of cytokinin and auxin for both the species of *Aristolochia.*

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil [30]. The auxins NAA and IBA were used singly to induce rooting from *in vitro* raised shoot lets. A range of concentration was tested (0.1, 0.5, 0.8 and 1.0 mg/l) for rooting. In the present study 1/2 strength MS basal medium and the two different auxins (NAA and IBA) were tried, the maximum results on rooting were obtained on half strength with IBA (0.5 mg/l) then NAA. The auxins, NAA and IBA were used singly to induce rooting from *in vitro* raised shootlets of *Dioscorea hispida* [31].

The well rooted plants were transferred to plastic cups containing soilrite for hardening and kept under controlled condition. Upon transferred to vermiculite medium plants started producing fresh shoots and roots after one week of transplanting. Later they were transferred to the field and the survival rate was 65% in case of *A. saccata* and 60% in case of *A. cathcartii*. The efficient micro propagation technique described here may be highly use full for raising quality planting material of *A. saccata* and *A. cathcartii* for commercial and off season cultivation which is not only help the ex-situ conservation but also help full in the restoration of genetic stock of the species.

Study of *in-vitro* propagation produced an efficient protocol for large scale multiplication and *ex situ* conservation of the medicinally important plant, *A. saccata* and *A. cathcartii* using nodal segments. It can also be used as a source of tissues for the biochemical characterization of medicinally active compounds and will increase the opportunities for the use of this medicinal plant in both traditional and modern medical health care. Wild medicinal plants are being depleted rapidly due to over-exploitation and unscientific methods of collection. Hence, in the present work, protocol for *in vitro* regeneration of the rare and endemic medicinal plant species *A. saccata* and *A. cathcartii* have been developed. These protocols could be used to make these plants available throughout the year for traditional healers, pharmaceutical usages, germplasm conservation, commercial cultivation, and also for the production of secondary metabolites.

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AUTHORS' CONTRIBUTION

All the authors contributed equally for the success of this research. The final manuscript has been read and approved by all the authors.

TRANSPARENCY DECLARATION

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

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Research Article

Proposal for screening of kidney disease in a random population based on World Kidney Day campaign

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ABSTRACT

Despite the advances on early screening techniques, getting to know the chronic kidney disease (CKD) prevalence in Brazil and worldwide remains a challenge for researchers. Aging, diabetes and hypertension are the main CKD causes in Brazil. The aim of the study was to evaluate the presence of urinary dipstick abnormalities in World Kidney Day campaign. This is a cross-sectional studyconducted at FMABC. This study was based on the answers to a kidney disease questionnaire and urinary dipstick test. A total of 205 patients were randomly invited to collect urine samples on World Kidney Day 2013. Among the 205 studied patients, 66.34% were women with mean age of 46.32 years. Around 34.14% of the patients were hypertensive and 9.75% diabetic. Urinalysis alterations were observed in 28.29% of patients. The group with urine alterations had older individuals (51.36 years) andmore diabetes (18.96%) with higher levels of glucose (143.2 mg/dl). Brazilian population is getting older and diabetic which represent risk factors for the onset of CKD. The necessity of an early detection by means of specific campaigns is thus of great importance. The use of dipstick test for screening is an important tool for kidney disease diagnosis.

Keywords: Proteinuria; Diabetes; Hypertension.

1. INTRODUCTION

Getting to know the real prevalence of chronic kidney disease (CKD) has been a challenging matter. However, this task has been made easier thanks to measures taken that focus on the disease screening. Today it is known that CKD has a growing incidence worldwide related to higher mortality rates owing to cardiovascular diseases, especially in developing countries [1].

It is estimated that between 8 and 16% of the world population suffer from CKD, having hypertension and diabetes as its main causes. Population aging and the expanded access to

diagnosis explain the increase in number of cases over the last years [2].

In Brazil, CKD represents 554 patients in dialysis per million of population, with around 84% of the total costs sponsored by the National Public Health Service [3].

An early diagnosis is important due to the possibility of providing immediate intervention and control of the conditions that lead to the deterioration of renal function. This screening may be done by the measurement of creatinine serum levels and the analysis of urine by reagent strips to detect proteinuria. Both methods are quite accurate when it comes to the detection of CKD [4].

The aim of this study was to evaluate the presence of urinary abnormalities by reagent strips to screen to kidney disease in the World Kidney Day campaign in São Bernardo do Campo, Brazil.

2. METHODS

2.1. Study design

This is a cross-sectional study conducted by the Discipline of Nephrology at Faculdade de Medicina do ABC on World Kidney Day. Patients were randomly invited to collect isolated urine samples for the investigation of proteinuria after signing the informed consent term. This work was approved by Institutional Ethics Committee (protocol number 09/2015) and is in accordance with the ethical principles of the Helsinki Declaration (1996).

2.2. Sample selection

Inclusion criteria consisted in being over 18 years-old. Women during their menstrual cycle and patients of both sexes who were not São Bernardo do Campo residents were excluded.

2.3. Variables and statistical analysis

Data regarding presence of hypertension, diabetes, heart disease, previous CKD, smoking and regular use of non-hormonal anti-inflammatories (NSAID) were collected.

Random capillary blood glucose was determined using an Accu-Check® glucometer (Roche, Switzerland), weight measured in kilograms (kg) with a Filizola PL 200 scale (Filizola®, Brazil) and blood pressure assessed with a Tycos® Aneroid sphygmomanometer (Welch Allyn®, USA) by auscultatory method. Mean blood pressure was calculated according the formula:

(2 x Diastolic Pressure + Systolic Pressure) / 3.

Once measurements were performed, for the sake of comparison, patients were divided into two groups according the presence of urinary dipstick abnormalities.

Results of quantitative and continuous variables were expressedas mean and standard deviation values. Qualitative variables were described by absolute and relative frequencies.

Student's t-test was used in order to compare the means between both groups, and whenever the assumption of data normality was rejected, the non parametric test of Mann-Whitney was applied. Homogeneity of proportions was tested by chi-square test; however, when the expected frequencies were less than five, Fisher's exact test was used. The significance level applied was p=0.05. The statistical package SPSS 17.0 for Windows (Microsoft, USA) was used for analysis.

3. RESULTS

A total of 209 patients were interviewed and they all collected urine samples. Among that number, four women were excluded owing to the fact they were in their menstrual cycle. Table 1 shows the demographic and clinical characteristics of the patients.

The mean age was 46.32 years, and there was a higher prevalence of women among the sample (66.34%). Around 34.14% of the subjects declared themselves to be hypertensive and only 9.75% diabetic. The mean capillary blood glucose was 118.2 mg/dl (± 48.52). Of the 205 patients, 28.29% showed alterations in the urinary screening test.

Table 2 highlights the division of patients into groups according to the presence or absence of abnormalities. A statistical difference could be observed concerning age, higher in the group with abnormal results (group 2), which suggests an association between higher age and abnormalities in the urine test.

The positive screening for kidney disease was related to the presence of diabetes, higher in group 2 (18.96%) with p=0,005. The mean capillary blood glucose in the group with positive screening outcome was 143.2 mg/dl (±77.98) whereas in the group with normal screening (group 1) result it was 108.22 mg/dl (±23.47), p=0.002.

4. DISCUSSION

Our results show the importance of a simple screening tool for the detection of kidney alterations in a random population. It was observed that only 13.79% of the individuals knew they had some sort of renal condition. Moreover, the great majority of patients who screened positively were not aware of the kind of condition they had.

Table 1. Clinical data and demographic characteristics of patients (n=205).

Variables	Values
Age (years)	46.32 ± 14.68
Gender (Male/Female) (%)	33.66 / 66.34
Weight (Kg)	73.64 ± 15.35
Hypertension (%)	34.14
Diabetes (%)	9.75
Self Report of Kidney Disease (%)	13.50
Heart Failure (%)	10.24
Smoking (%)	16.09
Sedentary (%)	48.29
NSAID* (%)	19.51
Mean Blood Pressure(mmHg)	93.75 ± 13.34
Capillary Glucose(mg/dL)	118.2 ± 48.52
Dipstick urine altered(%)	28.29
Proteinuria (%)	4.87
Hematuria (%)	13.17
Glicosuria (%)	6.87
Leucocituria (%)	12.19

Ω Data expressed as mean and standard deviaton for continuous variables and percentages to cathegorical ones. *NSAID: Non Steroidal Anti Inflammatory Drug.

Table 2. Comparison between groups with and without dipstick test altered among clinical data.

Variables	Group 1 (n = 147)	Group 2 (n = 58)	p
Age (years)	44.34 ± 14.86	51.36 ± 13.03	0.002*
Gender (male/female) (%)	35.38/ 64.62	29.32/ 70.68	0.407**
Weight (Kg)	73.19 ± 15.02	74.80 ± 16.26	0.514*
Hypertension (%)	31.97	39.65	0.296**
Diabetes (%)	6.12	18.96	0.005**
Self-report of kidney disease (%)	13.60	13.79	0.971**
Heart failure (%)	7.48	17.24	0.037**
Smoking (%)	17	13.79	0.572**
Sedentary (%)	46.25	53.44	0.353**
NSAID (%)	19.04	20.68	0.789**
Mean blood pressure (mmHg)	93.40 ± 13.10	94,63 ± 14	0.554**
Capillary glucose (mg/dl)	108.22 ± 23.47	143.2 ± 77.98	0.002*
Dipstick proteinuria (%)	-----	17.24	<0.001***
Dipstick hematuria (%)	-----	46.55	<0.001**
Dipstick glycosuria (%)	-----	24.13	<0.001***
Dipstick leucocyturia (%)	-----	32.75	<0.001***

*Student´s t-Test / **Chi Square Test / ***Fisher Exact Test.
NSAID: Non Steroidal Anti Inflammatory Drug.

CKD can be asymptomatic in its early stages and before it reaches its more advanced phases. Therefore, the American Diabetes Association reinforces the idea of an annual screening for all diabetics so that an early renal dysfunction may be detected [5].

Similarly, the US Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure recommends the screening for CKD in all patients before the onset of antihypertensive treatments [6].

Bastos et al call the attention to the need for intervention in CKD patients whose conditions may lead torenal replacement therapy [7]. The study concludes that the early diagnosis and prompt

referral to a nephrologist are wise measures for a better patient's outcome [7].

Upon performing dipstick screening on 38.721 individuals in São Paulo between the years 2005-2010, Lima et al. observed that the majority of the patients at the mean age of 46 years were of the female sex, a fact that is consonant with our findings [8]. On the other hand, proteinuria levels were lower in our group when compared with the results found by Lima, possibly given the large number of participating patients in his study [8].

Despite the importance of screening, the US Preventive Services Task Force, when conducting a systematic review on the screening and monitoring of CKD at early stages, found no controlled and randomized studies that evaluated screening for CKD and clinical outcomes [9, 10]. However, it is a fact that the Brazilian population is getting old and more diabetic as well, this association may represent risk factors for the development of CKD. The necessity of an early detection by means of specific campaigns is thus of great importance. In Europe, where population is also getting old, there is a substantial variation in CKD prevalence [11] and according to the European Kidney Health Alliance (EKHA), more than 10% of the population suffers from this desease [12]. CDK diagnosis is based on urine creatinine and albuminuria measurement and there is a great heterogeneity of laboratorial methodologies to address their values [11].

In conclusion, the use of urine reagent strips in kidney disease screening through proteinuria assessment in a random population is an important tool for diagnosis and subsequent follow-up to a nephrologist.

AUTHOR´S CONTRIBUTION

MRB and FLAF were responsible for conception and design and study supervision; VCRJ was responsible for analysis and interpretation of data; LYV, TCC and RRB were responsible for acquisition, analysis and interpretation of data; DRS and ACCM were responsible for development of methodology and data acquisition; LAA, ECP and BCAA were responsible for technical and material support. The final manuscript has been read and approved by all the authors.

TRANSPARENCY DECLARATION

The authors declare that they have no conflict of interest.

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Review Article

Incidence and significance of black aspergilli in agricultural commodities: a review, with a key to all species accepted to-date

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ABSTRACT

Black aspergilli (*Aspergillus* species of Section *Nigri*) present dark colonies, often black, and uniseriate or biseriate conidial heads. Currently 26 species and one variety are accepted within this section. They have been isolated from a wide variety of food worldwide and are considered as common causes of food spoilage and biodeterioration of other materials. They are commonly present in cereals and vineyards and have the ability to cause *Aspergillus* rot of black berry. Some species of this section, like *A. niger* and *A. awamori*, are a common source of extracellular enzymes such as amylases and lipases, and organic acids, such as citric and gluconic acid, used as additives in food processing and are used for biotechnological purposes. These products hold the GRAS (Generally Recognised as Safe) status. Other species are able to produce ochratoxins (OTA) and fumonisins. This review briefly shedlighted on the taxonomy of this important group of *Aspergillus* along with the species incidence, mycotoxin production in agricultural commodities as well as their significance as plant pathogens. A provisional key for identification (based on phenotypic characteristics) is provided for all described species to-date.

Keywords: Ochratoxins; Fumonisins; Biotechnology; *Aspergillus carbonarius*; Cereals; Grapes.

1. TAXONOMICAL OVERVIEW

Thom and Raper [1] and Raper and Fennell [2] published major monographic treatments on the genus *Aspergillus* and respectively accepted 89 and 150 species. Now the genus comprises 339 species [3] or 344 [4]. Many of these species can be conveniently separated into several distinct morphospecies, and several of these are based on colors according to the earlier classification [2]. However, phylogenetic analyses of sequence data resulted in separating the *Aspergillus* genus into eight subgenera [5]. Following these analyses, the economically important species that produce the ochratoxins were divided to include those species of the subgenus *Circumdati*, the sections *Circumdati* (=*Aspergillus ochraceus* group) and *Nigri* (*A. niger* group). There are no known teleomorphic species of section *Nigri*. In recent years, members of the *Aspergillus* section *Nigri* have undergone an

extensive taxonomic revision resulting in several new taxa. Mosseray [6] described 35 black aspergilli species, while Raper and Fennell [2] reduced this number to 12. Later, Al-Musallam [7] revised the taxonomy of the *A. niger* group and recognized seven species, based on morphological features, and described *A. niger* as an aggregate consisting of seven varieties and two *formae*. The black *Aspergillus* species were classified into the Section *Nigri* in the subgenus *Circumdati* by Gams et al. [8], formerly '*A. niger* species group' by Raper and Fennell [2]. They present dark colonies, often black, and uniseriate or biseriate conidiophores. In 1989, Kozakiewicz [9] suggested 17 taxa in the *A. niger* group and distinguished two groups: echinulate and verrucose, depending on their conidial ornamentations. In the past, it was very common that all *Aspergillus* isolates developing black colonies were identified as *A.niger* by non-taxonomists, because of the similarities in morphology. To solve this problem, Abarca et al. [10] published a review in the taxonomy of black aspergilla and proposed an identification key to distinguish the most common taxa based on uniseriate and biseriate character of the conidial heads. A provisional key of section *Nigri*, based on phenotypic characteristics, extrolites and β-tubulin sequencing, was also proposed [11] who accepted 15 species in this section: *A. aculeatus, A. brasilensis, A. carbonarius, A. costaricaensis, A. ellipticus, A. foetidus, A. heteromorphus, A. homomorphus, A. japonicus, A. lacticoffeatus, A. niger, A. piperis, A. sclerotioniger, A. tubingensis* and *A. vadensis*. Later on some more new species were described: *A. ibericus* [12], *A. aculeatinus, A. sclerotiocarbonarius* [13], *A. uvarum* [14], *A. saccharolyticus* [15]. Also in 2011, 4 additional species were described: *A. fijiensis, A. indologenus, A. eucalypticola, A. neoniger* and 2 others were validated; *A. violaceofuscus* and *A. acidus*, however *A. foetidus* was synonymized to *A. niger* based on molecular and physiological data and 2 other species described previously, *A. coreanus* and *A. lacticoffeatus*, were found to be colour mutants of *A. acidus* and *A. niger*, respectively [16]. Also in the study of Hubka and Kolarik [17] on β-tubulin paralogue *tubC*, stated that *A. japonicus* should be treated as a synonym with *A. violaceofuscus*, and *A. fijiensis* is reduced to synonymy with *A. brunneoviolaceus*. In 2012, two uniseriate species were described from indoor air (*A. floridensis* and *A. trinidadensis*) and *A. fijiensis* was confirmed as a synonym with *A. brunneoviolaceus* [18]. Currently and after these revisions, *Aspergillus* section *Nigri* is considered to comprise 26 defined species and one variety [5, 10, 11, 13, 14, 16-19] (refer to Table 1), although it remains under investigation, which may result in further changes.

2. DISTRIBUTION AND INCIDENCE OF THE BLACK ASPERILLI IN AGRICULTURAL COMMODITIES

It was indicated that most members of the genus *Aspergillus* occurred in the tropical latitudes below 25 degree north and south, with greater than expected frequencies in the subtropical to warm temperate zones at latitudes between 26 and 35 degrees [20]. Also, it was suggested that species abundance peaked in the subtropics is attributed to several biotic and abiotic interacting factors with the major factor temperature [20]. In general, the black species of aspergilli (particularly *A. niger* var. *niger*) were found to occur more frequently in forest and cultivated soils and less frequency in desert soils [20, 21]. *A.niger* is one of the most common species of the genus *Aspergillus*. It is one of the fungi that have been labelled with the GRAS (generally recognized as safe) status from the US Food and Drug Administration [22]. But instead of the safe categorization, *A. niger* has been found to be an opportunistic reason for infections of humans. If inhaled, in sufficient quantity it can cause severe lung problems i.e., aspergillosis in humans. It is also associated with various plant diseases resulting in huge economic loss. It is also reported to produce ochratoxin A and fumonisin B_2 in stored commodities [10, 23]. Black *Aspergillus* species were found as dominant in almost all agricultural commodities in all continents such as cereals (maize, wheat, barley, sorghum, millet, rye, oat, etc.), cereal products, beans, nuts (peanuts, almond and hazelnuts, coconut etc.), grape and grape products, fruits and fruit juices, and vegetables (refer to Table 2).

Table 1. List of species accepted to-date (ordered alphabetically).

1.	*A. aculeatinus* Noonim, Frisvad, Varga & Samson 2008
2.	*A. aculeatus* Lizuka 1953
3.	*A. brasiliensis* Varga, Frisvad & Samson 2007
4.	*A. brunneoviolaceus* Bat. & H. Maia 1955 (=*A. fijiensis* Varga, Frisvad & Samson 2011)
5.	*A. carbonarius* (Bainier) Thom 1916
6.	*A. ellipticus* Raper & Fennell 1965
7.	*A. eucalypticola* Varga, Frisvad & Samson 2011
8.	*A. floridensis* Ž. Jurjević, G. Perrone & S.W. Peterson 2012
9.	*A. helicothrix* Al-Musallam 1980
10.	*A. heteromorphus* Batista & Maia 1957
11.	*A. homomorphus Steiman*, Guiraud, Sage & Seigle-Mur. ex Samson & Frisvad 2004
12.	*A. ibericus* Serra, Cabanes & Perrone 2006
13.	*A. indologenus* Frisvad, Varga & Samson 2011
14.	*A. luchuensis* Inui 1901 (=*A. acidus* Kozak. 1989, =*Aspergillus awamori* Nakaz 1907)
15.	*A. neoniger* Varga, Frisvad & Samson 2011
16.	*A. niger* van Tieghem 1867 (=*A. foetidus* Thom & Raper 1945)
17.	*A. niger* var. *taxi* Zhou, Zhao & Ping 2009
18.	*A. piperis* Samson & Frisvad 2004
19.	*A. saccharolyticus* Sørensen, Lubeck & Frisvad 2011
20.	*A. sclerotiocarbonarius* Noonim, Frisvad, Varga & Samson 2008
21.	*A. sclerotioniger* Samson & Frisved 2004
22.	*A. trinidadensis* Ž. Jurjević, G. Perrone & S. W. Peterson 2012
23.	*A. tubingensis* (Schober) Mosseray 1934
24.	*A. uvarum* Perrone, Varga & Kozakiewicz 2007
25.	*A. vadensis* Samson, de Vries, Frisvad & Visser 2005
26.	*A. violaceofuscus* Gasperini 1887 (=*A. japonicus* Saito 1906)
27.	*A. welwitschiae* (Bres.) Henn. apud Wehmer 1907 (=*A. awamori* sensu Perrone et al. 2011)

3. OCHRATOXIN PRODUCTION IN AGRICULTURAL COMMODITIES AND BY THE ASSOCIATED BLACK ASPERGILLI

Ochratoxin A (OTA, Fig. 1) is a very strong nephrotoxin and potential carcinogen, teratogenic and immunosuppressive, classified as Group 2B by the International Agency for Research on Cancer [60]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established 100 ng kg^{-1} bw as the tolerable weekly intake (PTWI) recommended for OTA [61], which is also regulated by the European Commission. The regulation levels in food and feed products are established at 10 μg kg^{-1} in dry grapes, 2 μg kg^{-1} in grape juice, must and wine, and 0.5 μg kg^{-1} in food for babies and infants.

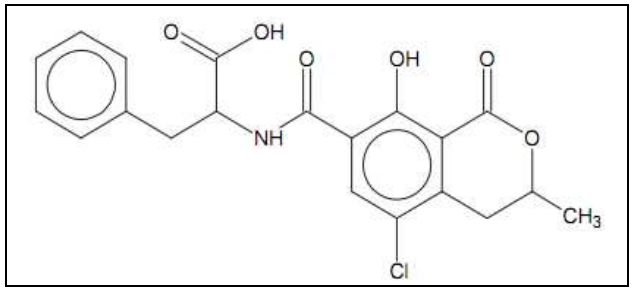

Figure 1. Chemical structure of OTA.

Dichotomous key for identification of species of section *Nigri* (based on phenotypic characteristics, designed by MA Ismail)

1.	Uniseriate (all species with no growth at 40 ºC) …….....	2
1.	Biseriate (growth at 40 ºC) …….....	9
2.	Versicle size up to 80 µm or more …….....	3
2.	Versicle size not exceed 45 µm …….....	7
3.	Stipe width up to 30 µm, conidia 3.5-5 µm, sclerotia if present cream, up to 0.5 mm diam	*A. aculeatus*
3.	Stipe width not exceed 20 µm ……..…….....	4
4.	Conidia large 4-7(8) x 3.5-7, up to 13 x 10 µm if from monophialide	*A. trinidadensis*
4.	Conidia small, less than 6 µm in length ……..…….....	5
5.	Conidia 2.5-4.5 µm, sclerotia if present white to cream, 0.4-0.6 mm diam ….....	*A. aculeatinus*
5.	Conidia smaller, globose to ellipsoidal 3.5-5.0(6) x 3.5-5.0 (5.5) µm ….....	6
6.	Sclerotia if present buff to orange brown up to 0.8 mm diam …….....	*A. brunneoviolaceus* (=*A. fijiensis*)
6.	Sclerotia if present buff yellowish, 0.2-1.1 mm diam …….....	*A. floridensis*
7.	Stipe width (5-)10-18 (-24) µm, vesicle 20-30 µm, conidia globose-ellipsoidal (3-) 4-7 (-9) x 3.0-7.0 µm, sclerotia if present dark brown to black, 0.5-0.8 mm diam ……..... ..	*A. uvarum*
7.	Not as above (stipe width and conidia smaller, vesicles larger)	8
8.	Stipe width 2-5 µm, vesicles 10-30 (-45) µm, conidia 3.5-4.0 x 4.0-5.5 µm, sclerotia if present white to cream, up to 0.5 mm diam. ….....	*A. violaceofuscus* (=*A. japonicas*)
8.	Stipe width 5-7 µm, vesicles 25-40 µm, conidia 5.0-6.2 µm, sclerotia absent	*A. saccharolyticus*
8.	Stipe width 5-11 µm, vesicles 20-45 µm, conidia 3-4 µm, sclerotia absent…….....	*A. indologenus*
9.	Conidial small, never exceed 5µm…….....	10
9.	Conidia large, exceed 5 µm…….....	20
10.	Vesicle not exceed 45 µm…….....	11
10.	Vesicles larger…….....	13
11.	No growth at 40 ºC, vesicles up to 30 µm, stipe width not exceed 7 µm; sclerotia 300-600 mm diam., white when young …….....	*A. heteromorphous*
11.	Growth at 40 ºC, vesicles up to 35 or 45 µm, stipe width up to 13 or 15 µm….....	12
12.	Vesicles not exceed 35 µm, stipe brown to black, short, not exceed 150 µm, sclerotia absent…….....	*A. vadensis*
12.	Vesicles up to 45 µm, stipe pale brown, long, up to 1700 µm, sclerotia produced by some strains, white …….....	*A. brasiliensis*
12.	Vesicles 30-55 µm, stipe hyaline, stipe width 8-14 µm, sclerotia absent, conidia globose 2.5-3.5 µm…….....	*A. eucalypticola*
12.	Vesicles 30-50 µm, stipe hyaline, stipe width 8-12µm, sclerotia absent, conidia 3.5-5.0 µm	*A. neoniger*
12.	Vesicle 20-40 µm, stipe hyaline, stipe width 10-13 (up to 30) µm, sclerotia absent, conidia 3.5-4.5 µm …….....	*A. luchuensis (=A.acidus)*
13.	Sporulation abundant & heavy, vesicles up to 80 µm…….....	14
13.	Sporulation poor …….....	18

Dichotomous key for identification of species of section *Nigri* (based on phenotypic characteristics, designed by MA Ismail)

14.	Sclerotia absent…….....	15
14.	Sclerotia present…….....	17
15.	Stipe length up to 1000 µm & width up to 12 µm…..…….....	*A. foetidus*
15.	Stipe width 8-12 µm, sclerotia absent, vesicle 30-50 µm …….....	*A. niger*
15.	Stipe width up to 14 µm, conidia 2.5-3.5 µm ……..…….....	*A. eucalypticola*
15.	Stipe width 10-13 (-30) µm, conidia 3.5-4.5 µm, vesicle 20-40 µm ….....	*A. luchuensis (=A.acidus)*
15.	Stipe longer, up to 3000 µm or more, stipe width up to 20 µm or more	16
16.	Stipe smooth, colorless or brownish only in the upper portion; stipe width 15-20 µm	*A. niger*
16.	Stipe very rough, brown on ageing; stipe width 20-33 µm…….....	*A. niger* var. *taxi*
16.	Stipes longer up to 6000 µm, smooth to coarse, brownish, stipe width 15-20 (-30) µm	*A. tubingensis*
17.	Sclerotia white, 1200-1800 µm; reverse yellow to orange to reddish brown in age, stipe width up to 12 µm …….....	*A. foitidus*
17.	Sclerotia white to pink to black, 500-800 µm, reverse white; stipe width 15-20 (30) µm .	*A. tubingensis*
18.	Sclerotia present, yellowish or pinkisk; stipes hyaline…….....	19
18.	Sclerotia absent, vesicle 40-65 µm, stipes orange brown…….....	*A. lacticoffeatus*
19.	Vesicle 40-55 µm, stipe width 7-10 µm, metulae 20-35 µm long …….....	*A. piperis*
19.	Vesicles 40-80 (-90) µm; stipe width 12-22 µm, metulae 30-60 µm long …….....	*A. costaricaensis*
20.	Growth at 40 ºC, sclerotia absent…….....	*A. ibericus*
20.	No growth at 40 ºC…….....	21
21.	Sclerotia absent……..…….....	22
21.	Sclerotia present…….....	24
22.	Conidia strongly ellipsoidal, 7-10 X 2.5-3, spinulose; vesicles 75-100 µm; stipe long up to 5000-8000 (-1 cm) X 12-20 µm…….....	*A. ellipticus*
22.	Conidia not ellipsoidal…….....	23
23.	Stipe width 35-40 µm, conidia globose, 7-9 µm, metulae length less than 15 µm, vesicle 40-80 (-100) µm…….....	*A. carbonarius*
23.	Stipe width9-15 µm, conidia 5-7 (-9) µm, metulae length less than 15 µm, vesicles not exceed 50-65 µm…….....	*A. homomorphus*
24.	Sclerotia cup-shaped with coiled setae; stipe width 8.5-13.5 µm, (with brownish stipe, vesicle, conidia, setae & sclerotia) …….....	*A. helicothrix*
24.	Characters not as above…….....	25
25.	Conidia strongly ellipsoidal, 7-10 X 2.5-3 µm, spinulose, sclerotia dull yellow to brown in age, 500-1500 µm…….....	*A. ellipticus*
25.	Conidia globose……..…….....	26
26.	Conidia 4.5-6.5µm, vesicle pyriform 30-50 µm, sclerotia yellow to orange to red brown; sporulation poor, stipe width less than 18 µm ….....	*A. sclerotioniger*
26.	Conidia up to 9 µm; vesicle up to 100 µm, stipe width wider …….....	27
27.	Sclerotia yellow to orange to red brown, no growth at 9 ºC, stipe width 13-27 µm …….....	*A. sclerotiocarbonarius*
27.	Sclerotia pink to yellow, growth at 9 ºC, stipe width 35-40, sporulation abundant	*A. carbonarius*

Table 2. Black aspergilli in agricultural commodities.

Commodity	Species	Country	References
Grape & grape products	*A. brasiliensis, A. niger, A. awamori, A. aculeatus, A. tubingensis, A. ibericus, A, carbonarius, A. japonicus, A. uvarum, A. acidus,*	Worldwide	[12, 14, 24-29]
Grapes	*A. carbonarius, A. tubingensis, A. japonicus, A. ibericus, A. niger* aggregate	Greece	[30]
Grapes	*A. carbonarius, A. niger* aggregate	Italy	[31, 32]
Wine grapes	*A. niger* var. *niger, A. niger* var. *awamori, A. foetidus*	Argentina	[33]
Maize	*A. japonicus, A. niger* var. *niger*	Worldwide	[27, 34-36]
Maize	*A. niger* aggregate	Portugal	[37]
Maize kernels	*A. heteromorphus, A. carbonarius, A. aculeatus, A. niger, A. japonicus, A. brasiliesis*	Kenya	[38]
Wheat	*A. niger*	Egypt	[39]
Sorghum	*A. niger*	Egypt	[36]
Milled rice	*A. niger*	Uganda & Pakistan	[40-42]
Paddy & mild rice	*A. niger*	Uganda	[43]
Peanuts	*A. japonicus, A. niger* var. *niger, A. carbonarius, A. niger var. awamori*	Worldwide	[27, 35, 44]
Peanuts	*A. niger, A. carbonarius*	Uganda & Kenya	[45]
Peanuts	*A. niger*	Egypt	[39]
Lentil & sesame	*A. niger*	Egypt	[36]
Coffee bean	*A. aculeatus, A. aculeatinus, A. carbonarius, A. sclerotiocarbonarius, A. sclerotioniger, A. niger, A. lacticoffeatus, A. japonicus, A. tubingensis*	Worldwide	[11, 27, 35]
Coffee beans	*A. niger* group	Colombia	[46]
Coffee beans	*A. niger, A. carbonarius*	Saudi Arabia	[47]
Beans, wheat, millet	*A. niger*	Nigeria	[48]
Cereal products (baby foods)	*A. niger, A. carbonarius*	Canada, England & Kenya	[49, 50]
Cereal products (baby foods)	*A. carbonarius, A. niger, A. phoenicis*	Uganda	[51, 52]
Spices	*A. niger* var. *niger*	Worldwide	[27, 35, 53]
Black pepper	*A. piperis*	Worldwide	[27, 35]
Desiccated coconut	*A. niger, A. carbonarius, A. japonicus*	Uganda & Kenya	[45]
Fruit juice & beverages	*A. niger, A. japonicus*	Egypt	[54]
Apricot, fig, grapes & plum	*A. awamori, A. carbonarius, A. japonicus, A. niger, A. tubingensis, A. sclerotioniger, A. aculeatus, A. aculeatinus*	Iraq	[55]
Cocoa bean, coffee bean & dried cassava	*A. carbonarius, A. niger, A. tubingensis, A. aculeatus*	Indonesia	[56]
Cocoa beans	*A. carbonarius, A. tubingensis, A. niger*	Sierra Leona, Equatorial Guinea & Ecuador	[57]
Olive oil	*A. niger*	Morocco	[58]
Vegetables	*A. brasiliensis, A. niger, A. japonicus, A. vadensis*	Egypt	[59]

Table 3. Ochratoxins produced naturally in agricultural commodities due to infection by black aspergilla (*A. carbonarius* and *A. niger*).

Commodities	Country	Reference
Grape	Worldwide	[28, 31, 73-76]
Grape	Italy	[32]
Grape juice	Europe	[77]
Wine	Europe, worldwide	[28, 74, 77]
Raisins	California, USA	[29]
Dried vine fruits	Worldwide	[7, 28, 76]
Cereals	Europe	[77]
Coffee	Europe	[74, 77]
Dry fruits	Europe	[77]
Cocoa	Europe	[77]
Figs	Central Europe	[74]
Peanuts	Argentina	[44]
Rice and rice products*	Worldwide	[78-83]
Cereal grains (wheat, barley, corn, oats, sorghum)*	Worldwide (UK, Italy, Ivory Coast, Japan, Tunesia)	[81, 84-88]
Cereal flour (wheat, rye, maize, oats)*	Worldwide	[78, 82, 88- 90]
Infant cereal food*	Worldwide	[86, 91, 92]

*Means that aspergilli and penicillia may be involved in ohratoxin production.

OTA is produced by fungi of the genera *Aspergillus* and *Penicillium*. The major species implicated in OTA production includes *Aspergillus ochraceus, A. sulphureus, Petromyces alliaceus, Penicillium verrucosum, A. carbonarius,* and to a lesser extent *A. niger* [62, 63]. Ueno et al. [64] were the first to report on ochratoxin A (OA) production by a black *Aspergillus* species, *A. foetidus*. This was later confirmed [33, 65].

OTA is a frequent natural contaminant of many foodstuffs such as cocoa beans, coffee beans, cassava flour, cereals, peanuts, dried fruits and wine [66]. Studies revealed that whenever OTA was detected in high levels, AFB1 was absent or present at very low levels and vice versa which suggests some sort of competition between these toxins at the production level in foodstuffs. OTA has also been reported as a contaminant of tiger nuts and fermented maize dough in West Africa [67]. Ochratoxin A contamination of agricultural products including cereals and grains influences chronic effect on human exposure [68]. Natural occurrence of mould infection and OTA contamination in maize and maize-based products is a worldwide problem [69]. *A. niger* is commonly isolated from maize [70] and a high incidence of *A. carbonarius* has been also reported [71]. Both species are the main source of ochratoxins in corn and other food products in both subtropical and tropical zones of the world [35] and to a lesser extent in grapes, wine, dried vine fruits and grape juice [72] (refer to Table 3).

A. carbonarius was recognized as the major OTA-producer [65, 93-96], near 100% of isolaes produce OTA when grown in pure culture [97-101]. The closely related species *A. niger* has also been reported reliably as a producer [64, 97, 98, 102]. However all reports agree that OTA production by *A. niger* is very uncommon. Also, it was observed that *A. niger* "aggregate", although the most common, showed a low percentage of OTA producing strains, from 4 to 10% [101, 103]; none of the strains belonging to *A. uvarum* was able to produce OTA [14]. *A. lacticoffeatus* and *A. sclerotioniger*, both isolated from coffee [11], and from raisin samples [104], are also reported as OTA producers (Table 4).

The most distinguishing characteristics to differentiate *A. niger* aggregate species (*A. niger*, *A. tubingensis* and *Aspergillus awamori*) from *A. carbonarius* are growth at 37°C and conidial diameter [19]. All 12 of the ochratoxigenic isolates of *A. carbonarius* showed restricted growth at 37°C, while all of the nonochratoxigenic isolates of *A. niger* aggregate grew well at 37°C. This effect was more pronounced at 40°C, at which the ochratoxigenic strains did not grow and the nonochratoxigenic strains grew well. In addition, all OTA-producing strains formed large (7-10 µm diameter), and all OTA-nonproducing strains formed smaller conidia (<4 µm diameter) [29] (refer to the Key).

Table 4. Ochratoxins produced by black aspergilli isolated from agricultural commodities.

Species	Ochratoxins	References
A. aculeatus	+	[10]
A. carbonarius	+	[11, 17, 19, 24, 25, 27-31, 35, 44, 47, 56, 57, 73, 74]
A. foetidus	+	[33]
A. japonicus	+	[27, 35, 44]
A. lacticoffeatus	+	[11]
A. niger var. *niger*	+	[11, 24, 27, 28, 33, 35, 44, 47, 56-58, 73, 74, 102]
A. niger aggregate/ Section *Nigri*	+	[31, 37, 106]
A. sclerotioniger	+	[11, 19]
A. tubingensis	+	[27, 30, 35, 57]
A. welwitschiae (=*A. awamori*)	+	[19, 28, 33, 44, 107]

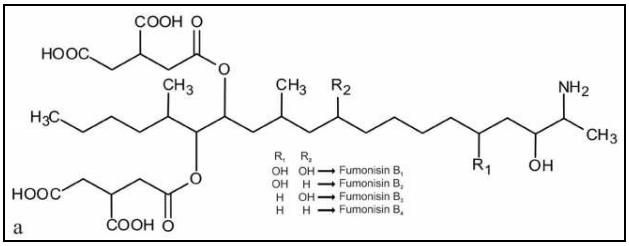

Figure 2. Chemical structures of fumonisins [113].

4. FUMONISINS PRODUCTION IN AGRICULTURAL COMMODITIES AND BY THE ASSOCIATED BLACK ASPERGILLI

Fumonisins (Fig. 2) were discovered in South Africa in 1988 [108, 109]. They are known to be produced by *Fusarium verticillioides* (formerly known as *F. moniliforme*), *F. proliferatum, F. oxysporum, F. globosum*, several other *Fusarium* spp., and *Alternaria alternata* f. sp. *lycopersici*. Fumonisins are frequently found in corn and corn-based foods [110, 111]. FB1 is the most commonly found, not only in corn (maize) and corn-based foods, but also in rice, sorghum, cowpea seeds, beans, soybeans and beer. FB1 can cause two diseases of farm animals: leucoencephalomalacia in horses and porcine pulmonary oedema. It is also carcinogenic, hepatotoxic, nephrotoxic and embryotoxic in laboratory animals. In humans fumonisins are associated with oesophageal cancer and neural tube defects based on studies in Transkei [109] and Texas [112]. The International Agency for Research on Cancer (IARC) designated FB1 in Group 2B as 'possibly carcinogenic to humans' [60].

Findings of fumonisins in agricultural commodities are shown in Table 5. In recent years fumonsins have been found in a wide variety of foods such as, cassava products in Tanzania [114], garlic and onion powders [115] and garlic bulbs [116], black radish [117], black tea [118, 119], figs in Turkey [120, 121], peanuts in Cote d'Ivoire, Cameroon and China [87, 122, 123], and soybeans in Japan [124]. Fumonisins have been found in dietary and medicinal wild plants in South Africa [125] and in other medicinal plants: leaves of orange tree, leaves/flowers of linden tree and chamomile in Portugal [118], mint and stinging nettle in Turkey [119]. Of particular note and interest is that for some foods, FB1 is not the major fumonisin as it is for maize and other grains. FB2 (without FB1) occurred in wine from several countries [126, 127], such as red wine must in Italy [126] and beer [128]. Table 5 shows some commodities contaminated with fumonisins.

Fumonisin production has also been proved by *A. niger* isolates originating from coffee beans and grapes [129, 130]. Further reports claimed that *A. niger* and *A. awamori* from grapes, raisins and coffee beans produced fumonisins particularly FB2 [129, 131], B2 and B4 [107, 126, 130, 132], although other isomers in smaller quantities [107] and a FB1 isoform, named FB6 were also detected [131]. No fumonisins were found in other black *Aspergillus* species from grapes, including *A. carbonarius* [126].

Whereas *F. verticillioides* produces fumonisins on agar media based on plant extracts such as barley malt, oat, rice, potatoes, and carrots, *A. niger* is able to produce fumonisins in high quantities on agar media with low water activity [63]. Recently, dried vine fruit samples (raisins, sultanas) were found contaminated with fumonisin-producing black aspergilli and several fumonisin isomers, including fumonisins B1-4, 3-epi-FB3, 3-epi-FB4, iso-FB1, and two iso-FB2,3 forms [107]. Several strains collected from figs, dates and onions were also able

to produce fumonisins, thus black aspergilla are suspected to be responsible for fumonisin contamination of grape-derived products, figs and onions. Figs and onions were also contaminated with low but significant amount of fumonisins [107]. Frisvad et al. [133] studied 180 strains of *A. niger* from various sources and found about 80% producing FB2 (refer to Table 6). Although the percentage of fumonisin-producing strains was very high, the absence of at least part of the fumonisin biosynthetic gene cluster has been reported in *A. niger* [134].

5. *ASPERILLUS NIGER* AS A PLANT PATHOGEN

A. niger has been identified as the responsible species in diseases of food crops, such as maize seedling blight, maize ear rot and seedling blight of peanuts. It causes also a disease called black mold on certain fruits and vegetables such as grapes, onions and peanuts [74, 141] (refer to Table 7).

Table 5. Fumonisins B1 and B2 produced naturally from some agricultural commodities due to infection by black aspergilli (*A. niger/A. awamori*).

Commodities	Country	Reference
Grape, raisins, figs, onion	Central Europe	[74]
Coffee beans	Central Europe	[63]
Grapes, raisins & wine	Central Europe	[127, 130, 135, 136]
Maize kernels	South Africa	[137]

Table 6. Fumonisins produced by black aspergilli isolated from agricultural commodities.

Species	Fumonisins	Reference
A. carbonarius	B_1, B_4 and several fumonisin isomers	[74]
A. niger var. *niger*	B_1, B_4	[27, 35, 63, 126, 130, 131, 138]
A. niger aggregate/ Section *Nigri*	B_2	[28, 37]
A. welwitschiae (=A. awamori)	+	[19, 107, 139, 140]

Table 7. Plant diseases caused by *Aspergillus niger*.

Disease & host	Reference
Almond chlorosis	[74, 142]
Apricot, peach ripe fruit rot	[74, 142]
Bulb (black) rot of onions & garlic	[74, 142]
Black rot of cherry	[143]
Carrot sooty rot	[74, 142]
Citrus black mold	[74, 142]
Crown rot of peanuts	[74, 142, 144]
Fig smut	[74, 142]
Fruit rot of banana	[145]
Fruit rot of grapes	[146]
Grape bunch rot	[74, 142]
Kernel rot of maize	[35]
Mango black mold rot	[74, 142, 147]
Pistachio fruit rot	[74, 142]
Rot of tomatoes	[148]
Stem rot of *Dracaena*	[149]
Strawberry fruit rot	[74, 142]
Tuber rot of yam	[150]
Vine canker	[74, 142]

6. CONCLUSION

This review outlines a taxonomic overview on all described and accepted *Asperillus* species in section *Nigri* up-to-date with a key for identification based on their phenotypic features, however these features are not enough for species delimitation and other tools (e.g. molecular techniques and/or some physiological and biochemical characteristics) are needed to support their identity. The incidence and implication of species in agricultural commodities are also discussed. Capabilities of some species of the section to produce ochratoxins and/or fumonisins are of special significance in these commodities due to their health hazard to human.

TRANSPARENCY DECLARATION

The author declares that has no conflict of interest.

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Review Article

Biological action of *Piper nigrum* - the king of spices

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ABSTRACT

Piper nigrum - the king of spices is originated in the Western Ghats of India. It has gained a global consideration because of its volume in the spice industry. It contains major pungent alkaloid piperine which is known to possess many interesting pharmacological actions. Medicinally black pepper can be used digestive disorder like large intestine toxins, different gastric problems, diarrohea and indigestion and also can be used against respiratory disorder including cold fever, asthama. Piperine exhibits diverse pharmacological activities like antihypertensive, antiplatelets, antioxidant, antitumor, antipyretic, analgesic, anti-inflammatory, anti-diarrheal, antibacterial, antifungal, anti-reproductive, insecticidal activities. *Piper nigrum* also found to decrease lipid peroxidation *in vivo*. It has reported to possess antioxidant activity that might be due to the presence of flavonoids and phenolic contents.

Keywords: *Piper nigrum*; Alkaloids; Antireproductive; Antioxidant; Flavonoids.

1. INTRODUCTION

Piper nigrum (Black pepper) is one of the most commonly used spices and considered as "The King of Spices" due to its trade in the international market [1, 2]. It is commonly known as Kali Mirch in Urdu and Hindi, Pippali in Sanskrit, Milagu in Tamil and Peppercorn, White pepper, Green pepper, Black pepper, Madagascar pepper in English [3]. Black pepper is used as medicinal agent, a preservative, and in perfumery [4]. The genus Piper has more than 1000 species but the most well known species are *Piper nigrum*, *Piper longum* and *Piper betli* [5]. Black pepper can be used for many different purposes such as human dietaries, as medicine, as preservative, as biocontrol agents [2, 6, 7]. Pepper is used worldwide in different types of sauces and dishes like meat dishes. It contains major pungent alkaloid piperine (1-peperoyl piperidine) which is known to possess many interesting pharmacological actions [8]. Tiwari and Singh [9] reported that this plant and its active components piperine can stimulate the digestive enzymes of pancreas and intestine and also increases billiary bile acid secretion when orally administered. Black pepper is important for its medicinal values [10]. Medicinally black pepper can be used digestive disorder like large intestine toxins, different gastric problems, diarrohea and indigestion and also can be used against respiratory disorder including cold fever, asthama [11-13]. Piperine exhibits diverse pharmacological activities like antihypertensive and anti-platelets [14], antioxidant, antitumor [15], antipyretic, analgesic, anti-inflammatory, anti-diarrheal, antispasmodic, hepato-protective [16], antibacterial, antifungal, anti-thyroids, anti-apoptotic, anti-spermatogenic, insecticidal and larvicidal activities etc. Piperine has been found to enhance the therapeutic efficacy of many drugs, vaccines and

nutrients by increasing oral bioavailability by inhibiting various metabolising enzymes [17]. In recent pasts, different therapeutic potentials of *Piper nigrum,* its extracts, or its important active chemical constituent "piperine" have been published in different international research journals. The current review is aimed to provide an updated literature review on recent research advancement of pharmacognosy, chemistry and pharmacological activities of *Piper nigrum* L. We have compiled a review on therapeutic potential of *Piper nigrum* by collecting updated scientific research information's from internet using Google search engine.

2. TAXONOMICAL CLASSIFICATION OF *PIPER NIGRUM*

Kingdom: Plantae
Class: Equisetopsida
Sub class: Magnoliidae
Super order: Magnolianae
Order: Piperales
Family: Piperaceae
Genus: *Piper*
Species: *nigrum*

3. GEOGRAPHICAL DISTRIBUTION

Black pepper is grown in many tropical regions like Brazil, Indonesia and India [3]. Geographically, it is confined to Western-Ghats of South India [18]. However, some reports of cultivation from Malaysia, Indonesia, Brazil, Sri-Lanka and West Indies are also available [19]. *P. nigrum* had been found in vast altitudinal regions and showed great adaptability to a wide range of environmental conditions which led to inter-species diversity [20]. "Black-pepper" as its generalized name is due to the color of the peppercorn. It is considered as the "king of spices" due to its trade in the international market [1, 2].

4. PHYTOCHEMISTRY

There are many biologically important phytochemicals are extracted from *P. nigrum* plants. They contain alkaloids, amides, propenyphenols, lignans, neolignans, terpenes, steroid, kawapyrones, piperolides, chalcones, dihydrochalcones, brachyamide, dihydropipericide, 3,4-dihydroxy-6 (N-ethyamine), benzamide, (2E, 4E)-N-eicosadienoyl pereridine, N-trans-feruloyltryamine, N-formyl piperidine, guineensine, (2E, 4E)-N-5[(4-Hydroxyphenyle)-pentadienoyl] piperidine, (2E,4E)-N-isobutyldecadienamide), (2E,4E)-N-isobutyl-eicosadienamide, (2E,4E,8Z)-N-isobutyl-eicosatrienamide, (2E,4E)-N-isobutyloctadienamide, piperamide, piperamine, piperettine, pipericide, piperine, piperolein, trichostachine, sarmentine, sarmentosine, tricholein, retrofractamide [21-27] (Figure 1). Pino et al. [27] observed that the major components of the essential oil obtained from the aerial parts of *P. nigrum* were gluulol, α-pinene, β-caryophyllene and α-terpinene. Piperine was the first amide to be isolated from piper species and it is the major active principle of black pepper, is closely related in structure to the known natural carcinogens-safrole, estragole and methylengenol which are also widely distributed in spices and plant oils [5]. Zheng et al. [28] studied that the fruits contain 1.0-2.5% volatile oil, 5-9% alkaloids, of which the major ones are piperine, chavicine, piperidine, and piperetine, and a resin. The terpenes, steroids, lignans, flavones, and alkaloids/alkamides have been identified as the primary constituents of the peppers [29]. Khan et al. [30] reported that most of the pharmacological properties of *P. nigrum* fruits are attributed to a piperidine alkaloid, piperine, which is present in the fruits in amounts of 1.7-7.4%. Piperine has also been shown to enhance the bioavailability of several drugs, for example sulfadiazine, tetracycline, streptomycin [31], rifampicin, pyrazinamide, isoniazid, ethambutol and phenytoin [30]. Vasavirama and Upender [5] concluded that due to its diverse pharmacological properties, piperine is important as a biomarker for standardization of fruit of *P. nigrum* and *P. longum* and of polyherbal formulations containing these raw materials.

5. ANTI-BACTERIAL ACTIVITY

Karsha and Laxmi [32] reported that anti-bacterial activity of black pepper (*Piper nigrum* Linn.) with special reference to its mode of action on bacteria and found that excellent inhibition on the growth of Gram positive bacteria like *Staphylococcus aureus,* followed by *Bacillus cereus* and *Streptococcus faecalis.* Among the Gram

negative bacteria *Pseudomo-nas aeruginosa* was more susceptible followed by *Salmonella typhi* and *Escherichia coli*.

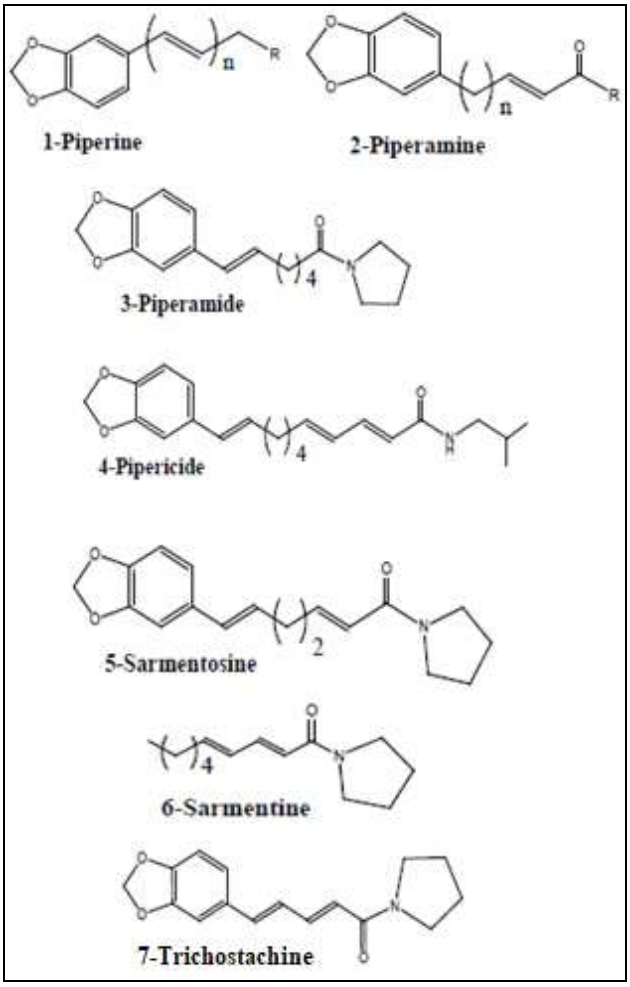

Figure 1. Structure of important chemical constituent of *Piper nigrum.*

Gram positive bacteria are more susceptible to the extracts due to antibacterial action appears to be loss of control over cell membrane permeability [33]. Khan and Siddiqui, [34] evaluated the antibacterial potential of aqueous decoction of *Piper nigrum* L. (black pepper), *Laurus nobilis* L. (bay leaf), *Pimpinella anisum* L. (aniseed), and *Coriandum sativum* L. (coriander) against different bacterial isolates from oral cavity of two hundred individual volunteers. Black pepper (aqueous decoction) showed strongest antibacterial activity comparable to aqueous decoction of *Laurus nobilis* and *Pimpinella anisum* at the concentration of 10 µl/disc. In a recent study, Palkumar et al. [35] experimented on *Piper nigrum* leaf and stem assisted green synthesis of silver nano-particles and evaluated its antibacterial activity against agricultural plant pathogens and observe that these silver nano-particles showed the excellent antibacterial activity against plant pathogens. Ganesh et al. [25] experimented photochemical analysis and antibacterial activity of *Piper nigrum* against human pathogenic bacteria and noted that presence of alkaloids, tannins, flavonoids, cardiac and cardiac glycosides shows antibacterial properties against the *Staphylococcus aureus, Salmonella typhi, Escherichia coli* and *Proteus sp.*

6. ANTIOXIDANT ACTIVITY OF BLACK PEPPER

Plants are important source of antioxidants [36]. Antioxidants completely stop or delay the process of oxidation [37]. Some *in vitro* studies revealed that piperine inhibited free radicals and reactive oxygen species, therefore known to possess protective effects against oxidative damage [3]. Free radicals cause many diseases [38]. Different free radicals attack on membranes causing oxidation of lipids, loss of different enzyme activities and may cause cancer [39]. *Piper nigrum* or piperine also found to decrease lipid peroxidation *in vivo* [40]. *Piper nigrum* reported to possess antioxidant activity that might be due to the presence of flavonoids and phenolic contents [41]. *Piper nigrum* was found to prevent the oxidative stress by inhibiting lipid peroxidation, human lipoxygenase and arresting hydroxyl and superoxide free radicals, decrease lung carcinogenesis in animal studies [42]. The memory enhancing and antioxidant proprieties of the methanolic extract of *Piper nigrum* were investigated in Alzheimer's disease model in rats [43]. The memory-enhancing effects of the extract were studied by means of *in vivo*. While, the antioxidant activity was evaluated by measuring activities of glutathione peroxidase, catalase, superoxide dismutase, and by measuring the total content of reduced glutathione, malondialdehyde, and protein carbonyl levels in the hippocampus [44]. Administration of the methanolic extract of *Piper nigrum* significantly improved memory performance and exhibited antioxidant potential. These studies suggest that methanolic extract of *Piper nigrum*

ameliorates amyloid beta (1-42)-induced spatial memory deterioration by depletion of the oxidative stress in the hippocampus of rats [3]. The anti-oxidant effect of three Piper species viz *P. nigrum, P. guineense* and *P. umbellatum* was evaluated for the protection of renal, cardiac, and hepatic anti oxidant status in atherogenic diet fed hamsters [42]. Piper species significantly inhibited the atherogenic diet induced increased lipid profile and alteration in antioxidant enzymes activities [45]. Ahmad et al. [47] reported that regenerated tissue of *Piper nigrum* like callus, *in vitro* shoots, roots, in vitro plantlets, possesses antioxidants activity which is probably due to the presence of flavonoids and phenolic contents. Piper species significantly inhibited the atherogenic diet induced increased lipid profile and alteration in antioxidant enzymes activities [45].

7. ANTI-CANCER ACTIVITY OF BLACK PEPPER

Piper nigrum had been reported to inhibit tumors formation in different experimental models [47]. Ahmad et al. [23] reported that piperine reduce the lung cancer by altering lipid peroxidation and by antioxidative protection enzymes activation. Piperine has distinct pharmacological activities along with anti-cancer activity [48]. Piperine was reported to inhibit G1/S transition and the proliferation of human umbilical vein endothelial cells (HUVECs), migration of HUVECs and *in vitro* formation of tubule and angiogenesis induced by collagen and breast cancer cell in chick embryos [49]. Landscron et al. [50] reported that piperine inhibits some of the pro-inflammatory cytokines that are produced by tumour cells, there by interfering with the signalling mechanisms between cancer cells, thereby reducing the chances of tumour progression. The anticancer activity of piperine against many cancer cell lines has been reported earlier. Therefore, the mechanisms of anticancer activity of piperine against both androgen independent and dependent cells of prostate cancer were investigated [51]. Piperine treatment was also found to induce apoptosis, by the activation of caspase-3 and by the cleavage of PARP-1 proteins in different prostate cancer cells like PC-3, DU-145 and LNCaP prostate cancer cells [29]. Treatment with piperine also found to disrupt the androgen receptor expression in LNCaP prostate cancer cells and cause significant diminution in the level of Prostate Specific Antigen in LNCaP cells [52]. The expression of phosphorylated STAT-3 and Nuclear factor-κB transcription factors were reduced in LNCaP, PC-3 and DU-145 prostate cancer cells after treatment of with piperine [28]. Piperine is non-genotoxic and found to possess anti-mutagenic and anti-tumor influences [3]. Dayem et al. [53] reported that *Piper nigrum* reduced lung cancer by modulating lipid peroxidation and through the activation anti oxidative protection enzyme.

8. DIGESTIVE ACTIVITY OF BLACK PEPPER

Many spices are known for their digestive stimulant action [54]. Srinivasan, [2] reported that black pepper enhances digestion by stimulation of the pancreatic enzymes and considerably decreases the food transit time of gastrointestinal tract. Ahmad et al. [23] reported that piperine increases the saliva production and gastric secretions, and increases the production and activation of salivary amylase. Platel and Srinivasan, [55] reported that orally administration of piperine or *P. nigrum* stimulate the liver to the secrete bile acids which in turn play key role in the absorption and digestion of fats

9. ANTIDEPRESSANT ACTIVITY OF BLACK PEPPER

Ahmad et al. [23] reported the antidepressant activity of piperine and its possible mechanisms was evaluated in corticosterone-induced model of depression in mice. Depression-like behavior in mice was developed after 3 weeks corticosterone injections. The depression was revealed by the significant reduction in sucrose utilization and augmentation in immobility time in the forced swim test and tail suspension test [55]. Further, the brain-derived neurotrophic factor protein and mRNA levels in the hippocampus were also significantly decreased in corticosterone-treated mice. Bai et al. [57] reported that corticosterone induced the behavioral and biochemical changes after treatment to animals with piperine. These results showed that piperine produces an antidepressant-like effect

in corticosterone-induced model of depression in mice [58].

10. INSECTICIDAL PROPERTIES

The phytochemical screening of black pepper fruit shows that it contains 4% alkaloids in the berry [13]. Awoyinka et al. [59] reported that the amide olefinic or alkyl isobutylamides compounds such as piperine, piperettine, tricostacine, peepuloidin, piplartin and trichonine contribute no small measure. These compounds have been demonstrated to be toxic to fruit flies, adzuki bean weevils, cockroaches and several other insect species [59]. Upadhyay and Jaiswal [60] evaluated the biological activities of *Piper nigrum* oil against *Tribolium castaneum* and found that oil had shown a dose response relationship as the larval and adult mortality increased while the larval survival and adult emergence decreased with increase in the concentration of essential oil. Khani et al. [61] reported that the *P. nigrum* extracts offer a unique and beneficial source of bio-pesticide material for the control of insect pests. The toxic effect of *P. nigrum* was reported against some test insects. *P. nigrum* was shown to be most toxic to *Callosobruchus chinensis*, *Acanthoscelides obtectus*, *C. cephalonica*, *Ephestia cautella* Hubn., followed by *Oryzaephilus surinamensis* (L.), *Sitophilus zeamais* Mosteh, *Rhyzopertha dominica* (Fab.) and *Tribolium castaneum* Herbst. The high toxicity effects of *P. nigrum* essential oils against *S. oryzae* adults and 3rd instar larvae of *C. cephalonica* are attributed to the presence of high concentrations of well-known toxic components piperine. Kraikrathok et al. [62] reported that bio efficacy of some piperaceae plant extracts against *Plutella xylostella* third instars under laboratory conditions and observed that the extracts of *Piper nigrum* plants was dose dependant and correlated to duration of exposure. The hexane extract of *P. nigrum* was active with an LD_{50} of 18435 ppm and mode of action of these extracts and effect on other developmental parameters was in progress. Scot et al. [63] reported the efficacy of extracts from two Piperaceae species. *Piper nigrum* and *P. tuberculatum* were evaluated using larvae and adults of the colorado potato beetle *Leptinotarsa decemlineata* and noted that young larvae and neonates were the most susceptible to 0.05% extract of *P. nigrum* reduced larval survival up to 70% within one week after treatment of potato plants. In the greenhouse, *P. nigrum* at 0.5% was as effective at reducing adult *L. decemlineata* feeding as combinations with 2 separate botanical mixtures, garlic and lemon grass oil. Under field conditions, the residual activity of the *P. nigrum* extracts was less than 3 h. When adult *L. decemlineata* were placed on treated plants exposed to full sunlight for 0, 1.5, and 3 h, leaf damage progressively increased as the main active compound, piperine, was found to degrade by 80% after 3 h. The results suggest that Piper extracts could be used effectively as contact botanical insect control agents to protect potato plants from developing *L. decemlineata* larvae at concentrations less than 0.1%. Paula et al. [64] noted that the natural lipophilic amides piperine and piperiline were isolated from *Piper nigrum* evaluated the contact toxicity of all synthetic amides, and also that of piperine and piperiline, at the dose 10 mg per insect, for the Brazilian economically important insects *Ascia monuste orseis* Latr, *Acanthoscelides obtectus* Say, *Brevicoryne brassicae* L, *Protopolybia exigua* DeSaus and *Cornitermes cumulans* Kollar. The results demontrated that the insects have different sensivities to the various amides, with mortality ranging from 0 to 97.5%, according to the compound and insect species. Samuel et al. [65] reported that the larvicidal effects of black pepper (*Piper nigrum* L.) and piperine against insecticide resistant and susceptible strains of *Anopheles* malaria vector mosquitoes and observed that Black pepper and piperine mixtures caused high mortality in the An. Gambiae complex strains, with black pepper proving significantly more toxic than piperine. It is concluded that black pepper shows potential as a larvicide for the control of certain malaria vector species.

11. ANTIPLATELET ACTIVITY

Srivastava et al. [66] reported that the valuable component of different piper species is piperine which is mostly responsible for various activities. Park et al. [67] reported that piperine possesses anti-platelet activity. Ahmad et al. [23] noted that the toxic effect of piperine on aggression of platelet in experimental rabbit induced by

different factors which activate platelet, by collagen and thrombin.

12. MOLLUSCICIDAL ACTIVITY

Srivastava et al. [66] reported that the effect of sublethal treatment (40% and 80% of 24h LC_{50}) of *Piper nigrum* fruit and *Cinnamomum tamala* leaf/bark and their different organic solvent extract, purified fraction singly and binary combination with synergist PB or MGK-264 (1:5) on level of different biochemical parameters viz. protein, amino acid, nucleic acids and phospholipids and rate of lipid peroxidation in nervous tissue of *L. acuminata.* Treatment of 80% of 24h LC_{50} of piperine caused maximum reduction in protein (12.95% of control), total free amino acid (10.33% of control), DNA (12.70% of control) and RNA (9.17% of control) levels in nervous tissue of *L. acuminata.* Maximum reduction (18.64% of control) in phospholipid levels and elevation of rate of lipid peroxidation (273.17% of control) were observed in the nervous tissue of snails treated with 80% of 24h LC_{50} of piperine. Treatment of 80% of 24h LC_{50} of purified fraction of *Cinnamomum tamala* leaf/bark caused significant reduction in protein (41.98% of control), total free amino acid (30.06% of control), DNA (43.71% of control) and RNA (16.42% of control), phospholipid (40.86% of control) level and increase the rate of lipid peroxidation (272.69% of control) in nervous tissue of *L. acuminata*. Binary combinations (1:5) plant products with PB or MGK-264 caused significant decrease in the different biochemical parameters. It is clear from the results that there is a significant elevation in lipid peroxidation levels with a reduction in phospholipid levels of nervous tissue of *L. acuminata* treated with different preparations of *P. nigrum* and *C. tamala* leaf/bark. Phospholipids are needed for the growth of endoplasmic reticulum or other cellular membranes [68]. It has been reported that all classes of phospholipids decrease markedly following high dose piperine treatment [69]. The enhancement of lipid peroxidation might be due to oxidative degradation of polyunsaturated fatty acids of the biomembrane leading to pathological infestation [70]. Formation of activated oxygen can have extremely detrimental consequence not only for phospholipids but also protein, nucleic acids, polysaccharides and inhibition of vital enzymes [71, 72]. The alkaloid, piperine, found in *P. nigrum* destroys the cytochrom P-450 and inhibits mono-oxygenase activity [73]. Some workers reported the effect of piperine activity in rat. Johri et al. [74] studied that the effect of piperine on the absorptive function of the intestine. *In vitro* experiments showed an increased rate of lipid peroxidation in the freshly isolated epithelial cells of rat jejunum. These results suggested that piperine may interact with the lipid environment to produce effect which leads to increased permeability of the intestinal cells.

13. ANTIREPRODUCTIVE ACTIVITY

Srivastava et al. [75] reported that the anti-reproductive activity of piperine against the snail *Lymnaea acuminata* and observed that piperine caused a significant reduction in the fecundity, hatchability and survival of the snail *Lymnaea acuminata* in each month of the year Nov. 2011 to Oct. 2012. Treatment with the piperine also prolong the hatching time of snails. Sublethal treatment of piperine caused a significant ($p<0.05$) reduction in protein, amino acids, DNA, RNA and AChE in the ovotestis/nervous tissue of treated snails with respect to control after 96h exposure period. Simultaneously, inhibition in acetylcholinesterase (AChE) activity in nervous tissue was also noted. The active component piperine (*Piper nigrum)* is an effective molluscicide against *L. acuminata*. Constituent of piperine *in vitro* inhibit enzyme activity which is responsible for leukotriene and prostaglandin biosynthesis; 5-lipoxygenase and COX-1 [76]. The cerebral neurosecretory caudo dorsal cells (CDCS) of the fresh water pulmonate snail *Lymnaea stagnalis* control egg lying, an event that involves a pattern of stereotyped behavior [78]. The CDCS synthesize and release multiple peptides, among which is the ovulation hormone (CDCS). It is thought that each peptide controls a specific aspect of the processes involved in egg laying [77].

The synthesis of protein in any of a tissue can be affected in two ways by a chemical, (1) it either affects the RNA synthesis at the transcription stage or (II) it somehow affects the uptake of amino acids in the polypeptide chain. Both these possibilities may account for the lower protein content in the

affected tissue. In the first case, the RNA synthesis would be inhibited resulting in reduced RNA as well protein content. In the second case, only the protein content would be affected [66, 78, 79]. Piperine inhibits P-glycoprotein and the major drug metabolizing enzyme CYP3A4 [81]. It seems that cumulative effect of molluscicide piperine on the level of protein, amino acids and nucleic acids in ovotestis of *L. acuminata* directly/or indirectly CDCs, which release ovulation hormone and ultimately affect the reproduction of snails in each month of the year. The AChE activity is one of the biomarker most frequently used in ecotoxicology. The enzyme is responsible for the breakdown of ACh in cholinergic synapses, preventing continuous nerve firing, which is vital for normal cellular neurotransmitter functioning [81]. The AChE inhibition result in accumulation of acetylcholinesterase at the nerve synapses so that the post synaptic membrane is in a state of permanent stimulation producing paralysis, ataxia and general lack of coordination in neuromuscular system and eventual death [82, 83].

14. COSMOPERINE ACTIVITY

Sabina Corporation [84] reported that cosmoperine prepared from piperine used in cosmetics, a natural bio-enhanser which improve the permeability of active compounds through skin. Cosmoperine activate and stimulate the natural power of skin to absorb nutrients [85, 86]. Cosmoperine isolated from piperine are non-irritant, interacts with the skin quantitatively and qualitatively in various means, furthermore, cosmoperine are pain relieving and causes skin reddening due to vascular engorgement as well as a slight skin tingling sensation.

15. CONCLUSION

In the present review we have made an attempt to congregate the botanical, phytochemical, toxicological information on *Piper nigrum* a medicinal herbs used in the Indian system of medicine, Survey of literature revealed that the presence of alkaloids, lignans, volatile oils and esters in different parts of this plants. Research on alkaloids has gained a special attention in recent times as several of them have shown promising activities like anti-inflammatory, hepatoprotective, stimulant effect, anti-amoebic and antibacterial etc. This review definitely helps for the researchers as well as practioners, dealing with this plant, to know its proper usage.

AUTHORS' CONTRIBUTION

Both authors contributed equally for the success of this review article. The final manuscript has been read and approved by both authors.

TRANSPARENCY DECLARATION

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

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Research Article

In vitro assessment of antimicrobial and anti-inflammatory potential of endophytic fungal metabolites extracts

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ABSTRACT

Endophytes are endosymbiotic microorganisms that act as reservoir of novel bioactive secondary metabolites with antimicrobial, cytotoxic and anti-cancer activities. In the present study, the extracts of 26 different endophytic fungal strains were screened for their antimicrobial and anti-inflammatory activities. The results showed a wide variety of antimicrobial activities against 12 target microorganisms including three Gram (+) bacteria, three Gram (-) bacteria, 3 yeasts, 2 dermatophytic fungi and one keratinophilic fungus. Four fungal extracts (*Aspergillus versicolor*, *A. awamori*, *A. niger* and *Penicillium funiculosum*) displayed a broader antibacterial spectrum and inhibited the growth of all Gram (+) and Gram (-) bacterial species. The extracts of 8 endophytic fungi inhibited the growth of the two tested dermatophytic strains (*Trichophyton mentagrophytes* and *T. rubrum*). Only eight fungal extracts have an inhibition activity against the keratinophilic fungal strain (*Chrysosporium tropicum*). The anti-inflammatory assay showed that the extracts of *Emericella nidulans*, *Pleospora tarda* and *Penicillium funiculosum* had good activities in inhibition of protein denaturation reached to 83%, 82.5% and 81.4%, respectively. Also, *Emericella nidulans* and *Pleospora tarda* recorded the maximum inhibition effect on bovine serum albumin denaturation reached to 95% and 90.7%, respectively. On the other side, *Emericella nidulans* showed the maximum inhibition activity (69.5%) out of all tested endophytic strains against humun red blood cells membrane stabilization assay. In conclusion some secondary metabolites of endophytic fungi have a promising potential as antimicrobial and anti-inflammatory compounds.

Keywords: Endophytes; Fungi; Antimicrobial; Anti-inflammatory; Drug discovery.

1. INTRODUCTION

World health problems caused by drug resistant bacteria and fungi are increasing. Many pathogenic microorganisms have developed resistance due to the misusage or long-term usage of the same class of antibiotics. Intensive search for more effective antibiotics to deal with these problems is now ongoing [1]. The isolation of novel secondary metabolites from the endophytes is a progressive research field [2]. Endophytic microbes are fungi and bacteria that colonize internal tissues of living plants without causing any adverse effects on its host [3-5]. Endophytes are endosymbiotic microorganisms that act as reservoirs of novel bioactive secondary metabolites, such as alkaloids,

phenolic acids, quinones, steroids, saponins, tannins, and terpenoids that are of interest for specific medicinal applications [6, 7]. The bioactivity of the secondary metabolites of endophytic fungi includes antimicrobial, anti- inflammatory, anti-proliferative or cytotoxic activity towards human cancer cell lines, and activity against plant pathogens [8-10].

The researchers are currently paying more attention to the drug development from the endophytic fungi [11-15]. The search for new antimicrobial compounds is important as bacterial and fungal infection remains the main cause for morbidity and mortality worldwide due to microbial resistance against the present commercially antimicrobial drugs [16]. Moreover, due to risk of adverse effects encountered with the use of synthetic antibiotics, endophytic fungi may offer an alternative source for antimicrobial agent with significant activity against pathogenic and infective microorganisms [17].

Inflammation is a normal protective response to tissue injury which damaged by microbial agents, physical trauma or noxious chemical. Inflammation is associated with pain, increase of vascular permeability, membrane alteration and protein denaturation due to release of lysosomal enzymes, kinins, prostroglandins and histamine [18]. The lysosomal enzymes released during inflammation produced a variety of disorders, so stabilization of lysosomal membrane is important in limiting the inflammatory response [19]. The search for anti-inflammatory properties has been on the rise due to their potential use in the therapy of various chronic and infectious diseases [20]. The prevailing non-steroidal anti- inflammatory drugs (NSAIDs) in the treatment of diseases associated with inflammatory reactions has adverse effects which pose a major problem in the clinical use. The greatest disadvantage in the presently available potent synthetic anti-inflammatory drugs lies in their toxicity [21]. Moreover, long-term use of NSAIDs can cause peptic ulcer [22]. Therefore, it is necessary to develop a novel anti-inflammatory agent that could overcome the disadvantages of NSAIDs. Furthermore, identification of such agent from natural origin could confer safety and efficacy for the treatment of inflammation [23].

For these reasons, in this study, the anti-bacterial, anti-dermatophytic and anti-keratinophilic fungi, anti-yeasts and anti-inflammatory activities of the extracts of 26 selected endophytic fungal strains were evaluated.

2. MATERIALS AND METHODS

2.1. Endophytic fungi

Total of 26 endophytic fungal strains were kindly provided by the Assiut University Myco-logical Centre (AUMC), Assiut University, Assiut Egypt. The fungal cultures were sub-grown on fresh slants have potato dextrose agar medium and incubated at 28 ± 2^0C for 10 days before used.

2.2. Preparation of fungal extract

Fungal strains grown on 50 ml of potato dextrose broth (PDB) in 250 ml Erlenmeyer flasks. Cultures were incubated for 10 days at 28±2ºC. The mycelia and the fermentation broth of each fungal strain were blended with 150 ml ethanol in electric blender; the extracts were filtered using filter paper to remove the mycelia. Mislabel extracts were, individually, transferred into rotatory evaporator under reduced pressure at 35ºC till semisolid residue was obtained.

2.3. Determination of antimicrobial activities

2.3.1. Bacterial strains

Three strains of Gram (+) bacteria namely: *Bacillus subtilis* AUMC B-101, *Bacillus cereus* AUMC B-70 and *Staphylococcus aureus* AUMC 6538, in addition to three strains of Gram (-) bacteria namely *Escherichia coli* NCCB 50028, *Serratia marcescens* AUMC B-89 and *Klebsiella* sp. AUMC B-77 were obtained from the cultures collection of the AUMC. Bacterial cultures were cultivated on nutrient agar (NA) slants and incubated at 37^0C for 24 h before using.

2.3.2. Dermatophytic and keratinophilic strains

Two strains of dermatophytic fungi named *Tricophyton mentagrophytes* AUMC 2360 and *Tricophyton rubrum* AUMC 10337 in addition to one strain of keratinophilic fungus named

Chrysosporium tropicum AUMC 1804 were used to determine the anti-dermatophytic and keratinophilic activities of the fungal extracts. Also, these strains were kindly provided by the AUMC and cultivated on sabouraud dextrose agar (SDA) medium and incubated at 28 ± 2^0C for 7 days before using.

2.3.3. Yeast strains

Two strains of pathogenic *Candida* species (*Candida albicans* AUMC 9212 and *Candida parapsilosis* AUMC 9163) in addition to one strain of *Saccharomyces cerevisiae* AUMC 203 were obtained from AUMC. Yeast cultures were grown on SDA medium and incubated at 30 ± 2^0C for 48 h before used.

2.3.4. Agar well diffusion assay

The antimicrobial assay was performed by agar well diffusion method [24]. A spore suspension of each of the different tested bacterial and fungal strains was prepared. Petri dishes have NA for bacteria and sabouraud dextrose agar for fungi was prepared. One ml of spore suspension of each of the different bacterial or fungal strain was transferred to suitable number of dishes containing the appropriate medium. A sterile swab was used to distribute bacterial or fungal suspension on the solidified agar plates. The plates were allowed to dry for 15 minutes. Wells were then prepared in the plates with the help of a cork-borer (1 cm). A total of 100 µl of the test endophytic fungal extract were introduced into the well. The plates were incubated overnight at 37^0C for bacterial strains at 30^0C for 48 h for yeast strains and at 28^0C for 5 days for both dermatophytic and keratinophilic strains. Microbial growth was determined by measuring the diameter of inhibition zone. Ethanol as a negative control and chloramphenicol and clotrimazole as positive control for antibacterial and antifungal, respectively were used. All results were recorded as mean values of three replicates ± standard deviation.

2.3.5. Determination of anti-inflammatory activities

2.3.5.1. Protein denaturation assay

The anti-inflammatory activity of the endophytic fungal extracts was tested by the protein denaturation method as described by Padmanabhan and Jangle [25] with some modification. Briefly, the reaction included 10mµl of the fungal extract and 3 ml of phosphate-buffered saline (pH 6.5) which was vortex with 0.5 ml of egg albumin and incubated at 25ºC for 15 min. A denaturation reaction was induced in a 65ºC water bath for 12 min. After cooling, absorbance was measured at 660 nm by spectrophotometer using double distilled water as the blank. The percentage inhibition of protein denaturation was appraised by the following formula:

Inhibition% = (Ac - As/Ac) × 100

where Ac and As represent control and sample absorbance, respectively. In this assay, diclofenac sodium a powerful NSAID was used as a standard. The samples were analyzed in triplicates.

2.3.5.2. Albumin denaturation assay

According to method of Williams et al. [26] and Shah et al. [27], with minor modifications, a solution of 0.2% W/V of bovine serum albumin (BSA) was dissolved in Tris buffer saline and pH was adjusted using to glacial acetic acid to 6.8. A total of 2.5 ml of the 0.2% W/V BSA was transferred to tube containing 50 µl of fungal extract in test tube and 50 µl of standard (diclofenac sodium) in standard tubes. The solution was heated at 72°C for 5 minutes and then cooled at room temperature for 15 minutes. The control was taken without the extracts. The absorbance of solution was read at 660nm in spectrophotometer against blank and the percentage of inhibition was calculated using the following formula

Inhibition% = (Ac - As)/Ac×100

where: Ac and As represent control and sample absorbance, respectively. The samples were analyzed in triplicates.

2.3.5.3. Membrane stabilization assay

Preparation of human red blood cells (HRBCs) suspension

The blood was collected from healthy human who had not taken any NSAIDs for 2 weeks prior to the experiment. The fresh whole human blood (10 ml) was centrifuged at 3000 rpm for 10 min and washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline [28].

Human red blood corpuscles (HRBCs) membrane stabilization method

Observations and conclusion for anti-inflammatory activity of fungal extracts were made on the basis of stabilization of HRBCs membrane by hypotonicity induced membrane lysis as recorded by Shing and Kumar [29] and Dhamodaran and Rajeswari [30] with minor modification. Assay mixture was prepared by mixing 50µl of fungal extracts with 1.5 ml phosphate buffer (pH 7.4, 0.15 M), 1.5 ml hyposaline (0.36%) and 50 µl HRBCs suspension (10% v/v). The mixture was incubated in water bath at 56ºC for 30 min then cooled under running tap water. The samples were centrifuged at 2500 rpm for 5 min. Diclofenac sodium was used as positive control. The absorbance was measured at 560 nm in spectrophotometer. Hemolysis produced in the presence of distilled water was taken as 100%. Percentage of HRBCs membrane stabilization/protection was calculated using the following formula:

Stabilization % = (Ac - As)/Ac×100

where: Ac and As represent control and sample absorbance, respectively. The samples were analyzed in triplicates.

3. RESULTS AND DISSCUSSION

3.1. Antimicrobial activities

The crude ethanolic extracts of 26 endophytic fungal strains exhibited a wide variety of antimicrobial activities against 12 tested organisms: 3 Gram (+) bacteria (*Bacillus subtilis, B. cereus* and *Staphylococcus aureus*), 3 Gram (-) bacteria (*Klebsiella* sp., *Escherichia coli* and *Serratia marcescens*), 3 yeasts, (*Candida parapsilosis, C. albicans* and *Saccharomyces cerevisiae*) and two dermatophyte (*Trichophyton mentagrophytes* and *T. rubrum*) and one keratinophilic fungus (*Chrysosporium tropicum*) by agar well diffusion method. The results of antibacterial activity assay showed that, extracts of 22 endophytic fungi (84.6%) produced bioactive compounds that exhibited antibacterial activity against at least one test bacterium with inhibition zones ranging from 5 to 45 mm. Four (15.4%) fungal extracts (*Aspergillus versicolor, A. awamori, A. niger* and *Penicillium funiculosum*) displayed a broader antibacterial spectrum and inhibited the growth of all positive and negative bacterial species (Table 1). This result is better than those recorded by other several studies. In a preliminary study recorded by Wang et al. [31] on screening of some endophytic fungi for production of antimicrobial activities found that more than 50% of isolates displayed antimicrobial activity against at least one tested microorganisms. Gong and Guo [32] reported that 56% of endophytic fungi inhibited growth of at least one of the test organisms and 8% showed broad spectrum inhibition. Crude extracts of 75% of tested endophytic fungi tested by Kharwar et al. [33] showed antibacterial potential against one or more clinical human pathogen. Siqueira et al. [34] reported that only 16 out of 203 (7.9%) endophytic isolates showed antimicrobial activities with a wider action spectrum inhibiting Gram (+) and (-) bacteria and fungi. Tong et al. [35] found that 66 of 72 (92%) endophytic fungal isolates exhibited a significant inhibitory activity at least against one test microorganism with diameters of inhibition zones ranging from 9 to 26 mm for the test bacteria.

The Gram (+) bacteria tested in the present study appeared to be more susceptible to the inhibitory effect of the crude extracts than Gram (-) bacteria. This result is in agreement with previous study by Chareprasert et al. [36] who found that most of the bioactive metabolite compounds from endophytic fungi were more effective against Gram (+) than Gram (-) bacteria and pathogenic fungi. The result in Table 1 showed that the negative control did not show any inhibition while antibiotic control showed mean zones of inhibition ranging from 5 to 45 mm in the agar well diffusion assay. Crude extract of *Aspergillus terreus* exhibited

inhibition zone of 10 mm against *Staphylococcus aureus* and 9 mm against each of *Escherichia coli* and *Klebsiella* pneumonia of 9 tested crude extracts of endophytic fungal species against the bacteria and fungi by well diffusion method. Moreover, endophytic fungi isolated from *Salvadora oleoides Decne* showed potent antimicrobial activity against *Salmonella typhi, Escherichia coli, Klebsiella pneumoniae* and *Aspergillus niger* [38]. Conclusively, Bugni and Ireland [39] found that *Aspergillus genera* are a major contributor of antimicrobial compound of fungal origin. Ogidi et al. [15] returned the antimicrobial activity of *Lenzites quercina* to the presence of fatty acids and other phytochemicals.

Table 1. Antibacterial activities of the ethanolic extracts of 26 endophytic fungi against three different strains of each of Gram (+) and Gram (-) bacteria

	Fungal strains	Gram negative bacteria			Gram positive bacteria		
		***Klebsiella* sp. AUMC B-77**	***E. coli* NCCB 50028**	***S. marcescens* AUMC B-89**	***B. subtilis* AUMC B-101**	***B. cereus* AUMC B-70**	***S. aureus* AUMC 6538**
Alternaria	*A. alternata* AUMC 6836	-ve	-ve	-ve	-ve	-ve	-ve
	A. alternata AUMC 8840	-ve	-ve	-ve	-ve	-ve	-ve
	A. alternata AUMC8841	-ve	-ve	-ve	-ve	-ve	-ve
Aspergillus	*A. awamori* AUMC 8855	15 ± 0.09	20 ± 0.07	30 ± 0.1	25 ± 0.3	25 ± 0.1	20 ± 0.2
	A. fumigatus AUMC8872	-ve	-ve	-ve	17 ± 0.02	29 ± 0.1	40 ± 0.2
	A. niger AUMC8852	20 ± 0.03	-ve	-ve	30 ± 0.4	30 ± 0.2	30 ± 0.007
	A. niger AUMC8856	20 ± 0.003	25 ± 0.1	35 ± 0.3	35 ± 0.02	43 ± 0.08	20 ± 0.1
	A. oryzae AUMC8863	30 ± 0.4	20 ± 0.02	-ve	-ve	25 ± 0.04	-ve
	A. versicolor AUMC6872	33 ± 0.03	40 ± 0.1	30 ± 0.04	4± 0.001	37 ± 0.2	20 ± 0.01
	Circinella muscae AUMC8861	-ve	-ve	30 ± 0.01	-ve	-ve	-ve
	Chaetomium globosum AUMC8862	-ve	-ve	40 ± 0.3	25 ± 0.05	15 ± 0.4	-ve
Fusarium	*F. lateritium* AUMC6833	-ve	-ve	5 ± 0.09	15 ± 0.01	20 ± 0.1	-ve
	F. oxysporum AUMC6827	-ve	-ve	-ve	5 ± 0.02	7.5 ± 0.05	-ve
	F. semitectum AUMC6816	-ve	-ve	-ve	10 ± 0.3	10 ± 0.01	-ve
	F. scirpi AUMC8858	-ve	-ve	-ve	15 ± 0.1	-ve	-ve
	F. subglutinans AUMC 8839	-ve	-ve	20 ± 0.08	-ve	-ve	-ve
	Gliocladium solani AUMC 6802	- ve	-ve	- ve	-ve	5 ± 0.2	-ve
Emericella	*E. nidulans* AUMC 8854	28 ± 0.2	-ve	15 ± 0.04	-ve	-ve	30 ± 0.02
	E. rugulosa AUMC8867	-ve	-ve	20 ± 0.05	-ve	-ve	-ve
	Exophiala costellanii AUMC8865	-ve	-ve	-ve	15 ± 0.1	-ve	-ve
	Papulaspora irregularis AUMC8843	-ve	-ve	-ve	-ve	-ve	-ve
Penicillium	*P. aurantiogriseum* AUMC8847	-ve	30 ± 0.07	40 ± 0.2	15 ± 0.05	20 ± 0.1	15 ± 0.3
	P. funiculosum AUMC8850	28 ± 0.1	15 ± 0.01	14 ± 0.2	16 ± 0.08	40 ± 0.001	40 ± 0.05
	P. raistrickii AUMC7265	-ve	15 ± 0.04	15 ± 0.1	15 ± 0.06	18 ± 0.01	-ve
	Penicillium sp. AUMC8859	18 ± 0.1	-ve	20 ± 0.006	18 ± 0.01	14 ± 0.06	-ve
	Pleospora tarda AUMC 8871	-ve	-ve	-ve	-ve	20 ± 0.03	-ve
Standard (chloromphanicol)		5 ± 0.001	45 ± 0.06	40 ± 0.01	32 ± 0.1	30 ± 0.05	25 ± 0.08

All results were recorded as mean values of three replicates ± standard deviation.

Table 2. Antifungal activities of the ethanolic extracts of 26 endophytic fungi against some strains of yeasts, dermatophytic and keratinophilic fungi

Fungal strains		Yeasts			Dermatophytic fungi		Keratinophilic fungi
		***C. parapsilosis* AUMC 9163**	***C. albicans* AUMC 9212**	***S. cerevisiae* AUMC 203**	***T. mentagrophytes* AUMC 2360**	***T. rubrum* AUMC 10337**	***C. tropicum* AUMC 1804**
Alternaria	*A. alternata* AUMC 6836	-ve	-ve	-ve	-ve	-ve	-ve
	A. alternata AUMC 8840	-ve	-ve	-ve	15 ± 0.1	-ve	-ve
	A. alternata AUMC 8841	-ve	-ve	-ve	-ve	-ve	-ve
Aspergillus	*A. awamori* AUMC 8855	-ve	-ve	-ve	30 ± 0.001	20 ± 0.3	20 ± 0.1
	A. fumigatus AUMC 8872	-ve	-ve	-ve	20 ± 0.005	35 ± 0.3	5 ± 0.03
	A. niger AUMC 8852	-ve	-ve	-ve	35 ± 0.02	30 ± 0.1	40 ± 0.09
	A. niger AUMC 8856	-ve	-ve	-ve	20 ± 0.1	15 ± 0.5	20 ± 0.01
	A. oryzae AUMC 8863	-ve	-ve	-ve	45 ± 0.07	35 ± 0.01	20 ± 0.3
	A. versicolor AUMC 6872	-ve	-ve	-ve	40 ± 0.03	-ve	-ve
	Circinella muscae AUMC 8861	-ve	-ve	-ve	-ve	-ve	-ve
	Chaetomium globosum AUMC 8862	-ve	-ve	-ve	-ve	-ve	-ve
Fusarium	*F. lateritium* AUMC6833	-ve	-ve	-ve	-ve	-ve	-ve
	*F. oxysporum*AUMC6827	-ve	-ve	-ve	40 ± 0.03	-ve	-ve
	F. semitectum AUMC6816	-ve	-ve	-ve	-ve	-ve	-ve
	F. scripi AUMC 8858	-ve	-ve	-ve	5 ± 0.03	-ve	-ve
	F. subglutinans AUMC8839	-ve	-ve	-ve	-ve	-ve	-ve
	Gliocladium solani AUMC 6802	-ve	-ve	-ve	-ve	-ve	-ve
Emericella	*E. nidulans* AUMC 8854	-ve	-ve	-ve	30 ± 0.2	25 ± 0.06	2.5 ± 0. 3
	E. rugulosa AUMC 8867	-ve	-ve	-ve	-ve	15 ± 0.1	-ve
	Exophiala costellanii AUMC 8865	-ve	-ve	-ve	20 ± 0.08	10 ± 0.04	-ve
	Papulaspora irregularis AUMC 8843	-ve	-ve	-ve	-ve	-ve	-ve
Penicillium	*P. aurantiogriseum* AUMC 8847	-ve	-ve	-ve	35 ± 0.05	-ve	5 ± 0.2
	P. funiculosum A UMC 8850	-ve	-ve	-ve	45 ± 0.1	25 ± 0.4	30 ± 0.02
	P. restickii AUMC 7265	-ve	-ve	-ve	-ve	20 ± 0.2	-ve
	Penicillium sp. AUMC 8859	-ve	-ve	-ve	-ve	10 ± 0.03	-ve
	Pleospora tarda AUMC8871	-ve	-ve	-ve	-ve	-ve	-ve
Standard (clotrimazole)		35 ± 0. 30	25 ± 0.10	10 ± 0.03	30 ± 0.07	25 ± 0.20	15 ± 0.01

All results were recorded as mean values of three replicates ± standard deviation

The extracts of 8 endophytic fungi (30.8% of tested strains) were affected on the two tested dermatophyte strains (*Trichophyton mentagrophytes* and *T. rubrum*) and gave inhibition zones ranged from 10-45 mm. Other 5 extracts were inhibited *Trichophyton mentagrophytes* only with inhibition zones ranged from 5-40 mm and other 3 fungal extracts were inhibited the growth of *Trichophyton*

rubrum with zones between 10-20 mm. Only 8 fungal extracts have an inhibition activity against the keratinophilic fungus (*Chrysosporium tropicum*). On the other hand, the extracts of all tested endophyte have no activities against any of the three tested yeast strains (Table 2). Seven endophytic extracts show inhibition zones against all the three tested dermatophytic and keratinophilic strains. *Penicillium funiculosum* and *Aspergillus oryzae* show the highest inhibition zone (45 mm) against *Tricophyton mentagrophytes*, *Aspergillus oryzae* and *Aspergillus fumigatus* appeared their highest activities against *Tricophyton rubrum* with inhibition zone reached to 35 mm. *Aspergillus niger* formed the highest inhibition zone against *Chrysosporium tropicum* which reached to 40 mm (Table 2).

Recently, studies were done on the effect of endophytic extracts against both dermatophytes and yeasts. Tong et al. [35] examined methanolic and ethyl acetate extracts of 72 endophytic fungal isolates against yeast and fungi and recorded that only moderate antiyeast and antifungal activities were observed for both with diameter of inhibition zone less than 16 mm on disc diffusion assay. Seddek [40] found that about 50% of the crude ethanol and aqueous extracts of 57 endophytic fungal isolates on the growth of 6 human pathogenic fungi representing 3 species of *Candida* (*C. albicans*, *C. glabrata* and *C. krusei*) and 3 dermatophytic fungi (*Trichophyton rubrum*, *T. mentagrophytes* and *Epidermophyton floccosum*) had no inhibition activities against all the 3 dermatophytic fungi while 72% of the extract affected the tested 3 species of *Candida*. Pharamat et al. [41] examined the antimicrobial activity of 73 endophytic fungi against *Saccharomyces cerevisiae* and *Candida albicans* and found that 11 (15.1%) and 7 (9.6%) of isolates produced inhibition zones ranged from 9 to 30 mm against the 2 tested yeast species, respectively. Kalyanasundaram et al. [37] reported that the crude extract of *Aspergillus terreus* inhibited *Trichophyton rubrum*, *Candida albicans* and *Trichophyton mentagrophytes* growth with inhibition zones reached to 8, 4 and 3 mm, respectively. Also, the inhibitory effect of *Clitocybe nebularis* on *Trichophyton mentagrophytes* with inhibition zones of 9-11 mm [42]. Medicinal product from plants and mushrooms could be continually sourced and adequately utilized to treat dermatophyte infections [43, 44]. This antifungal activity may be attributed to the presence of glucanase [45] or ganoduric protein [46].

3.2. Anti-inflammatory activity

In the present study, only 8 strains were selected for examined their anti-inflammatory activities (Table 3). The extracts of 3 of them (*Aspergillus niger*, *Aspergillus awamori* and *Penicillium funiculosum*) were recorded as effective on all Gram (+), Gram (-) bacteria and dermatophytic and keratinophilic fungi in the previous expriment. Other 3 extracts (*Aspergillus fumigatus*, *Aspergillus oryzae* and *Emericella nidulans*) appeared an inhibition effect on all dermatophytic and keratinophilic fungi and some of bacterial strains under study. The 7 selected extract (*Aspergillus versicolor*) was recorded as inhibition for all bacterial strains and only one of dermatophyte. The last selected extract (*Pleospora tarda*) had no inhibitory activity against all tested bacterial and fungal strains with exception of Gram (+) *Bacillus cereus.*

As part of the investigation on the mechanism of the anti-inflammation activity, ability of the selected 8 fungal extracts to denaturated protein was studied (Table 3). All the fungal extracts and the standard were tested at 10 µl/ml concentration. *Emericella nidulans*, *Pleospora tarda*, and *Penicillium funiculosum* extracts showed higher activities with inhibition % of protein denaturation reached to 83%, 82.5% and 81.4%, respectively. On the other hand, the extracts of all the 5 *Aspergilli* under study showed lower activities and inhibited protein denaturation by 65-79.9% (Table 3).
Standard diclofenac sodium recorded 77.4% inhibition of protein denaturation. Denaturation of proteins is well documented cause of inflammation and rheumatoid arthritis. Several anti-inflammatory drugs like salicylic acid have shown dose dependent ability to inhibit thermally induced protein denaturation [47]. The inhibitory effect on bovine serum albumin denaturation by the ethanol extracts of the tested endophytes is shown in Table 3. Maximum inhibition was 95% was observed by ethanol *Emericella nidulans* extract at 50 µl/ml followed by *Pleospora tarda* (90.7% at the same concentration).

Table 3. Anti-inflammatory activity of ethanolic extracts of selected endophytic strains

Fungal strains	Protein denaturation	Albumin denaturation	HRBCs membrane stibilization
	% inhibition	% inhibition	% inhibition
Aspergillus awamorii AUMC 8855	77.9 %	53.9 %	62.8 %
Aspergillus fumigatus AUMC 8872	75 %	62.7 %	52.8 %
Aspergillus niger AUMC 8856	71.7 %	82.4 %	59.3 %
Aspergillus oryzae AUMC 8863	79.9 %	86 %	66.8 %
Aspergillus versicolor AUMC 6872	65 %	69 %	63.4 %
Emericella nidulans AUMC 8854	83 %	95 %	69.5 %
Penicillium funiculosum AUMC 8850	81.4 %	57.7 %	52.7 %
Pleospora tarda AUMC 8871	82.5 %	90.7 %	56.5 %
Standard of diclofenac sodium	77.4 %	87.4 %	74.3 %

All results were recorded as mean values of three replicates ± standard deviation

The standard anti-inflammatory drug, diclofenac sodium showed 87.4% inhibition. The results recorded in this study are better than those recorded by Govindappa et al. [48] who found that methanol extract at 200 µg/ml concentration of *Aspergillus niger* showed 79% inhibition followed by *Aspergillus alternata* (78.6%) and *Penicillium* sp. 65.84%.

Since HRBCs membrane is similar to these lysosomal membrane components [49] and its stabilization implies that the extract may as well stabilize lysosomal membranes. So, the prevention of hypotonicity induced RBCs membrane lysis was taken as a measure in estimating the anti-inflammatory property of the secondary metabolites of fungi. Thus, HRBCs membrane stabilization has been used as a method in estimating the anti-inflammatory property [50, 51]. Stabilization of HRBCs membrane was studied for establishes the mechanism of anti-inflammatory action of ethanolic extracts of different 8 endophytes. All the tested extracts were effectively inhibiting the heat induced hemolysis. These results provide evidence for membrane stabilization as a mechanism of their anti-inflammatory effect. This effect may possibly inhibit the release of lysosomal content of neutrophil at the site of inflammation. The extracts inhibited the heat induced hemolysis of HRBCs to varying degree (Table 3). The maximum inhibition was 69.5% by ethanolic extract of *Emericella nidulans*. The diclofenac standard drug recorded 74.3% of inhibition. This result came in harmony with those recorded by Govindappa et al. [48] who showed that the maximum inhibition by methanol extract of *Aspergillus niger* was 78.42% followed by *Penicillium* sp. (77.61%) and *Aspergillus alternata* (77.98%). Also they found that the aspirin standard drug showed 85.92%. Results of our findings confirmed the use of some endophytic fungi such as *Emericella nidulans*, *Pleospora tarda*, *Aspergillus versicolor*, *Penicillium aurantiogriseum*, *Penicillium funiculosum*, *Aspergillus awamori*, *Aspergillus niger*, *Aspergillus oryzae* and *Aspergillus fumigatus* as sources of anti-microbial and/or anti-inflammatory drugs. This biological activity could be returned to the presence of phytochemicals like alkaloids, phenols, flavonoids, saponins, and terpenes in the endophytes [14]. The levels of phenolic and flavonoid compounds were correlated with the anti-inflammatory activity of the extracts [52]. The correlation between presence of flavonoids and their membrane stabilizing ability was approved by Sankari et al. [53]. Moreover, the main action of the anti-inflammatory agent is the inhibition of the cyclooxygenase system which is responsible for the biosynthesis of prostaglandins. NSAIDS like prostaglandins acts by inhibiting the lysosomal enzymes or by stabilizing the lysosomal membrane. Since RBCs membranes are similar to the lysosomal membrane components, the prevention of hypotonicity-induced HRBCs lysis was taken as measure of anti-inflammatory activity of drugs. The indomethacin drugs as inhibitor of prostaglandins biosynthesis act either by inhibiting these lysosomal enzymes or by stabilizing the

lysosomal membrane [54, 55]. Thus secondary metabolites of endophytic fungi have a promising potential to be included in antimicrobial and anti-inflammatory drug discovery program.

AUTHORS' CONTRIBUTION

All authors contributed in design, execution the research plan point to point, and writing of the manuscript. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interest.

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Review Article

Chrysin and its potential antineoplastic effect

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ABSTRACT

In 2012, in Europe, there were noticed over 3 million new cases of cancer and 1.75 million of deaths from cancer. Numerous anticancer agents are cytotoxic, can damage normal cells, and they can cause serious side effects. Currently, natural and non-toxic agents are being sought that reduce the cost of therapy, are more effective and targeted, and do not damage healthy cells. Chrysin which belong to flavonoids family as natural substance, has multiple anticancer activities. It has been reported that chrysin can induce apoptosis in tumour cells by different mechanism. In our work we demonstrated the potential use of chrysin in gastrointestinal, breast, cervical, and lung cancer. In conclusion it is proven that chrysin or combination of chrysin with other related drugs can effectively improve the effectiveness of anticancer therapy. Furthermore, new agents, such as nanoparticles, may show greater efficacy, and better targeting, hence, less side effects on healthy cells. Based on these results, nanochrysin it offers as new and effective drug delivery system. Moreover, it has been reported that chrysin is a potential antitumor but also an adjuvant agent that can be used in combination with other antimetastatic substances to reduce tumor metastasis.

Keywords: Chrysin; Flavonoids; Propolis; Anticancer activity.

1. INTRODUCTION

Propolis, or 'bee glue', is natural sticky plant product created by bees which colour varies from yellowish-green to dark brown, depended from its origin and age [1-3]. Propolis can be used as a built material and as biological weapon because of its antibacterial, antifungal, antiviral, cytotoxic, anti-oxidant, anti-inflammatory and immunomodulatory effects [4-9].

The chemical composition of propolis is complex and very different, as well as being dependent upon the geographic region, botanical origin and collecting bee species [1, 7, 10]. These factors have influence on its biological activity. In most European countries, propolis is collected by bees from black poplar, birch, alder, pine and willow species buds [2, 7]. Each source generates a different propolis. In the most common types, such as poplar propolis, flavones, flavanones, phenolic acids and their esters, predominate. In the birch propolis, flavones and flavonols (but not the same as in the poplar type) dominate. In the green propolis, we find mainly prenylated p-coumaric acids and diterpenic acids, while in red propolis, we see polyprenylated benzophenones [1].

Researchers working between 2000 and 2012, identified about 300 compounds in the various propolis, including flavonoids, terpenes, phenolics and their esters, lipid-wax substances, beeswax, sugars, hydrocarbons and mineral elements, vita-

mins, proteins, and amino acids [2, 10]. In general, however, raw propolis is composed of waxes (30%), resins and vegetable balsam (50%), essential and aromatic oils (10%), pollens and other substances (5%) [6, 7, 9, 10].

The flavonoids are a major chemical component of propolis. These flavonoids are a group of polyphenols with different structures and properties [2]. Flavonoids can be found in fruits, vegetables, grains, nuts, seeds, tea and herbs. In the plants, flavonoids are concentrated mainly in the leaves and flowers [11-14]. Chemically, they have structure of 15 carbon atoms, described as C6-C3-C6, with a benzoic ring and a phenylopropane unit [2, 11, 14-16]. The double band between C2 and C3 in the C ring influences the antioxidative activity of the flavonoids. This can be affected by way of glycolysation at position C3 [2, 11, 14, 17]. In accordance to the chemical structure, flavonoids may be classified into: flavones, flavonols, flavanones, flavanonols, chalcones, dihydrochalcones, isoflavones, isodihydroflavones, flavans,isoflavans, and neoflavonoids [10, 14, 16].

Chrysin (5,7-dihydroxyflavone or 5,7-dihydroxy-2-phenyl-4Hchromen-4-one) is one of the flavones which can be found in passion flower (*Passifloracaerulea),* in honey, in propolis and in bee pollen (Fig 1.) [12, 17, 18]. The chemical structure of chrysin, with the presence of a double band between C2-C3 in ring C, and the lacking of oxygenation at C3 (Fig 1.), is associated with numerous pharmacological properties. These include anti-microbial, anti-inflammatory, anti-spasmodic, anxiolytic, anthelmintic, anti-cancer, hypoglycemic, antiatherogenic, and anti-HIV [12, 14, 18-21].

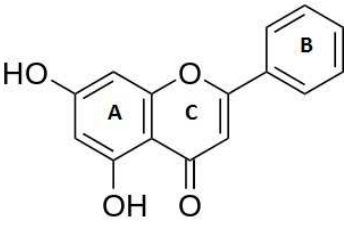

Figure 1. Structure of chrysin.

The effectiveness of all drugs and natural substances is dependent of their bioavailability and the solubility of these agents, but is also associated with their cytotoxicity [12]. Chrysin has a relatively low solubility and is poorly absorbed in the intestine which may limit its bioavailability and made it less useful during any therapy [22, 23]. The cytotoxicity of chrysin is dependent of used dosage [24].

In 2012, in Europe, there were noticed over 3 million new cases of cancer and 1.75 million of deaths from cancer. The most common types of cancer and most common causes of death were: female breast, colorectal, prostate and lung cancers [25]. Cancer as a multifactorial disease, may have a genetic background and be caused by harmful environmental factors [26, 27]. Numerous anti-cancer agents are cytotoxic, can damage normal cells, and they can cause serious side effects [26]. Currently, natural and non-toxic agents are being sought that reduce the cost of therapy, are more effective and targeted, and do not damage healthy cells. Flavonoids as natural substances, are regarded as safe and easy to obtain, so they are good candidates to anticancer therapy in clinical treatment [13].

The multiple anticancer activities of chrysin has drawn our attention. It has been reported that chrysin can induce apoptosis in different tumour cells [21, 26, 28-30], inhibit cell proliferation and block the cell cycle [31]. Moreover, it can activate notch 1 signalling [32], and it is a histone deacetylase inhibitor which can significantly inhibit tumour growth [33]. However, Song et al. [21] report that the addition of amino acids reduce the anti-cancer activity of chrysin.

In this review we looked for potential antineoplastic action of chrysin or chrysin in combination with other active substances in selected cancer diseases. In addition, evaluating and investigating the pathway and mechanism of action of this substance on cancer cells may be of great importance when planning antineoplastic therapy.

2. USE OF CHRYSIN IN SELECTED CANCER DISEASES

2.1. Chrysin in gastric cancer

According to published statistics, gastrointestinal cancers are one of the leading causes of cancer deaths in the world. One of the major clinical

problems is the late recognition and lack of effective antineoplastic therapy [25, 34].

Chrysin and triphenylgermanium Bromide (Chry-Ge) induced apoptosis in Colo205 cells by way of the intrinsic pathway. Moreover, it led to the reorganization of cytoskeleton and was evidenced of damaging the nucleus in Colo205 cells [35]. Other authors have investigated the effects of chrysin on MMP-9 (matrix metalloproteinase-9) expression and activity in AGS gastric cancer cells. In such studies, they found that chrysin can decrease cancer invasiveness in cells by controlling MMP-9 expression through the suppression of the JNK/c-Jun and ERK/c-Fos signaling pathways [36]. Moreover, chrysin can suppress RON (Recepteur d'origine Nantais) in the AGS cells, which, as a consequence, decreases cell invasion [37]. The phenolic compounds in New Zealand propolis, as well as chrysin alone, showed anti-proliferative and anti-inflammatory assays against three gastro-intestinal cancer cell lines; HCT-116 colon carcinoma, KYSE-30 oesophageal squamous cancer, and NCI-N87 gastric carcinoma [38]. In the Leòn et al. [39] study, the authors investigated the mechanisms of action of two flavonoids:silibinin (VOsil), and chrysin (VOchrys) in a human colon adenocarcinoma cell line (HT-29). Their results indicated that the complexation of the flavonoids inhibited the viability of HT-29 cells in a dose dependent manner. Moreover, the anticancer effects of VOchrys were mediated by a decrease of the GSH (glutathione) levels and by cell cycle arrest.

Chrysin treatment induced Tnfα and Tnfβ gene expression and activated multiple TNF-mediated signaling pathways in Colon (HCT116, DLD1) and rectal (SW837) cancer cell lines leading to apoptosis. In addition, it has been suggested that this comes about by way of a novel pathway in which the transcriptional factor AHR (Aryl Hydrocarbon Receptor) is required. The cell viability in all cell lines was decreased at 50 μM and 100 μM chrysin [40]. In HCT116 cells, chrysin induced cell death by DNA damage dependent of used dosage, as well as by mitochondrial membrane perturbation accompanied by cytochrome c release, down-regulation of Bcl-2, the activation of BID and Bax, and caspase-3 activation [41]. In related work, Mohammadian et al. [42] revealed that chrysin inhibits the growth of the AGS human gastric cell line. In this study, the authors used a PLGA-PEG-chrysin complex, as well as free chrysin. Herein, the value of inhibitory concentration 50 (IC50) was calculated for each case. Their results showed that the IC50 value was significantly decreased in nanocapsulatedchrysin, in comparison with free chrysin. This finding directly indicated that capsulated chrysin is more effective than free.

In the Li et al. study [43], the authors investigated the influence of the combination of chrysin and cisplatin on Hep G2 cancer cells, and saw increased apoptosis in Hep G2 cancer cells. Furthermore, the combination of chrysin and cisplatin treatment increased the expression of proapoptotic proteins (p53, Bax, and DR5), while it decreased the expression of the antiapoptotic protein Bcl-2. In addition, the combination of chrysin and cisplatin promoted both extrinsic and intrinsic apoptosis pathways by activating caspase-8 and caspase-9 in the Hep G2 cells. Similar results were obtained by Zhang et al. [30] and Huang et al. [44]. Huang et al. [44] used chrysin combined with apigenin and observed that this combination can reduce HepG2 and MDA-MB-231 proliferation and cell motility, as well as induce apoptosis.

2.2. Chrysin in female reproductive system

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among women [25, 45]. Among the cancers of the female reproductive system, however, cervical, endometrial and ovarian cancers are predominant. Indeed, cervical cancer is the third most common type among women, worldwide [46].

Lirdprapamongkol et al. [47] analyzed the effect of chrysin and tectochrysin on hypoxic survival of 4T1 mouse breast cancer cells in vitro. After application of 40-100 μM of chrysin, they saw a decrease in hypoxic cell survival, while the application of tectochrysin in a 60 μM concentration did not show any changes. Because tumor hypoxia is correlated with metastasis, the authors also examined the influence of chrysin on the 4T1 cell line, via the spontaneous lung metastasis model. They found out that administration of 100 and 250 mg/kg of chrysin by day, did not show any significant effect on primary tumor growth, but, the number of metastatic colonies in the lung was

decreased, as was the total number of metastases, while the size of metastases were significant suppressed in a dose-dependent manner.

It was showed that 5,7-dihydroxy-8-nitro-chrysin (NOC), a novel synthetic chrysin analog, induce apoptosis in MDA-MB-453 human breast cancer cell line intrinsically, via activation of caspase-9. In the tested cells, apoptosis were induced by activation of the Akt/FOXO3a axis (Forkhead box O3a transcription factor), with increased Bim (B cell lymphoma 2 (Bcl-2) - aninteracting mediator of cell death) expression [48]. Similar results were seen in a 2014 follow-up study. Herein, the authors reported that LW-214 (a new flavonoid sourced from chrysin), activated the intrinsic mitochondrial apoptotic pathway in human breast cancer MCF-7 cells. However, after 24 h, decreased expression of Bcl-2 and increased expression of Bax was observed, in a dose-dependent manner [49]. Zhao et al. [48] also used a nude mice model bearing an inoculated MCF-7 tumor to determine the influence of LW-214 in vivo. In this experiment, they saw that LW-214 inhibited tumor growth. In H&E staining, noted no morphological changes observed in the organs, and no significant difference was seen in the average body weight of mice treated by LW-214, compared with a control group. In conclusion, it suggest that LW-214 has anticancer effects in the MCF-7 cell line in vivo and in vitro [48, 49].

Another type of a new chrysin analog, 8-bromo-7-methoxychrysin (BrMC), induced intrinsic apoptosis in a time-dependent manner, via the Akt/FOXO3a axis in cisplatin (DDP)-sensitive (A2780) and -resistant (A2780/DDP) ovarian cancer cell lines. The effect of BrMCis greater that natural chrysin [50].

Mohammadinejad et al. [51], on a T47D breast cancer cell line, examined the effect of encapsulated chrysin in PLGA-PEG (poly (D, L-lactic-co-glycolic acid) and poly (ethylene glycol) as compared to pure chrysin. They saw that loaded chrysin in PLGA-PEG increases its solubility and tolerance, and decreases the side effects of the drug. In addition, the authors observed that pure chrysin and chrysin nanoparticles inhibited cell proliferation in a dose dependent manner. As a result of such, they suggested that placing the chrysin into nanoparticles improves its effectiveness on cell growth inhibition, and it strongly decreases the cyclin D1 expression. This study was continued by Eatemadi et al. [52] and Anari et al. [53]. Anari et al. [53] evaluated the cytotoxicity of chrysin nanoparticles and pure chrysin on two human breast cancer cell lines: T47D and MCF7. They confirmed the results of Mohammadinejad et al. [51] in that nanochrysin has a positive effect on the breast cancer cell lines (T47D and MCF7) in a dose-dependent and time-dependent manner. Still, they noted that the MCF7 cell line is less sensitive to chrysin than is the line T47D. Eatemadi et al. [52] have also shown that nanochrysin has a time-dependent cytotoxic effect, plus they found that it increased the expression of the BRCA1 gene and reduced the expression of the hTERT and FTO genes in the T47D cell line. By the way, chrysin inhibited the migration and invasion of MDA-MB-231 and BT-549 cell lines by way of the down-regulation of MMP-10 (matrix metalloproteinase-10). In both lines, MDA-MB-231 and BT-549, chrysin treatment increased the expression of E-cadherin, while it decreased the expression of vimentin, snail and slug. This suggests that chrysin has a reversal effect on the epithelial-mesenchymal transition [54].

Co-treatment therapy with chrysin and 1,2,3,4,6-penta-O-galloyl-b-D-glucose (5GG) induced apoptosis, cell cycle arrest, inhibited cell proliferation and colony formation in AU565 and MDA-MB-231 human breast cancer cells. The combination of chrysin and 5GG decreased the growth of tumor by down-regulation of the phospho-LRP6 (pLRP6) and Skp2 proteins [55]. Another combination of chrysin, chrysin and apigenin reduced cell viability and cell motility, as well as induced apoptosis in a dose- and time-dependent manner in MDA-MB-231 breast cancer cell line. Herein, co-treatment for 36 h synergistically decreased cell line motility but not viability, but significant cytotoxicity was observed for 72 h of co-treatment [44].

In another work, the authors examined the anticancer activity of *S. discolor* (*Scutellaria discolor* Colebr., SDE) on different cancer cell lines, and they isolated the substance which is responsible for this action. They discovered that SDE induced cell death in a concentration dependent-manner by up-regulation of APAF1, BAX, BCL2L11, caspase

9, DFFA, GADD45A and TP53, and increased the expression of caspase-3mRNA. In their work, they saw that the stronger effects were observed in the cervical cancer cell line, HeLa. This result was confirmed by utilizing the lines ME180 and Bu25TK. In addition, spectroscopic methods indicated that chrysin was the major compound of SDE that had showed the antiproliferative activity [56].

2.3. Chrysin in respiratory system

According to data, lung cancer is one of the leading causes of cancer death in developed countries [25, 57].

Shao et al. [58] reported that chrysin induces growth inhibition and apoptosis in the A549 cultured lung cancer cells. They also put forward that the actuation of AMP-activated protein kinase (AMPK) may have contributed to this process, as their Western-blot analysis results demonstrated a significant AMPK activation after chrysin treatment in A549 cells. Moreover, inhibition of AMPK by shRNA-mediated gene silencing, or by its inhibitor, diminished chrysin-induced A549 cell growth inhibition and apoptosis. Furthermore, forced activation of AMPK by introducing a constitutively active form of AMPKa (CA-AMPKa), or by its activators, mimicked the chrysin effect. In addition, as they found that chrysin inhibited the Akt/mammalian target of rapamycin (mTOR) activation, knocking down of AMPK by shRNA almost reversed this effect. Finally, they observed that a relative low dose of chrysin enhanced doxorubicin-induced AMPK activation, hence promoting A549 cell apoptosis.

Kasala et al. [59] investigated the chemo-preventive role of chrysin against benzo(a)pyrene [B(a)P] induced lung carcinogenesis in Swiss albino mice. In their work, they administered B(a)P orally (50 mg/kg body weight) twice a week for four weeks to induce lung cancer in the test mice. They reported that administration of B(a)P resulted in increased lipid peroxides and carcinoembryonic antigens, with concomitant decrease in the levels of both enzymatic and non-enzymatic antioxidants. Chrysin supplementation down-regulated the expression of PCNA, COX-2 and NF-kB and maintained cellular homeostasis. This confirmed the chemopreventive potential of chrysin against B(a)P induced lung cancer in Swiss albino mice [59].

In A549 cells, in vitro, Lim et al. [60] investigated the combination of chrysin and docetaxel (DTX). As a result of this study, they saw increased cytotoxicity, suppressed cellular proliferation and induced apoptosis in the post-treatment of chrysin following prior DTX treatment. Moreover, in vivo, chrysin enhanced the tumor growth delay activity of DTX and increased DTX-induced apoptosis by way of the A549-derived xenograft model. Furthermore, chrysin prevented DTX-induced edema in ICR mouse-subjects. These results indicate that chrysin administration strengthened the therapeutic efficacy of DTX and diminished the adverse effect of DTX. This outcome suggests that chrysin could be exploited as an adjuvant therapy for NSCLC.

Brechbuhlf et al. [61] reported that treatment with chrysin resulted in significant and sustained intracellular flavonoid-induced glutathione (GSH) depletion. What is more, the GSH enzyme network in the four cancer cell types was predictive of the severity of chrysin-induced intracellular GSH depletion. Their gene expression data also indicated a positive correlation between basal MRP1, MRP3 and MRP5 expression, and total GSH efflux before and after chrysin exposure. In addition, Brechbuhlf et al. [61] saw that in all the four investigated cell lines, co-treating the cells for 72 hours with chrysin (5-30 μM) and doxorubicin (DOX) (0.025-3.0 μM) significantly enhanced the sensitivity of the cells to DOX, as compared to 72-hour DOX alone treatment. In this experiment, the maximum decrease in the IC50 values of cells treated with DOX alone compared to co-treatment with chrysin and DOX was 43% in A549 cells, 47% in H157 and H1975 cells and 78% in H460 cells. Hence, chrysin worked synergistically with DOX to induce cancer cell death. This approach could allow for use of lower concentrations of applied chemo-therapy agents, by sensitizing cancer cells that are typically resistant to therapy to such agents.

Moreover, propolis extract and chrysin sensitizes A549 human lung adenocarcinoma and HeLa human cancer cell lines to TRAIL-induced apoptosis. Moreover, the TRAIL sensitization effect of chrysin is not mediated by inhibition of TRAIL-induced NF-κB activation or by glutathione

depletion. In actuality, immunoblot analysis using a panel of anti-apoptotic proteins, revealed that chrysin selectively decreases the levels of Mcl-1 protein, by down-regulating Mcl-1 gene expression as determined by qRT-PCR. The contribution of Mcl-1 in TRAIL resistance was confirmed by si-Mcl-1 knockdown. Indeed, among the signaling pathways that regulate Mcl-1 gene expression, only that constitutive of STAT3 phosphorylation was suppressed by chrysin. The proposed action of chrysin in TRAIL sensitization by inhibiting STAT3 and down-regulating Mcl-1 was supported by using a STAT3-specific inhibitor, cucurbitacin-I, which decreased Mcl-1 levels and enhanced TRAIL-induced cell death, in a manner similar to that observed with chrysin treatment [62].

Narayan and Kumar [63] explored the antineoplastic and immunomodulatory effects of chrysin (derived from an extract of *Achyranthes aspera*) (PCA) on urethane-induced lung cancer in vivo. In the study, PCA was fed orally to urethane (ethyl carbamate) primed lung cancerous mice at a dosage of 100 mg/kg body weight for 30 consecutive days. Herein, the enhanced activity and expression of the antioxidant enzymes GST, GR, CAT, SOD, as well as down-regulation of expression and activation of LDH enzymes in PCA were observed. What is more, PCA fed urethane-primed lung tissues showed down-regulated expression of the pro-inflammatory cytokines IL-1b, IL-6 and TNF, along with that of TFs, NF-jB and Stat3, while the expression of the proapoptotic proteins Bax and p53 was enhanced. In related experimental work, FTIR and CD spectroscopy data revealed that PCA resisted the urethane mediated conformational changes of DNA. This was made evident by the shift in guanine and thymine bands in FTIR, from 1,708 to 1,711 cm^{-1} and 1,675 to 1,671 cm^{-1}, respectively. The present study suggests that PCA components have a synergistic anti-cancerous and cytokine based immunomodulatory roll. Moreover, they have DNA conformation restoring effects.

Table 1. The type of active substances and the cell line on which they act.

Substances	**Cell lines of the digestive system**
Chrysin	Colo205, AGS, HCT-116, KYSE-30, NCI-N87, HT-29, DLD1, SW837
Chrysin and triphenylgermanium Bromide (Chry-Ge)	Colo205
Silibinin (VOsil)	HT-29
PLGA-PEG-chrysin complex	AGS
Chrysin and cisplatin combination	HepG2
Chrysin and apigenin combination	HepG2
Substances	**Cell lines of the female reproductive system**
Chrysin	4T1, T47D, MCF7, MDA-MB-231, BT-549
Chrysin and apigenin combination	MDA-MB-231
5,7-dihydroxy-8-nitrochrysin (NOC) (synthetic chrysin analog)	MDA-MB-453
LW-214 (flavonoid sourced from chrysin)	MCF-7
8-bromo-7-methoxychrysin (BrMC) (chrysin analog)	A2780, A2780/DDP
PLGA-PEG-chrysin complex	T47D, MCF7
Chrysin and 1,2,3,4,6-penta-O-galloyl-b-D-glucose (5GG)	MDA-MB-231, AU565
Scutellaria discolor Colebr., SDE*	HeLa, ME180, Bu25TK
Substances	**Cell lines of the respiratory system**
Chrysin	A549, lung cancer in Swiss albino mice induced by benzo(a)pyrene [B(a)P], urethane-induced lung cancer in vivo
Chrysin and docetaxel combination	A549
Chrysin and doxorubicin combination	A549, H157, H1975, H460

*Chrysin is a major compound of SDE with antiproliferative activity

3. CONCLUSION

The mechanism of action of chrysin is based on the induction of apoptosis in tumor cells, whereas in the initiation of this process various proteins and enzymes may be involved i.a.Bax, Bcl-2, caspases: 3, 8 and 9, p53 protein, and cytochrome C. In addition, chrysin exhibits an anti-inflammatory and anti-proliferative effects, it inhibited cancer cells growth and also reduces viability and motility of various tumor cells. Numerous studies have shown that the use of chrysin, chrysin analogues or chrysin combinations and other related drugs can effectively improve the effectiveness of anticancer therapy (Table 1.). Furthermore, new agents, such as nanoparticles, may show greater efficacy, and better targeting, hence, less side effects on healthy cells (Table 1.). Based on these results, nanochrysin offers new and effective drug delivery system. Moreover, it has been reported that chrysin is a potential antitumor but also an adjuvant agent that can be used in combination with other antimetastatic substances to reduce tumor metastasis.

AUTHORS' CONTRIBUTION

PC-W: concept of the work, collection and analysis of literature, text translation, wrote the manuscript. ML-S: collection and analysis of literature, preparation of literature, wrote the manuscript. BJ-J: critical evaluation of work, edited the manuscript. The final manuscript has been read and approved by all authors.

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The authors declare that they have no competing interests.

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Review Article

Managing phosphorus in terrestrial ecosystem: a review

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ABSTRACT

Increasing human population placed stress on the environment, as well as shifting in land use pattern to increase food production, significantly influence the dynamics of soil organic matter and associated nutrients (phosphorus) in terrestrial ecosystems. This review is based on the published work carried out in recent years and critically examines how the P cycling occurs within different terrestrial ecosystems, possible mechanisms involved in its transformation from one form to another and gaps to be investigated. In terrestrial ecosystems P mainly occurs as phosphate ion; generally precipitated with Ca, Al and Fe under varying pH conditions and become relatively immobile in soils. In agricultural fields, change in inorganic (Pi) and organic (Po) phosphorus are attributed due to fertilization and tillage while in forest and grasslands it is the matter of litter addition and its decomposition by microbes. Afforestation of grassland enhances the mineralization of organic matter and P availability through higher microbial activity, production of low molecular weight organic acids and root associations of mycorrhizae. Phosphorus losses primarily occur due to export in the form of erosion and product removal from ecosystem. Heavy export of P from terrestrial ecosystem accelerated the problem of eutrophication. Future studies should be focused on efficient practices to increase the use of accumulated surface P, estimating P bioavailability in soil and improved methods of runoff control to control P export into aquatic ecosystems. Optimization of practices and exploring novel approaches for sustainable production will maintain the enduring supply of this globally limited nutrient and reduce environmental consequences.

Keywords: Ecosystems; P dynamics; Organic P; Inorganic P; Land use; Litter; Soil microbes.

1. INTRODUCTION

Terrestrial ecosystems, particularly forests, are the major body expected to store a large amount of the increased atmospheric carbon (C) [1]. However, the extent of storage depends on different soil conditions of forests such as soil fertility, moisture and temperature [2]. Carbon (C) sequestration potential of vegetation, to sequester this rising level of CO_2 is checked by the low nitrogen availability in soil [3-4]. Many of the workers ignored P but it is likely to be a major obstacle in enhancing C sequestration, because low P availability can limit nitrogen (N) fixation and plant development [5-6], so it can be considered as a constraint in the sustainable management of ecosystem productivity [7-8]. However, in forest

ecosystems, fertilization is not a common practice, especially P fertilizers [9], so there is need to give more emphasis on appropriate management of P resources, as existing P reserves are limited and rapidly going to be used up. However, to cope up with the increased concentration of atmospheric CO_2, there is need to increase the forest productivity which results in depletion of P in surface soil and in response to P insufficiency, trees roots may mine soil deeper to acquire the same. Thus, understanding the P dynamics in soils is necessary to know the processes governing P availability. This manuscript synthesizes the available information regarding P content, factors affecting its dynamics and different fractions present in soils.

Phosphorus (P) is one of the most important macro-nutrient after nitrogen in terrestrial ecosystem productivity [10]. Phosphorus is an essential element and plays an important role in the functioning of all living bodies because, as it is the structural component of nucleic acids, co-enzymes, phosphoproteins, phospholipids and also determines many metabolic processes (provides energy as ADP and ATP). Low solubility of natural P-containing compounds and the slow natural cycle of P are the major constraints to check the availability of this essential nutrient and efficiency of the ecological unit [11-12].

2. FORMS OF PHOSPHORUS IN SOIL

Phosphorus in soils mainly comes from parental rock and fertilizers [10-13]. In soil, there are two major forms of P, inorganic and organic. Inorganic P forms are associated with hydrous sesquioxides and Al and Fe compounds in acidic soils whereas with Ca-compounds in alkaline soils. The inorganic phosphates in soils have been classified into easily soluble phosphate (ES-P), aluminium phosphates (Al-P), iron phosphates (Fe-P), reductant soluble phosphates (RS-P) and calcium phosphates (Ca-P) [14]. According to Brady and Weil [15], organic matter, calcium carbonate and sesquioxides are the key factors, controlling the distribution of different forms of P. Organic P (P_o) can account for 5-95% of the total P (TP) in the soil. P_o is derived mainly from manures, plant material, and products of microbial decomposition. P_o is highest under wetland soils, as characterized by high organic matter. Although a large proportion of TP occur in organic form, of which, only a small portion of this pool may be bioavailable.

There are many chemical fractionation schemes developed to assess the specific P form [16-17]. After that, Bowmen and Cole [18] developed method to fractionate various P_o forms. But there are some difficulties in identifying specific inorganic (Pi) and organic (Po) forms which include: modification of unidentified compounds from their original forms and also effects of the reagents on pure compounds and mineral associations [19-21]. To overcome these problems, Hedley et al. [22] developed a sequential fractionation scheme to differentiate available and non available form. This method has more advantages; like, extraction of both Pi and Po forms, extraction of microbial P during the process. Despite the limitation of time requirement and complexity, this method is more reliable and been in use from last 30 years. Major fractions, which can be extracted by this method, are: Resin P, Bicarbonate P, Hydroxide P, Acid P and Residual P (further description given in Table 1).

3. PHOSPHORUS CYCLING OR DYNAMICS

Phosphorus, one of the essential macro-nutrient limiting plant growth and development, especially in subtropical and tropical region [10, 23]. Major pools of P are present in terrestrial ecosystems, which generally account 100-3000 kg ha^{-1}, so its cycle is also termed as sedimentary cycle [11, 12, 24]. Sparingly soluble calcium phosphate i.e apatite, in rocks and other deposits are the major source of P in terrestrial ecosystems [10, 25]. Primary minerals of P, present in stratum rock are apatite, hydroxyapatite, and oxyapatite and their chief characteristic is that, they are water insoluble. But, in spite of this fact, they are also the principal source of P and under suitable environment, they can be solubilized and become available for living organisms. Inorganic phosphate are also found, in soils having higher or lower soil pH and P is rapidly converted to sparingly soluble amorphous and crystalline compounds, i.e. Ca^{2+} and Mg^{2+} phosphates in neutral to alkaline soils; variscite (Al-P) and strengite (Fe-P) in acid soils [26], which belong to the slowly cycling P pool and are not directly

available to biota [27]. Second major component of soil P is organic matter. The organic P pool, accounts for 15-80% of the total P pool [28-29] and can be greatly influenced by the quantity and quality of organic inputs and shifts in soil microbial community structure [30-32]. Organic P in soil is largely in the form of inositol phosphate, synthesized by microorganisms and plants and forms the most stable form of organic P (50% of the total organic P) in soil [33]. Soil P which occurs in equilibrium with the soil solution (bioavailable P) is referred as 'Labile P' and other P forms which are slowly available to plants are known as 'non-labile' [34].

Table 1. Forms of phosphorus extracted by Hedley et al. [22].

Sl. No	Form extracted	Form of P	Availability to plant
1.	Resin Pi	Adsorbed on surface of crystalline compounds	Soluble and easily available
2.	Bicarbonate - Pi	Adsorbed on surface of soil compounds	Available and remain in equilibrium with the soil solution
	Bicarbonate - Po	Labile Po inside the internal surfaces of soil aggregates	Available after mineralization and remain in equilibrium with the soil solution
3.	Hydroxide - Pi	Adsorbed on surfaces of secondary mineral (Al and Fe-P)	Low plant availability
	Hydroxide - Po	Extracts Po that is strongly held by chemisorption to Al and Fe components in the soil	Stable P involved with the long term transformation of soil P
4.	Acid - P	Associated with Ca and occluded within sesquioxides; acid extractable	Stable and low solubility
5.	Residual- P	Occluded and most recalcitrant P	Most stable, highly resistant and low bioavailability

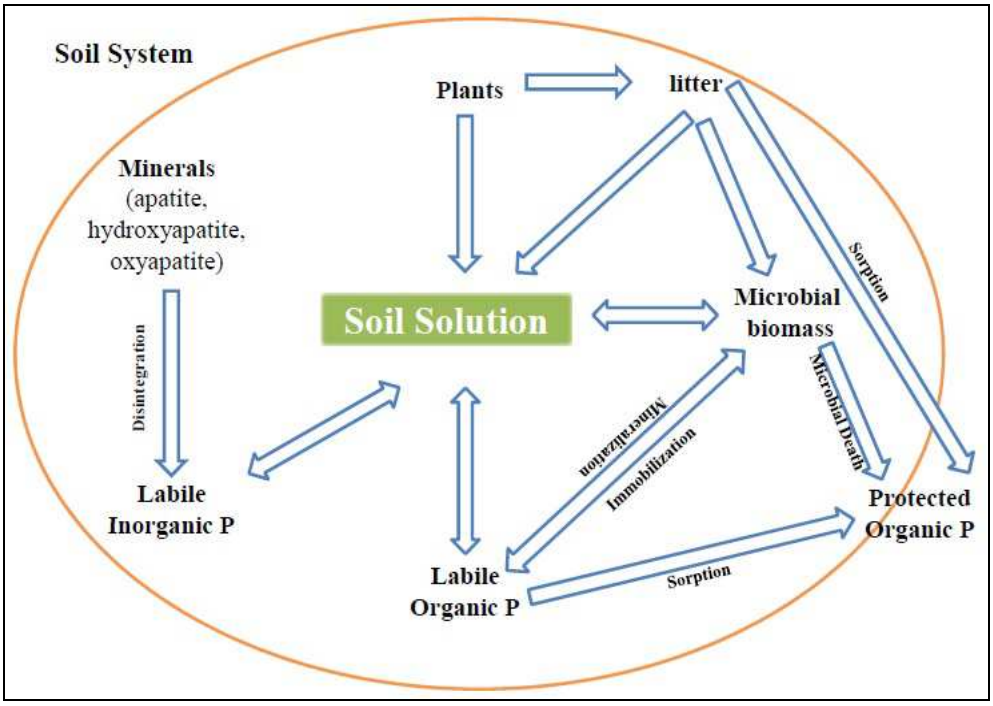

Figure 1. Phosphorus cycle in soil.

Phosphorus cycling or dynamics in soil can be defined as a series of processes influenced by the nature of the inorganic and organic solid phases present, the type and intensity of biological activity, the chemistry of the soil solution (pH, ionic strength, redox potential), and abiotic factors like texture and moisture content, [11, 34-35]. The natural P cycle starts with the disintegration (physical, chemical and microbial) of primary apatite P rocks and here, microbes play key role (oxidation and reduction of phosphorus compounds) so we can call them "forerunner of P cycle". After weathering, P comes in soil solution and incorporates into the system as different secondary Pi and Po form, which are of limited availability [10]. These forms are inter-exchangeable via different chemical and biochemical (sorption-desorption, oxidation-reduction and mineralization-immobilization) processes [11, 34].

3.1. Phosphorus cycling in agriculture ecosystem

P cycling is continuous in nature and governed by the need of users while in crop field it is disturbed due to addition and removal in the form of fertilizers and crop produce, respectively. It is quite necessary to understand P dynamics in agricultural soil, for managing the P usage, its consumption by roots according to their potential and ultimately to increase P use-efficiency by plants. P cycling in soil is governed by some biotic and abiotic factors, including adsorption, dissolution and microbial activity, respectively [36]. Mineralization of Po and its cycling is the main factor on which availability of P to plant depends [37]. Effect of P fertilization on availability of soil P had been studied from last century. Now, it is established that soils not getting P fertilizer had low total P (TP) while fertilized soil had have high TP [38-40]. Application of P fertilizers increases the inorganic P content [41-42] while addition of organic sources increases organic P content [43]. There is more inorganic (63 to 92%) P than organic (5 to 25%) P in manure and application of manure produces positive effect on content of P fractions in soil [44]. Relevance of manures has considerable impacts because there is progressive turnover of P into other forms [45] and higher application of manures increases the amount of labile Po, moderately labile Po, moderately resistant Po, highly resistant Po, Al-P, Fe-P, O-P and Ca-P in soil [46]. Application of P fertilizers will surly give more yield but it may also have long term effect on the P fractions [47] and especially labile Po pool in soil [48].

Lots of work have been done in past to study the effect of organic and inorganic P application on yield of crop, solely or in different proportions, their effects on different P pools, soil modification like pH, tillage and application of microbial inoculants just to increase the P use efficiency. Agricultural practices can also contribute in the composition of soil P like, the content of organic P in soil heavily depends on cropping system and tillage depth than the fertilizer used [49]. According to McLauchlan [50], tillage and crop removal have the tendency to reduce organic C of soil and concentration of the organic P in soil is directly proportional to organic matter content of soil [48, 51]. However, no-tilled surface soils have higher amount of organic C and available P in comparison to conventionally tilled soil [52], due to non-incorporation of applied P fertilizers. But in heavy soils like clayey ones, competition is there between organic anions and PO_4-P for the same sorption sites so, the availability of P is enhanced [53]. Another important factor which plays a role in deciding the P cycling is rhizosphere, association between plant roots, soil and microbial activity; where different exudates such as mucilage, organic acids, phosphatases modify the soil environment. According to Marschner [54], roots can decrease the pH of rhizoshpere by 2-3 units and increase the P availability. Rhizosphere pH can also be changed by uptake of cation and anion like in case of nitrogen, where ammonium uptake causes acidification while nitrate causes alkalization. pH change in the rhizosphere is mainly affected by uptake ratios and nitrogen assimilation. Now in recent years rhizospheric P management became a novel approach and Jing et al. [55] reported that by using P plus ammonium, maize growth improved in a calcareous soil due to rhizosphere acidification. Similarly, faba bean (*Vicia faba*) can also acidify its rhizosphere [56]. Rhizospheric microorganisms like arbuscular mycorrhizal fungus (AMF), phosphorus solubilizing microorganisms (PSM) and plant growth promoting rhizobacteria (PGPR) are also

known to increase P cycling [57] and it has been found that combined usage of AMF and PSM showed the positive response in P uptake [58].

These results with respect to the effect of different strategies on P cycling or improvement are not clear-cut and it can be supported with the findings of Jalali and Ranjbar [59] who suggested that different P pools, like Ca-P, Fe-P, Al-P and organic P, are highly active and their content depend on the actual properties of the soil. So management practices for increasing the organic C should be imparted in field to maintain availability of P. Many studies are there on the effects of pH modification on P availability, but a consistent plan to manage soil phosphorus for sustainable crop production and to minimize P loss from soils have not been fixed. Different forms of P are available in soil due to the inherent properties of soil, which are not available to plants and changing these characteristics of soil on long term basis may be difficult to achieve.

3.2. Phosphorus cycling in forest ecosystems

Soil nutrients are the key drivers of any ecosystems, however in forest ecosystems; they play an important role in development and maintenance of the ecosystem sustainability [60-61]. In forests, the nutrient cycle is maintained by itself, as there is development of thick forest floor due to addition of litter. Litter fall is in form of branches, leafs, bark and fruit, which contain an appropriate amount of nutrient and by their decomposition nutrient are returned back in soil [62-63]. But released nutrients may be immobilized or mineralized, depending on the site conditions [62-64]. Nutrient use efficiency in any forest depends on the amount of nutrients content in litter, root and woody biomass of trees [65]. In areas having permanent vegetation, like forests, Po fractions are present in higher proportions [29, 66]. According to Chen et al. [67], amount of dry matter produced per unit of P is scientifically inferior in temperate forests as compared to tropical ones. There is always difference in organic matter deposition and nutrient cycling, between forests and other ecosystem, because, both affect the mineralization and immobilization processes and show significant impact at ecosystem level.

First report on P dynamics in forest ecosystem was published by Fisher and Stone [68], they observed that under pine plantation mineralization of organic P was higher as compared to the adjacent abandoned fields and larch plantations. In New Zealand, several workers also reported that under recently established forest, there was increased mineralization of organic P but the level of microbial biomass P and enzyme activities responsible for organic P mineralization is lower [67, 69]. This may be attributed to lower inputs of organic matter and in addition due to decrease in soil pH [67]. Davis [69] also found that concentrations of total and organic P were lower under the *P. radiata* stand, which is attributed due to enhanced nutrient uptake and decompostion of organic matter by the pines. Chiu et al. [70] reported that concentration of bioaviliable inorganic P was greater in soils under Chinese hemlock (*Tsuga chinensis*) as compared to the dwarf bamboo (*Yushania niitakayamensis*) and in NMR analysis, they found that inorganic orthophosphate monoesters was the major forms of P extracted by trees. Decline in the content of orthophosphate monoesters under pine vegetation is mainly due to the utilization of these compounds by conifers through root-microbe symbiotic interactions [67, 71]. Plant root-microbial association is important activity in any terrestrial ecosystem, because it plays most vital role in alteration or decomposition of soil organic matter and release of associated nutrients. Roots are the secretors of various exudates in form of chemical compounds into the soil [67, 72], which become signals for microbes to initiate the transformation process of soil organic matter and associated nutrients [11]. There are many reports defining the ability of different bacterial species (*Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Microccocus, Aerobacter,* etc.) to solubilize insoluble inorganic phosphate compounds [73]. Microorganism associations like mycorrhizae are known to modify root structure and their functions also [74-75] and mediating the availability of soil P to associated plants. There are ample reports suggesting that mycorrhizae releases low molecular organic acids such as citric, oxalic, maleic, and acetic acid, to solublise the organically bound P [76]. Chen et al. [67] also reported that mineralization of organic P was higher under pine

forest due to the symbiotic association between pine roots and ecto-mycorrhizae. As ecto-mycorrhizae, releases organic acids and cause acidification of the rhizosphere zone, thus promoting the solubilisation of inorganic and organic P.

The studies mentioned above provided background information about the studies conducted by the various researchers. Therefore, from the above studies, it has been concluded that, in forests there is dominance P_o (Table 2), its mineralization mainly depends on microbial aspects and availability or transportation from soil to plant is governed by mycorrhizae. However, it can also be assumed that in P scarcity, trees have to absorb from inside the deeper layers of soil to take more nutrients and in this case, there is more below ground development of roots which may hamper the aboveground canopy development. To overcome this, there is need to improve our knowledge regarding to the processes controlling P availability in surface and deep soil layers. There is also need to develop certain strategies which can enhance the mineralization of P_o, as it is the major P pool in forest ecosystem.

Table 2. Literature reports on soil P fractions (mg kg^{-1}) in surface soil (0-15 cm) under different vegetation types.

Sl. No	Study Area	Vegetation type	pH (H_2O)	SOC (g kg^{-1})	TP	Po	Pi	MBP	Reference
1.	Daqinggou National Nature Reserve, Inner Mongolia, China	Elm (*Ulmus macrocarpa*) savanna with dense grasses	7.3	8.9	149.0	94.8	54.3	4.78	[77]
		Grassland	6.5	3.6	107.0	70.7	36.3	2.89	
		Mongolian pine plantation	6.7	4.0	79.9	47.7	32.2	2.69	
		Chinese pine plantation	6.7	3.5	73.1	38.6	34.5	2.10	
		Poplar plantation	6.7	4.3	109.5	69.7	39.9	3.53	
2.	Qingyuan Experiment Station, Institute of Applied Ecology, China	Natural secondary forest	5.82	50.45	741	475	272	40.3	[78]
		Larch *(Larix olgensis*) plantation	5.55	34.70	1025	543	481	26.1	
3.	Rio Paja Forest plot, Panama Canal watershed, central Panama	Tropical rain forests	3.55	-	45	27	18	-	[79]
4.	Campo Chagres Forest plot, Panama Canal watershed, central Panama	Tropical rain forests	7.00	-	824	494	330	-	[79]
5.	Cave Stream forest, Craigieburn research area, central south island, New Zealand	Mixed stand of Ponderosa pine (*Pinus ponderosa*) and Corsican pine (*P. nigra*)	-	-	839	552	287	37.4	[76]

SOC: soil organic C; TP: total P; Po: organic P; Pi: inorganic P and MBP: microbial biomass P

3.3. Phosphorus cycling in grassland ecosystems

Grasslands, a biological community, characterized by mixed herbaceous (non-woody) vegetation cover, with high biodiversity due to high plant species diversity [80-81]. Plant diversity is a key element in grasslands because: increased forage production [82-83], stability against disturbances [84] and also improves nutrient cycling [82]. In grasslands, nutrient addition includes atmospheric inputs, fertilizers and animal feed while removal of nutrients through animal product, harvested forage and via off-site nutrient transport including leaching and surface runoffs [86-88]. Among the nutrients, P has a tremendous influence on species richness [89] after nitrogen. In grassland soils, total P content

varies between 200 to 1100 ppm and concentration depends upon the age of soil [90]. According to Kemp et al. [87], in grasslands or pastures, most of the nutrients taken up by plants are returned to the soil in the form of litter and root residues (10-70%) or animal excreta (50-95%). In grazed pastures, cycling of P is secured in comparison to N, as it is less soluble and mobile, so surface runoff is a major loss pathway through which P is lost Whitehead [91]. According to Parfitt [92], from intensively fertilized plot receiving 38 kg P ha^{-1}, 4 kg P ha^{-1} yr^{-1} could be lost due to runoff. Timmons and Holt [93] reported that from an ungrazed, unfertilized, native prairie in Minnesota, P loss through runoff is 0.1 kg P ha^{-1} yr^{-1}. The plant mycorrhizal symbiosis absorbs P from the soil solution, which comes from hydrolysis of labile ortho-P or mineralization of Po [94]. Plant litter and animal excreta are the main source of P in grazed pasture. Whitehead [91] reported that only 100 to 250 g P kg^{-1} of P in the diet of animals is converted into live-weight gain or milk, while rest is recycled to soils in form of plant residues and animal excreta, so mineralization of organic compounds is the key process in grasslands with respect to the P dynamics. There are more chances of net P immobilization in tropical grasslands because P content in grasses is < 2.0 ppm [95].

Land use change is the key activity which can alter the rate of mineralization or cycling of P in grasslands. The most common land use change occurred in all over the world is the afforestation of grasslands with conifers and this enhances the rate of mineralization of organic matter and associated P and hence, P availability in topsoil is increased [67]. Till date, most of the work has been focused on changes in land use and its effect on P cycling. There is need to know, what changes can occur in soil microbial community due to the particular land use change [67, 96-97]. We can't ignore the changes in the structure and activity of soil microbial community, as they are the fore-runners or key drivers in the mineralization of P.

4. VARIABLES INFLUENCING P DYNAMICS IN TERRESTRIAL ECOSYSTEM

Phosphorus occurs in soils in various forms, organic and inorganic which can be further divided into labile and non-labile P [98]. Therefore, soil P can be considered in terms of 'pools' of varying availability to the plants. A major portion of soil P exists as insoluble and fixed forms including primary phosphate minerals, humus P, microbial biomass P, insoluble phosphate of Ca, Fe and Al and also P fixation by hydrous oxides and silicate minerals. This fraction is known as non-labile P and is the largest pool of soil P in terms of quantity [99]. Whereas, labile P is the readily available fraction that exhibits a high dissociation rate and is in rapid equilibrium with solution P [99-100]. Soil P moves among these pools and remains in continuous dynamic equilibrium. Phosphorus may also move among pools as shown by the conversion of organic P into inorganic P via mineralization by microbial and root-released phosphatases [101]. The factors which influence these equilibrium reactions are discussed in the following sections.

4.1. pH

In highly weathered soil solution, P concentration primarily depends upon the soil pH levels that indicate how certain minerals iron and, aluminum, interact with phosphorus in the soil, and it is the interaction that affects the phosphorus availability in soil [102]. The inorganic P compounds mainly couple with amorphous and crystalline forms of Al, Fe, depending upon the acidity of the soil [22]. Because surface adsorption of P increases with decreasing pH, these adsorption processes would often be expected to be more influential at low pH [103] resulting in a "positive" pH dependence (i.e. increased solution P level at higher pH), provided that adsorption is fully reversible within the time scale of interest. However, precipitation of solution P with Ca is expected in calcareous-alkaline soil with higher pH. A number of Ca-P minerals may form, such as amorphous calcium phosphate (ACP), octacalcium phosphate (OCP) and apatite (hydroxyapatite or fluorapatite). Precipitation/dissolution of these minerals will cause "negative" pH dependence (increased solution P level at lower pH) [104]. Murrmann and Peech, [105] performed back titrations for two soils and found decreasing P solubility with increasing pH until about pH 5.5 to 6, at which pH minimum solubility occurred.

At higher pH, P was more increasingly dissolved again. At very high pH, however, (>8-9) P solubility decreased due to Ca-P mineral precipitation.

4.2. Nature and amount of clay

4.2.1. Hydrous oxides of Fe/Al

Fe/Al oxides and hydrous oxides are abundant in acid soils and high P retention in these soils is attributed to active Al and Fe associated with organic (mainly Al-humus complexes) and mineral fractions (ferrihydrite), which form in the course of soil development [100, 106]. These oxidized secondary minerals can bind P making it temporarily unavailable for plants and microbes through the formation of labile and non-labile P [107-108]. P ions bind to the Fe/Al oxide surface by interacting with OH- and/or OH^{2+} groups on the mineral surface in two steps, a mononuclear adsorption followed by a binuclear

4.2.2. Calcium carbonate

The solubility of P in Ca rich calcareous soil is mainly controlled by the solid phase dicalcium phosphate of chemisorption of P on calcite, with the formation of a surface complex of calcium carbonate bound P with a defined chemical composition [109]. Impure and/or, amorphous calcium carbonate with large specific surface area exhibits greater P adsorption and more rapid precipitation of Ca-P minerals. Calcareous soils with highly reactive calcium carbonate and high Ca-saturated clay content will exhibit very low solution P levels, since P can readily be precipitated or adsorbed [100]. The lower the Ca:P ratios of the Ca phosphates the higher their solubility in water. The equilibria of Ca phosphates from solution P to the highly insoluble hydroxyapaptite is shown below [110].

$H_2PO_4^- + Ca^{2+} \Leftrightarrow CaHPO_4 + H^+$

$3\ CaHPO_4 + Ca^{2+} \Leftrightarrow Ca_4H(PO_4)_3 + 2H^+$

$Ca_4H(PO_4)_3 + Ca^{2+} + H_2O \Leftrightarrow Ca_5(PO_4)_3OH + 2H^+$

From these equilibria it is clear that H^+ promotes solubility of Ca phosphates in the soil and Ca^{2+} has the reverse effect in calcareous soil. The hydroxyapaptite formed in this reaction has very low water solubility, thereby depleting the solution P concentration to the greatest extent.

4.2.3. Silicate minerals

Soils derived from volcanic ash (Andisol soil) are characterized by unique property of high phosphorus retention capacity, with main constraint for plant growth being usually the low solution P and its availability [106, 111]. Allophones (Si-Al-Fe-O-OH-OH_2) have a large surface negative charge which is partly or, entirely balanced by the complex aluminium cations. Phosphorus gets adsorbed by reacting with such aluminium cations [112]. In this way, some phosphate of the labile pool is continuously being transferred to non-labile P and thus becomes immobile.

4.3. Soil organic matter (SOM)

SOM is the major source of organic P pool and that, in highly weathered and high P-sorbing soils, the P maintained in organic pools may be better protected from loss via fixation than by P flowing through inorganic pools [113]. The organic P compounds are associated with rapidly to slowly decomposable organic molecules, such as nucleic acids, phospholipids, sugar phosphates, inositol phosphates, and recalcitrant humic substances [22]. Different organic anions produced from OM decomposition form stable complexes with Fe/Al, preventing the formation of non-labile P by reacting with phosphate anions. These complex ions exchange for P are adsorbed on Fe/Al oxides. Anions such as oxalate, citrate, tartrate and malate are found to be most effective in doing such [100]. In addition to that, SOM may be sorbed to soil particles at non-specific sorption sites, which would increase the surface negative charge of the particle. This would reduce the electrostatic attraction of P to the soil and keep more P in solution [114].

4.4. Microbial biomass

The soil microbial biomass plays a central role in soil phosphorus dynamics, especially in the dynamics of soil organic P [115]. The soil microbial biomass has two main roles in the dynamics of P in soil: i) the principal driver for the transformation of organically-bound phosphorus to plant-available phosphate (solution and labile P), and ii) the accumulator of a significant pool of P [116].

Additionally, microbes indirectly affect P availability by changing the soil pH and via organic molecules released during decomposition of organic materials (Fig. 2), which may block P sorption sites and complex Fe, Al and Mn [107,117]. Microbial biomass P responds rapidly to the addition of C substrate to the soil. In the short term, net mineralization will occur if the amount of soluble P in added residues is in excess of that taken up by the microbial biomass. However, residue P content is often insufficient to meet the requirements of the growing microbial biomass. Under such circumstances, the microbial biomass will take up P from the solution and labile pools in soil; leading to net immobilization of soil P, thereby depleting those pools of soil P [116]. In addition to that, the microbial biomass has a high capacity to acquire P from non-labile pools that are generally not considered to be plant-available, and will be more competitive than plants for solution and labile P [118-120].

4.5. Anaerobic condition

Under anaerobic conditions, reductive dissolution of ferric hydroxides carrying P is an important mechanism of P release into the solution [121]. Thus, redox status of a soil is important determinant of the potential role of a soil to retain P. Other mechanisms include dissolution of occluded P, which increases the mineralization of organic P in acid soils, and also increases the solubility of Ca-P in calcareous soils, and maximizes P diffusion [100]. Alternate drying/rewetting, freezing/thawing, and associated microbial activity tend to destroy organo-mineral complexes and kill microorganisms, often resulting in releases of dissolved phosphorus from the affected soils [42].

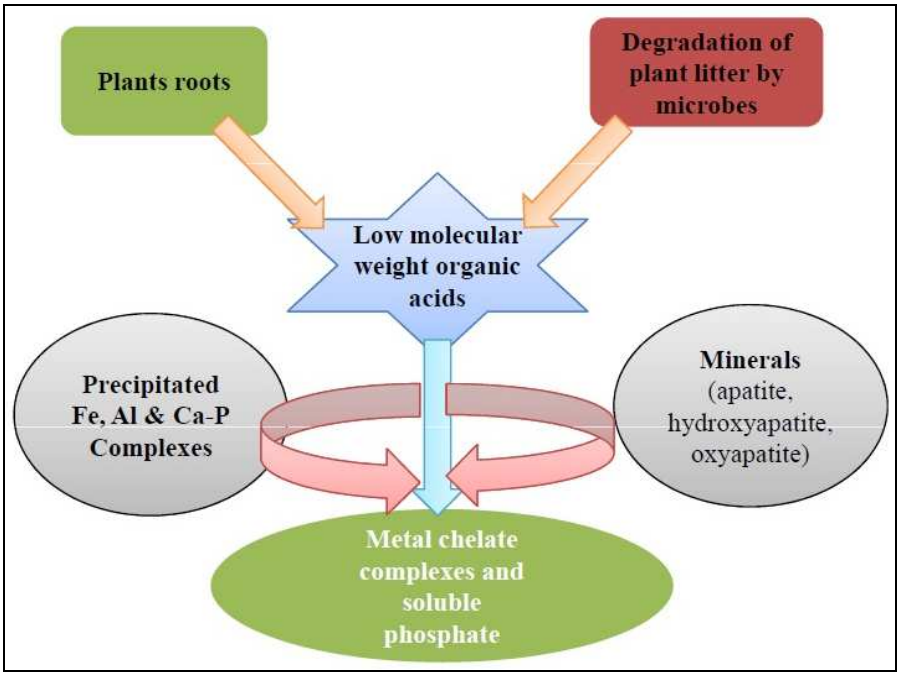

Figure 2. Release of P through the action of low molecular weight organic acids and other naturally occurring chelates.

4.6. Plants

Absorption of P by plant roots causes depletion in the solution P concentration, and labile P rapidly replenishes the solution P, but at a very slow rate depletion of labile P causes some non-labile P to become labile. However, the depletion rate of different P fractions in the root rhizosphere varies significantly among different plant species and different genotypes within a given species [22, 123-125]. Rhizospheric pH may be changed by imbalance uptake of cations and anions by plants, which can affect the P dynamics in the soil [126]. Organic anions secreted from plant roots (Fig. 2),

increase the solution P by desorbing inorganic P (labile P) from a mineral surface and chelating or complexing cations, such as Al^{3+}, Fe^{3+},and Ca^{2+} ions that are bound to non-labile P [127]. Some enzymes secreted from plant roots, such as phosphatase, can catalyze hydrolysis of organic P. It has been suggested that higher phosphatase in the rhizosphere, compared to the bulk soil, can induce significant depletion of organic P in the rhizosphere [124, 128].

Management of soil P bioavailability is one of the main challenges for many regions of the world. The main processes and/or factors controlling soil P bioavailability are P interactions with Al, Fe, and Ca hydrous oxides, amorphous, and crystalline complexes, along with organic P mineralization [129]. The rate and extent to which these processes occur are greatly influenced by agricultural management practices including rate of P fertilization, nature of fertilizer, and method of fertilizer addition, tillage, and drainage etc. The phosphatic fertilizer in current use scenario requires a greater input that cannot be afforded by the small to marginal farmers of the developing nations. Therefore, improved methods of phosphate application like application in granular form or, as bands in close proximity to the roots and fertigation, as well as liming acid soils, can definitely increase soluble P in soil and provide enough time to crops for its uptake and, reduce the influence of these factors on P availability in soil. Thus, these management practices can reduce the rate of expensive superphosphates application and maintain better soil health and sustainable production in terrestrial ecosystem.

5. LONG-TERM ECOSYSTEM MANAGEMENT

Primary productivity of any ecosystem depends on nutrients; like in terrestrial ecosystems, nitrogen (N) and phosphorus (P) are the most common limiting elements, both individually and in combination, while in aquatic ecosystem, P become most problematic as it causes eutrophication. In agriculture ecosystem, strategic P addition is important and it should be based on quantity of P available in soil, how much is going to be fixed and upto what extent crop can take. Combination of strategic P application and germplasm with high uptake capacity will provide agricultural sustainability, better P status in soil and increased P use efficiency. In forest ecosystem, litter produced is the major source of P, during the decomposition of woody debris by microbes, P is released in the soil. But in grassland ecosystems, long term accumulation of animal excreta is the major source of P and represents serious environmental concern. Quite well-organized nutrient recycling can be promoted in grazing land systems by using efficiently organized strategies like regular shifting of animal feed, supplying sufficient fertilizers and maintaining suitable population of animals can potentially improve P use efficiency by plants and restrict environmental pollution. In both, forest and grassland ecosystems, P cycling depends on the decomposition of organic matter and it can be restricted due to immobilization by plants and animal production. It can be assumed that to overcome the reduction there is release of P from organic pool or from weathering of rocks. To maintain the P cycling, intermixing of grassland and forest can be done. It will also lead to greater productivity of grasses and subsequently improved SOM and structural integrity. There is need of long term comparative studies on strategic P inputs, improved methods for P application and P efficient germplasms in agricultural ecosystems, controlled grazing practices and impact of intermixing of grassland and forest ecosystems on soil health and P cycling, as it will provide better solutions for P management.

6. FURTHER RESEARCH

In future, studies will be focused on mechanisms to increase the P use efficiency and associated processes under different ecosystems:

- Rate of P release from root and leaf litter inputs and its efficient utilization;
- Changes occurring in soil microbial community under different ecosystems using nucleic acid based techniques, including production of low molecular weight organic acids and their transport processes;
- Relationship between plant root and VAM associations;
- Understanding the effects of subsurface placements of P resources under different ecosystems

to arrest the P export and eutrophication;

- Development of mathematical models simulating temporal changes in residual soil and organic P;
- Practices to control export of P at their source, as it is most beneficial and effective.

7. CONCLUSION

Increased human activity influences the nutrient cycles in ecosystems as agriculture and forestry removes nutrients from these ecosystems and also increases the transport of P to aquatic ecosystems. There is a considerable association between the type of land use and export of P, as it has been proved that the alteration of forest into agriculture ecosystems quadruples phosphorus export. The key factors for controlling P export are geology, land use, SOM, pH and microbial aspects and by using these, it is easy to predict the P export and cycling within any ecosystems. However, heavy use of P fertilizers accelerated the problem of eutrophication, so there is need of efficient practices to increase the use of accumulated surface P, estimating P bioavailability in soil and improved methods of runoff control to control P export into aquatic ecosystems. There is need of information with respect to the effects of conservation on P cycling in long term basis. There are models available which simulates the changes in P availability in short times, using first order kinetics, but does not for long term changes. Therefore, use of these models is limited in order to estimate the loss of P and research should be directed towards the development of model, to recognize well-organized soil and management practices that may increase P use efficiency and reduces the export of P into water bodies.

AUTHORS' CONTRIBUTION

All authors contributed equally for the success of this review article. The final manuscript has been read and approved by both authors.

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interests regarding the publication of this paper.

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