ISSN 2449-8955
EJBRAT 8(2) 2018

Volume 8
Number 2
April-June 2018

European Journal of Biological Research

MNiSW points 2017: **11**
Index Copernicus 2017: **100**

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European Journal of Biological Research

ISSN 2449-8955

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ISSN 2449-8955

European Journal of Biological Research

Research Article

Diversity of inulinase-producing fungi associated with two Asteraceous plants, *Pulicaria crispa* (Forssk.) and *Pluchea dioscoridis* (L.) growing in an extreme arid environment

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Received: 15 January 2018; **Revised submission:** 09 March 2018; **Accepted:** 21 March 2018

DOI: http://dx.doi.org/10.5281/zenodo.1205649

ABSTRACT

Inulinases are potentially valuable enzymes catalyze the hydrolysis of plant's inulin into high fructose syrups as sweetening ingredients for food industry and ethanol production. The high demands for inulinase enzymes have promoted interest in microbial inulinases as the most suitable approach for biosynthesis of fructose syrups from inulin. Arid land ecosystem represents a valuable bioresource for soil microbial diversity with unique biochemical and physiological properties. In the present study, we explored the fungi diversity associated with the rhizosphere and rhizoplane of two desert medicinal plants namely *Pluchea dioscoridis* and *Pulicaria crispa* growing in the South-Eastern desert of Aswan, Egypt. A total of 180 fungal isolates were screened based on their ability to grow on potato dextrose agar medium supplemented with 1% inulin. The isolated fungal colonies were morphologically identified according to cultural characteristics and spore-bearing structure. In addition, the inulinase activity of the isolated fungi was examined spectrophotometrically. Among these, *Aspergillus terreus* var. *terreus* 233, *Botrytis cinerea*, *Aspergillus aegyptiacus*, *Cochliobolus australiensis* 447 and *Cochliobolus australiensis* exhibited high inulinase activity ranging from 5.05 to 7.26 U/ml. This study provides a promising source of microbial inulinase, which can be scaled up for industrial applications.

Keywords: Microbial inulinase; Arid land; *Pluchea dioscoridis*; *Pulicaria crispa*.

1. INTRODUCTION

Fungi are a diverse group of microorganisms comprising seven known phyla, including Ascomycota, Basidiomycota, Microsporidia, Glomeromycota, Blastocladiomycota, Chytridiomycota and Neocallimastigomycota [1]. Fungi communities are essential soil components as both decomposers and plant symbionts, playing critical roles in the ecological and biogeochemical processes in natural environments [2-5]. Fungi communities can undergo

transitory variations in its structure due to the selective pressure exerted by the surrounding environment [6, 7]. For example, it is well known that production and diffusion of root exudates are affected by plant health and development, and these exudates exercise a selective microbial stimulation/ inhibition that varies according to the unique rhizosphere nutrient pool [3, 5, 7, 8]. In this context, plant roots represent a favorable habitat for diverse fungi populations, including those colonize the soil zone around the root (Rhizosphere) as well as the root surface (Rhizoplane) [8-10]. Rhizosphere and rhizoplane areas are always affluent in various fungal populations with vigorous activities in close association with host plant [11]. Therefore, the co-evolution of plant roots and soil fungi plays a significant role in soil physical and biological processes that increase biodiversity and soil fertility in agricultural and natural systems [3, 4, 7].

Inulin is a widespread naturally occurring storage polysaccharides found in the roots of several plants belonging to Gramineae and Asteraceae families [12, 13]. Inulin composed of fructose unit chains with various length, linked by β-2,1-D-fructosyl-fructose bonds, and generally terminated by a single glucose unit connected by the α-D-glucopyranosyl bond [13, 14]. Inulinases (2,1-β-D-fructan fructanohydrolases) are potentially useful enzymes catalyze the conversion of plant inulin into high fructose syrups as sweetening ingredients for the food industry and ethanol production [13-15]. Although inulinases were first isolated from plants, it is very difficult to separate plant inulinase in sufficient quantities for industrial and biochemical applications [12]. In addition, the acid hydrolysis of inulin to fructose displays several drawbacks, which resulted in difructose anhydrides as a final result with no sweetening properties [12, 16]. These drawbacks have forced interest in microbial inulinases as the most suitable method for biosynthesis of fructose syrups from inulin [13, 14, 17]. There are several fungal species can produce inulinase enzymes, among which, *Aspergillus*, *Penicillium*, and *Khyveromyces* species are the most common fungi that used for inulinase industrial production [18]. The increasing potential of inulinase applications promoted the screening of new inulinase-producing fungi with high thermostable character. In the present study, we isolated, identified rhizosphere and rhizoplane fungi from two desert medicinal plants including *Pluchea dioscoridis* and *Pulicaria crispa* growing naturally in the South-Eastern desert of Aswan governorate, Egypt. The isolated fungi were screened based on their ability to grow on potato dextrose agar (PDA) medium supplemented with 1% inulin as the only carbon source, followed by quantitative analysis of their inulinase enzymatic activity using spectrophotometric analysis. The obtained results demonstrated the potential of the desert-adapted plants like a rich bioresource for various fungal stains with high inulinase activity, which can be used for industrial purpose.

2. MATERILAS AND METHODS

2.1. Study area

This study was performed at Aswan University, Aswan governorate, Egypt (24° 5' 15" N 32° 53' 56" E). The rhizosphere and rhizoplane samples from *P. dioscoridis* and *P. crispa* were collected from three different locations including Aswan University campus (Location I, sites 1-6), Aswan airport road (location II, sites 7-8) and Aswan dam road (Location III, sites 9-10). The climatic conditions in this region ranged from very hot dry summer (30 to 50°C) to moderately cold dry winter (10 to 25°C) [3].

2.2. Preparation of rhizosphere and rhizoplane samples

In each study site, three replicates were established with one-meter distance from each other, at 20-35 cm depth from the surface. *P. dioscoridis* and *P. crispa* roots were carefully uprooted, and roots with adhering soil particles were placed in sterilized plastic bags and transported to the laboratory. The rhizosphere samples were collected according to Timonin [19], and Moubasher and Abdel-Hafez [20]. One gram of roots was lightly scraped to collect the firmly adhering soil particles to the root surface by using **s**terilized hairbrush and spatula. The collected rhizosphere-soil samples were subjected to serial dilution (10^{-4}) using sterile distilled water (SDW). One ml of the rhizosphere soil suspension was transferred to PDA plates amended with chloramphenicol (500 mg l^{-1}). The

plates were incubated in an incubation chamber at 28 ± 1°C for 7 days. The number of fungal colonies (cfu g^{-1} soil) formed on PDA dilution plates in each sample was calculated.

The rhizoplane samples were collected from plant roots by washing one-gram of roots with SDW and cut into approximately 1 cm pieces. Root pieces were allowed to surface-dry under sterile conditions prior to placement into PDA plate. Five pieces of root were placed on PDA plates amended with chloramphenicol (500 mg l^{-1}) and incubated at 28 ± 1°C for 7 days [21]. The number of fungal colonies (cfu g^{-1} root) formed on PDA dilution plates in each sample was calculated.

2.3. Identification of fungi

All the isolated fungal colonies were morphologically identified according to cultural characteristics (color, texture, and pigmentation), and spores and spore-bearing structure using standard identification manuals [22-28].

2.4. Screening of inulinase-producing fungi

All isolated strains from the rhizosphere and rhizoplane samples were screened for their ability to grow on Czapek's agar medium supplemented with inulin as the only carbon source and were incubated at 28 ± 1°C for 7 days. A total of 180 isolates were able to grow on inulin-Czapek's agar medium, and these strains were further used for inulinase assay.

2.5. Inulinase assay

A loop of the selected fungal isolates was inoculated separately in 5 ml fermentative medium consisted of inulin 10.0, $NaNO_3$ 3.0, K_2PO_4 1.0, $MgSO_4$ 0.5, KCl 0.5 and $FeSO_4$ 0.001 g/l. The inoculated-cultures were incubated at 30°C for 12 days. The fermented broth was centrifuged at 10000 rpm, for 20 minutes at 4°C, and the supernatant was filtered and used as the crude enzyme extract. Inulinase activity was carried out spectrophotometrically according to Singh et al. [29]. The reaction mixture consisted of one ml crude enzyme extract and 0.8 ml of 2% (w/v) inulin dissolved in 0.05 M sodium acetate buffer (pH 5.5). The reaction mixture was incubated at 37°C for 60 min, then 2 ml dinitrosalicylic acid (DNS) reagent was added to the reaction. The mixture was boiled for 10 min, immediately cooled on ice and absorbance was measured at 540 nm. One enzyme unit was defined as the amount of enzyme that produces 1 μmol of fructose from inulin per min under standard assay conditions.

3. RESULTS AND DISCUSSION

Since the early 90s, the plant-soil fungi associations have been the subject of intensive research, which enable us to gain an insight into the critical role of soil fungal symbionts in plant ecology and physiology [30]. However, little is known about the plant-rhizosphere and rhizoplane fungi associations in the desert ecosystems where water availability is limited. In our recent studies, we were able to illustrate the significant roles of *Trichoderma longibrachiatum* isolated from desert soil in Egypt that confer beneficial agronomic traits to onion (*Allium cepa*) [3], and the eco-physiological role of *Thermomyces* endophyte CpE-mediated heat stress tolerance in cucumber, which will facilitate the cultivation of heat-tolerant cucumber (*Cucumis sativus*) [4]. This study extended the previous work to isolate prospective rhizosphere and rhizoplane fungi from two desert medicinal plants *P. dioscoridis* and *P. crispa* and their potential for inulinase enzyme production.

3.1. Fungal occurrence and diversity

A total of 180 fungal isolates were isolated from the rhizosphere and rhizoplane of *P. dioscoridis*, and *P. crispa* based on their ability to grow on PDA medium supplemented with 1% inulin as the only carbon source (Figs. 1-2, Supplementary Fig. S1). The morphological identification of all fungal isolates was confirmed based on the anamorph and teleomorph characters using a light microscope (Fig. 3). A total of 62 fungal isolates were obtained from rhizoplane of *Pluchea dioscoridis*, while 28 fungal isolates were isolated from rhizosphere of *P. dioscoridis* (Fig. 4A). In addition, five isolates were overlapped between *P. dioscoridis* rhizosphere and rhizoplane (Fig. 4A).

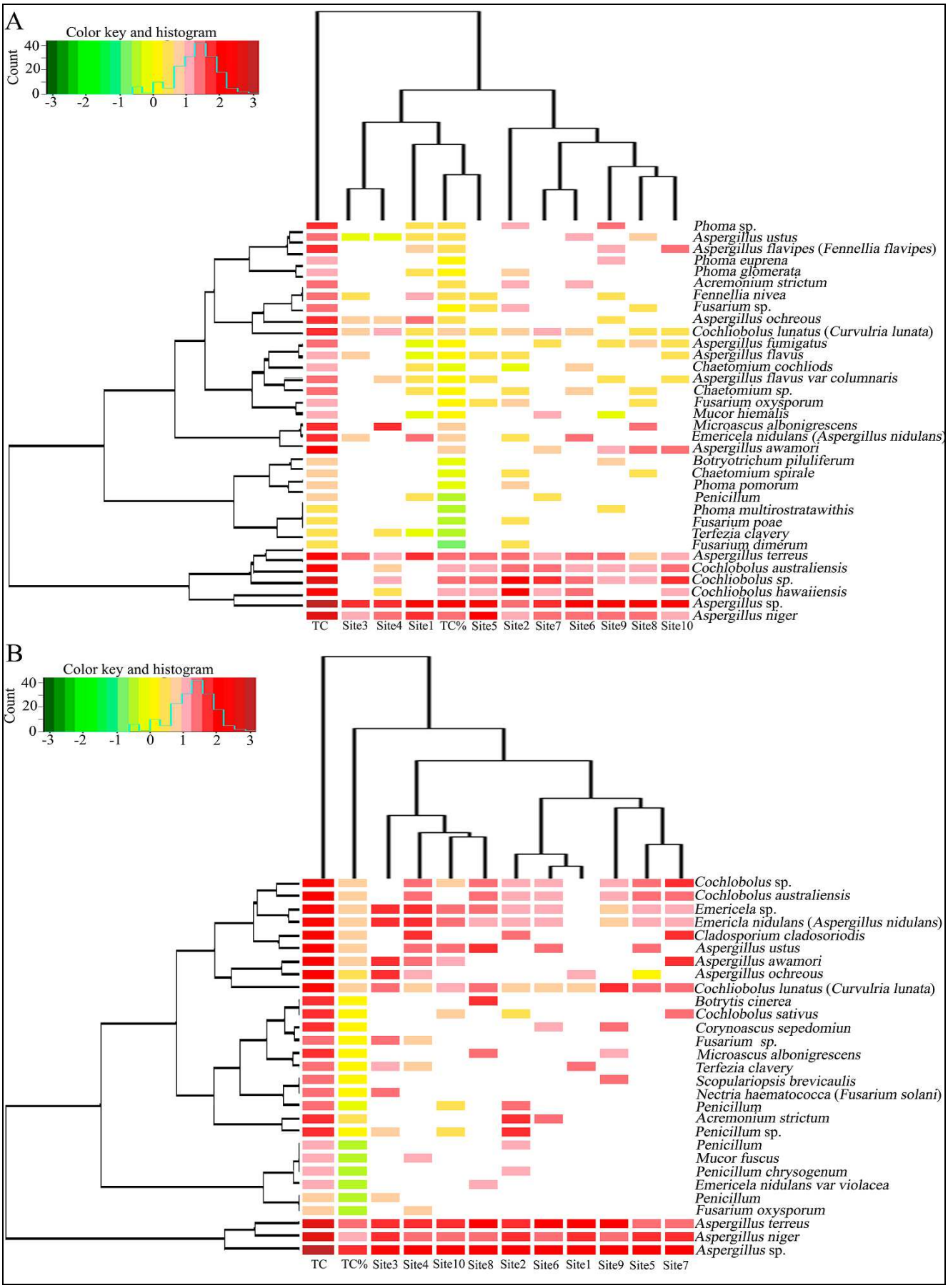


Figure 1. Heatmap clustering of the total number of fungi species isolated from *Pluchea dioscoridis*-rhizoplane (A) and *P. dioscoridis*-rhizosphere (B) at different sites (1-10). To construct heatmap color scale, the fungal counts were normalized using logarithm of base 10. TC, total count.

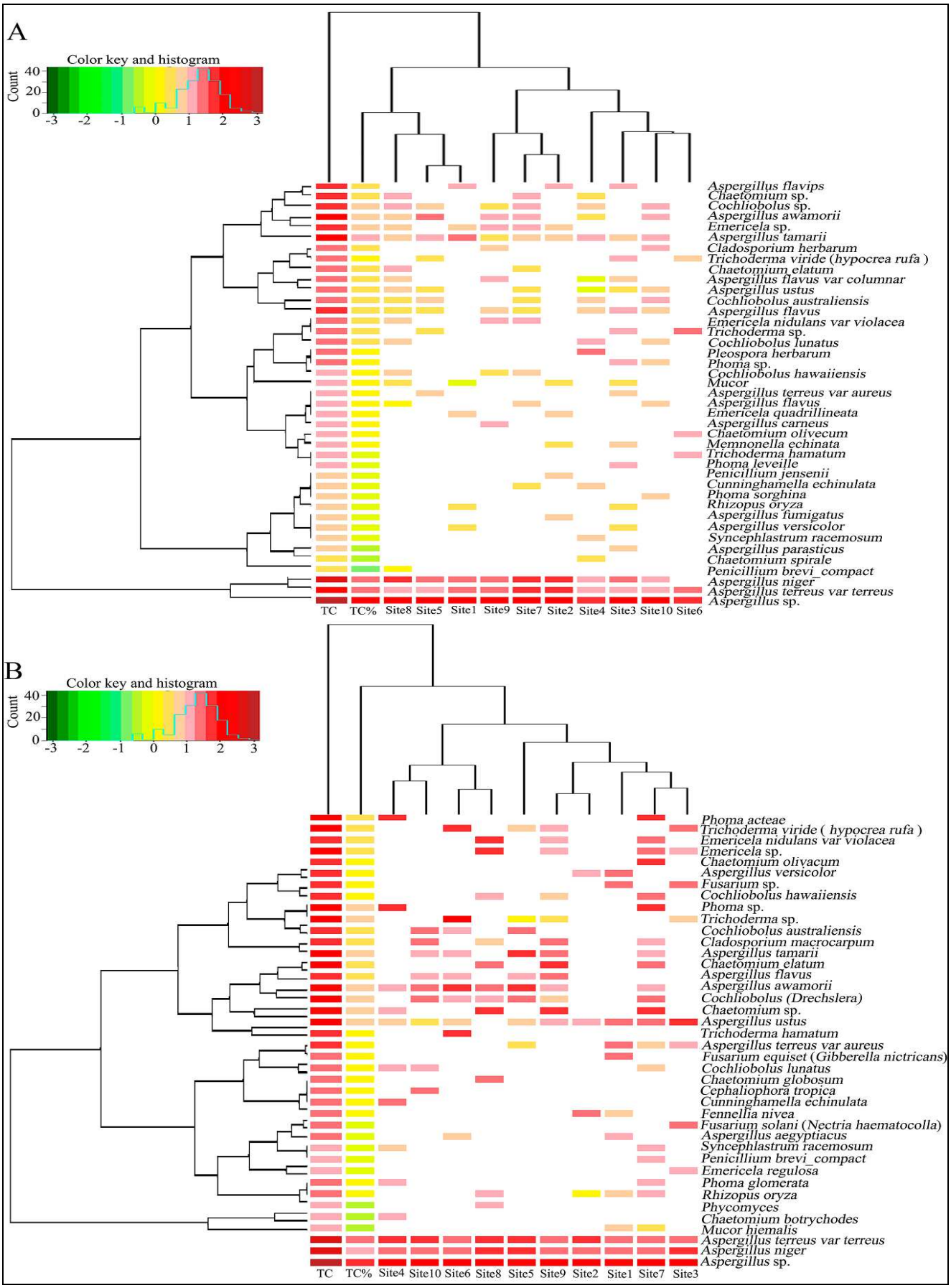
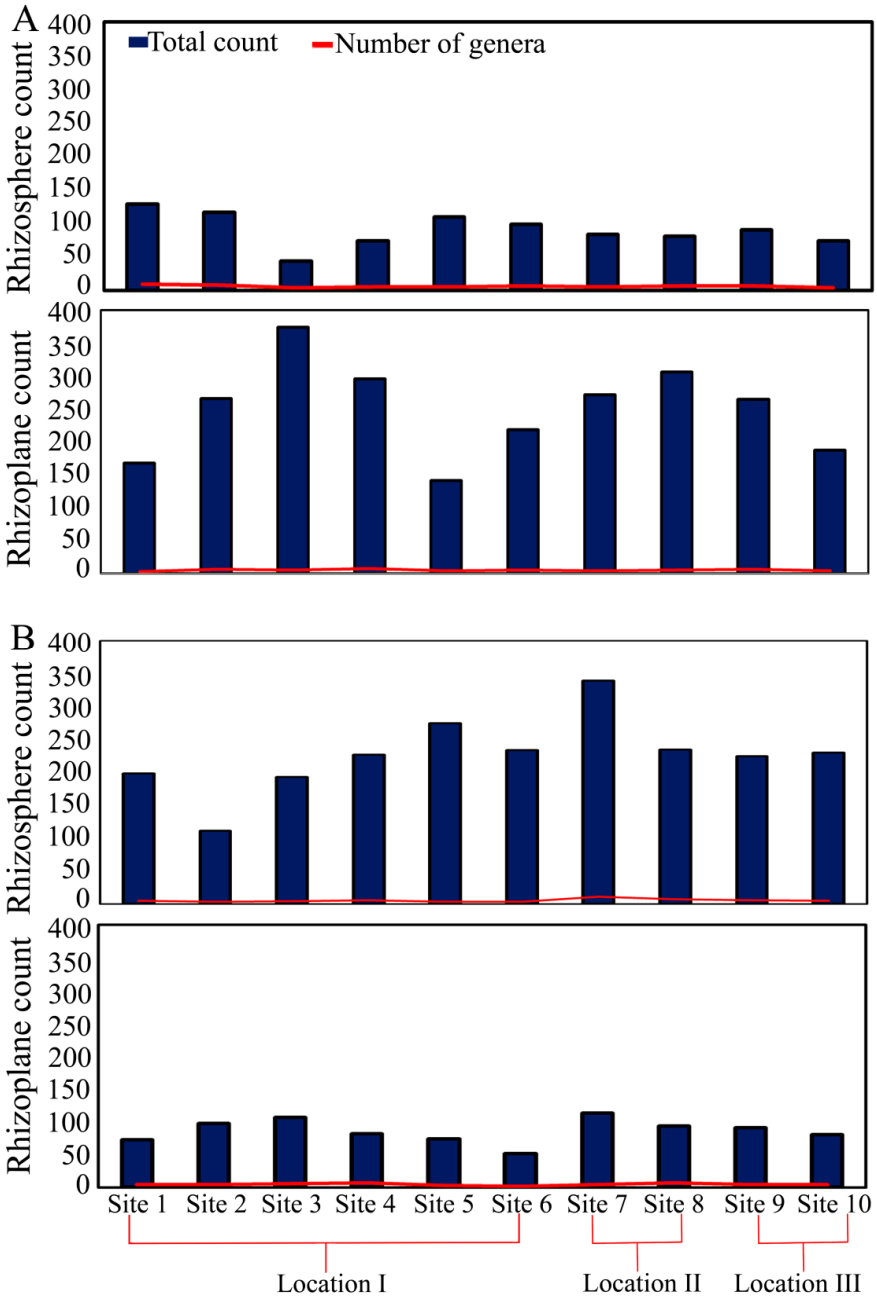


Figure 2. Heatmap clustering of the total number of fungi species isolated from *Pulicaria crispa*-rhizoplane (A) and *P. crispa*-rhizosphere (B) at different sites (1-10). To construct heatmap color scale, the fungal counts were normalized using logarithm of base 10. TC, total count.



Supplementary Figure S1. Total count of fungal isolates isolated from *Pluchea dioscoridis*-(A) and *Pulicaria crispa*- (B) rhizoplane and rhizosphere at studied sites.

Similarly, the total number of fungal isolates isolated from *Pulicaria crispa*-rhizoplane and rhizosphere was 46 and 44, respectively, whereas nine fungal isolates were overlapped (Fig. 4B). The high number of fungi species obtained from the rhizoplane and rhizosphere of desert medicinal plants indicates the significant role of root exudates in soil fungal diversity and dynamics, which are important for plant adaption to the arid land ecosystem [4]. Our data was in accordance with the early reports that showed a high diversity of fungal species were detected in the rhizosphere and rhizoplane of *Hyoscyamus muticus* and *Hordelymus europaeus* relative to other soil samples [31, 32]. In addition, *Aspergillus niger*, *A. terreus* and *Aspergillus* sp. were the most dominant fungi in the all sample sites (Figs. 1-2). *Aspergillus* spp. were highly abundant in the all sample sites represented by 13 identified species and three varieties, followed by *Chaetomium*, *Emericella*, and *Phoma* that were represented by six identified species (Figs. 1-2).

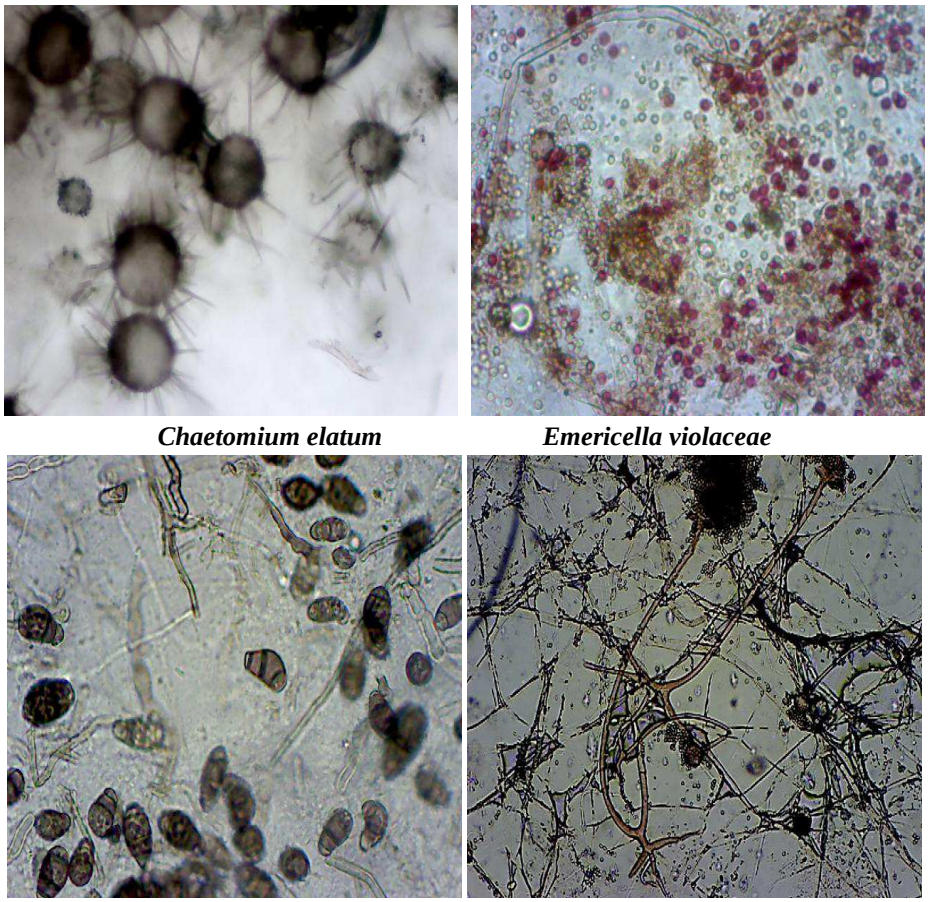


Figure 3. Morphology of some isolated fungi.

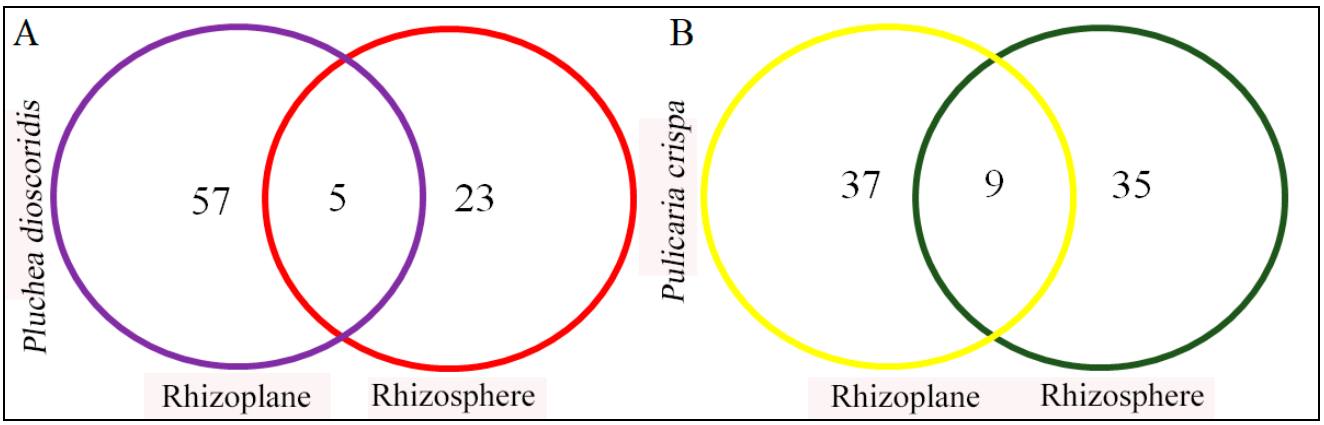


Figure 4. Venn diagram of the total number of fungal isolates isolated from *Pluchea dioscoridis*- (A) and *Pulicaria crispa*- (B) rhizoplane and rhizosphere.

Our data was in accordance with the study by Lima et al. [33] demonstrating that *Aspergillus* spp. were highly abundant in the rhizosphere and rhizoplane of *P. crispa* and *P. dioscoridis*. *Aspergillus* spp. have a wide range of optimum growth temperature ranging from 25 to 40°C and minimum growth temperature around 10°C compared with other fungi [34]. The dynamic range of *Aspergillus* growth temperature is an important physiological character, which enables *Aspergillus* spp. to survive under different environmental conditions. Among all recovered species during this work, there were twenty three identified species and one variety belonging to 14 terrestrial fungal genera were recovered from *Pluchea dios-*

coridis in rhizosphere samples. In addition, 31 identified species and 2 varieties appertaining to 17 genera were recovered from *Pulicaria crispa*. While, 27 species and two varieties belonging to 13 genera were isolated and identified from *Pluchea dioscoridis*, whereas thirty one and three varieties appertaining to 15 genera were recovered from *Pulicaria crispa* in rhizoplane samples. Out of all recorded species in this investigation, 12 identified species were isolated from rhizoplane only of the two plants were; *Aspergillus carneus*, *A. flavus* var. *columnaris*, *A. fumigatus*, *A. parasiticus*, *Botryotrichum piluliferm*, *Chaetomium cochliodes*, *Phoma euprena*, *Phoma* sp., *P. pomorum*, *Emericella quadrillineata*, *Memoniella echinata* and *Pleospora herbarum*. In Pakistan, Qureshi et al. [35] isolated *Fusarium solani*, *Cochliobolus australiensis*, *Macrophomina phaseolina* and *Rhizoctoinia solani* from rhizoplane of 65 plants belonging to 58 genera and 19 families. The following 14 fungal species; *Aspergillus egyptiacus*, *Botrytis cinerea*, *Cephaliophora tropica*, *Cladosporium cladosporioides*, *C. macrocarpum*, *Chaetomium bostrychodes*, *C. globosum*, *Cochliobolus sativus*, *Emericella rugulosa*, *Gibberella intricans*, *Mucor fuscus*, *Nectaria haematocoa*, *Phoma acteae* and *Phycomyces* sp. were isolated only from rhizosphere of the two tested plants. Most of the fungal genera that were recorded in this investigation were repeatedly reported for rhizosphere of different wild and cultivated plants in the South-Eastern desert of Egypt and different parts of the world by [29, 32, 36-39]. In the rhizoplane of *Pluchea dioscoridis*, there were four species found to be dominant are; *Aspergillus ustus*, *Aspergillus flavus* var. *columnaris*, *Aspergillus flavus* and *Aspergillus fumigatus*, *Phoma viride*, *Fusarium* sp. and *Chaetomium cochloides* were showed an intermediate frequency while remaining isolated were found in low frequencies whereas, in rhizoplane of *Pulcaria crispa*, *A. tamarii*, *A. awamori*, *A. flavus* and *Aspergillus ustus*, *Cochliobolus australiensis* and *Emericella violacea* were found to be dominant. *Mucor hiemalis* and *Rhizopus oryzae* were showed an intermediate frequency while, remaining isolates were found in low frequency, these genera were represented by few species that did not exceed five species for each genus. *Acremonium*, *Botryotrichum*, *Fusarium* and *Microascus* which were recovered from *Pluchea dioscoridis* in moderate and low incidence, were completely missed in *Pulicaria crispa*. Whereas, *Cladosporium*, *Cunninghamella*, *Memnoniella*, *Pleospora*, *Rhizopus*, *Syncephlastrum* and *Trichoderma* which were represented by two or one identified species and present in moderate to low incidence, were recovered from *Pulicaria crispa* and completely absent in *Pluchea dioscoridis*. All fungal genera and species which were recovered during this work were previously recovered from soil, rhizosphere, rhizoplane and endophytic fungi at different parts of the world and on different habitats by many investigators [35, 38, 40-45].

3.2. Inulinase activity of fungal isolates associated with Asteraceous plants

A total of 180 fungal isolates were able to grow on PDA medium supplemented with 1% inulin. Out of which, 23 fungal isolates (12.77%) exhibited high inulinase activity ranging from 5.05 to 7.26 U/ml (Figs. 5-6). Whereas 67 fungal isolates (37.22%) displayed moderate inulinase activity, ranging from 3.01 to 4.99 U/ml (Figs. 5-6). *Aspergillus terreus* var. *terreus* 233 isolated from *P. dioscoridis*-rhizoplane, and *Botrytis cinerea* isolated from *P. dioscoridis*-rhizosphere exhibited the highest inulinase activity ranging from 6.28 to 6.41 U/ml (Fig. 5A-B). On the other hand, *Chaetomium cochliods* isolated from *P. dioscoridis*-rhizoplane, and *Emericla nidulans* var. *nidulans* isolated from *P. dioscoridis*-rhizosphere displayed the lowest inulinase activity, ranging from 0.17 to 0.34 U/ml (Fig. 5A-B). Similarly, *Aspergillus aegyptiacus* isolated from *P. crispa*-rhizoplane, and *Cochliobolus australiensis* 447 isolated from *P. crispa*-rhizosphere exhibited highest inulinase activity ranging from 7.10 to 7.26 U/ml (Fig. 6A-B). However, *Mucor hiemalis* isolated from *P. crispa* rhizoplane and rhizosphere displayed the lowest inulinase activity of 0.85 U/ml (Fig. 5A-B). In general, our results indicated that the isolated fungal species exhibited significant differences in inulinase activities, and several *Aspergillus* spp. isolated in this study showed high inulinase activity in comparison with other fungi species. The high inulinase activity of *Aspergillus* spp. seems to be a physiological characteristic of this species to enable them to extract nutrition under severe environmental condi-

tions. Similar studies have also indicated the high inulinase activity derived from *Aspergillus* spp. [46, 47]. The histogram analysis of inulinase activity of fungal isolates isolated from *P. dioscoridis*-rhizosphere and rhizoplane exhibited a right-skewed/asymmetrical distribution due to the high number of fungi showed low inulinase activity (Fig. 7).

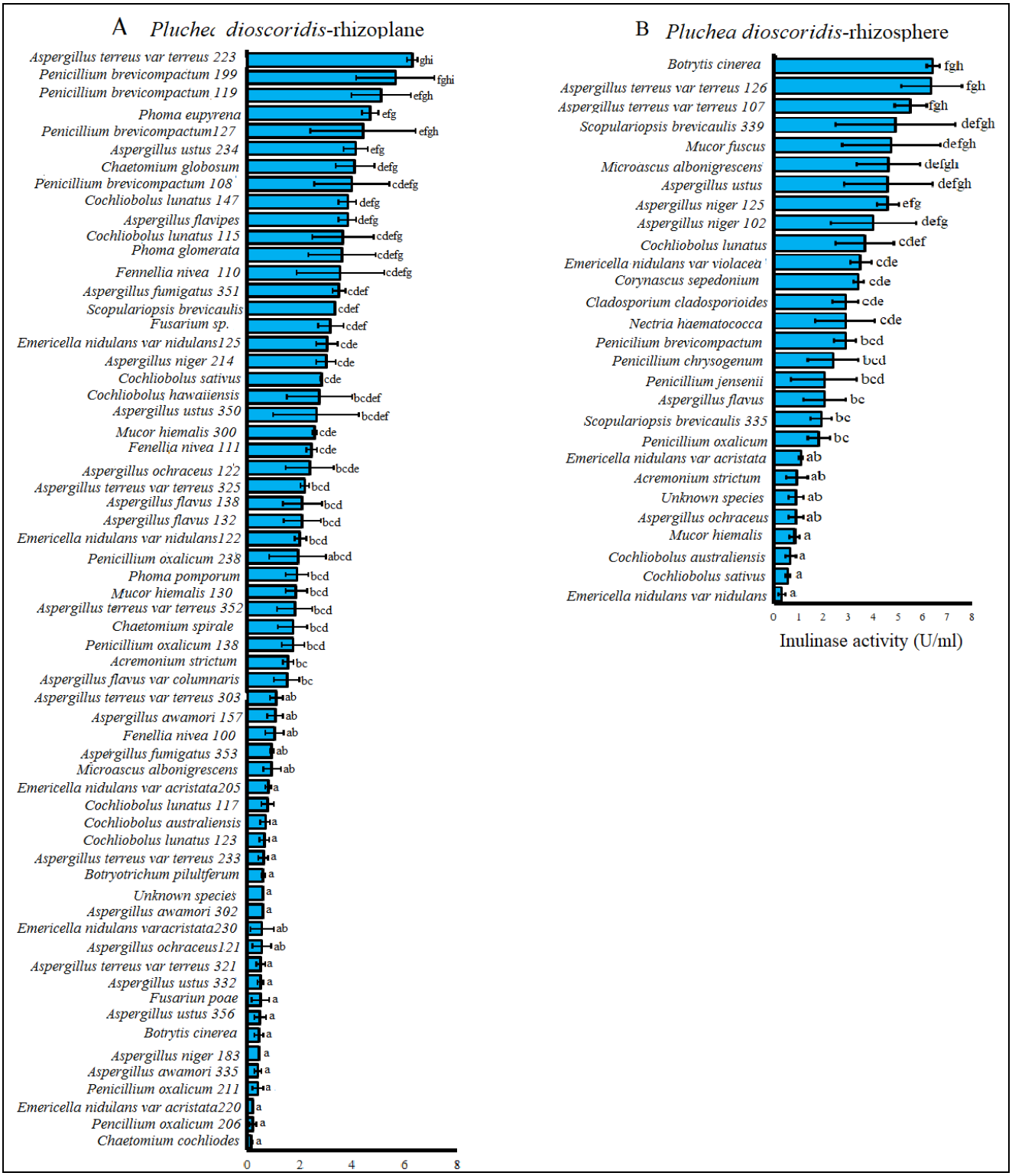


Figure 5. Inulinase activity (U/ml) of fungi species isolated from *Pluchea dioscoridis*-rhizoplane (A) and rhizosphere (B).

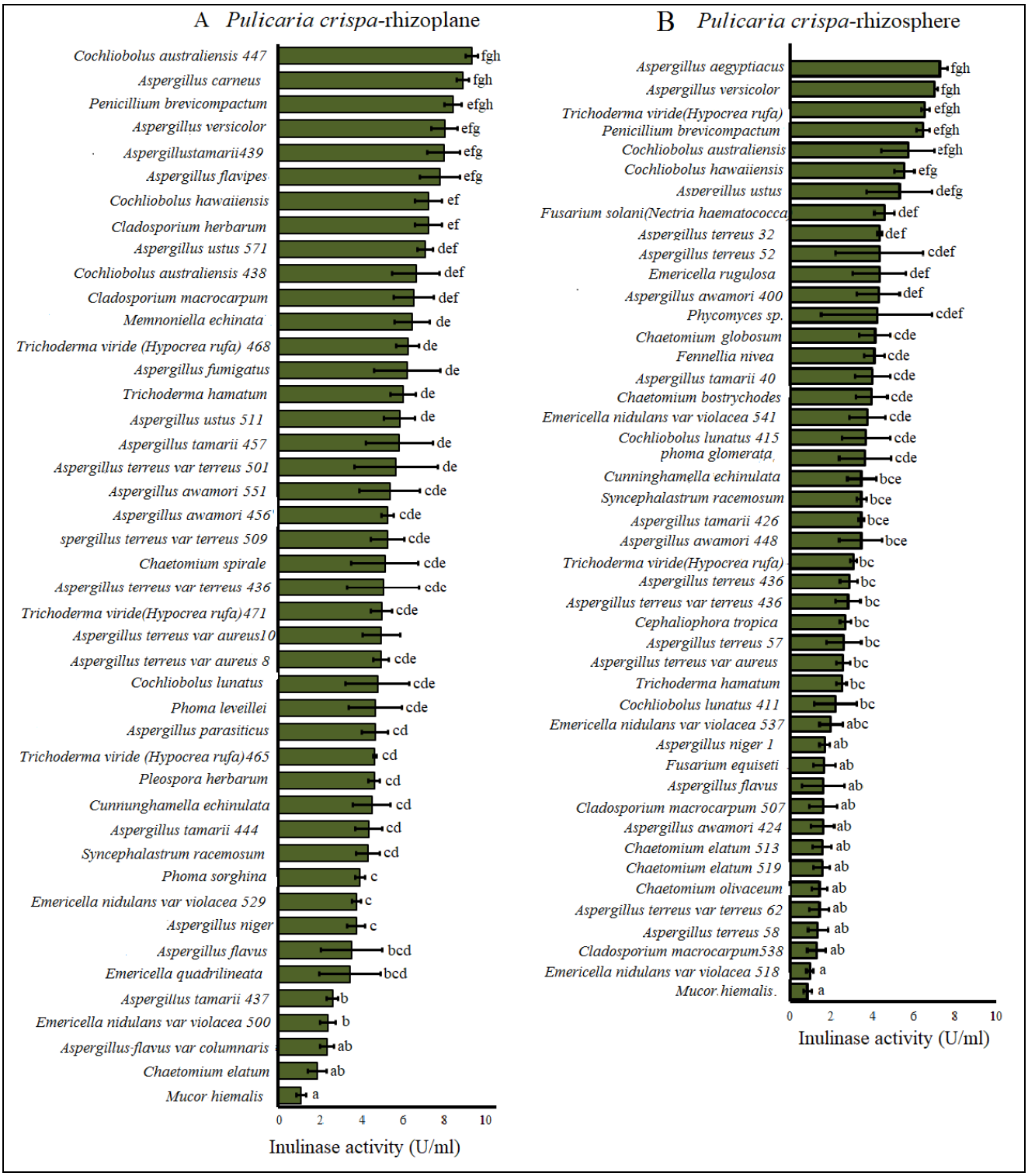


Figure 6. Inulinase activity (U/ml) of fungi species isolated from *Pulicaria crispa*-rhizoplane (A) 0020 and rhizosphere (B).

On the other hand, the histogram analysis of inulinase activity of fungal isolates isolated from *P. crispa*-rhizoplane and rhizosphere exhibited normal distribution due to the high number of fungi showed moderate inulinase activity (Fig. 7). The high inulinase activity for *Aspergillus terreus* var. *terreus* 233, *Botrytis cinerea*, *Aspergillus aegyptiacus* and *Cochliobolus australiensis* 447 observed in this study was in accordance with previous reports [41, 48, 49]. For examples, Coitinho et al. [49] demonstrated the high efficiency and thermostability of inulinase enzyme purified from *Asper-*

gillus terreus using sugarcane bagasse as a substrate. *B. cinerea* xylanase activity has been reported as an essential component for their virulence effect [50]; however, this is the first report about *B. cinerea* inulinase activity, which might be a starting point for further in-depth studies about its role in the plant-pathogen interaction. Souza-Motta et al. [41] also demonstrated the ability of filamentous fungi isolated from rhizosphere to hydrolyze inulin.

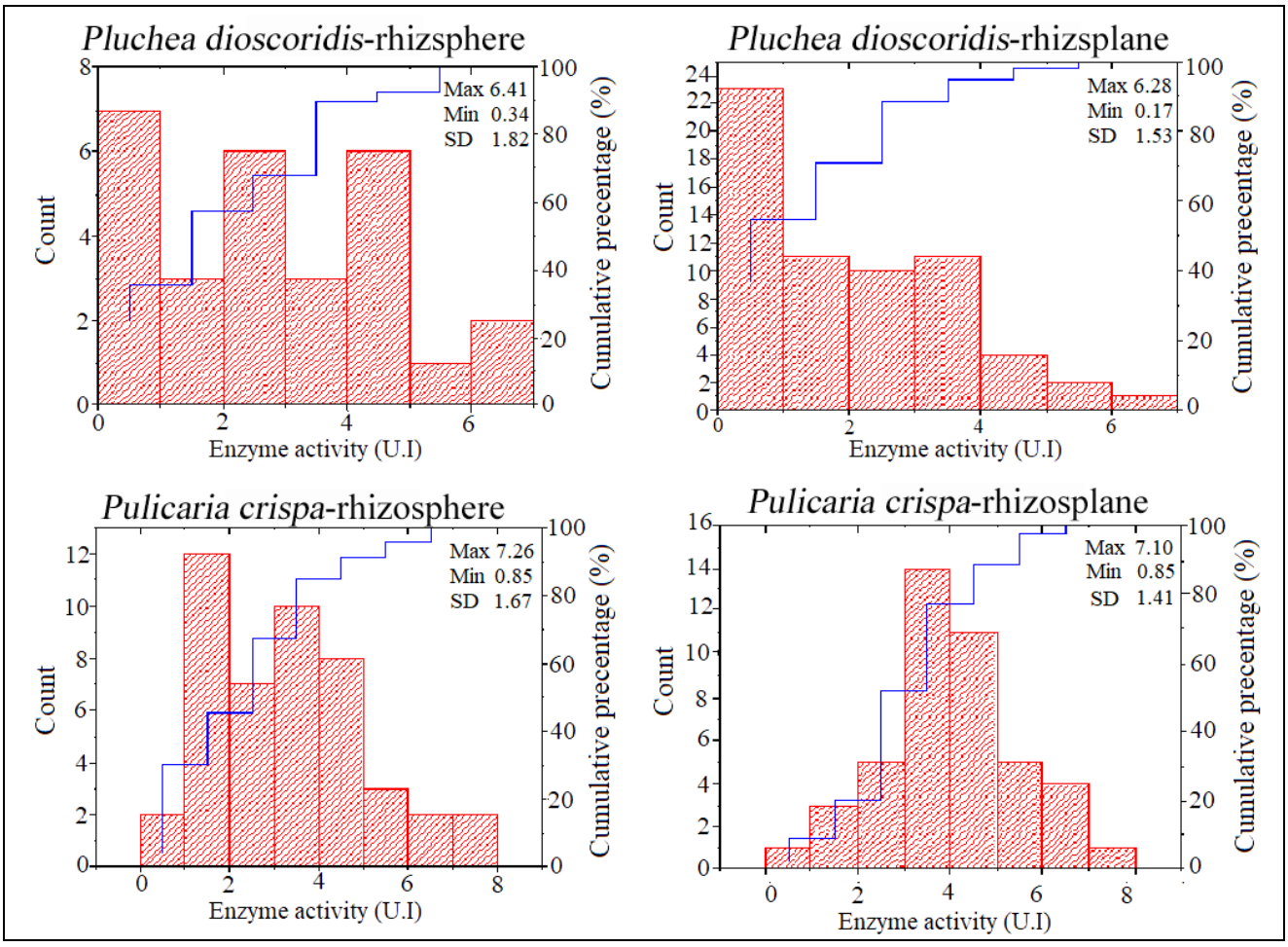


Figure 7. Histogram analysis of inulinase activity on the X-axis and total fungal isolates on the Y-axis isolated from *Pluchea dioscoridis*- and *Pulicaria crispa*-rhizoplane and -rhizosphere.

4. CONCLUSION

In conclusion, we were able to isolate and identify 180 fungal isolated from the rhizosphere and rhizoplane of two desert medicinal plants *P. dioscoridis* and *P. crispa* in the South-Eastern desert of Aswan governorate, Egypt. We also examined the inulinase activity of the isolated fungi, revealing high ability of several fungal isolates including *Aspergillus terreus* var. *terreus* 233, *Botrytis cinerea*, *Aspergillus aegyptiacus*, *Cochliobolus australiensis* 447 and *Cochliobolus australiensis*. These fungal isolates could be a potential bio-resource for microbial inulinase production. Optimization experiments are needed for exploring the best conditions for increasing the enzyme productivity by these isolates for potential commercialization.

AUTHOR'S CONTRIBUTION

MA, SE: Project supervisors, research design, wrote and revised the manuscript; DKh, MS: experimental work, MAR: statistical analysis, Figures and wrote the first draft. All authors read and approved the final manuscript.

TRANSPARENCY DECLARATION

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

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ISSN 2449-8955

European Journal of Biological Research

Research Article

Optimization of kojic acid production conditions from cane molasses using Plackett-Burman design

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Received: 04 February 2018; **Revised submission:** 23 March 2018; **Accepted:** 03 April 2018

DOI: http://dx.doi.org/10.5281/zenodo.1211517

ABSTRACT

Fungal synthesis of kojic acid has gained more interest in these days as an alternative way to chemical synthetic. The aspect of the microbial fermentation process is to develop a suitable culture medium to obtain the maximum amount of kojic acid using statistical methods. In this study; different selected three isolates of *Aspergillus flavus* (No 1, 2 and 3) were screened for their ability to produced kojic acid and the isolate No 3 was the highest kojic acid producer one. The capability of *A. flavus* No 3 to produce kojic acid was improved using Plackett-Burman design. From ten different agro-industrial wastes cane molasses recorded the highest kojic acid productivity with 2.24 g/l^{-1} day^{-1} and was the most effective parameter plays a crucial role in Plackett-Burman design. Maximum kojic acid production (24.65 g/l) by *A. flavus* (No. 3) obtained under the fermentation conditions: incubation temperature at 25°C, incubation time 9 days, pH 3, inoculum size 0.5%, shaking rate at 150 rpm and medium constituents: Cane molasses 60 g/l, yeast extract 7 g/l, KH_2PO_4 2 g/l, $ZnSO_4 \cdot 7H_2O$ 100 µg/l and $MgSO_4 \cdot 7H_2O$ 1 g/l with regression analysis (R^2) 99.45% and 2.33-fold increase in comparison to the production of the original level (10.6 g/l).

Keywords: Kojic-acid; Agro-industrial wastes; Optimization; Plackett-Burman; *Aspergillus*.

Abbreviations: Czapek's dextrose agar medium (CZD), kojic acid (KA), Consuming sugars (CS), Dry mass (DM).

1. INTRODUCTION

Kojic acid (5-hydroxy-2-hydroxymethyl-l-pyrone) is an organic acid has a weak acidic property crystallizes in form of colorless and prismatic needles [1]. The melting point of kojic acid ranges from 151-154°C. Kojic acid is soluble in water (3.95 g/100 ml at 20°C), ethanol and ethyl acetate. On the contrary, it is less soluble in ether, alcohol ether mixture, chloroform and pyridine [2-4].

Kojic acid is a major secondary metabolite can be produced from carbohydrates by using different carbon and nitrogen sources, also using agriculture wastes under aerobic fermentation strategies. Kojic acid is produced by *Aspergillus* spp.

belonging mainly to the section *flavi*: *Aspergillus flavus* [5-9], *Aspergillus oryzae* [10-14], *Aspergillus oryzae* var *effusus* [15], *Aspergillus tamarii* [16] and *Aspergillus parasiticus* [6, 7, 14, 17-19], as well as *Penicillium* sp. and certain bacteria [14, 20, 21].

Glucose, sucrose, acetate, ethanol, arabinose and xylose have been used as carbon sources for kojic acid production. Glucose is the best carbon source for kojic acid production due to the similarity of its structure to that of kojic acid. It has been suggested that, during the fermentation, kojic acid is formed directly from glucose without any cleavage of the carbon chain into smaller fragments [5, 22, 23]. Utilization of agro-industrial wastes or by-products for the fungal production of useful products has been recommended by many investigations such as cheese whey [24-26], sugar cane molasses [27-30], fruits, vegetables, corn steeps liquor [9, 31].

Kojic acid is a natural antibiotic agent, early as 1934 it was reported that kojic acid inhibited the growth of Gram-negative more strongly than that of Gram- positive bacteria [32]. This property was rediscovered much later and the antibiotic action of culture filtrates of several fungi was shown to be due to the presence of kojic acid and it is used in the medical field as a pain killer and anti-inflammatory drug [33]. In the food industry, KA used as one of the precursors for flavor enhancers [34]. Kojic acid has the ability to prevent the undesirable melanosis (blackening) of agricultural products by inhibiting polyphenol oxidase [35], and used as a skin care product for whitening [4] and as a protective against U.V. light. It has been used for the production of miso, soya sauce and sake in Japan for a long time [36, 37].

Agro-industrial wastes, include wastes generated during the industrial processing of agricultural or animal products or those obtained from agricultural activities in the form of straw, stem, stalk, leaves, husk, shell, peel, lint, seed, pulp, legumes or cereals (rice, wheat, corn, sorghum and barley), bagasse' from sugarcane or sweet sorghum milling, spent coffee grounds, brewer's spent grains, and many others. These wastes are mainly composed of sugars, fibers, proteins, and minerals. The chief constituents of such agro-industrial wastes include cellulose, hemicelluloses and lignin, collectively being called "lignocellulosic materials" [38]. Cheap agro-industrial sources such as wheat bran, soy bean meal, corn steep liquor, sugarcane bagasse', whey, etc. have been used as carbohydrate as well as nitrogen sources in the lieu of synthetic ones [39]. Different types of treatments (physical, chemical, and enzymatic) can be given to these by-products in order to make them easily consumed by microbes [40].

A classical method of optimizing the fermentation conditions and medium constituents depends on single parameter whilst all the other factors are maintained at a fixed level. However, statistical planned experiments effectively explained the interaction of parameters and minimize the error in determining the effect of parameters [41, 42]. The design of experiment reduces the number of experiments and increases process efficiency [43, 44]. Statistically designed experiments are used for optimization strategies such as screening experiments and optimization for targeted response [45]. Plackett-Burman design used which greatly enhance the yield of product, reduces time, cost, process variability and has been successfully used to optimize many bioprocesses [46, 47]. The Plackett-Burman design was developed by Plackett and Burman in 1946. It is two-level fractional design for studying up to k=N-1, where k are variables and N is the number of runs. These designs have complex alias structures, and hence, this design was generally preferred for screening of significant factors [48].

The main objective of this work was to test the ability of different *Aspergillus flavus* isolates to produce kojic acid on both glucose and agro-industrial wastes media, secondly to improve the production by investigating the effect of several variables on the KA production process and recorded the optimum fermentation conditions for the highest KA production using Plackett-Burman design as a statistical approach.

2. MATERIALS AND METHODS

2.1. Microorganisms

Three isolates of *Aspergillus flavus* proved previously as highly kojic acid producers [9] and proved as non-toxigenic producers (data not recorded here) were selected for this study. These isolates were maintained on Czapek's dextrose agar medium (CZD) aerobically and stored at 4±1°C until

using (sub-cultured every 30 day). Prior to the experiments *A. flavus* isolates grown on CZD medium at 28±1°C for 4 days aerobically. Homogeneous spore suspension obtained by scrapping fungal hyphae and suspended it in sterilized distilled water containing 0.01% (v/v) tween 80 until spore suspension 3×10^6 spore/ml and stirred for 30 min. then using it as inoculum.

2.2. Medium and culture conditions.

Modified Czapek's dextrose liquid medium used for kojic acid production [49] containing (g/l): glucose, 100.0; yeast extract, 5; KH_2PO_4, 1.0 and $MgSO_4.7H_2O$, 0.5. These contents were dissolved in 1000 ml distilled water with initial pH adjusted to 3 before autoclaving. After sterilization in an autoclave at 121°C and 1.5 atm pressure for 20 min. chloramphenicol, 250 mg/ml was sterilized separately by membrane filtration, using a membrane of pore size 0.22 mm and added as bacteriostatic agent. Incubation was carried out at 28±1°C on a rotary shaking (150 rpm) for 7 days. All the experiments were carried out independently in triplicates. After the incubation period, mycelium was recovered by filtration through dried and weighed Whatman filter paper (No. 113), washed with distilled water three times and then dried at 70 °C overnight for dry mass (DM) determination. The supernatants were used for quantitative determination of kojic acid (KA) and consuming sugars (CS).

2.3. Screening for kojic acid production on agro-industrial wastes

Ten agro-industrial wastes collected from Agriculture Research Centre and sugar factories were used in this experiment, namely beet molasses, cane molasses, mixed of cane and beet molasses, bagasse, starch water, corn steep liquor, rice straw, *Zea mays* waste, onion waste and peanut waste. The hard wastes were washed to remove dust, separated, dried, ground and sieved through 1-mm mesh screen. All wastes were prepared in concentration 40 g/l. Incubation was carried out at 28±1°C on a rotary shaking (150 rpm) for 7 days. All the experiments were carried out independently in duplicates. The chemical analysis of beet molasses and cane molasses showed in Table 1.

Table 1. Chemical composition of Abo-Qurqas sugarcane and beet molasses, Egypt.

Test	Cane molasses	Beet molasses
Brix	86.50±1.0	82.50±1.0
pH	5.1±0.1	8.1±0.1
Ash %	12.30±0.5	10.50±0.5
Total sugar %	56.0±1.0	57.50±1.0
Non-fermentable sugar %	4.50±0.2	2.00±0.2
Fermentable sugar %	51.50±0.1	55.50±0.1
Reducing sugar %	24.90±0.1	1.82±0.1
Nitrogen %	0.61±0.1	1.3±0.1
Protein %	3.81±0.1	8.12±0.1
Color % brix	22500	16060
CaO %	1.58±0.1	2.00±0.1
P_2O_5 %	0.3±0.01	0.1±0.01
SO_4 g/l	19.0±2.0	5.4±2.0

2.4. Optimization using Plackett-Burman design

Plackett-Burman design was used to screen the fermentation parameters that influenced kojic acid (KA) production [50]. Eleven trails carried out by Plackett-Burman design for screening the fermentation parameters with respect to their main effect and without interaction effects between various constituents of the medium is shown in Table 2. Each independent variable was tested at two levels, high (+1) and low (−1). In each column and row should contain equal number of negative and positive signs. Plackett and Burman design was used to screen and evaluate the important medium components that influence the response. Kojic acid yields are explained by the following polynomial equation:

$$Y=bo + \sum biXi + \sum bijXiXj + Ei \quad (1)$$

Where, Y; the variable dependent response; i; the regression coefficient; X; the independent variable level; b0 is offset term; bij is interaction effect and E; the experimental error. The experimental data were statistically analyzed to determine the significant difference ($p \leq 0.05$) in response under different conditions. The response surface graphs were also plotted using the same software. The quality of fit for the regression model equation was

expressed as R^2. The program Sigma XL (Version 6.12) was used to analyze this experiment.

Table 2. Plackett-Burman design for screening kojic acid production using different variables by *Aspergillus flavus.*

Variable code	Variable	Unit	Level	
			Low (-1)	High (+1)
A	Incubation temperature	C°	25	35
B	Incubation time	D	5	9
C	Fermentation type		Shaking	Static
D	Inoculums size	%	0.5	2
E	Initial pH		3	5
F	Molasses	gl^{-1}	20	60
G	Yeast extract	gl^{-1}	3	7
H	KH_2PO_4	gl^{-1}	0.5	2
J	$ZnSO_4.7H_2O$	μgl^{-1}	0	100
K	Glycine	μgl^{-1}	0	100
L	$MgSO_4.7H_2O$	gl^{-1}	0.1	1

2.5. Analytical analysis

Kojic acid was determined spectrophotometrically using ferric chloride reagent; the developed purple-red color was measured quantitatively against substrate-free blank at 540 nm [2, 20, 51]. The residual sugar was analyzed spectrophotometrically by anthron method using T60 UV with a split beam UV visible spectrophotometer covers a wavelength range of 190-1100 nm [52].

3. RESULTS AND DISSCUSSION

3.1. Kojic acid production by *Aspergillus flavus* isolates

Three isolates of *A. flavus* (No. 1, 2 and 3) were screened for their ability to produced kojic acid on fermented medium. All the isolates grown on the production medium and showed various degrees of dry mass and kojic acid production. A wide variation in KA production on the screening medium ranged from 8.5±0.01 to 10.6±0.01 g/l in submerged cultures and 7.4±0.02 and 8.9±0.01 g/l in static cultures. The highest fungus dry mass and kojic acid producer was *Aspergillus flavus* (No. 3) giving 10.58±0.01 g/l KA (with productivity 1.51 g/l/day) and 6.1±0.53 g/l dry mass so it was selected for the further experiments. Several species of *A. flavus* group were estimated as kA producers such as *A. flavus*, *A. oryzae* and *A. parasiticus* [9, 14, 23, 53-58].

Brief description of kojic acid highly producer *Aspergillus flavus* Link (No. 3); Growth on CZD medium 60 mm in one week, Texture floccose becoming granular, Color bright yellow-green; occasionally yellow-brown, cream reverse. Conidiophores roughened; vesicles globose with radiate or columnar spore production; phialides arising directly or produced on medullae in others; conidia round to elliptical, 3-6 μm; smooth or finely roughened (Fig. 1).

3.3. Screening for kojic acid production on agro-industrial wastes by *Aspergillus flavus* Link

Ten agro-industrial wastes were tested as a carbon source for the growth of *A. flavus* (No. 3) and KA production was illustrated in Fig. 2. *Aspergillus flavus* growth and KA production were largely impressed by the type of waste. The results indicated that cane molasses promoted both fungal growth and kojic acid production (15.71 g/l KA, 20.2 g/l DM) followed respectively by potato waste water (11.4 g/l KA, 17.5 g/l DM), onion wastes (9.49 g/l KA, 9.05 g/l DM) and mixed molasses (9.23 g/l KA, 15.6 DM). It is worthy to mention that bagasse, corn steep liquor, peanut wastes and rice straw contribute low production of kojic acid matching 0.87, 2.37, 3.62 and 3.98 g/l kojic acid, respectively. The current study clearly proved that *A. flavus* could grow well on cane molasses and produced a large amount of kojic acid. Utilization of different carbon sources such as glucose, starch, sucrose, maltose and cellulose by different *Aspergillus* species for kojic acid production were studied by Rosfarizan and Ariff [59]. El-Aasar [19] reported that glucose also has yield the highest kojic acid production by *A. parasiticus* and followed by sucrose and beet molasses. Several quantities of KA

produced by fungi were recorded previously by several researchers such as: Manabe et al. [60] recorded 40 g/l kojic acid by *A. flavus*; El-Sharkawy [57] obtained 60 g/l kojic acid with immobilization technique by *A. flavus* ATCC 9179. Kwak and Rhee [11] produced 80 g/l kojic acid using, also, immobilized cells of *A. oryzae*. Ogawa et al. [61] produced 20g/l KA by *A. oryzae* NRRL 484 using shaking culture. Wakisaka et al. [62] produced 24 g/l KA from the same previous isolate on different medium.

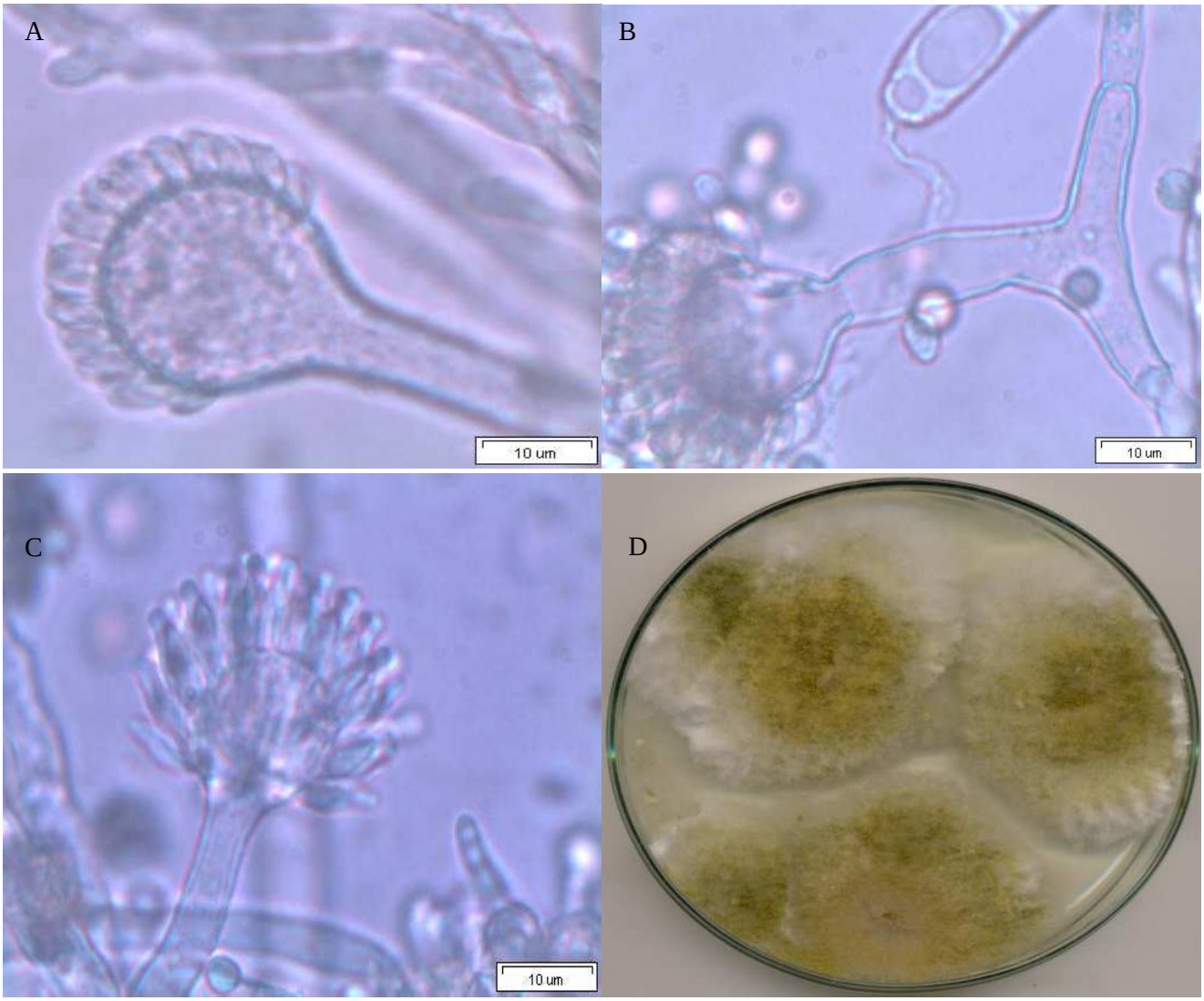

Figure 1. *Aspergillus flavus*, **A, B**: Biserriate phialide (Bi); Conidiophore (Co) and hypha (Hy); **C**: Monoserriate phalide (Mo); Bars, 10 µm; **D**: Fungus growth on Czapek's dextrose agar medium.

3.3. Optimization of KA production using Plackett-Burman design

The highly kojic acid producer (*A. flavus* No.3) was chosen for screening the effects of different parameters on KA production using Plackett-Burman design. Each variable was studied at two levels (-1, 1) as declared in Table 1. Relationship between the response and the screened variables was expressed by the following polynomial equations:

KA (g/l) = (11.98) + (-6.38) * A + (1.35) * B + (-6.36) * C + (-0.54) * D + (-1.67) * E + (7.98) * F + (1.44) * G + (4.31) * H + (-1.06) * J + (-0.388) * K + (4.8) * L. (2)

CS (%) = (8.52) + (-1.89) * A + (-0.96) * B + (-1.05) * C + (-0.48) * D + (1.32) * E + (2.05) * F + (-0.4) * G + (1.32) * H + (1.89) * J + (-0.49) * K +

(0.82) * L. (3)

DM (g/l) = (15.37) + (-0.55) * A + (0.25) * B + (-2.1) * C + (1.74) * D + (1.48) * E + (4.79) * F + (1.77) * G + (0.92) * H + (-0.27) * J + (0.083) * K + (3.47) * L. (4)

The results obtained in Table 3 indicated that there was a wide variation in kojic acid production of 0.82 to 24.65 g/l, consuming sugar 27.33 to 89.87% and dry mass varied between 3.6 and 28.2 g/l indicating the important effect of both medium components and environmental factors on the production of KA. The ANOVA results are shown in Table 4 showed that among the eleven variables, D (inoculums size), K (glycine) in kojic acid production; B (incubation time), J ($ZnSO_4.7H_2O$), K (glycine) in dry mass; G (yeast extract) in consuming sugars were found to be non-significant ($p>0.05$). Among the tested parameters, cane molasses was the most effective parameters plays a crucial role in KA production, dry mass and consuming sugars with 3.99, 4.79 and 7.98 coefficient effect as shown in Pareto-Plot (Fig. 3).

All the predicted values of Plackett-Burman design were located in close proximity to experimental values. This supports the hypothesis that the model Eq. (2, 3, and 4) is sufficient to describe the response of the experimental observations of KA production, dry mass and consuming sugars (Fig. 4). The main effects of different parameters on kojic acid production by *A. flavus* showing effect of two variables (other variables were kept at zero in coded unit indicated in Fig. 5.

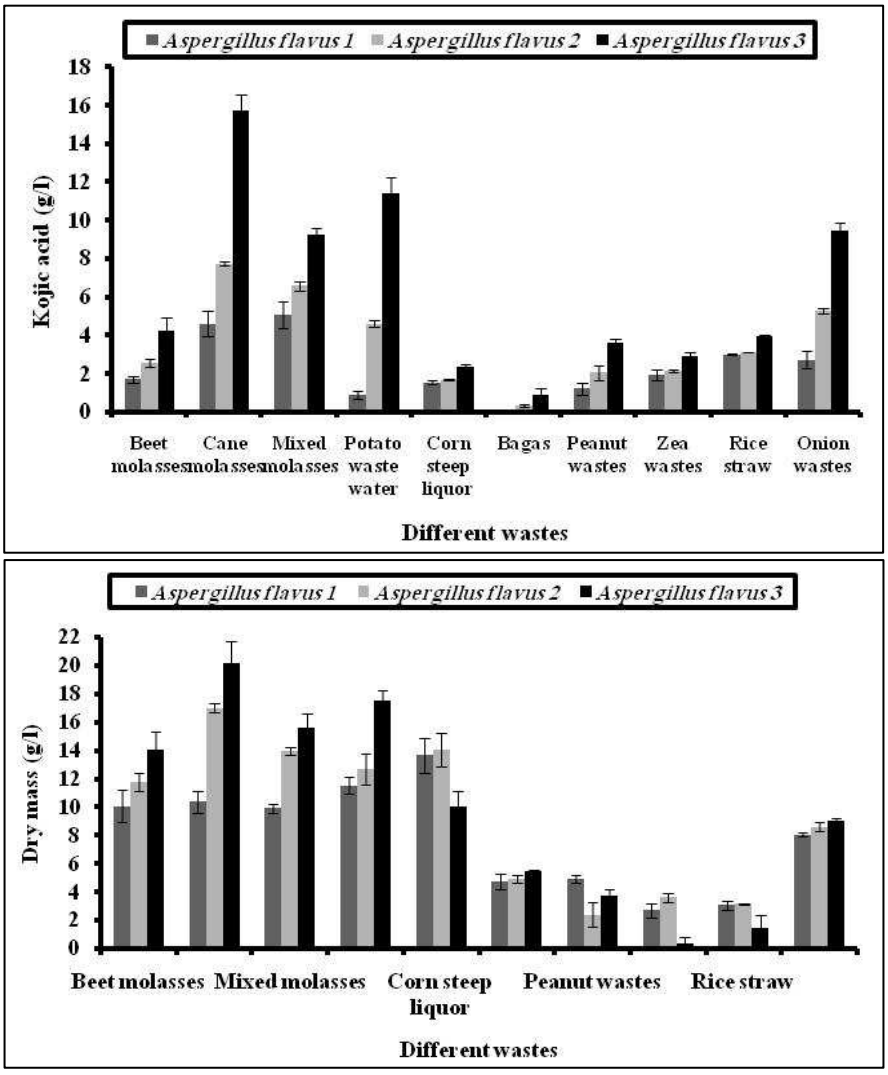

Figure 2. Screening for kojic acid production on different agro-industrial wastes by three species of *Aspergillus*.

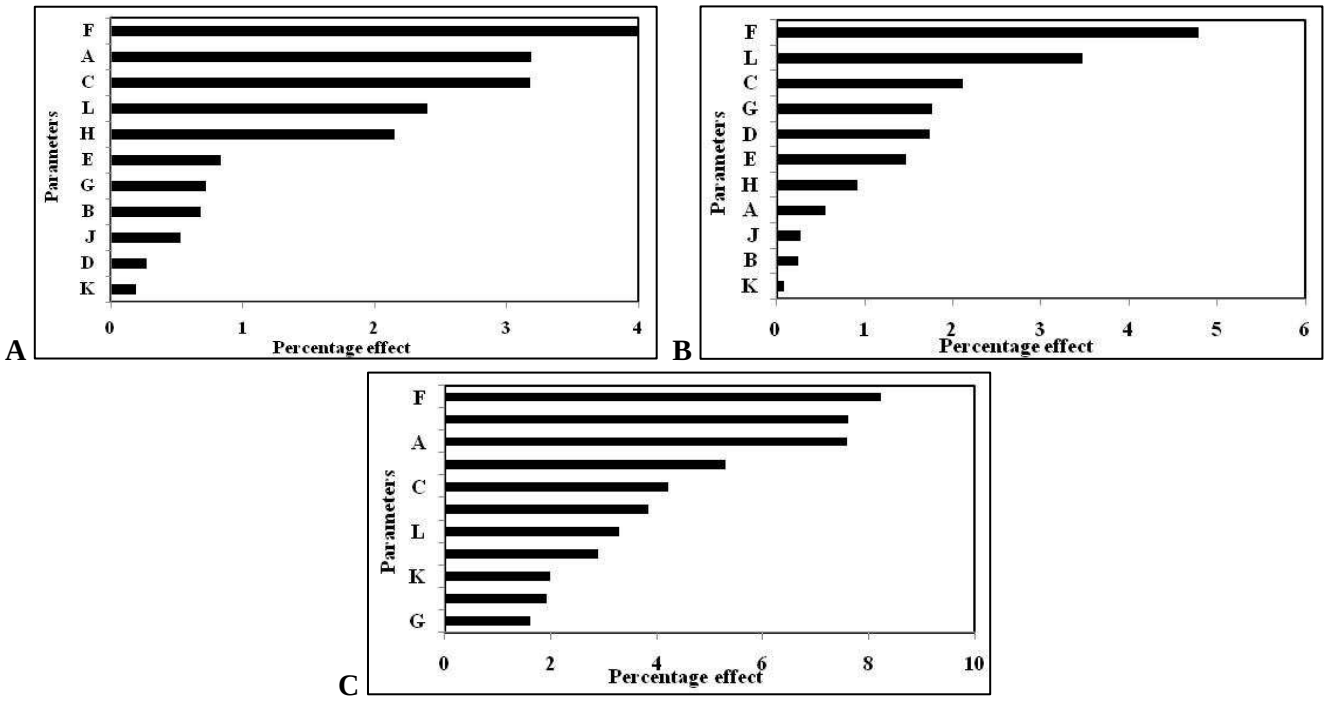

Figure 3. Pareto-Plot for Plackett-Burman parameter determines the effect of each parameter on kojic acid produced by *Aspergillus flavus* (3), A: Kojic acid (g/l); B: Dry mass (g/l) and C: Consumed sugars (%).

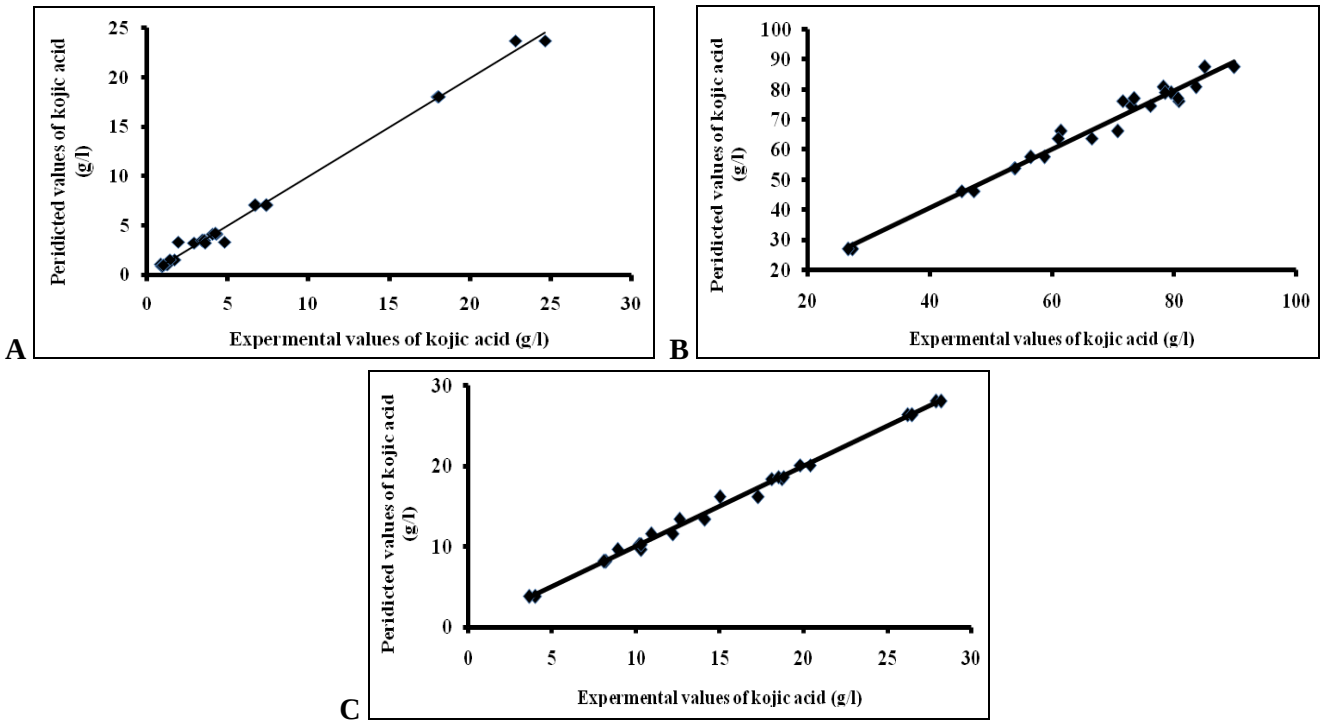

Figure 4. Comparison between kojic acid (g/l) experimental and predicted values of the Plackett-Burman design by *Aspergillus flavus* (3), A: Kojic acid (g/l); B: Dry mass (g/l) and C: Consumed sugars (%).

Three-dimensional response surface curves were generated to study the interaction between each two variables (Fig. 6). The Model F value of KA (value is calculated as ratio of mean square regression and mean square residual due to the real error) was 197.79 ($p<0.05$), DM was 207.74 ($p<0.05$) and CS was 44.09 ($p<0.05$) implies that the model is significant. The R^2 value was 99.45%,

99.48% and 97.59% for KA, DM and CS, respectively indicated that the entire variation was explained by the model. The adjusted R^2 value was 98.95%, 99% and 95.37% for KA, DM and CS, respectively.

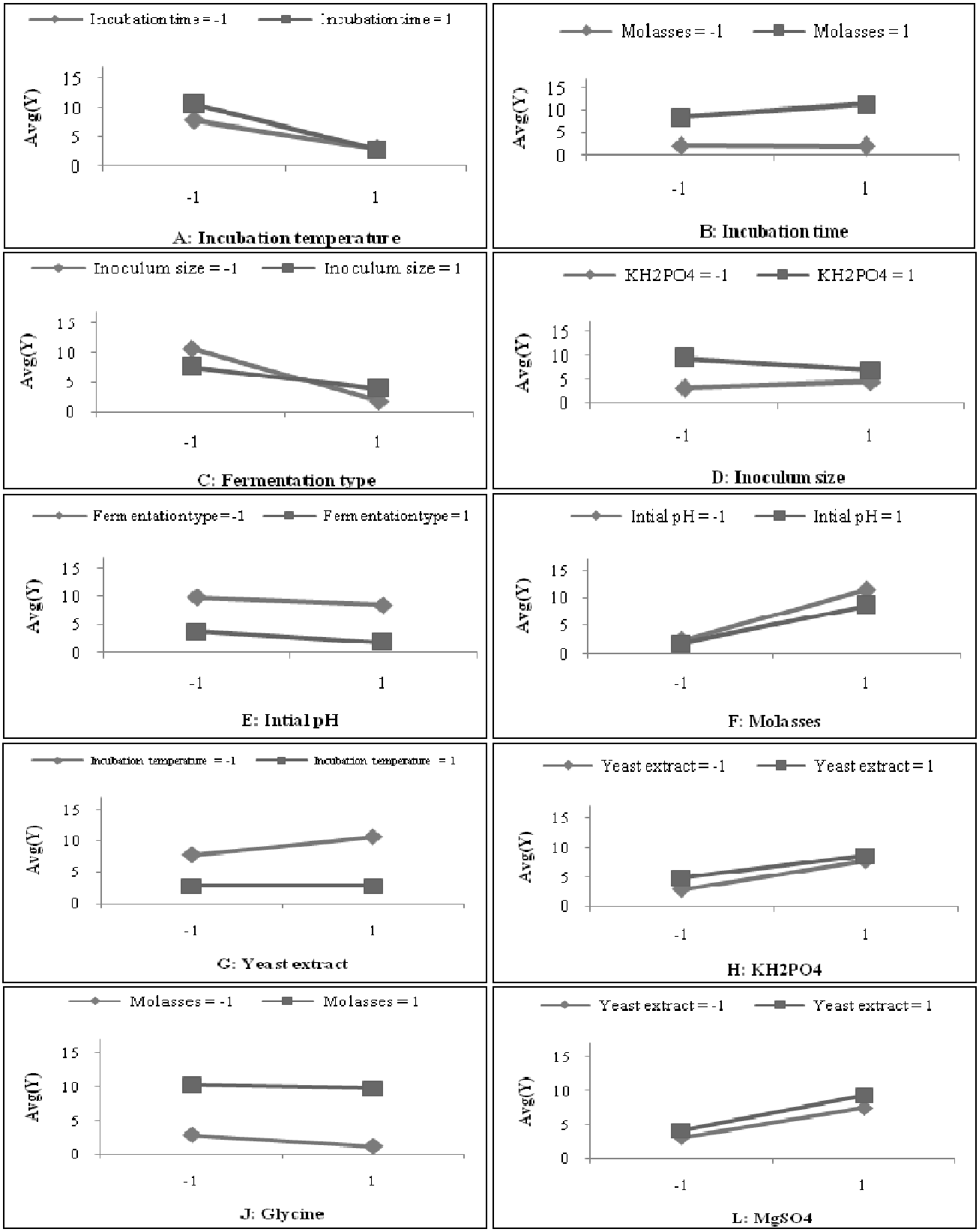

Figure 5. Main effects of different parameters on kojic acid production by *Aspergillus flavus* (3) showing effect of two variables (other variables were kept at zero in coded unit).

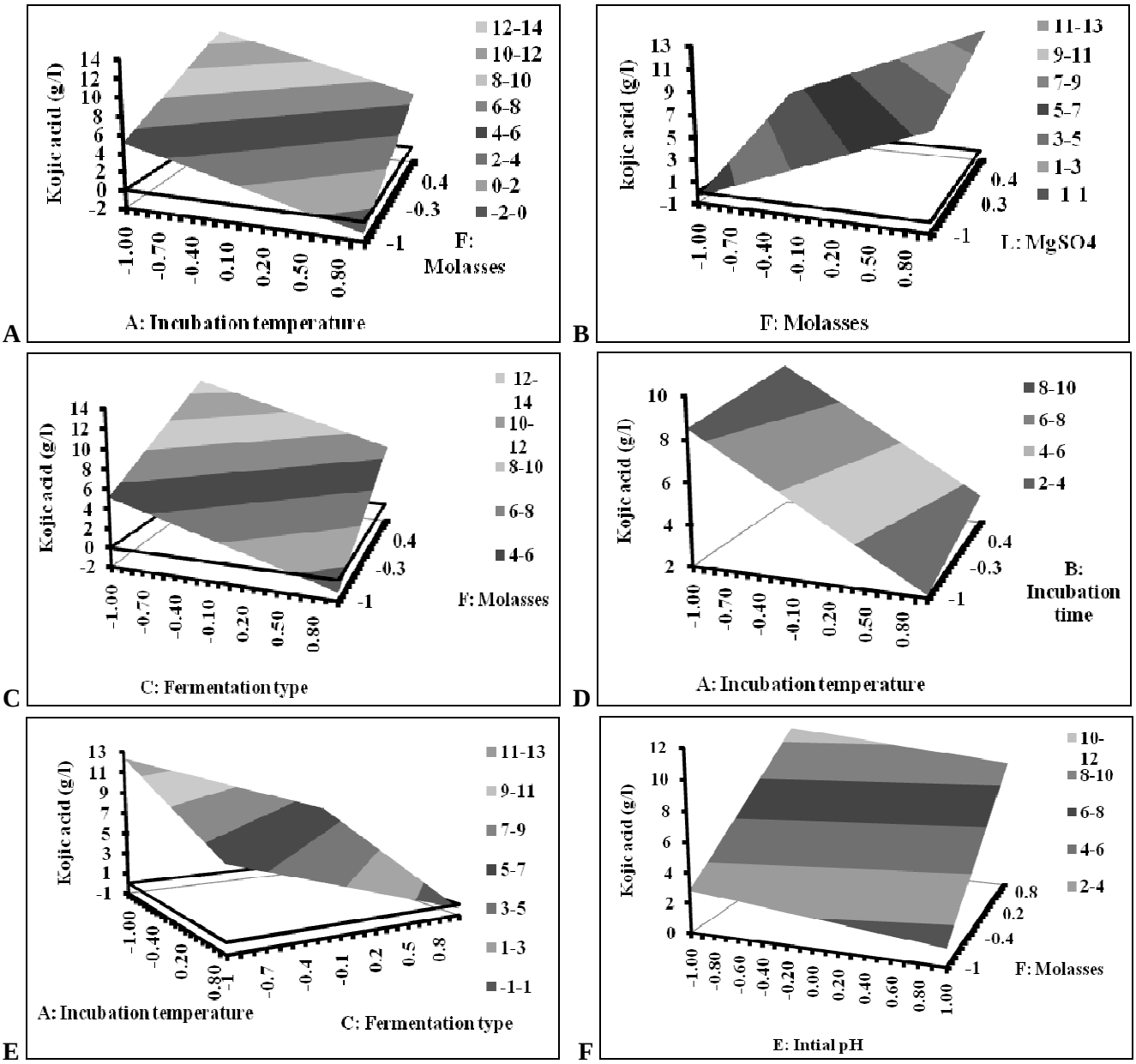

Figure 6. Response surface plots of kojic acid production by *Aspergillus flavus* (3) showing the effect of two variables (other variables were kept at zero in coded unit) : (A) Molasses and Incubation temperature, (B) Molasses and $MgSO_4$, (C) Molasses and fermentation type, (D) Incubation temperature and incubation time, (E) Incubation temperature and Fermentation type, (F) Molasses and initial pH.

Maximum KA production (24.65 g/l) by *A. flavus* obtained under the fermentation conditions: incubation temperature at 25°C, incubation time 9 days, pH 3, inoculums size 0.5%, shaking rate at 150 rpm and medium constituents: Cane molasses 60 g/l, yeast extract 7 g/l, KH_2PO_4 2 g/l, $ZnSO_4.7H_2O$ 100 µg/l and $MgSO4.7H_2O$ 1 g/l. In agreement with our results; Lin et al. [17, 63] showed that the optimal pH values for the production of kojic acid were 4.5, 6.2 and 6.5 by *A. flavus*, *A. parasiticus* and *A. oryzae*. Optimal pH for producing KA 3 obtained by strains of *A. oryzae*, this could be explained by the optimal pH of the KA-producing enzymes is around pH 3.5 [21, 59]. Also, secondary metabolites produced in the late log-stationary phases, in which the cultural medium has already been acidified by various acidic primary metabolites (itaconic acid or citric acid) [64, 65]. Optimum temperature for kojic acid production by fungi in the most of the cases was found to be 25-30°C [63, 66].

Table 3. Plackett-Burman design variables with kojic acid production by *Aspergillus flavus* as response.

Trials	A	B	C	D	E	F	G	H	J	K	L	Kojic acid (g/l)		Dry mass (g/l)		Consumed sugars %	
												Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	-1	-1	1	1	1	-1	1	1	-1	1	-1	1.25	1.04	12.20	11.55	78.25	80.90
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	1.68	1.54	8.20	8.15	85.06	87.46
3	1	-1	-1	-1	1	1	1	-1	1	1	-1	4.30	4.17	18.10	18.40	45.28	46.28
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	4.24	4.25	4.00	3.80	61.48	66.15
5	1	1	-1	1	-1	-1	-1	1	1	1	-1	1.39	1.54	8.10	8.15	89.87	87.46
6	1	1	-1	1	1	-1	1	-1	-1	-1	1	2.94	3.25	19.80	20.10	79.46	78.98
7	-1	-1	1	1	1	-1	1	1	-1	1	-1	0.82	1.04	10.90	11.55	83.55	80.90
8	-1	1	1	1	-1	1	1	-1	1	-1	-1	6.70	7.06	15.00	16.15	58.76	57.65
9	1	-1	-1	-1	1	1	1	-1	1	1	-1	4.04	4.17	18.70	18.40	47.28	46.28
10	1	1	1	-1	1	1	-1	1	-1	-1	-1	3.44	3.48	12.60	13.35	71.61	76.23
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	22.82	23.74	26.20	26.35	66.60	63.82
12	1	1	1	-1	1	1	-1	1	-1	-1	-1	3.52	3.48	14.10	13.35	80.84	76.23
13	1	-1	1	-1	-1	-1	1	1	1	-1	1	0.99	1.00	10.20	10.25	80.62	77.05
14	-1	-1	-1	1	1	1	-1	1	1	-1	1	18.04	18.06	27.90	28.05	27.33	26.96
15	-1	1	1	-1	1	-1	-1	-1	1	1	1	0.88	0.93	8.90	9.60	53.81	53.89
16	1	1	-1	1	1	-1	1	-1	-1	-1	1	3.57	3.25	20.40	20.10	78.50	78.98
17	-1	1	-1	-1	-1	1	1	1	-1	1	1	24.65	23.74	26.50	26.35	61.04	63.82
18	-1	1	1	1	-1	1	1	-1	1	-1	-1	7.41	7.06	17.30	16.15	56.55	57.65
19	1	-1	1	1	-1	1	-1	-1	-1	1	1	1.92	3.36	18.50	18.65	73.07	74.60
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	4.25	4.25	3.60	3.80	70.81	66.15
21	-1	1	1	-1	1	-1	-1	-1	1	1	1	0.97	0.93	10.30	9.60	53.96	53.89
22	1	-1	1	-1	-1	-1	1	1	1	-1	1	1.01	1.00	10.30	10.25	73.47	77.05
23	-1	-1	-1	1	1	1	-1	1	1	-1	1	18.09	18.06	28.20	28.05	26.58	26.96
24	1	-1	1	1	-1	1	-1	-1	-1	1	1	4.80	3.36	18.80	18.65	76.13	74.60

The sign +1 and −1 represent the two different levels (high and low) of the independent variable under investigation. A: Incubation temperature, B: Incubation time, C: Fermentation type, D: Inoculums size, E: Initial pH, F: Molasses, G: Yeast extract, H: KH_2PO_4, J: $ZnSO_4.7H_2O$, K: Glycine and L: $MgSO_4.7H_2O$.

Table 4. Statistical analysis of Plackett-Burman design of each variable at two levels for kojic acid production by *Aspergillus flavus*.

Variable code	Variable	Coefficient			t value			P value		
		Kojic acid (g/l)	Dry mass (g/l)	Consumed sugars %	Kojic acid (g/l)	Dry mass (g/l)	Consumed sugars %	Kojic acid (g/l)	Dry mass (g/l)	Consumed sugars %
	Constant	5.990	15.367	65.830	40.055	104.901	87.615	0.0000*	0.0000*	0.0000*
A	Incubation temperature	-3.189	-0.550	7.602	-21.325	-3.755	10.118	0.0000*	0.0027*	0.0000*
B	Incubation time	0.677	0.250	3.842	4.526	1.707	5.113	0.0007*	0.1136 ^{N}	0.0003*
C	Fermentation type	-3.179	-2.108	4.223	-21.258	-14.393	5.620	0.0000*	0.0000*	0.0001*
D	Inoculums size	-0.271	1.742	1.928	-1.811	11.890	2.566	0.0952 ^{N}	0.0000*	0.0247*
E	Initial pH	-0.833	1.475	-5.292	-5.574	10.069	-7.043	0.0001*	0.0000*	0.0000*
F	Molasses	3.989	4.792	-8.241	26.675	32.711	-10.968	0.0000*	0.0000*	0.0000*
G	Yeast extract	0.720	1.767	1.617	4.815	12.060	2.151	0.0004*	0.0000*	0.0525 ^{N}
H	KH_2PO_4	2.153	0.917	2.906	14.400	6.258	3.868	0.0000*	0.0000*	0.0022*
J	$ZnSO_4.7H_2O$	-0.529	-0.267	-7.616	-3.541	-1.820	-10.137	0.0041*	0.0937 ^{N}	0.0000*
K	Glycine	-0.194	0.083	1.995	-1.299	0.569	2.655	0.2184 ^{N}	0.5799 ^{N}	0.0210*
L	$MgSO_4.7H_2O$	2.401	3.467	-3.282	16.058	23.665	-4.369	0.0000*	0.0000*	0.0009*

t – student's test, p – corresponding level of significance,* Significant at $p \leq 0.05$, ^{N}, non-significant at $p \geq 0.05$.

4. CONCLUSION

From the outcome of our investigation it is possible to conclude that non-toxigenic *Aspergillus flavus* can be highly recommended in industrial production of kojic acid. Also using statistical method in optimization for improving the production has a great potential for applications and was very effective in our study as the production of KA (24.65 g/l) in this paper increase with 2.33-fold in comparison to the production of original level (10.58 g/l) using Plackett-Burman design.

AUTHORS' CONTRIBUTIONS

A-NAZ and GAEM designed the research plan, drafting and revised. GAEM and RAH carried out the research point by point. All authors helped in collected, re-identifying the fungal strains and approved the manuscript. All authors read and approved the final manuscript.

TRANSPARENCY DECLARATION

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

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ISSN 2449-8955

European Journal of Biological Research

Review Article

Pesticides and food safety in Africa

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Received: 01 February 2018; **Revised submission:** 13 April 2018; **Accepted:** 30 April 2018

DOI: http://dx.doi.org/10.5281/zenodo.1237542

ABSTRACT

African countries have experienced nonconformance in the levels of pesticides for local consumption and export. Sometimes this leads to rejects and other forms of embarrassment from the importing countries. Economic challenge and lack of awareness heighten the overall cost of interventions in pesticide-related food safety management. For example, not a few of the infractions were a result of incorrect ways of pesticide application. The hazard accompanying chemical pesticide application has left open a window of biological alternatives which this review article seems to explore**.** The bio-alternatives, including green pesticides cancel out the adverse effect of residual chemicals on crops in farm and store and so make it more attractive.

Keywords: Pesticides; Food security; Human health; Green-pesticide; Africa.

1. INTRODUCTION

With an annual growth rate of 1.2%, the world population is estimated to reach 9 billion by 2050 [1, 2]. United Nations (UN) estimates, indicate that 95% of this increase in world population will occur in the developing countries and regions such as sub-Saharan Africa [3, 4], hence the need to step-up food production through increase in agricultural productivity. In Africa, crop losses caused by pests and diseases are two major barriers to increase in agricultural produce. This has led to the overzealous application of agrochemicals or pesticides to farm crops [2, 5], and this in turn has brought its own set of problems both to the farmers and the environment [6-10]. Pesticides are chemical substances used to kill, repel or control pests or used to prevent the damage the pests may cause. They are commonly used to control a variety of agricultural pests that are likely to damage farm crops and livestock, leading to a substantial reduction in farm productivity. Initially, with little insight into the long term effect, pesticides use seemed to be a success, until the incidence of resistance. Hitherto, easily controlled pests became uncontrollable due to adaptation leading to application of higher amounts to ensure effectiveness. The development of new chemicals resulted in undesirable side effects both to the farm produce and the environment [2].

2. CLASSIFICATION OF PESTICIDES

The level of toxicity by pesticides is classified by WHO [11] into 4 categories. These are; class I: very toxic, class II: toxic, class III: slightly toxic and class IV: unharmful. Some common categories of pesticides include insecticides, algicides, herbicides, biocides, fungicides, molluscicides, nematicides, and rodenticides. Pesticides are also grouped according to their chemical properties and these include the organophosphates, organochlorines (chlorinated hydrocarbons), carbamates and thiocarbamates, and pyrethroids (Table 1). Pesticides formulations and

presentation are in solid, liquid and gaseous forms.

Just before the commencement of the current millennium, new techniques were introduced which are mainly biological (chemical free) and include microbial pesticides, biochemical pesticides and genetically modified organisms (GMOs) to salvage the situation of massive spread of agro-chemicals in agricultural fields. However, in the tropical regions of sub-Saharan Africa, the use of pesticides (agro-chemicals) is still the common practice. Chemical compounds such as DDT, HCH and Lindane that are environmentally recalcitrant are today banned from use in farms in developed countries of the world but tragically remains in popular use in developing countries [2]. The persistent pesticide residues therefore readily contaminate food and disperse in the environment. This is particularly prevalent in regions of lack of progress and economic challenge. Food quality becomes compromised and the environment adversely affected. Agbohessi [12] stated that the growing use of pesticide in agriculture contributes significantly to environmental pollution. Velisek [13] had viewed the role of pesticides to environmental pollution to be on par with emissions from industrial sources. Only about 0.1% of the farm sprayed pesticides reach the target pests, the excess is dispersed in the ecosystems where it contaminates the land, water, and air [14] thus endangering lives. Even at non-lethal doses, these chemicals disrupt the nervous system, liver, hormonal regulation, reproduction, embryonic development and growth in fish [15-20]. In South Africa, agricultural residue pesticides such as DDT, malathion, cypermethrin, aldrin, and endrin have been in the waters of Western Cape. They express bio-concentration and bio-magnification along the food value chain with possible dire health consequences to man and livestock [21].

In West Africa sub region, farmers use large quantities of pesticides, sometimes not appropriate in doses and efficacy [12]. The use of banned pesticides is not uncommon, for example, Endosulfanis still in use though it was banned in 2007 due to its ability to pollute the environment and poison human beings [22]. Endosulfan (6,7,8,9,10, 10-hexachloro 1,5,5a,6,9,9a-hexahydro-6,9-methano-2,3,4-benzodioxathiepine-3-oxide, CAS No.115-29-7) is an organochlorine pesticide rated as the most hazardous to the environment due to its non-biodegradable nature and possibility of bio-magnification as it migrates along the food chain [23]. Tihan, a milder and more environmental biodegradable pesticide has been developed to replace Endosulfan, however, the two are still in use simultaneously in West Africa [12] thereby putting not only the environment but also our food crop at perpetual risk.

Sodium chlorate and sulphuric acid were in use in the 1940s and in the late 1940s synthetic pesticides such as DDT, BHC, Aldrin, Dieldrin, Endrin, Chlordane, Parathion, Captan, and 2,4-D were developed and widely used. These new products were cheap, effective and generally accepted. However, in 1962, the problems and danger of the indiscriminate use of pesticides to the environment was highlighted. Even when many African countries are familiar with, and possibly signatories to many global initiatives like FAO code of conduct on distribution and use of pesticides, Codex, Cartagena Protocol, Montreal Protocol, Stockholm convention, needless application leading to environmental pollution and the concerns about health of living organisms still subsists. Reasons for these include inadequate expertise [24]; conscious use of obsolete pesticides [25] and different monitoring capacities that vary from one location to another [26].

In 1970s-1980s, many new products were born out of research including the popular and greatest selling herbicide ‘glyphosate’, third generation insecticides and new spray treatments. From the 20th to the 21st century, an entirely new family of pesticides or agrochemicals has been birthed. Modern research and advances in chemistry has made these products safer, more selective and much more environmental friendly, with effective usage rate only requiring grams rather than kilograms per hectare [27]. These modern agrochemicals have helped farmers boost productivity significantly, particularly in regions like Sub-Saharan Africa (SSA) where crop yields have been low. In a Kenya survey, the commonly used insecticides on vegetables included dimethoate (WHO II), used by 48% of farmers, lambda cyhalothrin (WHO II, 27%), cymoxanil (WHO II, 22%), cypermethrin (WHO II, 22%), cyfluthrin (WHO Ib, 20%), mancozeb (WHO U, 18%), and deltamethrin (WHO II, 14%). Lack of Personal Protective Equipment (PPE), reduced literacy level and geographical location contribute to

pesticide-related cost of illness (COI). When morbidities arise from such exposure, cultural therapists deploy milk, lemon juices, honey, and herbs [28].

3. TYPES OF PESTICIDES

There are various types of chemical pesticides. Their characteristics and mechanism of action are as shown in Table 1.

4. PESTICIDES AND FOOD SAFETY IN AFRICA

Agriculture remains the major occupation in most African countries and dominated by small and medium holder farmers with minimal education. Pesticide use in Africa has therefore been reported as low compared to other continents. Agrow [30] reported a range of approximately 2-4% of global pesticide market of US$ 31. Similarly, Repetto and Baliga [31] reported average pesticide use in Africa per hectare as 1.23 kg/ha, while 7.17 kg/ha has been recorded in Latin America and 3.12 kg/ha in Asia. One conclusion could be that most farmers in Africa being small holder farmers, and their output in terms of food production is low, do not usually consider the use of pesticides [32]. Pesticides are preferably used on large scale, commercial farming of cash crops such as cotton, cocoa, oil palm, coffee and vegetables. The fact remains that whether pesticide use in Africa is low or not, the risk and impact arising from the toxicity of the chemicals where used, cannot be overemphasized. Previous studies have reported poor pesticide practices by African farmers [32-34] and these practices include use of unsuitable products, poor handling, wrong dosage, timing and targeting of application, non-calibrated equipment, and application equipment that is poorly maintained [35]. Others are the use of banned products, mixture of products, mixing with bare hands, splashing pesticides on crops using brushes or twigs, lack of minimal protective clothing and even tongue-testing to assess concentration strength has been reported [34, 36-38]. In 2018, South Sudan played host to a technical team from the UN due to the invasion of the farms in 8 states by army worm [39]. A cocktail of pesticides is being proposed which may go with the attendant consequences, if misapplied.

This misuse of pesticide results not only in the control of the intended pests but also has serious impact on the operator, the farm crops and livestock, the health of the consumer, soil organisms, and contamination of the entire environment [40, 41]. United Nations report [42] has highlighted the growing health and environmental hazards from chemicals and stated that the potential cost of pesticide-related illnesses in sub-Saharan Africa between 2005-2020 could reach $90 billion. United Nations Environmental protection (UNEP) estimated the cost of lost work, medical treatment and hospitalization due to pesticide poisonings among small scale farmers in 37 African countries to be $4.4 billion. In 2010, Uganda's National Environment Management Authority reported that each farmer loses 24.6 days per year due to pesticide poisoning, 9.4 days due to respiratory illnesses and 15.2 days due to skin infections [43].

In 2015, the EU banned some agricultural food items meant for export from Nigeria due to the presence of high levels of dichlorvos pesticide. The food items include beans, sesame seeds, melon seeds, dried fish and meat, peanut chips and palm oil [44]. The European Food Safety authority (EFSA) claimed that the rejected food items were found to contain between 0.03-4.6 mg/kg of dichlorvos pesticide while the acceptable Maximum Residue Limit (MRL) is 0.01 mg/kg. This pesticide is usually applied during storage while the products are being prepared for export. In 2013, EU issued fifty (50) notifications of border refusals to Nigerian beans exporters over high level of unauthorized pesticide. In 2015, EU issued thirteen (13) border rejections alert to the same beans exporters. Generally, between 2008 and 2013, there has been a significant number of border refusals of food imports by the EU due to non-compliance of exporting countries with its food safety standards, which amount to about 9233 rejections between 2008 and 2013 [45]. Fruits and vegetables are the most commonly affected product usually refused entry into EU markets as a result of the exporters failing to meet EU standards. The surveillance studies on vegetables in Ghana presented a mixed bag. While residues were found at concentrations not particularly considered a health problem with occasional exceedances in respect of chlorpyrifos, diazinon and permethrin [46], an earlier investigation by the Christian Aid [47] and

NPA [48] the use and misuse of pesticides indeed constituted a major threat to the lives of farmers because of exceedance of EU MRLs. Although raw agro produce are particularly primary targets, industrial products like soft drinks and fast foods are not spared [49, 50].

Table 1. Various types of pesticides (insecticides, fungicides and herbicides), examples, their characteristics and mechanism of action.

S/No	Type: Chemical	Examples	Characteristics	Mechanism of action
1.	Organophosphates	• Chlorpyrifos • Dimethoate • Fenthion • Naled • Temephos • Trichlorfon	- Made from phosphoric acid - Most are insecticides - They are highly toxic - It breaks down faster in the soil, food and feed	These control pest by acting on the nervous system, interfering with the nerve impulse transmission, disrupting the enzyme cholinesterase that regulates acetylcholine (a neurotransmitter)
2.	Organochlorines (chlorinated hydrocarbons)	• Aldrin • Chlordane • Dieldrin • Endolsulfan • Endrin	- Generally persistent in the soil, food and in human and animal bodies. - They can accumulate in fatty tissues - Traditionally used for insect control and mites. - They do not break down easily. - Some such as DDT and Chlordane are no longer in use because they stay in the environment for a long time	They control pests by disrupting nerve impulse transmission
3.	Carbamates and Thiocarbamates	1.Insecticides • Carbaryl (banned due to health risks) • Propoxur • Methomyl • Carbofuran • Thiodicarb 2. Herbicides • Barban • EPTC • Propham • Triallate 3. Fungicides • Nabam	- They are made from carbamide acid - They are less persistent in the environment - Mild health hazards to human and animals especially the herbicide and fungicide range. However, health risk is higher with insecticides	They control pest by acting on the nervous system, interfering with the nerve impulse transmission, disrupting the enzyme cholinesterase that regulates acetylcholine, with enzyme effect usually reversible
4.	Pyrethrin (synthetic version of Pyrethrin, modified to increase stability in the environment)	• Cyhalothrin • Cypermethrin • Deltamethrin • Esfenvalerate • Permethrin	- Stable in sunlight (do not degrade quickly)	Disrupts nerve impulse transmission (increases sodium flow into axon), which stimulates nerve cells and eventually causes paralysis

Source [29].

Acute or chronic exposure of humans to dichlorvos can have dire consequences. It inhibits an enzyme, acetylcholinesterase [51] with neurotoxic effects such as difficulty in breathing, diarrhea, abdominal cramps, vomiting, salivation, sweating, nausea, convulsions, dizziness, weakness, tightness in the chests, blurred vision, eye and skin irritation, eye pain, runny nose, wheezing, laryngospasm, cyanosis, and at high concentrations convulsion and coma. As at date, there is no conclusive information on the carcinogenic effects of dichlorvos on humans. Symptoms in animals include ataxia, salivation, dyspnea, tremors and diarrhea. Toxicological studies on animals showed an increase in the incidence of tumors of the pancreas, mammary glands and stomach.

According to Varo [51], dichlorvos has been found to be a highly toxic pesticide. In a study to determine the residual levels of the commonly used dichlorvos on small and large scale vegetables farms in Lusaka, Zambia, it was reported that the levels of dichlorvos was significantly above the MRL [52]. Similarly, in Northeastern Nigeria, research showed that farmers and traders use locally formulated pesticide which contains high levels of dichlorvos (about 7.7% w/v) in trade [53]. Therefore, consumers of such vegetables and other commodities with such levels of dichlorvos residues in Zambia and Nigeria, and in Africa as a whole are at risk to the health issues outlined above in the long run, since they export to other African countries.

5. PESTICIDE STANDARDS REGULATION

Just like drugs, pesticides are subject to regulation. The safety of pesticides is reviewed by the authorities before they are allowed to be used on crops. In Nigeria, the Standards Organization of Nigeria (SON) and NAFDAC are responsible for setting these standards, controls and enforcement. These standards are synchronized with the Codex Alimentarius (Codex Alimentarius Commission) which was established by FAO and WHO in 1963. The commission ensures coordination of all food standards work embarked upon by international governmental and non-governmental organizations.

The use of pesticides is usually authorized only after a risk assessment has been done and checked that any residue remaining after correct use of the pesticide will not lead to any consumer concern. The potential residues on a harvested crop are regulated by a maximum residue level (MRL) which is set As Low As Reasonably Achievable; the ALARA principle [54]. MRL usually include wide safety margins that are well below the level that could pose any adverse effect on consumers' health and safety. Maximum Residue Levels (MRLs) are part of Good Agricultural Practices (G.A.P). MRLs are primarily trading standards, which are applied to help ensure that residue levels do not pose unacceptable risks for consumers of such food. Pesticides regulation in the EU, is governed by Directives (EC) No 396/2005. This Directive which came into effect in 2008 establishes the MRLs of pesticides allowed in products of plants and animal origin intended for consumption, based on scientific evidence from risk assessments. This Directive replaced all pesticides standards among EU member states which existed prior to 2008. Figure 1 illustrates how residue levels are measured.

The ADI and ARfD are obtained through animals such as mice testing. These are set based on the highest dose where no recognizable harmful effects are observed; the No Observable Adverse Effect Level (NOAEL). The international practice is that the NOAEL is divided by an uncertainty factor of at least 100 to compensate for potential differences between animals and humans; and for differences between individuals [54]. Since the NOAEL may differ for chronic (long term) and acute (short term) effects, the ADI and ARfD may be set at different levels.

When MRLs are exceeded, it does not necessarily imply a risk to health but an indication that a pesticide has been incorrectly used. Food products which have residues exceeding MRL cannot be sold. When a farmer uses a pesticide according to the label instructions and Good Agricultural Practice (GAP), the residues in crop at harvest do not normally exceed the Maximum Residue Level established in the country of use. However, since MRLs are not harmonized world-wide, MRL exceedances can occur when products are exported to a country with a lower MRL for the specific pesticide and crop combination.

In South Africa, according to Mutengwe [26] the implicated pesticides that exceeded established MRLs were imazalil (37.71%), prochloraz (28.69%),

and iprodione (5.74%). The unregistered pesticide residue most often found on grapes and avocados was also imazalil (62.23%) and, on nectarines diphenylamine (11.15%) and the exceedances of MRL values involved oranges (43.44%), avocados (27.87%), grapefruits (7.38%), and lemons (6.56%). This led to a change of mind and negative perception by the public and reconsideration by the government on how to control pesticide use globally. It is not in all cases that residues went as higher than the MRL set by regulating bodies as observed in the survey carried out in Sudan [55]; although high levels sometimes are controlled by biological means [56]. In Libya, organochlorines and other pesticides from the field, were detected in some fish at concentrations higher than the permissible limit, according to FAO where concentrations were calculated in mg/kg BW of fish [57].

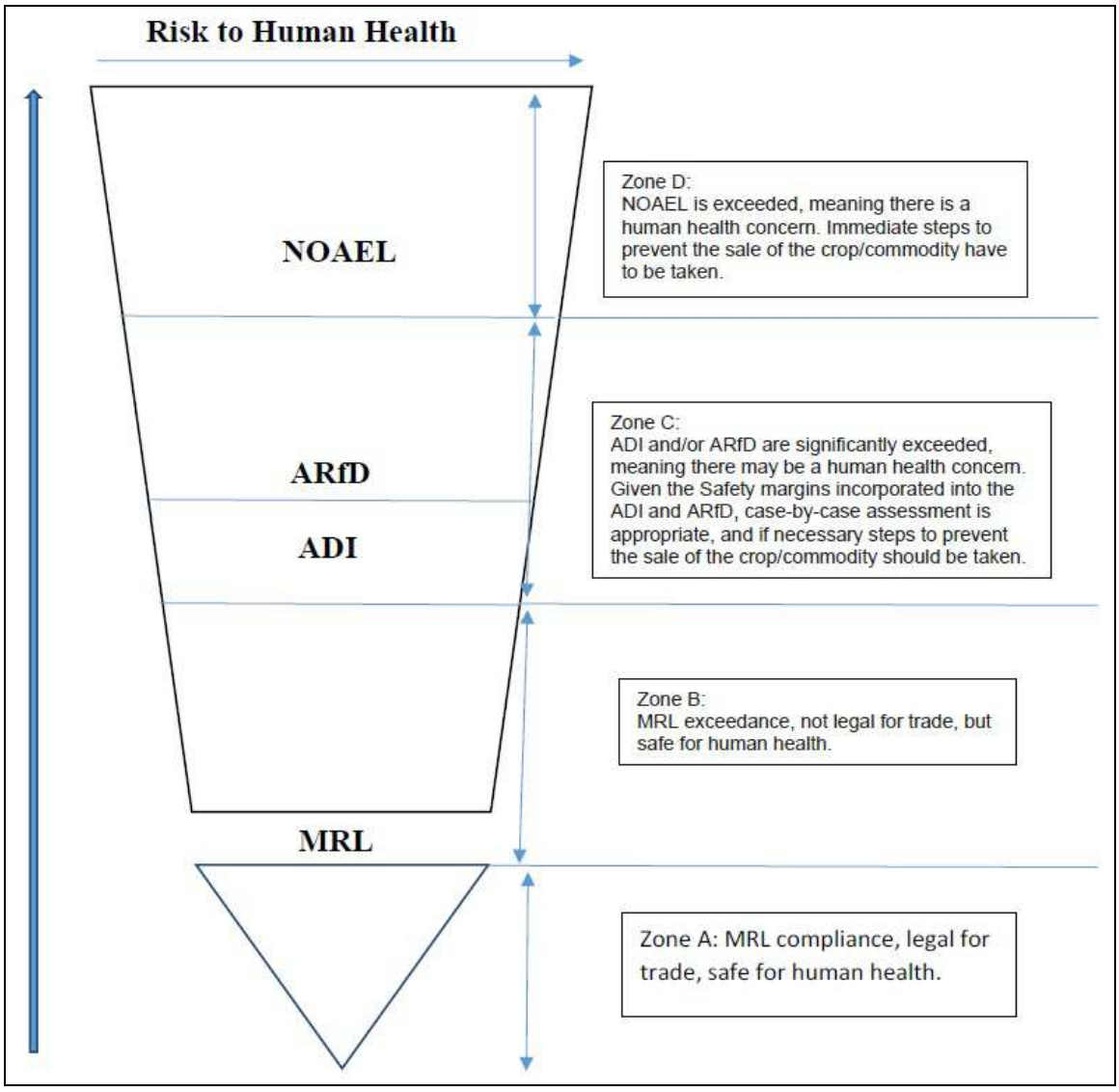

Figure 1. Measuring Residue levels [54].

Legend:

NOAEL (No Observable Adverse Effect Level): the highest exposure level at which no adverse effects can be identified in tests.

ARfD (Acute Reference Dose): a toxicological safety limit specifying the amount of a substance which can be ingested on a single day without any effects on the health of the consumer.

ADI (Acceptable Daily Intake): a toxicological safety limit specifying the amount of a substance which can be ingested every day over an entire lifetime without any recognizable risks to the health of the consumer.

MRL (Maximum Residue Level): a legally fixed maximum concentration for a particular active ingredient in a particular crop. A trade standard, intended primarily to check that a pesticide has been applied correctly.

6. HEALTH EFFECTS DUE TO CONSUMPTION OF FOOD WITH PESTICIDE RESIDUES

Pesticides are potentially toxic and hence are hazardous to humans and animals resulting in both acute and chronic health effects depending on the dosage and ways in which persons are exposed. Exposure can be through contact with the skin, ingestion or inhalation. When people consume food with large quantities of pesticides, above safe limits, it can lead to acute poisoning or long term health effects including cancers. Chronic toxicity occurs when subjected to small doses over a period of time. Imazalil is among the persistent pesticides on edible fruits such as grapes, with an allowed MRL of 5 mg/kg. When consumed over a long of time in concentrations above the MRL, it can cause mortality issues and significant brain enzyme depression [58]. The numerous suspected health effects that have been associated with chemical pesticides include dermatological, gastrointestinal, neurological, carcinogenic, respiratory, reproductive and endocrine effects [59-62]. In some cases can lead to hospitalization and death [59, 63]. Pesticide residues and food safety has also been an issue of concern from the consumers perspective, although the rating seemed to be far below food safety caused by bacterial pathogens. In 2011, a workshop organized by EU to capture opinions of stakeholders on food safety issues in fresh foods reported that consumers ranked bacterial pathogens as the first, followed by foodborne viruses, with Pesticides residues and mycotoxins taking the third and fourth positions respectively in the ranking [64]. This shows that though pesticide residues cause serious health effects on consumption of foods contaminated with them, other more serious causative agents such as bacterial pathogens and viruses are of more importance to the consumers and should as a matter of priority be the main focus. In the same workshop, these consumers proposed as control measures good agricultural practices, good hygiene practices and food safety management system certifications.

7. CURRENT TRENDS IN FOOD AND CROP PROTECTION

In the last decade, there has been new development in food and crop protection. They are the genetic engineering of organisms, the organic-chemical-free agriculture and green pesticides.

1. Genetically modified organisms (GMOs) such as engineered soybeans, maize, and tomatoes came as a solution to food security and revolutionized agriculture [65]. These crops were modified to be tolerant to glyphosate, a common herbicide. South Africa in recent times delved into the development of alternative methods of pest control in order to reduce environmental levels of organic and inorganic pesticides. One of these developments is genetically modified (GM) crops, such as GM maize and cotton. More than 90% of farmers plant GM maize and cotton and South Africa is currently ranked 9th worldwide in planting GM crops [66]. There are many advantages of using GM crops for pest control. First, the crop is protected continuously in the field and the time used to detect pest infestation is reduced. Secondly, there is protection of the plant part that is difficult to reach with insecticide spraying. Thirdly, control is no longer affected by the weather. The crop is protected even if the field conditions are not suitable for aerial or ground application of insecticides [67]. Also, there is general reduction in insecticide use. Although GM crops have become a major component of insect control strategies, a proper perspective of its potential demands a close look at limitations and uncertainties that may reduce its future impact on agriculture such as development of resistance of target pest and effect on potential non-target organism [21].

Secondly, sprays of the bacteria, *Bacillus thuringiensis*, have been used to control pests. The crystalline protein produced by these bacteria kills certain insect species and have limited effects on most non-target species [68]. The use of commercial *Bacillus thuringiensis* sprays have, however, been limited due to their relatively high cost, poor crop coverage, rapid environmental inactivation, and less desirable level of pest control, when compared with less expensive conventional chemical insecticides [69]. Toxin-encoding genes from *B. thuringiensis* have been expressed in transgenic crop plants, providing protection from some key pests [68].

In South Africa two of these key pests of maize are the lepidopteran stem borers, *Busseola fusca* and *Chilo partellus* which are of economic

importance throughout Southern and Eastern Africa. Large-scale planting of *Bacillus thuringiensis* (Bt) crops to control these pests started in South Africa in 1998. Bt cotton for control of the boll worm complex, particularly the African bollworm, *Helicoverpa armigera* was also introduced into South Africa during the same period. Generally, there is reduction in insecticide use. For example, reduced insecticide use was reported from the Makathini Flats region of Kwa-Zulu Natal, South Africa, where 95% of smallholder (1-3 hectares) cotton producers grew rain-fed Bt cotton. Farmers that adopted Bt cotton reported reduced insecticide use and a reduction in labour [70]. However, in India there have been reports of Bt cotton failures and claims of mass suicide due to significant financial losses by the farmers [71]. The government of India claims the pink bollworm, a major pest that attacks cotton crop has already developed resistance to the new technology, as observed in 2015/16 crop year, where most cotton crops were significantly damaged by the bollworm and whitefly in most of the farms in India [72]. In 2002, after field trials with Bt cotton became successful, Indian government officially approved the commercial release of Bt cotton to farmers and by 2010, almost all the farmers in India had migrated from non-Bt cotton to Bt cotton because of the advantages of high income gained through better pest management at lower cost. Expenditure on chemical pesticides was drastically reduced. Bt cotton technology gave a boast to Indian cotton business and helped India come to be the second largest producer of cotton in the world. The recent development of pest resistance to Bt cotton technology has become a serious threat to Indian cotton business. Burkina Faso in Africa, has completely rejected the introduction of Bt Cotton technology to its farmers both for political and economic reasons. Herring and Rao [71] in their study of Bt cotton failure reported that each hybrid of cotton consist of different germ plasm and the mechanism for obtaining the Bt transgene which confer insect resistance trait on them is not exactly the same, leading to variations in results in yields comparisons.

2. Organic agriculture:

The development of organic agriculture which respects the normal functioning of the ecosystem, avoids the use of pesticides and leads to food free of synthetic chemicals and thus healthier. However organic agriculture is limited in scope and does not have potential for mass production needed to feed the world [73]. Organic agriculture thus improves food safety but cannot cope with food security.

3. Green pesticides:

These are nature-oriented and beneficial pest control materials used to control pest populations thus increasing food production. Green pesticides are botanical and natural materials that are used to reduce pest population and increase food production. They are safe and eco-friendly, and are compatible with environmental components than synthetic pesticides. They include substances such as plant extracts, hormones, pheromones, and toxins of organic origin. It also includes many other aspects of pest control such as microbial, entomophagus nematodes, plant derived pesticides, secondary metabolites of microorganisms and mineral-based controls used to express resistance to pests. More recently, under this umbrella of green pesticides, are extremely biodegradable synthetic and semi-synthetic products in pest management.

Green pesticides are attractive alternative to chemical pesticides because they reduce the negative impacts to human health and the environment, the reason it is now a contemporary issue [6]. However, their use in Nigeria and other parts of Africa is still hampered by some challenges. First, there is still no appropriate application technology particularly the use of oils and dust formulations [75]. Secondly, the residual effect of green insecticides is short-lived compared to synthetic chemicals, hence repeated applications are required to obtain reasonable crop protection. Thirdly, they are yet to be available to farmers in commercial quantities. Fourthly, there is the problem of farmers' acceptability of this new technology in pest control [76] which calls for training and promotion of the use of these green pesticides in integrated pest management by the relevant authorities. Awareness campaigns and farmer-friendly capacity building can resolve most of these issues.

Table 2. Types of green pesticides (botanicals, biological and mineral-based controls).

Types	Description	Target
1. Botanical pesticides	These are plant extracts used as insecticides to control insects. They are usually harvested by macerating plant tissues high in active ingredients and distilling the specific compound. The advantage of using botanical pesticides is their rapid degradation in the environment.	
i. Neem	Insecticidal extract (namely azadirachtin) from seed and bark of the Neem tree (widely found in India), which act as insect repellant, anti-feedant and interferes with growth. Neem can also be used as systemic insecticide when applied directly to the soil and taken up by the plant and transported to the shoots and leaves. Multiple applications are required as it degrades easily (with 3-7 days).	Effective against caterpillars, flies, whitefly, scales, and aphids.
ii. Pyrethrium	Easily broken down by stomach acid of mammals, hence toxicity is very low except if application doze is increased above recommended on label.	Effective against soft bodied garden pest such as scales, whitefly, mealybugs and thrips but ineffective against mites.
iii. Horticultural oils	They work by disrupting insect feeding and egg laying. Egg covered with oil suffocates the developing pest. Have minimal phytotoxic effects on plants when used properly.	All insects
iv. Dormant & summer oils	They can be applied to plants during growing season.	Effective against eggs, nymph, larva, and adult leaf rollers, aphids, mites, and scales.
v. Traditionally used botanical insecticides e.g. Nicotine, Rotenone, Ryania, Sabadilla, and pyrethrum	Nicotine and tobacco have long history of use and is very effective against insects but also has high toxicity against mammals. Hence being considered for regulatory phase out. Rotenone made from isoflavonoid, an extracts of the tropical legumes *Derris* and *Lonchocarpus*. It is highly toxic to insects and fish but also moderately toxic to mammals. Rotenone has been widely used on ornamental crops, but has been phased out in the US and Canada during regular re-evaluation. However, its use is being continued in other countries. Sabadilla is an extract from the seed of *Schoenocaulon officinale*, a neo-tropical lilywhich contains veratridine alkaloids with a neurotoxic mode of action. It high toxicity as contact insecticide and low mammalian toxicity. Ryania is an extract from *Ryania* sp., a South American shrub. It contains the active ingredient di-terpene alkaloid ryanodine, which is a contact and ingested insecticide against horticultural and ornamental crop pests. Pyrethrum is an extract of *Chrysanthenum cinerariaefolium* plant. It is toxic to both mammals e.g. cats, fish and also insects	Effective against agricultural insects such as lepidoptera, leafhoppers, and thrips. Also mosquitoes, fleas, flies, moths ants bees,

Types	Description	Target
2. Natural (biological) products	These are living organisms used to control pests and are called biological controls or biological agents. When a microorganism is packaged and sold to control a pest, it is legally considered a bio pesticide and is regulated as such [74]. However, Nematodes are not regulated pesticides though equally used as bio pesticides.	
i. *Bacillus thuringiensis (Bt)*	*Bt* is a naturally-occurring bacterium. Commercial Bt products are formulations of the bacterial toxin and are non-living. Bt can be sensitive to ultraviolet light (sunlight) and is most effective when applied in overcast conditions or late in the day. Most Bt products degrade within 24 hours regardless of sunlight conditions or temperature, giving them a very short period of effectiveness once they have been applied. Multiple applications are thus needed for sufficient management of pests.	Feeds on the larval stages of insect pests such as mosquitoes, Colorado potato beetles, and cabbage loopers. *Bt.* var. *kurstaki*feeds on caterpillars, commonly found on vegetables and fruits.
ii. *Beauveria bassiana*	*Beauveria bassiana* is a soil borne fungus. *It is* applied to the target pest as a spore. Once the spores have contact with the insect exoskeleton, they grow hyphae that secrete enzymes, which in turn dissolve the cuticle. These fungal hyphae then grow into the insect, feed on its body tissue, produce toxins, and reproduce. It takes up to seven days for the insect to die. If moist conditions (92 percent humidity or greater) are present during this time, *B. bassiana* will "bloom" and release more spores into the environment to repeat the cycle on other pest insects.	Effective against thrips, aphids, whitefly, caterpillars, beetles, and subterranean insects like ants and termites
iii. Nematodes	Nematodes are multi cellular organisms commonly referred to as microscopic worms.	Effective against soil-dwelling insect pests such as root weevils and cutworms, and can also control pests that pupate or hibernate in the soil such as codling moth larvae
iv. *Nosema*	*Nosema* are protozoans.	*Nosema locustae* is used to manage grasshoppers
v. Fermented microbes e.g. abermectin	Abermectin, is a fermented product of *Streptomyces avermitilis*	Used in baits for household insect pests. The fermented product is very toxic to caterpillar pests such as cabbageworm, cabbage looper, diamondback moth, armyworm, and cutworm, as well as fruit flies such as spotted wing drosophila.
3. Mineral Controls	Insecticides developed from mineral resources mined from the earth. The toxicity of mineral-based insecticides depends on the chemical properties of the mined elements. Some, such as sulfur are registered for organic use and have relatively low toxic effects on people and non-target organisms. In	

Types	Description	Target
	contrast, lead arsenate is a natural mineral product that was cancelled as a pesticide in 1988 due to its toxicity and persistence in the environment.	
i. Diatomaceous earth	Fine particle dust comprised of fossilized diatoms.	Effective against slugs and soil-dwelling insects
ii. Elemental Sulphur	Elemental sulfur is a finely ground powder that can be applied either as a dust or a spray. This mineral is one of the oldest pesticides known, and reported pest resistance is rare. Sulfur acts as a metabolic disruptor on insects.	Effective against aphids, thrips, and spider mites
iii. Iron phosphate	They come in pellets and liquid formulations.	Effective against slugs and snails when combined with baits.
iv. Kaolin	Kaolin is fine clay that is sprayed on plant foliage or fruit to deter feeding and egg laying of insects.	Effective against apple maggot, codling moth, and leafhoppers.
v. Soap	Natural soaps are derived from plants or animal fat.	Effective against aphids, scales, whitefly, mealy bugs, thrips, and spider mites.

Source: [6].

8. CONCLUSION

From the fore-going, there are several evidences that green pesticides are generally safe and effective, although they come with their challenges as already highlighted above. In spite of the seeming shortcomings, green pesticides still remain the attractive option and an alternative for the future. Vendors, consumers and policymakers need to be made aware of the higher quality and safety of products treated with green pesticides. They are equally eco-friendly and extremely biodegradable. Therefore, carry over into food and food products is unlikely, thus reducing food safety risks. This approach looks attractive in solving the issue of incessant pesticide exceedance in food trade involving Africa and Europe.

AUTHORS' CONTRIBUTION

SOF suggested the topic and provided the technical guide. AAA did extensive literature search. Both authors read and approved the final manuscript.

TRANSPARENCY DECLARATION

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

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ISSN 2449-8955

European Journal of Biological Research

Research Article

Pectin coating of titanium and polystyrene surfaces modulates the macrophage inflammatory response

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Received: 19 April 2018; **Revised submission:** 11 May 2018; **Accepted:** 20 May 2018

DOI: http://dx.doi.org/10.5281/zenodo.1250541

ABSTRACT

Titanium has been used with success for bone anchoring of dental implants. However, when implant surfaces are exposed to the oral environment, the progression of peri-implantitis triggered by specific oral bacteria has been reported. Bacterial colonization of implants leads to prolonged immune cell activation and bone resorption. A new strategy to improve implant biocompatibility and prevent peri-implantitis is to develop pectin surface nanocoatings. These plant-derived polysaccharides are promising candidates for surface nanocoatings of titanium implants due to their osteogenic and anti-inflammatory properties. Therefore, the aim of the study was to evaluate the *in vitro* effect of nanocoating with plant-derived rhamnogalacturonan-I (RG-I) on pro- and anti-inflammatory responses of primary human monocyte-derived macrophages (HMDMs) induced by *Escherichia coli* LPS and *Porphyromonas gingivalis* bacteria. In the present study, two different types of surface materials, tissue culture polystyrene (TCPS) plates and titanium (Ti) discs, coated with pectic polysaccharides, potato unmodified RG-I (PU) and potato dearabinanated RG-I (PA), have been examined. The inflammatory responses of HMDMs after *E. coli* LPS/*P. gingivalis* stimulation were investigated through gene expression measurements of pro- and anti-inflammatory cytokines. The results showed that PU and PA decreased expression of the proinflammatory genes tumour necrosis factor-alpha (*TNFA*), interleukin-1 beta (*IL1B*) and interleukin-8 (*IL8*) in activated HMDMs cultured on TCPS/Ti surfaces. In contrast, the effects on anti-inflammatory interleukin-10 (*IL10*) gene expression were not significant. The results indicate that RG-Is should be considered as a candidate for organic nanocoatings of titanium implant surfaces in order to limit host proinflammatory responses and improve bone healing.

Keywords: Rhamnogalacturonan-I; Titanium; Nanocoating; *Porphyromonas gingivalis*; LPS; Macrophage; Inflammation.

1. INTRODUCTION

The gold standard material for endosseous dental implants is titanium (Ti) due to its favorable physiochemical, mechanical and biological properties [1-3]. However, when dental implant surfaces are exposed to the oral environment, spontaneous progression of inflammation with bone and soft tissue destruction has been reported [4]. Therefore, titanium implant surface modifications are continuously developed to limit inflammation and enhance bone healing process following implant placement [5-7]. Nanocoating with organic molecules, such as proteins and polysaccharides, is one of the methods used to improve biocompatibility of dental implants. Plant-derived polysaccharides, mainly represented by rhamnogalacturonan-I (RG-I) from pectins, have been proposed as potential candidates for surface nanocoating of titanium implants due to their osteogenic and anti-inflammatory properties [8]. The structure of RG-I can easily be modified with various enzymes, which results in different physicochemical properties and their effect on the cellular response [8-12]. The results of *in vitro* studies showed increased proliferation and metabolic activity, as well as decreased proinflammatory response of different cells cultured on enzymatically modified RG-I with short arabinan side chains [12-15].

The success of biomaterial implantation depends on the outcome of the bone healing process following implant placement. Initially, implantation induces an acute inflammatory response to the implanted biomaterials, followed by repair processes resulting in bone healing [16, 17]. Macrophages, monocyte-derived cells, are one of the major cellular players in the host inflammatory response. Macrophages have recently come to the forefront in biomaterials research not just as mediators of tissue debris removal, capable of secreting proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8), but also potentially as key players in promoting new bone tissue formation at the implant surface through growth factor secretion [18, 19]. However, bacterial factors such as lipopolysaccharide (LPS, a cell wall component of Gram-negative bacteria) are potent macrophage activators, which inhibit bone formation and stimulate production of proinflammatory mediators [20]. Prolonged macrophage exposure to bacterial LPS results in peri-implant inflammation and leads to tissue destruction around the dental implant.

The peri-implantitis is associated with biofilms comprising predominately of Gram-negative and anaerobic species of periodontal pathogens, such as *Porphyromonas gingivalis* [21]. This oral bacterium is capable of generating an arsenal of specialized virulence factors that contribute to its pathogenicity, including LPS, fimbriae, hemagglutinin and cysteine proteinases (gingipains) [22]. The *P. gingivalis* LPS is a stimulator of host proinflammatory response and bone resorption. It stimulates proinflammatory cytokine production of IL-1α, IL-1β, IL-6, IL-8 and TNF-α in monocytes, as demonstrated *in vitro* [22, 23]. However, certain bacterial species, including *Escherichia coli*, that are less regularly detected in periodontitis could frequently be found at peri-implantitis site [24-26]. *E. coli* is an opportunistic pathogen in extensive variety of human infections and has impact on general host immune response, causing e.g. septic shock [27, 28]. It has been suggested that *E. coli* could migrate via the blood circulation from infections elsewhere in the human body and colonize peri-implant sites. These bacteria can favor the development of peri-implantitis, especially in immunocompromised host with a high risk of bacterial infection [25].

It has been reported that RG-Is inhibit inflammatory cell response in LPS-stimulated macrophages *in vitro* [8, 29]. However, knowledge of the effect of coating of titanium with RG-I molecules on pro- and anti-inflammatory responses of macrophages is still very sparse. Therefore, the aim of the present study was to evaluate the *in vitro* effect of nanocoating of titanium and polystyrene surfaces with potato unmodified RG-I (PU) and potato dearabinanated RG-I (PA) on pro- and anti-inflammatory responses of primary human monocyte-derived macrophages (HMDMs) stimulated by *E. coli* LPS and *P. gingivalis* bacteria. The expression levels of pro- and anti-inflammatory cytokines were investigated to determine inflammatory responses of activated HMDMs. The results of the present study could contribute to development of plant-derived pectin nanocoatings to prevent

inflammation and improve the bone healing process following implantation, especially in immuno-compromised patients with poor tissue healing capacity and a high risk of bacterial infection.

2. MATERIALS AND METHODS

2.1. Isolation, modification and nanocoating of RG-I

RG-I was prepared as described previously by Gurzawska et al. [7] by treatment of potato pulp (P) with enzyme preparations. The arabinan side chains of potato RG-I were removed with α-L-arabinofuranosidase and endo-arabinanase (Novozymes, Bagsvaerd, Denmark). The chemical properties, monosaccharide composition and linkage analysis of potato unmodified RG-I (PU) and potato dearabinanated RG-I (PA) have been presented in previous studies [7, 10]. Briefly, the results showed that the PU and PA is a homogeneous coating of 6-10 nm thick, defined as a nanocoating [10]. To evaluate the *in vitro* effects of PU and PA nanocoatings on cellular responses, two different types of material substrates were used: tissue culture polystyrene (TCPS) plates (Techno Plastic Product, Trasadingen, Switzerland) with a diameter of 60 mm and commercially pure (grade 2) machined titanium (Ti) discs (Dentsply, Mannheim, Germany) with a diameter of 60 mm. PU and PA (128 µg/ml) were coated on the surface of 6-well TCPS plates and on the surface of the Ti discs placed in 6-well TCPS plates. The reaction was carried out at room temperature overnight in sterile conditions on a shaking platform (IKA-Werke GmbH & Co. KG, Staufen, Germany) at 100 rpm and then the plates and Ti discs were extensively rinsed in sterile water and dried in a laminar flow hood before *in vitro* experiments.

2.2. Detection of PU and PA nanocoatings

PU and PA RG-Is nanocoatings on Ti surface were visualized using immunofluorescence labeling and confocal microscopy. Uncoated and PU, PA-coated Ti discs were placed separately in polystyrene 6-well plate and blocked with 1 ml/well of 5% skimmed milk (5% solution of fat-free milk powder in phosphate-buffered saline (PBS), pH 7.2) (Applichem, Darmstadt, Germany). After 15 minutes skimmed milk was removed. Anti-(1→4)-β-galactan LM5 (Plant Probes, Leeds, UK) was diluted 1:10 in 5% skimmed milk and applied 1ml/well. The plate was placed on a shaker for 2 hours. All Ti discs were washed three times with 5% skimmed milk. 1 ml/well of secondary antibody, goat anti-rat IgG for LM5 linked to FITC (fluorescein isothiocyanate) (Sigma-Aldrich, Munich, Germany) diluted 1:200 in 5% of skimmed milk was added. The plate was covered with aluminum foil and placed on the shaker for 2 hours. Subsequently, Ti discs were washed three times with 1 M PBS. Confocal images were done with a Leica TCS-SP5 II confocal laser scanning microscope (Leica Microsystems, Exton, PA, U.S.A.) with PL Fluotar 10/×0.30 DRY objective. Excitation 488 and 633, zoom 1.8; beam splitter TD458/514/594, respectively emission bandwidth 500–600 nm; 633 nm laser; scan speed 100 Hz; emission bandwidth 644-714 nm.

2.3. *In vitro* studies

The TCPS and Ti discs with PU and PA nanocoatings were compared with control uncoated TCPS and Ti discs. Peripheral blood for preparation of mononuclear cells for all *in vitro* experiments was obtained from healthy volunteer donors. Written informed consent was obtained from all volunteer donors and the study was approved by the local research ethics committee of the Dental School, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK (approval number 14/SW/1148).

2.3.1. Cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized (10 U/ml) blood by centrifugation on Ficoll-Paque™ Plus (Amersham Biosciences, Little Chalfont, UK) as previously described [18]. PBMC were resuspended at a density of 1×10^6 cells/ml in Iscove's-modified Dulbecco's medium (Sigma-Aldrich, Poole, UK) supplemented with 2.5% human AB serum (BioSera, Ringmer UK), antibiotics (100 µg/ml streptomycin, 100 U/ml penicillin) (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich). PBMC were

seeded at a cell density of 1×10^6 per well in PU-/PA-coated and uncoated 6-well TCPS plates and on PU-/PA-coated and uncoated Ti discs placed in 6-well TCPS plates. The cells were incubated for 2 h at 37°C with 5% CO_2 to obtain adherent cells (monocytes). After 2 hours, the medium containing non-adherent cells was removed and replaced with fresh medium. The adherent monocytes were then incubated for 5 days at 37°C with 5% CO_2 to allow differentiation into macrophages. The cell morphology was observed before and after stimulation with LPS/*P. gingivalis* by inverted microscopy (Primovert, Zeiss, UK). Microscopic images were registered with a Zeiss AxioCam ERc 5s video camera.

2.3.2. E. coli LPS treatment and P. gingivalis invasion assay

After 5 days of incubation, adherent human monocyte-derived macrophages (HMDMs) cultured on tested and control TCPS and Ti surfaces were treated with *E. coli* serotype O26:B6 LPS (Sigma-Aldrich L5543; Sigma-Aldrich) at 100 ng/ml or heat-inactivated *P. gingivalis* bacteria (*Pg*; ATCC 33277) at a multiplicity of infection (MOI) of 100 (10^8 bacteria/well) and incubated for 6 h at 37 °C with 5% CO_2 to RNA isolation.

In the present study *P. gingivalis* strain ATCC 33277 (Manassas, Virginia, USA) was cultivated under anaerobic conditions at 37°C as previously described [14]. After cultivation, the bacteria were harvested by centrifugation, washed in sterile PBS and heat-inactivated for 10 min at 100°C. The number of bacteria was determined by measuring the OD at 600 nm and appropriate dilutions were made to obtain the desired MOI.

2.3.3. RNA isolation, reverse transcription and real-time polymerase chain reaction (PCR)

The RNA extraction was carried out using TRI reagent (Sigma-Aldrich) and the RNeasy Mini Kit (Qiagen, Hilden, Germany). The protocol was followed according to the manufacturer's specification. The concentration of RNA was determined by UV spectrometry at 260 nm (Eppendorf, Hamburg, Germany). The RNA was reversed transcribed to cDNA using one-step high-capacity cDNA RT kit (Applied Biosystems, Warrington, UK). Real-time PCR reactions were performed using the Light Cycler 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany), utilizing Roche SYBR Green reagents according to the manufacturer's instructions. The primer sequences (Sigma-Aldrich) for the specific target genes including tumour necrosis factor-alpha (*TNFA*), interleukin-1 beta (*IL1B*), interleukin-8 (*IL8*) and interleukin-10 (*IL10*) and for beta-2-microglobulin (*B2M*) as the housekeeping gene are described in Table 1. Real-Time PCR reactions were carried out in 10 µl volumes in a 96-well plate (Roche Diagnostics GmbH) containing 1 µl of cDNA and 9 µl reaction mixture, according to the manufacturer's instructions. PCR conditions consisted of an initial denaturation step of 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s.

The comparative $2^{-\Delta\Delta Ct}$ method was performed for analysis of relative gene expression data, as previously described by Livak et al. [30]. Relative expression levels were calculated for each sample after normalization against the housekeeping gene *B2M*. A pool of all the cDNA was used as calibrator in our study.

Table 1. Sequences of real-time PCR primers.

Target gene	Forward (5'-3')	Reverse (5'-3')
B2M	ACCCCCACTGAAAAAGATGA	ATCTTCAAACCTCCATGATG
TNFA	ATCCTGGGGGACCCAATGTA	AAAAGAAGGCACAGAGGCCA
IL1B	TTCGAGGCACAAGGCACAA	AAGTCATCCTCATTGCCACTGT
IL8	CTCCTTGGCAAAACTGCACC	CAGAGACAGCAGAGCACACA
IL10	TGCCTTCAGCAGAGTG	GGGAAGAAATCGATGA

2.4. Statistical analyses

Descriptive statistics were used and mean values were calculated. Data are shown as mean ± SEM and were analyzed using one-way ANOVA and post hoc Bonferroni test (IBM SPSS Statistic 22; IBM Corporation, Armonk, NY, USA). As significance level, a p-value of 5% was used throughout the study.

3. RESULTS

3.1. PU and PA nanocoatings of titanium surfaces visualized with confocal microscopy

The confocal images showed presence of PU and PA nanocoatings on the coated titanium disc surface compared to uncoated titanium disc surface (Figure 1).

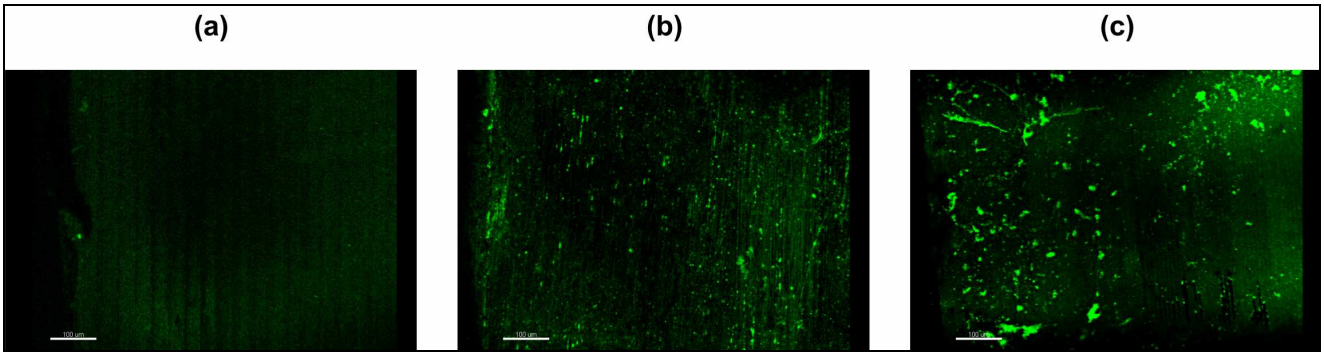

Figure 1. Representative confocal images of PU and PA nanocoating visualized with immunofluorescence labeling on (a) titanium (Ti) surface without coating (C), on (b) Ti surface coated with unmodified RG-I (PU), and on (c) Ti surface coated with dearabinanated RG-I (PA).

3.2. PU and PA nanocoatings of polystyrene surfaces decrease HMDM spreading

The morphology of HMDMs cultured on TCPS before and after stimulation with LPS/*P. gingivalis* is presented in Figure 2. The morphology of the unstimulated HMDM grown on the uncoated TCPS and PU, PA-coated TCPS surfaces looked quite similar, the cells were round and aggregated. LPS as well as *P. gingivalis* caused less cell spreading on surfaces coated with PU and PA.

3.3. PU and PA nanocoatings of polystyrene surfaces influence HMDM gene expression

We investigated the effect of RG-I (PU and PA) nanocoatings on pro- and anti-inflammatory gene expression in 5-day HMDMs cultured on TCPS surfaces. The relative expression of proinflammatory cytokine genes (*TNFA*, *IL1B*, *IL8*) and anti-inflammatory *IL10* gene was examined in HMDMs stimulated with *E. coli* LPS/*P. gingivalis* bacteria and in unstimulated HMDMs.

In general, proinflammatory *TNFA*, *IL1B* and *IL8* gene expression in activated HMDMs was highest on control TCPS surfaces and the lowest on PA-coated TCPS surfaces (Figure 3). PA significantly decreased *TNFA* ($p < 0.001$) and *IL8* ($p < 0.01$) expression in both *E. coli* LPS- and *P. gingivalis*-stimulated HMDMs compared with the TCPS control, while significant decrease of *IL1B* expression ($p < 0.001$) in the presence of the PA nanocoating was observed only in HMDM cultures stimulated with *P. gingivalis* bacteria. PU significantly decreased *TNFA* ($p < 0.01$) expression in HMDMs activated with *E. coli* LPS compared to the non-coated TCPS surfaces. No statistically significant differences were found between expression levels of proinflammatory genes in unstimulated HMDMs cultured on PU-/PA-coated and control surfaces.

As shown in Figure 3, neither nanocoating with PU nor PA significantly influenced the expression of *IL10* in unstimulated and *E. coli* LPS/*P. gingivalis*-stimulated HMDMs as compared to the corresponding TCPS control.

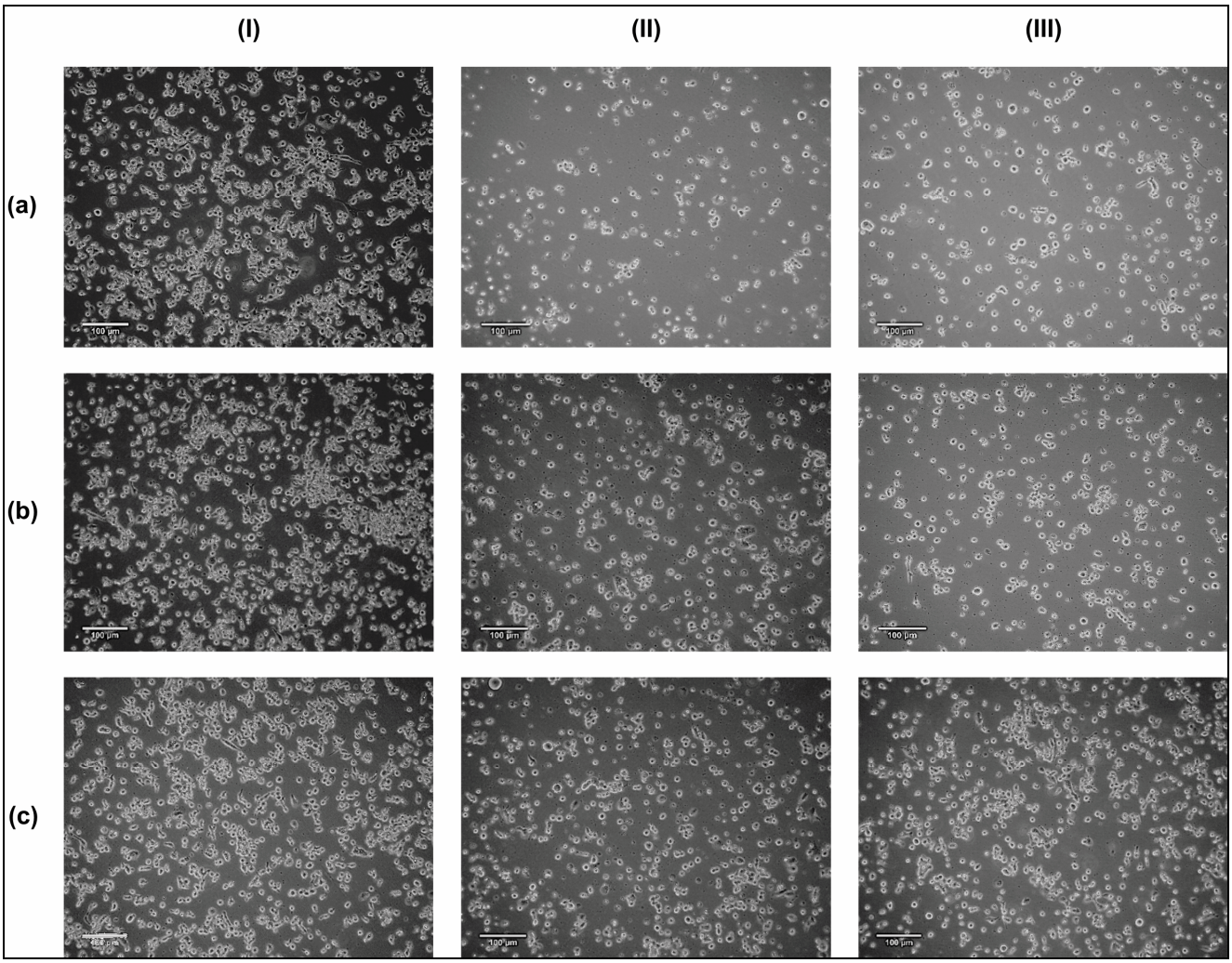

Figure 2. Representative images of HMDM morphology before (I) and after stimulation with LPS (II) and *P. gingivalis* (III) on (a) tissue culture polystyrene (TCPS) surfaces without coating (C), on (b) TCPS surfaces coated with unmodified RG-I (PU), and on (c) TCPS surfaces coated with dearabinanated RG-I (PA).

The level of *TNFA*, *IL1B*, *IL8*, and *IL10* expression, in general obtained higher values in HMDMs stimulated with *E. coli* LPS, rather than *P. gingivalis* bacteria. The lowest level of proinflammatory and anti-inflammatory gene expression was observed in unstimulated HMDM cultures.

3.4. PU and PA nanocoatings of titanium surfaces influence HMDM gene expression

The effect of RG-I (PU and PA) nanocoatings on Ti surfaces on pro- and anti-inflammatory gene expression was investigated in 5-day HMDMs. The relative expression levels of proinflammatory (*TNFA*, *IL1B*, *IL8*) and anti-inflammatory *IL10* genes were determined in unstimulated and *E. coli* LPS/*P. gingivalis*-stimulated HMDMs.

Generally, the expression of proinflammatory *TNFA*, *IL1B* and *IL8* genes in activated HMDMs was the highest on control Ti surfaces and the lowest on PA-coated Ti surfaces (Figure 4).

PA significantly reduced *IL8* expression in both *E. coli* LPS ($p < 0.01$) and *P. gingivalis* ($p < 0.05$) activated HMDMs compared to Ti control. The significant decrease of *TNFA* expression, in the presence of PA nanocoatings, was observed only in HMDM cultures stimulated with *P. gingivalis* bacteria ($p < 0.01$) and in unstimulated HMDMs ($p < 0.05$) compared to the non-coated Ti surfaces. No significant differences between PU-coated and control Ti surfaces were observed for proinflammatory gene expression in both stimulated and unstimulated HMDMs.

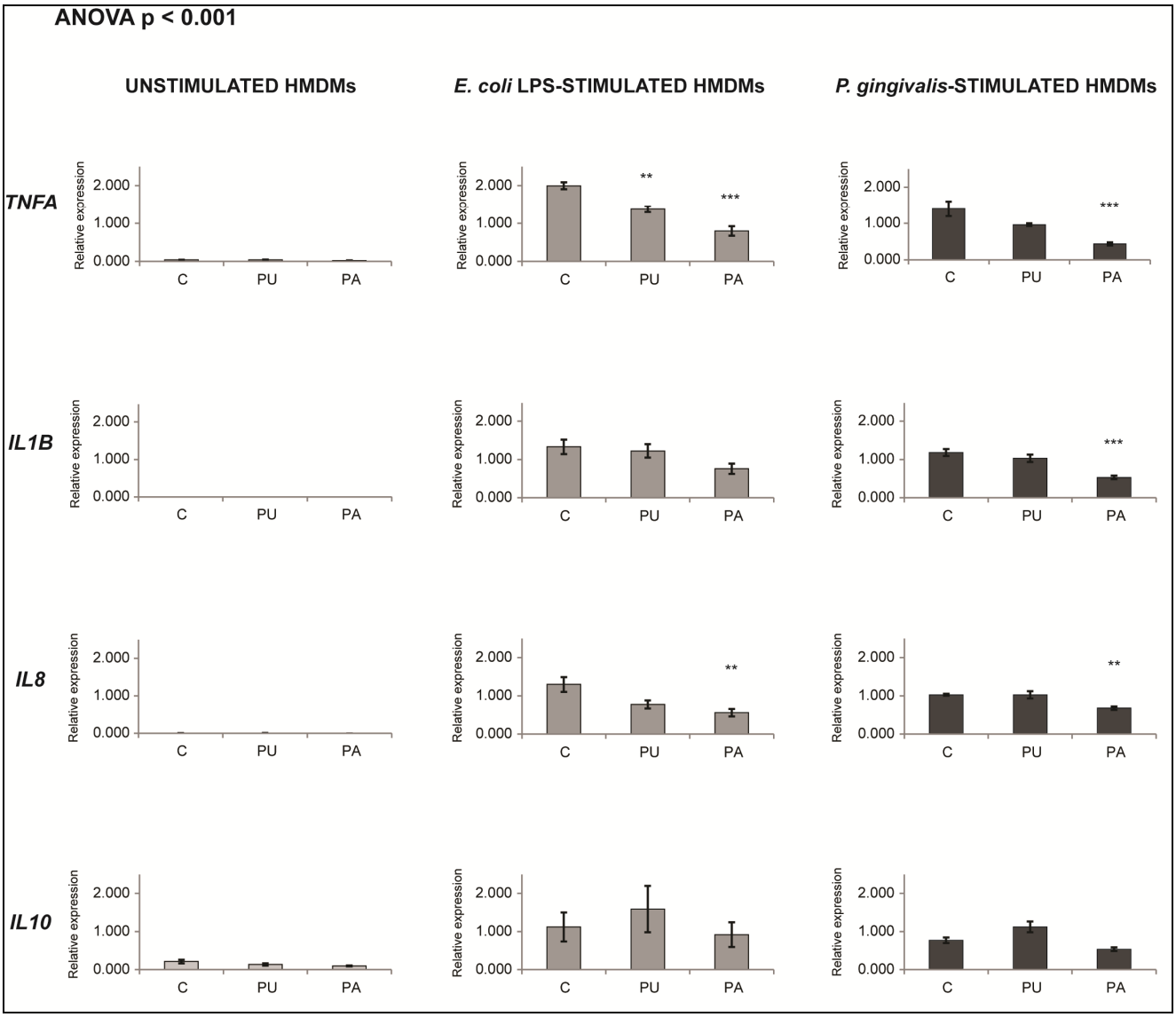

Figure 3. Relative expression of *TNFA*, *IL1B*, *IL8* and *IL10* in unstimulated and *E. coli* LPS/*P. gingivalis*-stimulated human monocyte-derived macrophages (HMDMs) cultured on tissue culture polystyrene (TCPS) surfaces without coating (C), on TCPS surfaces coated with unmodified RG-I (PU), and on TCPS surfaces coated with dearabinanated RG-I (PA). Data are given as means +/- SEM (n = 9) and were statistically analyzed using one-way ANOVA with Bonferroni's for multiple comparisons. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). RG-I: rhamnogalacturonan-I.

The expression of *IL10* in *E. coli* LPS/ *P. gingivalis*-stimulated HMDMs was not significantly affected by different Ti surfaces analyzed, whereas in unstimulated HMDMs, PA nanocoatings significantly decreased *IL10* expression.

As shown in Figure 4, the relative expression level of *TNFA*, *IL1B*, *IL8* and *IL10*, in general resulted in higher values in HMDMs stimulated with *E. coli* LPS rather than *P. gingivalis* bacteria.

4. DISCUSSION

Pectins from a variety of plants have been shown to possess immunomodulatory activity, acting on immune cells such as macrophages [8, 29, 31, 32]. Macrophages play a key role in mediating the host inflammatory in response to initial biomaterial implantation and in peri-implant infections [8, 33]. Thus, we examined the potential biomedical use of potato unmodified RG-I (PU) and potato modified RG-I (PA) as a nanocoating for Ti implant surfaces. For *in vitro* examination of organic nanocoatings, tissue culture polystyrene (TCPS) plates and Ti discs are frequently used for testing before *in vivo* studies [9]. Therefore, in the present study, adherent human monocyte-derived macro-

phages were cultured on coated/non-coated TCPS and Ti surfaces and activated in two different ways: (i) with *E. coli* LPS, a bacterial endotoxin which is a powerful macrophage activator [20], and (ii) by *P. gingivalis* invasion. Both *E. coli* and *P. gingivalis* bacteria have been frequently isolated from inflamed peri-implant tissues [2, 21, 24-26]. Moreover, it has been reported that *P. gingivalis* and *E. coli* induce the host proinflammatory response through different Toll-like receptor (TLR)-independent mechanisms. While the LPS of *E. coli* is a strong activator of TLR4 responses, *P. gingivalis* LPS is predominately a TLR2 activator [34].

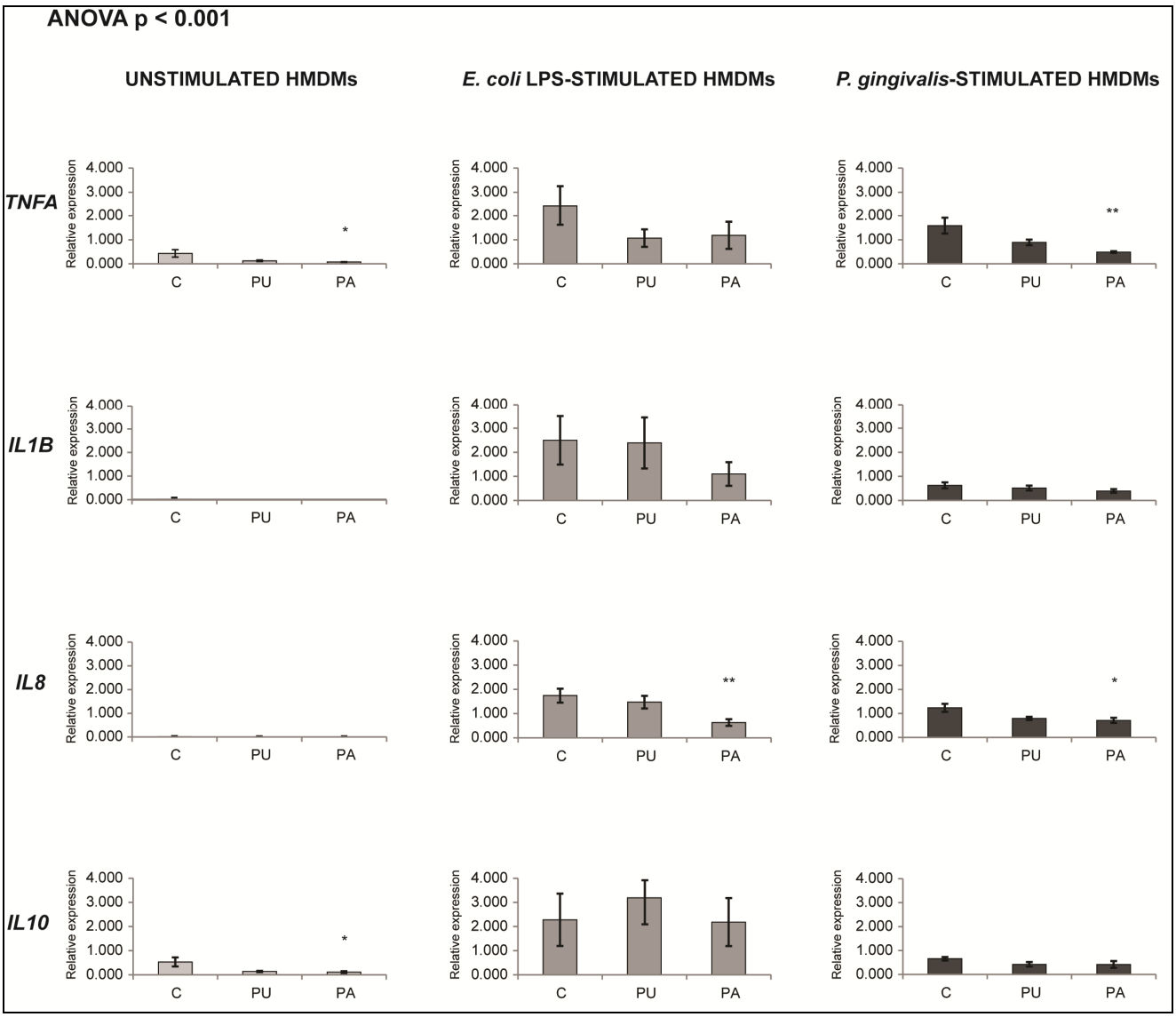

Figure 4. Relative expression of *TNFA*, *IL1B*, *IL8* and *IL10* in unstimulated and *E. coli* LPS/*P. gingivalis*-stimulated human monocyte-derived macrophages (HMDMs) cultured on titanium (Ti) surfaces without coating (C), on Ti surfaces coated with unmodified RG-I (PU), and on Ti surfaces coated with dearabinanated RG-I (PA). Data are given as means +/- SEM (n = 9) and were statistically analyzed using one-way ANOVA with Bonferroni's for multiple comparisons. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). RG-I: rhamnogalacturonan-I.

The results of morphology detection showed that PU and PA nanocoatings prevent HMDM spreading after LPS of *E. coli* as well as *P. gingivalis* stimulation. This findings is of great importance as it has been demonstrated that the spreading of macrophages on different surfaces is a marker of activation [8].

We assessed the ability of PU and PA

nanocoatings to inhibit proinflammatory and stimulate anti-inflammatory response of activated HMDMs. To investigate inflammatory responses of HMDMs after *E. coli* LPS/*P. gingivalis* stimulation, gene expression levels were measured for pro- and anti-inflammatory cytokines. TCPS and Ti without PU and PA nanocoatings were used as control surfaces (positive controls). In parallel, non-activated HMDMs were cultured on tested and control TCPS/Ti surfaces (negative controls) to exclude the possibility that PU and PA molecules activate the inflammatory response of HMDMs.

Taken as a whole, our results clearly demonstrate that HMDMs are activated by *E. coli* LPS and *P. gingivalis* bacteria on TCPS/Ti surfaces with and without pectin nanocoatings. In general, nanocoatings with PU and PA decreased expression of genes coding for proinflammatory cytokines in *E. coli* LPS/*P. gingivalis*-stimulated HMDMs on TCPS/Ti surfaces, compared with uncoated controls.

In response to bacterial products such as LPS and other inflammatory stimuli, macrophages release large quantities of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-8 and IL-12 [35]. Increased level of these proinflammatory cytokines activates osteoclasts, bone-resorbing cells and leads to destruction of periodontal and peri-implant bone tissue [36, 37]. Therefore, the expression of proinflammatory genes: *TNFA*, *IL1B* and *IL8* in activated HMDMs was assessed on both PU and PA coatings. The expression of examined genes was downregulated in *E. coli* LPS/*P. gingivalis* activated HMDMs cultured on PU- and PA-coated TCPS and Ti. As our data showed, PU and PA nanocoatings decrease the expression of proinflammatory cytokines, it is possible that nanocoating with RG-I could inhibit macrophage activation, osteoclast recruitment and prevent bone destruction during bacterial infection. Recently, enzymatically modified RG-I from apple (MHR-α) has been shown to decrease secretion of pro-inflammatory TNF-α, IL-6 cytokines and nitrites in J774.2 murine macrophages activated by bacterial LPS [8, 29]. The results of another study also demonstrate that Guar gum, a plant-derived polysaccharide, strongly inhibits nitric oxide generation and cytokine TNF-α secretion in LPS activated RAW 264.7 murine macrophages [31]. In contrast, some pectins have the capability to induce proinflammatory responses in macrophages. Stimulatory effects of polysaccharide from *Solanum nigrum* on proinflammatory RAW 264.7 murine macrophage response have been reported [32]. The polysaccharide fraction isolated from *S. nigrum* promoted the secretion of TNF-α and IL-6 in RAW 264.7 cells. Such different results indicate that plant-derived polysaccharides regulate macrophage activation by various biochemical mechanisms, possibly caused by differences in RG-I structure [8].

To evaluate the anti-inflammatory effects of PU and PA nanocoatings, the expression of the anti-inflammatory cytokine gene *IL10* was also examined in HMDMs stimulated with *E. coli* LPS/*P. gingivalis* bacteria. IL-10 is an important immunoregulatory cytokine that inhibits the expression and production of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-8 and IL-12 [35]. Generally, the expression of *IL10* in activated HMDMs was not affected by different surfaces analyzed. There were no significant differences in *IL10* expression between PU/PA-coated and control surfaces. Based on our *IL10* gene expression results, PU and PA appear to have no effect on the anti-inflammatory cytokine responses of activated HMDMs. The results may be due to different activation of molecular pathways. However, more *in vitro* studies need to be done to completely exclude anti-inflammatory activities associated with RG-I. A variety of cytokines with anti-inflammatory properties as well as several different signal transduction pathways are involved in suppression of inflammatory reaction [35].

In accordance with our previous studies, the results obtained from proinflammatory cytokine gene expression in activated HMDMs clearly indicate that PU and PA lead to different biological effects *in vitro*. PA, when compared to PU, seems to have a strong ability to reduce cellular proinflammatory responses. The sugar composition of PU and PA differs mainly in arabinose and galactose content. In this study, we also visualized PU and PA nanocoatings on Ti discs by immunofluorescence staining using the primary LM5 antibody, which specifically binds to galactan side chains. The results from confocal microscope detection showed higher amount of galactose on Ti surfaces coated with PA compared to PU. Galactose in known to be a specific high-affinity ligand for

galectin-3, a powerful proinflammatory mediator [38, 39]. Therefore, it can be speculated that the higher amount of galactose, a galectin-3 ligand, in PA's structure leads to more effective blocking of galectin-3 binding sites and attenuation of proinflammatory responses in activated HMDMs when compared to PU with lower amount of galactose residues. However, the biochemical mechanism underpinning inhibition of cellular proinflammatory response by PA nanocoatings requires further investigation.

Based on our examination of proinflammatory cytokine gene expression in non-activated HMDMs cultured on pectin coated TCPS/Ti surfaces, PU and PA molecules do not possess proinflammatory activity. The expression of *TNFA*, *IL1B* and *IL8* was downregulated in unstimulated HMDMs in the presence of PU and PA, when compared to negative controls, however the differences were not significant. The lack of proinflammatory properties of RG-Is is potentially of great importance for biomaterials applications.

5. CONCLUSIONS

The host immune response is a key factor influencing peri-implant bone regeneration. Our results indicate that it is possible to modulate cellular proinflammatory responses with plant-derived RG-I nanocoatings applied to titanium, a standard material used in dental implantation. Nanocoatings of TCPS and Ti surfaces with RG-Is (PU and PA) have the capacity to inhibit *in vitro* proinflammatory response of HMDMs stimulated with *E. coli* LPS and *P. gingivalis* bacteria, through downregulation of cytokine gene expression of *TNFA*, *IL1B* and *IL8*. Based on *IL10* gene expression results, PU and PA did not modulate anti-inflammatory response of activated HMDMs. However, due to RG-I's capacity to limit proinflammatory cellular responses, PU and PA nanocoatings are innovative candidates with considerable potential for improving the biocompatibility of implants and preventing immunopathological damage of peri-implant tissues. Better understanding of interactions between RG-Is and cells as well as *in vivo* investigation of RG-I's compatibility is needed to engineer novel immunomodulatory plant-derived RG-I biomaterials able to suppress immune responses and promote regenerative processes.

AUTHORS' CONTRIBUTION

AM: Conception and design, Development of methodology, Acquisition of data, Analysis and interpretation of data, Writing of the manuscript. JF: Development of methodology, Acquisition of data, Analysis and interpretation of data, Review of the manuscript. BB and OA: Study supervision, Administrative, technical and material support, Review of the manuscript. KG: Conception and design, Study supervision, Administrative, technical and material support, Review of the manuscript. All authors read and approved the final manuscript.

FUNDING

The work was supported by Marie Sklodowska-Curie grant (grant number FP-7 # 290246).

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interest.

ACKNOWLEDGMENTS

The authors thank RAPID (Rheumatoid Arthritis and Periodontal Inflammatory Disease) research group for valuable assistance and Bodil Jørgensen and Peter Ulvskov from University of Copenhagen for providing RG-Is.

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ISSN 2449-8955

European Journal of Biological Research

Research Article

Effect of selenium on nutritive value of purslane (*Portulaca oleracea* L.)

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Received: 19 April 2018; **Revised submission:** 26 May 2018; **Accepted:** 04 June 2018

DOI: http://dx.doi.org/10.5281/zenodo.1283418

ABSTRACT

Purslane (*Portulaca oleracea*) one of the auxiliary plants was traditionally consumed in many parts of the world for its nutritional and medicinal benefits. The nutrient components of purslane such as total protein, total carbohydrates and mineral content such as macro elements (Na, K, Ca and Mg) and micro elements (Fe, Cu, Pb and Zn) were estimated at different concentrations of selenium which treated in soil where the plant cultivated. The protein and carbohydrate contents of leaves as well as protein of stems increase with increasing the selenium concentration, while protein and carbohydrate of roots as well as carbohydrate of stems decrease with increasing Se concentration. The mineral content was also affected by Se concentration, Fe, Cu and Zn of leaves decreased with increasing Se concentration, while K, Ca, Mg and Na are directly proportional with Se concentration. In stems, Zn only is inversely proportional with Se concentration. In roots, Fe, Cu, Mg and K are inversely proportional with Se concentration, while Na, Ca and Zn are directly proportional. The findings of this study revealed that carbohydrates, protein and mineral contents of purslane can be affected and controlled by selenium concentration.

Keywords: Purslane; Selenium; Food value; Mineral content.

1. INTRODUCTION

Portulaca oleracea (L.) belongs to family Portulacaceae, annual herb with succulent leaves may grow prostrate or erect depending on light availability [1], which distributed all over the world. It grows well in diverse geographical environment [2, 3]. World Health Organization considered purslane as one of the most useful medicinal plants so that named “Global Panacea” [4]. *Portulaca oleracea* is a nutritious vegetable used for human consumption [5], it can be eaten raw or cooked. It is consumed in many different parts of the world such as China, India, and Middle East countries, South East Asia, Netherlands, Mexico and United States. According to Mohamed and Hussein [6], in Middle East, purslane can be consumed raw as salad or soups. The seeds may be ground into flour as ingredient in mush bread. It is rich in antioxidant vitamins and omega-3 fatty acids [7]. Like spinach, the succulent parts of the plant, leaves and stems are edible with slightly acidic and salty taste; recently purslane become has highly nutritional value more than the major cultivated vegetables due to higher content of beta-carotene, ascorbic acid and alpha-linolenic acid [8]. Additionally, purslane with antioxidant properties and high nutritive value is considered as power food [9]. Pharaohs mentioned in Egyptian texts, purslane the earliest vegetable consumed by human [10]. In China, fresh leaves of

the plant given in liver disease, diarrhea and applied to abscesses while, in North America, seeds used to be anthelmintic and considered a cooling diuretic [11]. Purslane named pigweed, used as complementary for growth of children due to its highly content of protein and carbohydrate [12].

Humans, animals and some other microorganisms need selenium because it is essential for normal and healthy life [13]. Selenium a metalloid mineral micronutrient becomes deficient (< 40 µg/day) and toxic levels (> 400 µg/day) [14]. Low Se intake has been associated with a number of deficiency syndromes, particularly cardiomyopathy and osteoarthritis, recent research demonstrates the importance of Se to human health [15].

So far little information is available on the nutrient composition of *Portulaca oleracea*, the aim of this research was evaluate the selenium concentration on the food value of purslane that may considered this plant one of the more important foods of the future.

2. MATERIALS AND METHODS

The seeds of *Portulaca oleracea* were selected from agricultural research center of Egypt and cultivated in agricultural land which situated 2 km west of Zagazig city, Sharkia, Egypt. The agriculture was done in the time for the plant growth during summer season (May 2016). Before cultivation, land was equipped by plowing and leveling.

By following the land, germination occurred after 15 days of planting where one pair of leaves appeared then consequently growth occurred. The land was cleared from weeds weekly. Land was divided into 16 stands involving control, the area of each stand (1 m x 1 m). Two types of plant extracts (A and B) were added to soil with 3 weights (5, 7.5 and 10 g). The first extract (A) was from pollen grain of *Poa annua* carried on the seed, while the second (B) was from germinated pollen grain of *Bubleurum lancifolium*.

Each stand applied with one treatment of extracts, making combinations from different weights of these extracts to give 15 treatments represent Se concentrations, soil without selenium called control as shown in Table 1. Treatments were added 5 times with irrigation of soil, the concentration of Se in the extract was evaluated according to Khedr and Hend [16]. Experiment carried out in triplicate for each treatment of Se and control.

Table 1. Classification of stands with selenium concentrations.

Stands no.	Treatment	Se added (mole.dm^{-3})
1	A1 (5 g of A)	3
2	A2 (7.5 g of A)	4.5
3	A3 (10 g of A)	6
4	A1+B1 (5 g of A + 5 g of B)	11
5	A1+B2 (5 g of A + 7.5 g of B)	15
6	A1+B3 (5 g of A + 10 g of B)	19
7	A2+B1 (7.5 g of A + 5 g of B)	12.5
8	A2+B2 (7.5 g of A + 7.5 g of B)	16.5
9	A2+B3 (7.5 g of A + 10g of B)	20.5
10	A3+B1 (10 g of A + 5 g of B)	14
11	A3+B2 (10 g of A + 7.5 g of B)	18
12	A3+B3 (10 g of A+10 g of B)	22
13	B1 (5 g of B)	8
14	B2 (7.5 g of B)	12
15	B3 (10 g of B)	16
16	-	0

Plant samples were collected at the end of season and separated into root, stem and leaf then cleaned with fresh and distilled water for removal of soil and other particles.

2.1. Determination of mineral content

Samples were digested in 10 ml acids mixture (1 HNO_3 + 3 HCl) according to Prakash et al. [17] and the elements in samples were measured by an atomic absorption and flame photometer Shimadzu Model AA640F (Japan).

2.2. Total carbohydrates content

Total carbohydrate content was estimated by anthrone method according to Hedge and Hofreiter [18].

2.3. Total protein content

Total protein content was estimated according to Bradfort [19] by borate buffer solution (pH 8.5) and protein reagent (Coomassie brilliant blue G250).

2.4. The statistical analysis

This analysis applied here is the Two Way Indicator Species Analysis (TWINSPAN) according to Ter-Braak [20]

3. RESULTS AND DISCUSSION

3.1. Plant nutrients

In roots**,** it is clear that the content of Na in roots is higher than other macro nutrients and the highest content at (A3 + B1) treatment which contains (350.14 ppm) while, the content of Fe is higher than other trace elements and the highest content was at control (1.23 ppm) (Table 2). The ability of the plant to absorb the nutrients, rate of their absorption and distribution to functional sites affect the normal and adequate nutrition of plants [21]. The uptake and accumulation of mineral nutrients important for plant metabolism affected by the presence of selenium which causing inhibition in the absorption of K leading reduction in the K content of plants because of the harmful effect of Se on plasma membrane of root cells [22].

Table 2. Elemental analysis (ppm) in roots of *Portulaca oleracea.*

Stand no.	Se added (mole.dm^{-3})	Fe	Cu	Zn	K	Ca	Mg	Na
1	3	1.195	0.0920	0.0059	136.74	35.12	53.8	79.8
2	4.5	1.092	0.1027	0.1097	129.48	39.81	49.1	169.17
3	6	0.2638	0.0846	0.0281	76.76	36.44	91.7	194.14
4	11	0.3856	0.1060	0.0315	187.5	20.74	76.8	261.3
5	15	1.0532	0.0781	0	215	20.63	93.1	72.02
6	19	0.1550	0.0847	0	195.22	35.71	49	269.7
7	12.5	0.0356	0.0757	0.0511	108.26	28.21	48.8	233.2
8	16.5	0.8498	0.099	0	148.88	26.01	36.5	146.34
9	20.5	0.2000	0.080	0	49.01	25.42	38.2	122.52
10	14	0.0292	0.074	0	80.25	34.49	50.1	350.14
11	18	0.1333	0.079	0	61.29	52.77	93.7	142.42
12	22	0.0808	0.072	0	101.85	55.2	90.9	178.32
13	8	0.7491	0.085	0	94.89	20.24	87.9	111.17
14	12	0.0277	0.058	0	95.15	24.84	91.5	179.21
15	16	0.0802	0.090	0	57.77	24.09	85.5	204.6
16	0	1.2313	0.1228	0	163.63	32.64	143.9	67.87

In stems, the content of K in stems is higher than other macro nutrients and the highest content recorded at (A1 + B1) treatment which contains (715 ppm) while, the content of Fe is higher than others and the highest content was at (A2 + B1) treatment with (1.25 ppm) (Table 3).

Table 3. Elemental analysis (ppm) in stems of *Portulaca oleracea.*

Stand no.	Se added (mole.dm^{-3})	Fe	Cu	Zn	K	Ca	Mg	Na
1	**3**	0.1666	0.0976	0.0109	613.5	41.47	108.7	150.56
2	**4.5**	0.1802	0.0851	0.0125	266.3	43.005	50	219.2
3	**6**	0.8379	0.0944	0.0470	495.1	40.516	91.6	189.99
4	**11**	0.0915	0.084	0	715	34.50	142.2	220.54
5	**15**	0.2147	0.0813	0	484	52.22	91.4	210
6	**19**	0.077	0.0836	0	136.7	77.9	40	130.69
7	**12.5**	1.253	0.1034	0.0425	313.1	47.73	65.3	211.9
8	**16.5**	0.503	0.0996	0	275.5	30.387	61.9	198.8
9	**20.5**	0.1177	0.1087	0.0313	250	49.83	73.6	241.9
10	**14**	1.0327	0.0847	0.116	211.5	12.73	89	268
11	**18**	0.676	0.0945	0.0424	93.5	51.119	36.4	173.07
12	**22**	0.1348	0.0998	0.1115	279.6	28.94	93.6	230.83
13	**8**	0.1315	0.1045	0.2012	284.7	21	121.1	232.3
14	**12**	0.4128	0.0982	0.1309	376.8	24.856	86.4	176.6
15	**16**	0.5947	0.0854	0.0320	248.7	35.060	87.7	178.9
16	**0**	0.255	0.0587	0.266	417.2	33.35	74.2	148.31

Table 4. Elemental analysis (ppm) in leaves of *Portulaca oleracea.*

Stand no.	Se added (mole.dm^{-3})	Fe	Cu	Zn	K	Ca	Mg	Na
1	**3**	0.114	0.0916	0.0050	421.6	44.434	184	99.59
2	**4.5**	0.0633	0.099	0.0452	334.4	49.49	145.7	117.86
3	**6**	0.1528	0.1029	0.0155	349.1	44.634	181.5	75.42
4	**11**	0.0849	0.0828	0	331.1	43.488	117.8	86.8
5	**15**	0.044	0.0856	0.0013	461.7	43.88	155	109.1
6	**19**	0.0019	0.0769	0	248.8	37.08	46.3	105.99
7	**12.5**	0.0353	0.0952	0	516.3	44.34	198.5	105.96
8	**16.5**	0.115	0.0889	0	410.2	10.63	122.54	90.85
9	**20.5**	0.350	0.0969	0	366.9	19.36	101.4	60
10	**14**	0.022	0.0849	0	408.9	51.015	141.8	130.4
11	**18**	0.0129	0.0940	0.0166	214.1	50.549	63.1	130.89
12	**22**	0.0377	0.0868	0	539.5	48.58	155.9	178.99
13	**8**	0.0481	0.1030	0	348.5	28.34	118.1	110.44
14	**12**	0.0022	0.0943	0	295.5	28.808	123	165.85
15	**16**	0.0960	0.063	0	275.8	35.01	111.3	149.12
16	**0**	0.0635	0.1070	0.055	327.2	34.27	121.8	42.98

In leaves, the content of K in leaves is more than any other element and the highest amount was at (A3 + B3) treatment which contains (539.5 ppm) while, the content of Fe is higher than other elements as well as in stems and roots and the highest content was at (A2 + B3) treatment with (0.35 ppm) (Table 4).

The nutrient composition of purslane depends on its growth stages and organs [6]. They also reported that total P, Fe and Mn content in leaves was significantly higher than those found in stems. According to [23], Ca, Mg and S tend to accumulate in leaves, while K tends to accumulate in the stem.

Ions can interact with the soil and plant in different ways, which can lead to deficiency or toxicity phenomena that affect growth and development [24, 25]. The ionic uptake by the cell is affected by the environmental salinity, which affects the relative availability of the ions in the area surrounding the root [24, 26].

In the present study, the differential accumulation of the Na^+, K^+, Ca^{2+} and Mg^{2+} in plant organs agreed with [23]. Se concentration as well as salinity, when increased, K^+ concentrations of roots and stems decreased, while Na^+ concentrations increased. The increased Na^+ with the concomitant decreased the K^+ in plant. This might be attributed to the competition and resultant selective uptake between K^+ and Na^+, which causes increase in the uptake of Na^+ at the cost of K [27-31].

3.2. Carbohydrates

The amount of carbohydrates in roots is more than in leaves and stems of purslane [6]. The highest amount of carbohydrates in roots was at (A2 + B2) treatment which contains (51.56 mg/g dry wt) while, the highest amount in stems was at (A3 + B1) treatment with (49.29 mg/g dry wt). The highest amount of carbohydrates in leaves was recorded at (B3) treatment (51.2 mg/g dry wt) as shown in Table 5.

Table 5. Amount of total protein and total carbohydrate (mg/g dry wt) in leaves, stems and roots of *Portulaca oleracea*.

Stand no.	Se added (mole.dm^{-3})	Leaves		Stem		Roots	
		Protein	Carbohydrate	Protein	Carbohydrate	Protein	Carbohydrate
1	3	44.4	25.55	32	45.59	36.06	44.6
2	4.5	38	31.3	49	36.44	45.57	24.74
3	6	38.6	15.96	31.4	35.5	32.8	26.99
4	11	58.4	39.4	34	15.64	37.67	24.94
5	15	44	44.49	34.1	28	35.2	47.49
6	19	45	17.94	43.7	18.67	18.3	31.39
7	12.5	57.2	15.7	46.2	23.72	40.4	42.56
8	16.5	45	39.4	27.5	43.94	47.3	51.56
9	20.5	48.6	19.6	33.7	43.65	32.6	15
10	14	31.8	36.9	34.5	49.29	46.2	31.5
11	18	37.5	45.22	30.3	31.19	23	39.5
12	22	37.4	24	40.7	34.19	14	21.47
13	8	46	32.6	31.2	42.3	48.7	41.79
14	12	60	49.55	32.1	46.5	43.2	32.93
15	16	42.2	51.2	40	27.7	46.7	42.15
16	0	39.5	48.5	32	37.7	43.2	36

3.3. Proteins

The amount of proteins in leaves is more than stems and roots of purslane. The highest amount of proteins in leaves was recorded at (B2) treatment with (60 mg/g dry wt) while, the highest amount in the stems was recorded at (A2) treatment with (49 mg/g dry wt) and the highest amount of proteins in roots at (B1) treatment with (48.7 mg/g dry wt) as shown in Table 5.

The protein levels in purslane cultures (control plants) were similar to or higher than those of other forage, vegetable and food crops. These high crude protein values were also reported by [32, 33] and placed purslane above alfalfa, which has a crude protein content of 17% DW, and is currently the most important commercial vegetable crop in the USA.

3.4. Effect of Se on nutrients

The correlation between selenium concentration (treatments) and elements is indicated on the ordination diagram produced by Canonical Correspondence Analysis (CCA) of the biplot of element- concentrations. The length and direction of an arrow representing a given variable provide an indication of the importance and direction of the gradient of concentration, for that variable, within the set of samples measured. The angle between an arrow and each axis is a reflection of its degree of correlation with the axis, as shown in Figures 1-3.

In roots, the canonical correspondence analysis (CCA) ordination show protein, carbohydrates and Zn are separated at the right and upper side of the CCA diagram closely related to 8, 4.5, 11 and 16 mole.dm^{-3} of Se. Cu, Fe, K and Mg are separated at the right and lower side of the CCA diagram. Protein and carbohydrates are separated at the lower and left side of CCA diagram exhibit a close relationship with 3 and 15 mole.dm^{-3} of Se. Ca is separated at the left and lower side of CCA diagram affected by 18 and 22 mole.dm^{-3} Se concentrations while Na is separated at the upper and left side of CCA diagram closely related to 12.5, 14, 12 and 20.5 mole.dm^{-3} Se concentrations as shown in Figure 1.

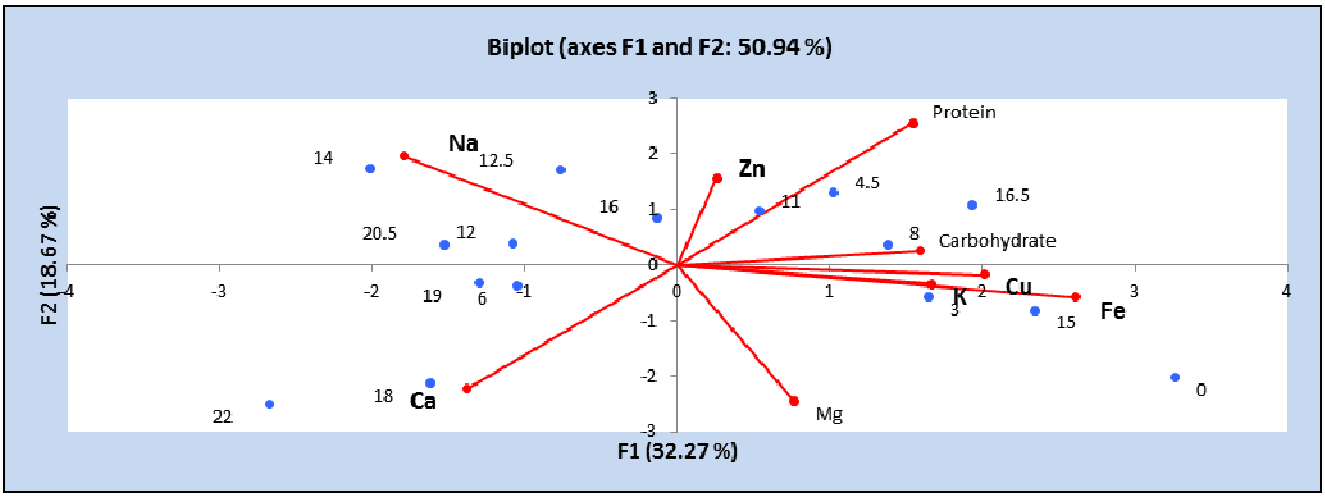

Figure 1. Canonical Correspondence Analysis (CCA) ordination diagram of elemental content in roots and the selenium concentrations.

The content of Ca, Zn, carbohydrates and proteins in roots increase with an increase in Se concentration, while K, Cu, Fe, Mg and Na decrease with increasing the Se concentration.

In stems, (CCA) ordination show Ca is separated at the right and upper side of the CCA diagram closely related to 16 and 19 mole.dm^{-3} of Se. Protein is separated at the right and lower side of the CCA diagram affected by 4.5, 18 and 12.5 mole.dm^{-3} Se concentrations. Carbohydrates, Na and Zn are separated at the lower and left side of CCA diagram exhibit a close relationship with

14, 20.5, 12, 16.5 and 22 mole.dm^{-3} of Se. K and Mg are separated at the left and upper side of CCA diagram affected by 11, 3 and 6 mole.dm^{-3} Se concentrations as shown in Figure 2.

The content of Ca, Na, Zn and carbohydrates in stems increase with an increase in Se concentration, while K, Fe, Mg and protein decrease with increasing the Se concentration.

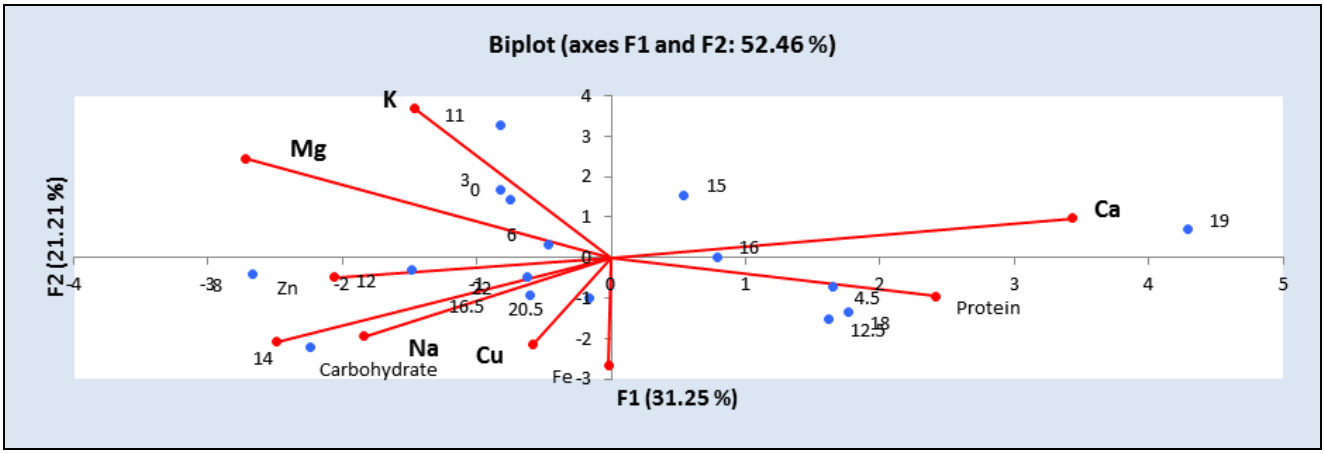

Figure 2. Canonical Correspondence Analysis (CCA) ordination diagram of elemental content in stems and the selenium concentrations.

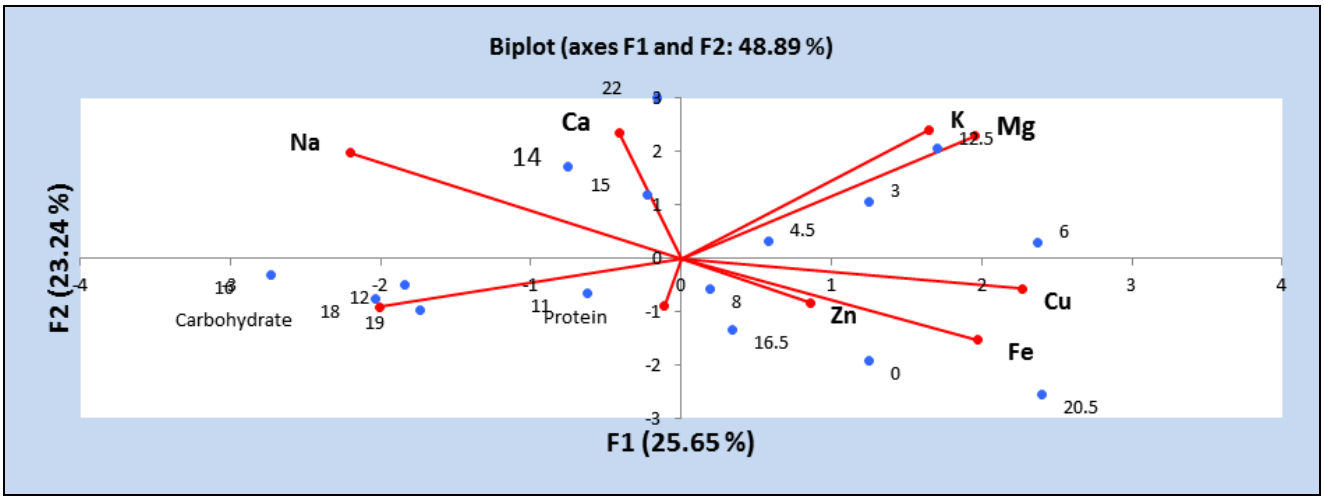

Figure 3. Canonical Correspondence Analysis (CCA) ordination diagram of elemental content in leaves and the selenium concentrations.

In leaves, (CCA) ordination show K and Mg are separated at the right and upper side of the CCA diagram closely related to 12.5 mole.dm^{-3} of Se. Cu, Fe, Zn are separated at the right and lower side of the CCA diagram affected by 16.5 and 8 mole.dm^{-3} Se concentrations. Protein and carbohydrates are separated at the lower and left side of CCA diagram exhibit a close relationship with 12, 18 and 19 mole.dm^{-3} of Se. Na and Ca are separated at the left and upper side of CCA diagram affected by 14 and 15 mole.dm^{-3} Se concentrations as shown in Figure 3.

The content of K, Mg, carbohydrates and proteins in leaves increase with an increase in Se concentration, while Cu, Fe, Zn, Ca and Na decrease with increasing the Se concentration.

Selenium with high level acts as a prooxidant and cause damage to plants however, at low level it has positive effect on growth of plants, counteracting many types of environmental stresses such

as heavy metals and stimulating plant growth [34]. There are studies carried out on different Se fertilization methods as well as different crops such as common purslane [35].

4. CONCLUSION

In conclusion, the carbohydrates and protein of leaves and stems were increased with increasing the selenium concentration, while in roots decreased with increasing Se concentration. The mineral content was also affected by Se concentration, Fe, Cu, and Zn in leaves decreased with increasing Se concentration, while Na, Ca, K and Mg are directly proportional with Se concentration.

ACKNOWLEDGEMENTS

The authors acknowledge of the Zagazig University, Faculty of Science, Department of Botany and Microbiology for helping providing laboratory facilities and help to analysis of research work.

AUTHOR'S CONTRIBUTION

KHF, HM: are supervisors of Ph.D thesis of SHA, wrote and revised the manuscript; SHA: experimental work; KHF, HM: statistical analysis, figures and wrote the first draft. All authors read and approved the final manuscript.

TRANSPARENCY DECLARATION

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

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ISSN 2449-8955

European Journal of Biological Research

Research Article

Histomorphological responses to aqueous crude leaf extract of *Alafia barteri* on prefrontal cortex, heart, kidney, liver and testis of adult male Sprague-Dawley rats

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Received: 19 April 2018; **Revised submission:** 30 May 2018; **Accepted:** 06 June 2018

DOI: http://dx.doi.org/10.5281/zenodo.1284554

ABSTRACT

Phytonutrients present in *Alafia barteri* leaves include antioxidants which serves to protect cells and tissues against detrimental effects of reactive oxygen species and other free radicals. This research work was targeted at investigating the activities of oral administration of aqueous leaf extract of *Alafia barteri* on the histology of the prefrontal cortex, heart, kidney, liver and testis of adult Sprague Dawley rats. Twelve (n=12) adult male Sprague Dawley rats weighing between 170-200 g (4-6 weeks old) were used for this study; they were divided into 2 groups of six rats each. The control group A received 2 ml/kg normal saline and treated group B received 500 mg/kg body weight aqueous extract of *Alafia barteri* for twenty eight days. The gross anatomical parameters of the selected organs and their histology were assessed. The gross anatomical and histological observation of the prefrontal cortex, heart, kidney, liver and testis revealed no visible distortion in *Alafia barteri* extract treated group when compared with control. Aqueous leaf extract of *Alafia barteri* thus has no deleterious effects on the histological profile of the prefrontal cortex, heart, kidney, liver and testis of the rats.

Keywords: *Alafia barteri*; Frontal cortex; Heart; Kidney; Liver; Testis.

1. INTRODUCTION

The importance of herbs in the treatment of diseases is almost universal among non-industrialized societies, and is often more assessable and affordable compared to modern pharmaceutical drugs. The World Health Organization (WHO) estimated that 80 percent of the populations of some Asian and African countries presently use herbal medicine to treat various ailments. Biological compounds present in *Alafia barteri* leaves include antioxidants which serves to protect cells and tissues against detrimental effects of reactive oxygen species and other free radicals. Protective agents from plant origin with anti-peroxidative and antioxidant properties play an important role in protecting the liver against toxicity [1, 2].

Alafia barteri has been used in traditional medicine to treat various diseases in Nigeria and other African countries since time immemorial. *Alafia barteri* Oliv, Apocynaceae, is a climbing

shrub distributed widely in the tropics. It is valued for its efficacy in the traditional medicine system in Nigeria and other African countries, as an anti-inflammatory and fever remedy. The infusion of the leaves and twining stem are used for the treatment of inflammation and fever [3, 4]. The decoction of root and leaves of the plant is also taken internally or applied externally to treat rheumatic pain, toothache and eye infection [5]. The extracts of the leaves were found to have antibacterial and antifungal activities [6, 7]. The aqueous leaf extract was reported to display potent antiplasmodial activity [8], antinociceptive and anti-inflammatory activities [9].

In South-Western Nigeria (specifically in Lagos), *Alafia barteri* has been used for the treatment of malaria [10]. Apocynaceae is quite a large family with about 200 genera and 2000 species known, including genus like *Alafia*, *Catharanthus*, *Alstonia*, etc. [11]. Plants in the Apocynaceae are poisonous, rich in alkaloids, glycerides and flavonoids obtained from the leaves, seeds, stems, roots and latex and are known source of anti-malarial activities [12, 13]. *Alafia barteri* Oliver (Hook F. Icon) is a tropical rainforest plant, native to the West and Central Africa, stretching from Guinea Bissau to Cameroon, Congo and Nigeria [11]. *Alafia barteri* is called agbari-etu by the natives of South-Western Nigeria (Lagos), meaning instant fever remedy. Leaf infusion and root decoctions from *Alafia barteri* are used in Nigeria and other African countries as a remedy for malaria [14]. In Nigerian traditional medicine, the stem and root decoctions of *Alafia barteri* are used for treating rheumatic pains, toothache, eye infection and sickle-cell anaemia [10, 12]. Polyphenols, flavonoids and alkaloids have been reported for wide varieties of pharmacological activities, including antiplasmodial activity [15-17]. High levels of polyphenols and flavonoids reported in the roots and leaves fractions of *Alafia barteri* could be responsible for its antiplasmodial activity [8].

To this end, we employed histological methods to evaluate the safety use of *Alafia barteri* leaf extracts on selected vital body organs. The rationale is that histological observations would provide a more assertive and reliable results of the effects produced as a result of the interactions between phytochemicals and body tissues than *in vitro* tests and analysis of the highly dynamic biochemical activities as contained in extracted tissue fluids. In addition, the use of Histological methods of assessment of the effects of *Alafia barteri* leaf extract on body tissues is important since literatures are comparatively scarce on such methods of investigation of the plant's extracts' effects.

Present study therefore focused on the effects of *Alafia barteri* leaf extract on histo-architecture of prefrontal cortex, heart, kidney, liver and testis of male Sprague-Dawley rats.

2. MATERIALS AND METHODS

2.1. Collection of plant material

The leaves of *Alafia barteri* were collected in December 2017 at Ipale forest, Irawo, Atisbo Local Government, Oyo State, Nigeria. The plant sample was authenticated by professor Ogunkunle of the Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso, Nigeria. Voucher specimen deposited in the same unit for reference purpose.

2.2. Preparation of the plant extract

The leaves were thoroughly washed in sterile water and were air dried to a constant weight in the laboratory. The air-dried leaves were weighed using Gallenkamp (FA2406B, England) electronic weighing balance and were milled with automatic electrical Blender (model FS-323, China) to powdered form. The powdered plant sample (500 g) was extracted with 96% ethanol for 24h, at room temperature with constant stirring. This process was repeated twice for complete extraction. The extract was filtered through cheese cloth and then through Whatman #1 filter paper, the filtrate was concentrated using a rotary evaporator (Rotavapor® R-210) at 42-47°C.

2.3. Animals and treatment

Male wistar rats 8 weeks old, weighing 170 ± 200 g were obtained from the animal facility of Department of Anatomy, Ladoke Akintola Uni-

versity of Technology, Ogbomoso, Nigeria. The animals were kept in polypropylene cages under room temperature (25°C), with 12 h light and 12 h dark cycle and were allowed to acclimatize for two weeks. The animals were fed with grower's mash (Farm support services Ltd, Ogbomoso, Nigeria) at a recommended dose of 100 g/kg as advised by the International Centre of Diarrheal Disease Research, Bangladesh (ICDDR, B) daily. They had access to water *ad libitum*. Twelve male wistar rats (n=12) were used for the investigation. They were divided in two groups of Control (A) which received only 2 ml/kg normal saline and Treated (B). A daily dosage of 500mg/kg body weight of *Alafia barteri* extract was administered orally to the treated Group B for 28 days. Twelve hours after the administration of the last *Alafia barteri* dose, the rats were at the time of sacrifice first weighed, blood samples were collected through ocular artery and centrifuged at 1,500 g/min at 4°C for 10 min to obtain serum then animals were sacrificed under high ether anaesthesia. All experimental protocols followed the guidelines for Care and Use of Laboratory Animals in Biomedical Research of the National Institutes of Health of the United States [18] and Department of Anatomy, Ladoke Akintola University of Technology, Ogbomoso, Nigeria Ethical Committee guide line.

2.4. Histology preparation of the organs

The organs were harvested and fixed in formaldehyde for 24 h after which it was transferred to 70% alcohol for dehydration. The tissues were passed through 90% and absolute alcohol and xylene for different durations before they were transferred into two changes of molten paraffin wax for 1 hour each in an oven at 65°C for infiltration. They were subsequently embedded and serial sections cut using rotary microtome at 5 microns. The tissues were picked up with albumenized slides and allowed to dry on hot plate for 2 min. The slides were dewaxed with xylene and passed through absolute alcohol (2 changes); 70% alcohol, 50% alcohol and then to water for 5 min. The slides were then stained with haematoxylin and eosin. The slides were mounted in DPX. Photomicrographs of the tissues were taken.

3. RESULTS

3.1. Morphological observations

There was increased in body weight in both the experimental animals administered with *Alafia barteri* extract and control group throughout the duration of the experiment. In addition, there were no morphological alterations in the appearance of the prefrontal cortex of the brain, heart, kidney, liver and testis of the animals in the treatment groups compared to those in the control groups twenty four hours after the organs were harvested. The prefrontal cortex, heart, kidney, liver and testis (with all their component parts) of the animals in both the treatment and control groups appeared morphologically normal.

3.2. Histology observations of prefrontal cortex, heart, kidney, liver and testis tissues

The neurohistological assessment of the frontal cortices of the rats in the extract treated group displayed normal histological profile, degenerative changes such as cytoarchitectural distortions, vacuolations and evidence of necrotic bodies were absent in the frontal cortices of the extract treated rats. The sections obtained in the control group shows numerous intact pyramidal cells with their nuclei (Fig. 1).

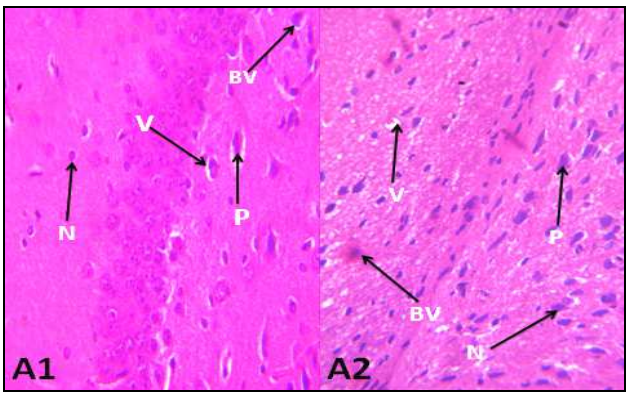

Figure 1. Images of the prefrontal cortex of the animals in control A1 and tread A2 groups. (H&E, x 400). V = vacuolations, P = pyramidal cells, Bv = Blood vessels, N = neurons. Numerous intact pyramidal cells with their nuclei.

The cardiac histology in both control and extract treated group revealed a normal appearance showing normal and centrally arranged nucleus, connective tissue also appeared normal the cardiac muscle fibers are well arranged (Fig. 2).

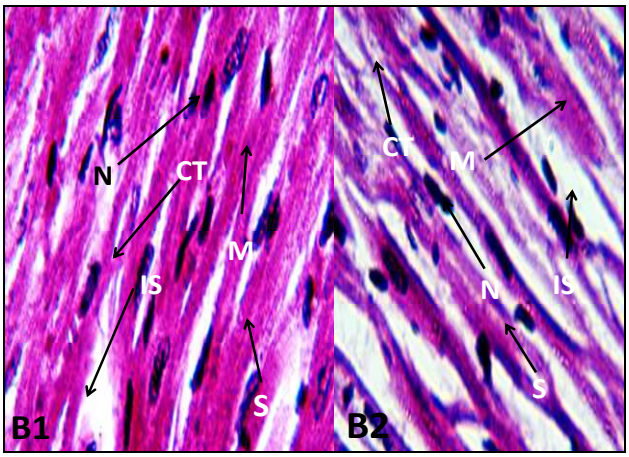

Figure 2. Histological demonstration of the photo-micrograph of section of the heart in control B1 and treated group B2 at light microscope level using H&E staining techniques (x 400) showing, normal Nucleus (N), normal space striation (S), normal connective tissue (CT), normal muscular fibre (MF) were well arranged.

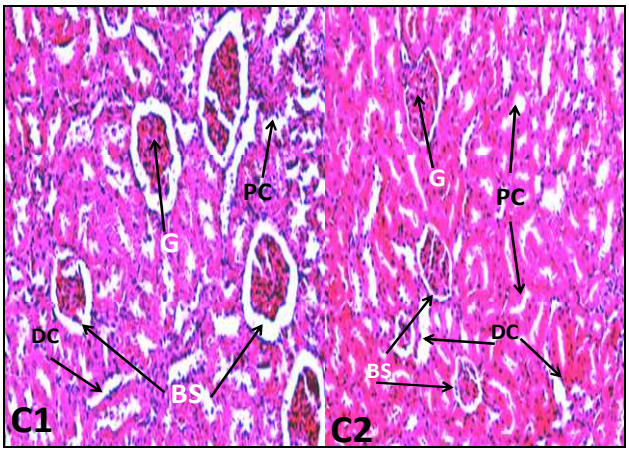

Figure 3. Histological demonstration of the photo-micrograph of section of the Kidney in control C1 and treated group C 2 at light microscope level using H&E staining techniques (x 400) showing normal and preserved histological outline with normal glomerulus (G), Bowmen's space (BS), proximal convoluted tubules (PC) and distal convoluted tubules (DC).

The histological outline of the kidney of the rats in the treated and control group appeared normal and preserved (Fig. 3). The sections of the liver of the rats in both the extract treated and the control groups also displayed well preserved histological profile with evidence of normal hepatic cytoarchitecture with visible terminal hepatic lobules consisting of terminal hepatic venules, hepatocyies with intervening sinusoidal spaces radially accentuated (Fig. 4).

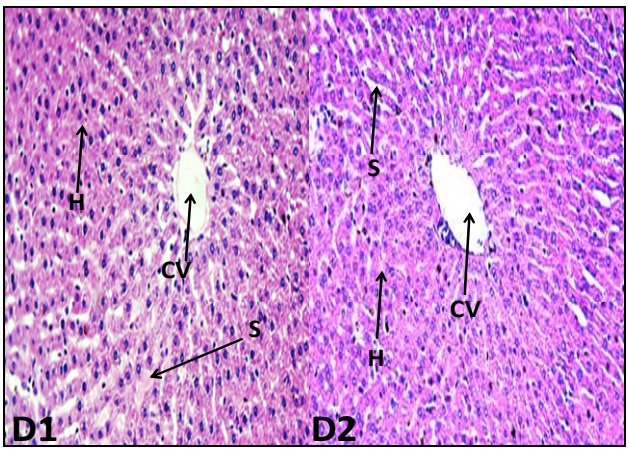

Figure 4. Histological demonstration of the photo-micrograph of section of the Liver in control D1 and treated group D2 at light microscope level using H&E staining techniques (x 400) showing the normal central vein (CV) sinusoids (S) and hepatocytes (Hc).

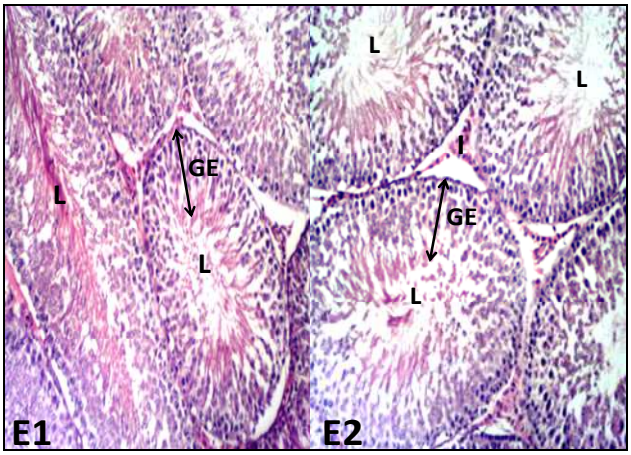

Figure 5. Histological demonstration of the photo-micrograph of section of the testes in control E1 and treated group E2 at light microscope level using H&E staining techniques (x 100) showing normal cellularity in germinal epithelium (GE), lumen (L) filled with sperm cells and interstitial cells of Leydig in the interstitium (I).

The histological section of the testes of the rats in both the extract treated and the control groups devoid of histo-pathological abnormalities

and revealed a normal cellular composition in their germinal epithelium with sperm cells in the lumen and a normal interstitium (Fig. 5).

4. DISCUSSION

Alafia barteri, which is the plant of interest in this study, is used by some African populations for its nutritional and pharmacological properties [19]. In this study, we investigated some of the effects of the aqueous leaf extract of *Alafia barteri* on prefrontal cortex, heart, kidney, liver and testis in order to elucidate some of the possible implications that could occur following its consumption.

Histomorphology observation on the prefrontal cortex following administration of aqueous extracts of *Alafia barteri* revealed normal histoarchitecture with intact cells and their nuclei, this could be ascribed to the presences of bioactive constituents present in the *Alafia barteri* extract such as flavonoid, terpenoids, saponin, tannins, steroid and cardiac glycoside which are antioxidant agent, this concur with the report of Makajuola et al. [2] that plant with antioxidant constituents improved histomorphology of the prefrontal contex. The histomorphology of the heart of the control and *Alafia barteri* extract treated group demonstrates normal morphology which is in consonances as reported by Ajibade et al. [20] that the cardiac histology of the rats treated with physiological saline revealed a normal appearance showing normal and centrally arranged nucleus, connective tissue also appeared normal and cardiac muscle fibers are well arranged. The kidney's functional integrity is to maintain total body homeostasis through its role in the excretion of metabolic wastes and in regulation of intracellular fluid volume, electrolyte composition, and acid-base balance [21]. This therefore implies that any harmful effect on body metabolism could be suggestive of toxic insult to the kidney [22]. The histological observations seen in the sections of the kidney of the experimental rats in the treated groups stained with H&E revealed that oral administration of the aqueous leaf extract of *Alafia barteri* has no deleterious effects on the histological outline of the kidney in this study. Therefore histological appearance of the control and treated group is consistent with normal histology. The histological observations seen in the sections of the liver in the control and *Alafia barteri* extract treated group revealed normal hepatic cytoarchitecture with evident of visible terminal hepatic lobules consisting of terminal hepatic venules, hepatocyies with intervening sinusoidal spaces radially accentuated. Since *Alafia barteri* has antioxidant components it can protect and alter any damage done to the liver by heavy meters or microorganism. This is in line with the report of Ibegbu et al. [23] that the results of histological observations showed normal architecture of the liver with central vein, hepatic cords and sinusoidal spaces in groups of animals treated with physiological saline.

There is no any observable lesion in the histology of the testes in the extract groups when compared with the control. This is in accordance as reported by Cody et al. [24], Harborne and Williams [25] that plants containing flavonoids are effective in prevention of lesion, mainly because of their antioxidant properties. However, in test groups, there was an observed increased in spermatogenic activity towards the lumen of the seminiferous tubule. This increased cellular activity was from the basement membrane up to the lumen of the seminiferous tubules of the testes. This was evidenced by the reduced number of primary spermatogonia cells. This is an indication that they might have differentiated to next level of spermatogenic cells mainly due to the presence of potent antioxidant like flavonoids that scavenge free radicals and increase testosterone formation by the interstitial cells of Leydig [26]. Our observations are therefore concur with the report of Muhammed et al. [27], Ofusori et al. [28] and Adekomi [29]. Cell death occurred pathologically or accidentally is regarded as necrotic and could result from extrinsic implications or disturbances to the cell which may include toxic or traumatic effects [30]. Processes involved in cellular necrosis may lead to cell death include compromise or disruption of the structural and functional potentials of the various membranes in the cell. Necrosis of the cell is not induced by intrinsic stimuli to the cells as observed in programmed cell death, but by an abrupt environmental disturbances and deviation from the normal physiological conditions, factors and functions. The type of cell loss and the particular part of the organ affected determines the symptoms associated with individual disease [31]. This study thus shows that

oral administration leaf extract of *Alafia barteri* has no disruptive and toxic impacts on cellular characteristics of the frontal cortex, heart, kidney, liver and testis of Sprague Dawley rats. To the best of our knowledge, this is the first study reporting the impact of *Alafia barteri* on the histological profile of the selected organs of study in Sprague Dawley rats.

AUTHORS' CONTRIBUTIONS

SA, OB and OD contributed in collecting plant samples and identification, running the laboratory work and analysis of the data. SA and OB contributed to biological studies and analysis of the data. SA and OD contributed to critical reading of the manuscript. SA designed the study, supervised the laboratory work and wrote manuscript. All authors read and approved the final manuscript.

FUNDING

This study did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

TRANSPARENCY DECLARATION

Authors have declared that no competing interests exist.

ACKNOWLEDGEMENTS

Authors are grateful to the Prof. Ogunkunle for the identification and authentication of the plants used for this research work.

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