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Research Article

Prevalence of intestinal parasitic infections among school children of Al-Mahweet Governorate, Yemen

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ABSTRACT

Intestinal parasitic infection is one of the ten top major public health problems in developing countries, including Yemen. Epidemiological parasitology study of the prevalence and distribution of intestinal parasitic infections among primary schools pupils (aged 7-15 years), was conducted in six primary schools in the period between March and November 2012 in Al-Mahweet Governorate, Yemen in order to determine the prevalence of intestinal parasitic infections and associated factors among primary school children. 200 pupils were selected by using multi-stages sampling technique with the targeted schools in the study area. Stool samples were collected and examined by the Kato-Katz technique and direct method. A semi-structured questionnaire was administered to the study subjects and microscopic examination of stool was done. Chi-square was used to determine if there was any relationship between age and sex on the occurrence of the intestinal parasitic infections. The overall prevalence rate in the present study was 90%. Nine species of intestinal parasitic were identified. The most common diagnosed were *Entamoeba histolytica* cysts (64%), *Schistosoma mansoni* (36.5%), amorphous amoebae (22.5%), *Trichuris trichiura* (18%) and *Enterobius vermicularis* (13%). Multiple intestinal parasitic infections were recorded (75.5%) having the highest prevalence among the children. Male (46.5%) were more infected than female (43.5%). Also, there was a difference in the percentage of infections observed among the different age groups of the studied children. The study revealed that poor hygienic practices and unsanitary condition were responsible for the high prevalence of intestinal parasites. Deworming of the primary school children and health education on proper hygiene are recommended.

Keywords: Prevalence; Schoolchildren; Intestinal parasitic infections; Yemen.

1. INTRODUCTION

Intestinal parasitic infections are endemic worldwide and have been described as constituting the greatest single worldwide cause of illness and disease [1]. These infections are one of the major health problems in several developing countries [2], including Yemen. Rates of the infection prevalence in these countries range from 30-60%, as compared to < 2% in the developed countries [3].

WHO has estimated about 3.5 billion people to be affected with these parasites worldwide, and 450 million people fall ill as a result of these infections, with the majority being children [4].

These infections represented more than 40% of the burden of all the tropical diseases, excluding malaria [5].

Several environmental and socioeconomic factors have been identified to be responsible for the continued persistence of intestinal parasite infections in children [6]. These infections continue to be a global health problem, particularly among children in poor communities in developing countries [7-9]. Also, in Yemen, intestinal parasitic infections are common varying from one area to another, depending on the degree of personal and community hygiene sanitation and climatic factors [10].

School-age children are the group with the highest prevalence and infection intensities and are also very vulnerable to the effects of worm infection, including nutritional deficiencies which aggravate malnutrition and worse the rates of anemia and impaired physical and mental development contributing significantly to school absenteeism [11-14]. About 400 million school-age children around the world are infected with roundworm, whipworm and hookworm [15]. In low-income countries, children aged 5-14 years has 12% of the total disease burden of intestinal worms infections [16]. Peak levels of these infections typically occur in hosts aged between 10 and 14 years in endemically infected communities [11]. This study was designed to determine the prevalence of intestinal infections and to identify risk factors associated with intestinal infections among the schoolchildren of Al-Mahweet Governorate, Yemen.

2. MATERIAL AND METHODS

2.1. Study area

This study was carried out during March and November 2012 in Al-Mahweet Governorate (Fig. 1). Al-Mahweet Governorate located to the north-west of the capital Sana'a, between longitude 43-44 to the east and latitude 15-16 to the north and rises from sea level 2100 m. It is away from the capital Sana'a a distance of 111 km.

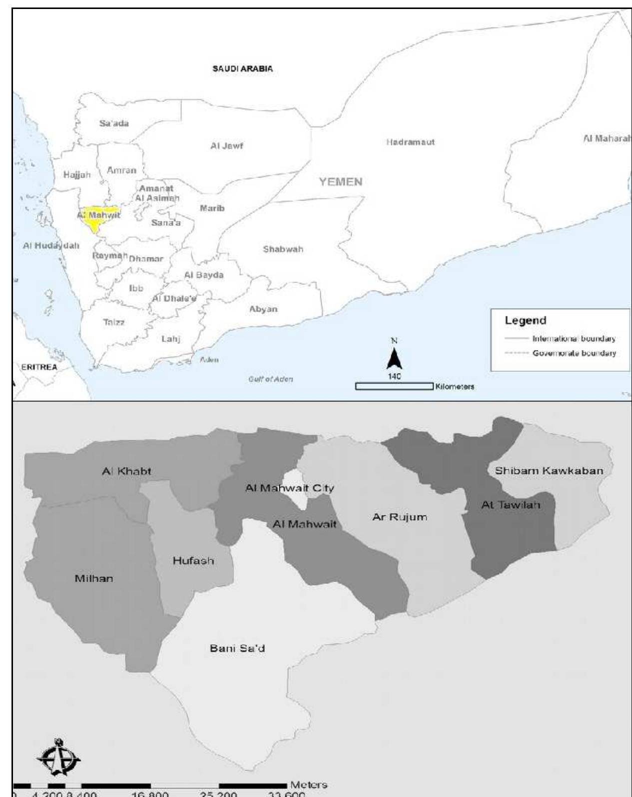

Figure 1. Geographic maps of Al-Mahweet Governorate (study area) in Yemen.

2.2. Study population and sample size

For the present study, six schools were selected on the basis of their location in the four administrative divisions (Al-Mahweet city, Shebam-Kaokaban, Al-Taweela, and Al-Rogum). Two representative primary schools were randomly selected from the division of Al-Mahweet city, two representative schools were randomly selected from the division of Shebam-Kaokaban. One representative school was randomly selected from the division of Al-Taweela and one representative primary school was randomly selected from the division of Al-Rogum. The study population included schoolchildren attending years 1-9 at the selected schools. Children aged between 7 and 15 were selected randomly from a list provided by each school.

This study was approved by the local Ethical Committee. To begin the study, members of our team visited a selected of primary schools. The teams are familiar with the cultures and geography of Al-Mahweet Governorate. Four of the total schools were from the rural area while the others two schools were from the urban area, which were selected using a systematic selection method by

calculating the sampling interval. Further discussions were held with an administration of the selected schools and with local health officials to secure their approval and cooperation. A random cluster sample of urban and rural schoolchildren were chosen separately. The total sample size was 200 children, 100 children of the total sample was from rural areas and the others 100 children was from urban areas.

2.3. Data collection

After obtaining written consent from the parent of the child, a pre-tested interviewer-administered structured questionnaire was used to collect data, such as students' age, sex, school year, personal hygiene and social determinants. Students from grades 1 to 3 were given the questionnaire to be completed by their parents.

At the beginning of the study, each student was weighed and height measured. The students were measured wearing light uniforms, without shoes, belts, caps or any other material that could tamper with their actual heights and weights. Each student was assigned an identification number.

2.4. Collection and analysis of stool sample

Stool samples were collected using a sterile, labeled, clean, dry, wide-mouthed plastic containers with identifying marks, which were given to students on the day of the study, after thoroughly explaining the way of collection. Each container was labeled to correspond with the number of the questionnaires given to them. Collected stool samples were transported to the laboratory of the medical centers under the ministry of health in each area, as soon as possible.

The examinations of stool samples were performed immediately by experienced personnel in the laboratory, for analysis by using the Kato-Katz technique [17, 18]. Duplicate Kato-Katz slides were prepared from each stool specimen. By a systematic manner, the Kato-Katz slides were examined microscopically for intestinal parasitic eggs.

2.5. Data quality control and microscopic examination

To assure the reliability of data collected in the study the questionnaire was prepared and before the questionnaire was used in the actual data collection it was pre-tested at 2 schools. Stool sample collection and investigation was made according to a standard procedure without any delay for more than 30 min after collection of the samples. Microscopic reading was made by laboratory technologists and results were confirmed by them a microscopic examination.

2.6. Statistical analysis

The collected data were tabulated and analyzed through computer facilities using the Statistical Package for Social Science (SPSS) for windows version 11.5.

3. RESULTS

Of the 200 children, 180 (90%) were infected. 87 (43.5%) of them were females while 93 (46.5%) of them were males. The infected children of age group 7-9 years were recorded (11.5%). While the age group (10-12 years was recorded (43%), and the age group 13-15 were recorded (35.5%). The positive infection was high in almost of the different areas, and the high positive infection was in the children who use stream water as a source of drinking water (Table 1).

Eleven types of intestinal parasitic species were encountered in the study, these are: *Entamoeba histolytica* cyst (64%), *Schistosoma mansoni* (36.5%), amorphous amoebae (22.5%), *Trichuris trichiura* (18%), *Enterobius vermicularis* (13%), *Ascaris lumbricoides* (10%), *Hymenolepis* sp. (5.5%), *E. histolytica* trophozoite (3.5%), *Giardia lamblia* (3%), *Taenia saginata* (1%) and *Giardia lamblia* trophozoite (0.5%) (Fig. 2).

Both single and multiple infections were observed. A few children had one intestinal parasitic species concurrently (14.5%), while a greater percentage of the children were infected with a multiple intestinal parasitic species (75.5%) among study participants who had an intestinal parasitic infec-

tion. Cases of multi-infection were higher in both of male and female children (Table 2).

3.1. Comparison of prevalence for each species in all the four locations

Table 3 shows the prevalence of intestinal parasitic infection among children in the four locations. The frequency of infection with each Parasites and Helminths is shown separately. In general, the prevalence of intestinal parasites at Al-Taweela and Al-Mahweet city were present in appreciable numbers. The prevalence of intestinal parasites at Al-Rogum and Shebam-Kaokaban were present in lowest numbers. At Al-Taweela location the prevalence of *Schistosoma mansoni* is highest of others parasites, and at Al-Mahweet city location the prevalence of *E. histolytica* cyst, amorphous amoebae and *Trichuris trichiura*, are highest of others parasites. While at Al-Rogum and Shebam-Kaokaban locations the prevalence of *E. histolytica* cyst is highest of the others intestinal parasites. The prevalence of *Giardia lamblia* trophozoite and *Taenia saginata* were very low at all the four locations.

The data in locations where intestinal parasitic infections are highly endemic, it is common to find a significant proportion of subjects who are infected with two or more of the parasites. That was true of the children in Al-Taweela and Al-Mahweet city locations as shown in Table 3.

To identify levels of childhood personal hygiene that contribute to the risk of Yemeni children acquiring infections with intestinal parasites, every child was interviewed by a member of the research team and each was observed and checked with personal hygiene. The combined results enabled a search for evaluating a level of each children' personal hygiene, that most likely placed a child at risk of becoming infected. The findings of this study showed that 30.5% of the sample children were in the level of bad personal hygienic and 7.5% were to the level of so bad personal hygiene. While 62% of their were in the level of a good compare to other levels.

Table 1. Association between socio-demographic characteristic and intestinal parasitic infection.

Factor	Parasites positive	Parasites negative	Total	Coefficient association value
Gender:				
Male	93 (46.5%)	13 (6.5%)	106	
Female	87 (43.5%)	7 (3.5%)	94	0.269
Total	180	20	200	
Age group:				
7-9 years	23 (11.5%)	0 (0)	23	
10-12 years	86 (43%)	8 (4%)	94	
13-15 years	71 (35.5%)	12 (6%)	83	0.727
Total	180	20 (10%)	200	
Administrative divisions:				
Al-Mahweet city	50 (25%)	0 (0%)	50	
Shebam-Kaokaban	34 (17%)	16 (8%)	50	
Al-Taweela	50 (25%)	0 (0%)	50	
Al-Rogum	46 (23%)	4 (2%)	50	0.401
Total	180 (90%)	20 (10)	200	
Source of drinking water at home:				
Ponds	10 (5%)	1 (0.5%)	11	
Streams	120 (60%)	11 (5.5%)	131	
Well	33 (16.5%)	3 (1.5%)	36	
Dams	17 (8.5%)	5 (2.5%)	22	0.152
Total	180 (90%)	20 (10%)	200	

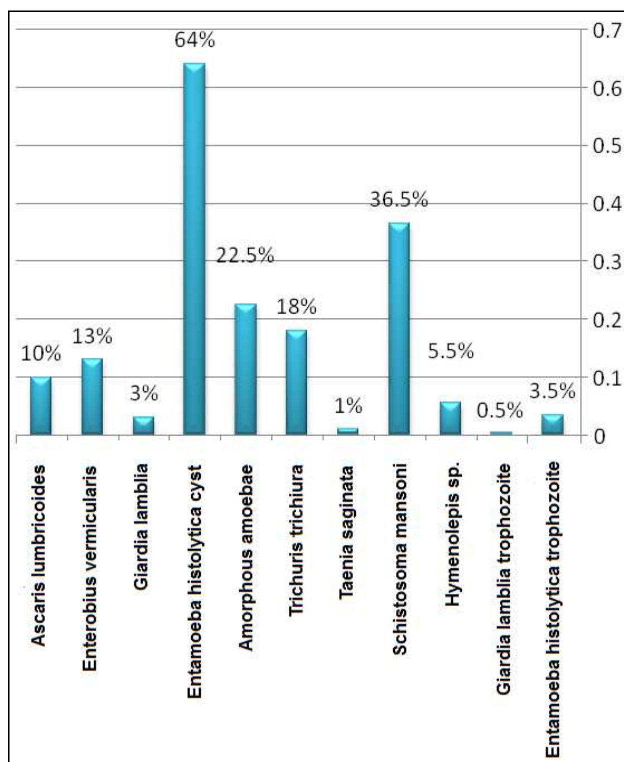

Figure 2. Types and percentages of intestinal parasites that appeared amongst the sample.

Table 2. Association between gender and kind of infection**.**

Gender	Kind of infection		Total
	Single	Multi	
Male	15 (7.5%)	78 (39%)	93 (46.5%)
Female	14 (7%)	73 (36.5%)	87 (43.5%)
Total	29 (14.5%)	151 (75.5%)	180 (90%)

Nature of a toilets facilities used by study sample showed that the children who used pit latrines had the highest prevalence (57.5%) of intestinal parasitic infection, followed by children who used modern latrines (32%), while children that used pit, recorded the least prevalence (9.5%) of intestinal parasitic infection.

3.2. Effects of intestinal parasitic infections on children growth and development

In order to evaluate the effect of intestinal parasitic infections on the growth and development of schoolchildren, the body weights and heights were measured. Weight (kg) and height (m or cm) data were analyzed separately as well as combined to compute weight/height ratios (W/H) and body mass index (BMI) (kg/m^2). Our results have shown that 22% of schoolchildren were in the level of a stunting and 67.5% were in the level of an underweight body while only 10.5% were in the level of the normal body.

4. DISCUSSION

The overall prevalence of intestinal parasites in the present study was 180 (90%) of all stool samples examined. Such a combined rate was not reported in any previous literature in the country. However, rates exceeding 50% were reported from Haja town (54% *Trichuriosis*), Al-Mahweet (61%) and Maitam (53%) both for *Ascariosis* [19].

Table 3. Prevalence of intestinal parasitic infections among children in all the four locations of Al-Mahweet Governorate.

Parasites	Al-Taweela	Al-Rogum	Shebam-Kaokaban	Al-Mahweet city	Total	
					No	%
E . histolytica trophozoite	3	3	0	1	7	3.5
Giardia lamblia trophozoite	1	0	0	0	1	0.5
Hymenolepis	4	4	2	1	11	5.5
Schistosoma mansoni	50	14	7	2	73	36.5
Taenia saginata	1	0	1	0	2	1
Trichuris trichiura	1	8	1	26	36	18
Amorphous amoebae	0	2	0	43	45	22.5
E. histolytica cyst	10	44	27	47	128	64
Giardia lamblia	4	0	2	0	6	3
Enterobius vermicularis	12	8	6	0	26	13
Ascaris lumbricoides	2	12	3	3	20	10
Total	88	95	49	123		

The high prevalence of intestinal parasites recorded in the study area could be attributed to exposure of the children to predisposing factors to intestinal parasitic infections; such as (poor sewage disposal system, unsafe sources of water, poor sanitary conditions, poor housing and lack of awareness on the part of the parents and children). This finding agreed with the study conducted in Kenya showed that the overall, 91.6% of the children were infected [20]. And also, was close to findings of other studies. A study conducted in Amhara region, North West Ethiopia showed that the overall intestinal parasite in the present study was (84.3%) [21]. The study conducted in South-eastern Nigeria reported that schoolchildren (75.7%) were infected with at least one helminth parasite [6].

The others studies reported the opposite finding. A previous study conducted in Jeddah, KSA reported that the overall prevalence of the parasitic infection was 48% [22]. A study conducted in Morocco showed that the mono- or poly-parasitism was detected in 34.5% of the children [23]. A study conducted in Ethiopia showed that the overall prevalence of intestinal helminths was 51.5% [24]. A study conducted in South Ethiopia reported that the overall prevalence of intestinal helminthic infection was 26.9% [25]. A study conducted in Nigeria showed that only 15.75% were positive for parasitic infection [26]. Another study conducted in Nigeria showed that 45.5% were positive for parasitic infection [13]. Another study conducted in Ondo state, Nigeria showed that 48% were observed to be infected [27]. A study conducted in Nepal reported that 23.71% of the rural public school children were found to be harboring one or more intestinal parasites [4]. Another study conducted in Eastern region of Nepal showed that the overall intestinal protozoan infection was found to be 18.5% [28]. Possibly the difference might be due to the geographical difference, the living and the socioeconomic nature of the study subjects.

The present study findings showed that the infection rate of intestinal parasites were different between male (46.5%) and female (43.5%). A previous study conducted in Al-Mahweet, Yemen mention that, the infection rates were significantly higher among the boys than in the girls [19]. This finding agreed with previous studies in different countries. A study conducted in Jeddah, KSA showed that the infection in females (48.7%) more than male (47.8%) [22]. A study conducted in Nepal reported that the prevalence of intestinal parasitic infections among boys (28.2%) was higher compared to that of girls (20.2%), but the difference was not statistically significant (*P-value* = 0.191) [4]. Another study conducted in Nepal reported that the Protozoan infections were in 18.4% of males and 18.6% of females, but the difference was not significant (*P-value* = 0.984) [28]. A study conducted in Nigeria reported 52.3% of infected females, while 47.7% of males [27]. Another study conducted in Nigeria reported that the helminthic infection rate was higher among males (18.5%) than female pupils (13.2%) [26]. Another study conducted in Ebonyi State, Nigeria reported that the helminthic infection rate was higher among males (27.7%) than females (23.6%) [29].

Whereas, other studies have indicated the opposite finding. A study conducted in Yemen reported that the infection rate of protozoa between male (33%) and female (9%) has a significant (*P-value* = 0.001) [10]. A study conducted in Morocco showed that the females showed a higher prevalence of intestinal parasitic infection (41,3%) than the males (26,4%), this is statistically significant (*P-value* = 0.02) [23]. A study conducted in South Ethiopia reported that the overall prevalence of infection was 7.7% for girls and 17.1% for boys and the difference was statistically significant (*P-value* = 0.006) [25]. This indicated that the gender may or may not play a role in Parasitosis depending on the region and other environmental or behavioral factors.

The results of this study also clearly showed that there was a difference in intestinal parasitic infection rate between the age groups sampled. The prevalence of infection was higher in the age group 10-12 year were recorded (43%). This may be as a result of children paying little or no attention to personal and general hygiene; walking barefooted, dirty hands are used to share foods, passing viable ova to one another. The 13-15year age group recorded (35.5%). This is so because children in this group of years are more actively involved in carrying out activities in and out of their immediate environment thereby exposing them to infection. Similar study tackled the pattern of infection in

children of Yemen showed that the age interval, 9-13 years, was the most affected group in Al-Mahweet area, whereas it was 10-12 years in the Taiz area [19].

Other studies have agreed with this study result. A study conducted in Morocco showed that the age distribution of the prevalence of infection showed that the infection rate was highest among the children aged more 10 years (54.5%), this observed difference in prevalence by age was statistically significant (*P-value* = 0.02) [23]. A study conducted in Nepal showed that the highest among children aged less than 6 years (37.5%) followed by children aged 6-10 years (31.6%) and children aged more than 10 years (16.8%), this prevalence of intestinal parasites was found to be statistically different among the age groups [4]. A study conducted in Nigeria showed that the pupils within the group 8 to 9 years had the highest prevalence (42.0%) which decreased with increase in age [26]. Another study conducted in Nigeria showed that pupils between 8 and 10 years of age were the most infected [13].

The present study results also showed that the *E. histolytica* cyst (64%), *Schistosoma mansoni* (36.5%), amorphous amoebae (22.5%), *Trichuris trichiura* (18%) and *Enterobius vermicularis* (13%) infections, were the highest prevalence among schoolchildren in Al-Mahweet Government area, Yemen. Other studies have indicated the opposite finding. A study conducted in Egypt showed that identified the prevalence of intestinal parasitic infections are *Entamoeba coli* (19.3%), *Ascaris lumbricoides* (3.8%), *Hymenolepis nana* (12.5%), *Enterobius vermicularis* (5.7%) and *Giardia lamblia* (12.5%) in school pupils [15]. A study conducted in India reported that the predominant parasite detected was *Ascaris lumbricoides* (54.9%) followed by *Trichuris trichiura* (32.5%), *Taenia saginata* (9.1%), *Enterobius vermicularis* (2.6%) and *H. nana* (2.05%) [30]. A study conducted in Nigeria showed that four different types of helminths were encountered namely: 33 (52.4%) *Ascaris lumbrioides*, 14 (22.2%) hookworm, 12 (19.0%) *Taenia* spp. and 4 (6.3%) *Schistosoma mansoni* [26]. Another study conducted in Ondo state, Nigeria reported that the parasite species encountered in it were: *Ascaris lumbricoides* (45.5%), *Strongyloides stercoralis* (26.1%) and hookworm (21.6%) [27]. A study conducted in southeastern Nigeria showed that the intestinal helminth parasites including *Trichuris trichiura* (34.5%), hookworms (33.7%), *Ascaris lumbricoides* (22.7%) and *Strongyloides stercoralis* (3.6%) were encountered in the faecal samples examined [6]. Another study conducted in Zamfara state, Nigeria reported that the common intestinal worms in the area are *Ascaris lumbricoides* (32.23%), *Enterobius vermicularis* (21.05%), *Trichuris trichiura* (20.39%), hookworm (13.81%) and *Taenia* spp. (12.50%) [31]. Another study conducted in Nigeria showed that *Ascaris lumbricoides* were encountered in 46% of the infected specimens, hookworms in 23%, *Trichuris trichiura* in 9% and *Strongyloides stercoralis* in 11% [13]. A study conducted in Amhara region, north west Ethiopia reported that the most prevalent intestinal parasites were hookworm (71.2%), *Entamoeba histolytica/dispar* (6.7%) and *Strongyloides stercoralis* (2.4%) [21].

The reason for the difference might be the geography of the place or the socioeconomic condition of the study area and the habit of the study participants in relation to hygienic circumstances.

This study results also showed that the anthropometrical measurements revealed that the children of Al-Mahweet Governorate are shorter and lighter than WHO standards. Which, this study results showed that more than 67% of the schoolchildren were found to be underweight and 22% stunting.

According to toilet facilities, this study results showed the high percent of the children (57.5%) using pit latrine toilet. The use of pit latrine protect against intestinal helminths, it must be provided alongside with an adequate water supply to ensure personal cleanliness and cleanliness of the latrine. But where the provision of the latrine is not accompanied by an adequate supply of water the chances of fecal contamination become higher. Also, the higher frequency of infection recorded among children may be attributed to the improper usage, poor quality hygiene of the toilet and unacceptably higher numbers of persons per toilet (overcrowding). A previous study conducted in Yemen showed that latrines are at hand for 49% of people, not available for 30%, while 22% had latrines but did not use them all the time [10].

In this study, the prevalence and distribution of intestinal parasites among the schoolchildren of different locations varied according to where the children lived. The prevalence of intestinal parasites at locations of Al-Taweela and Al-Mahweet city were present in appreciable numbers. While the prevalence of intestinal parasites at locations of Al-Rogum and Shebam-Kaokaban, were present in lowest numbers. Therefore, knowledge of the prevailing distribution and intensity of intestinal parasitic infection in each local area was essential for planning, implementing, and evaluating intervention programs.

5. CONCLUSIONS

The study revealed a high level of intestinal parasitic infection burden, a situation which is not too good for the physical, mental and cognitive development of the children.

This study highlights the need for periodical school deworming interventions to control child morbidity associated with intestinal parasitic infections. Appropriate health education regarding hygienic practices along and de-worming interventions are recommended to reduce worm burden among schoolchildren in Al-Mahweet Governorate. Yemen government should embark on measures to control the spread of intestinal parasitic infection among schoolchildren in Al-Mahweet Governorate. Also, Non-Governmental Organizations (NGOs) should be practically involved in the control of intestinal parasitic infection through public enlightenment on the undesirable consequences of the infection in children. The use of free chemotherapy, health education campaigns in the communities and improved socioeconomic conditions will no doubt enhance the control of intestinal parasitism and morbidity caused by these worms. There is a necessity for further study to investigate in large sample allowing a better understanding of the risk factors associated with intestinal parasites.

The major limitation of the current study was a low sample. But, it was thought that the results were still important because there is little knowledge on the data of the region.

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AUTHORS' CONTRIBUTION

Both authors have equally contribution in conducted studies and manuscript preparation. The final manuscript has been read and approved by both authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Research Article

The decline of the white-tailed jackrabbit (*Lepus townsendii*): carbohydrate and soil texture analysis

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ABSTRACT

A decline of the white-tailed jackrabbit, (*Lepus townsendii*), has been occurring throughout the species natural range. This has provoked the need for research and a greater understanding of the reasons behind the decline. Literature suggests that the white-tailed jackrabbit forage quality may not be sufficient, which is important for pre-natal nutrition and further, that the metabolism of the jackrabbit is higher in the winter; however, the amount of carbohydrates available to jackrabbits has not been investigated. Prairie grasses and soils from white-tailed jackrabbit inhabited areas in central and western South Dakota, were sampled from three counties from the fall of 2013 until the spring of 2015. The results of this study suggest that the carbohydrate concentration (glucose and fructose) of grasses are low during the fall and winter when pre-natal nutrition for the first litter is important and the concentrations of glucose, fructose, and soil texture between all three counties were significant ($p<0.001$). Jackrabbits were also found in areas with a higher clay concentration for soils. Jackrabbit biochemical studies coupled with physiological research is needed to help portray a better understanding of white-tailed jackrabbit population health.

Keywords: White-tailed jackrabbit; *Lepus townsendii*; Glucose; Fructose; Soil texture; Vegetation.

1. INTRODUCTION

The white-tailed jackrabbit (*Lepus townsendii*) is an endemic species located in the north-central to north-western United States to as far south as northern New Mexico to upwards into Canada [1]. This species has diffused dispersal into eastern Iowa and Wisconsin; however, the range has retracted [2-4]. The white-tailed jackrabbit is listed as a species of "special concern" for several states including Iowa, Wisconsin, Nevada, Washington and Oregon. However, the jackrabbit is listed as a predator or varmint status in other states such as Wyoming and South Dakota. Also called the Prairie Hare, the white-tailed jackrabbit preferred habitat includes pasture, cropland [5], prairie [3], sagebrush steppe [6]. To date, it has been thought that jack-rabbit declines are due to land use intensification [3] human intrusion, predators [7], while others have suggested fragmentation of habitat and monocul-tures of crop plants [4].

Past literature suggests that the jackrabbit distribution was associated with cultivation or settlement activities (e.g. [2, 8, 9]) and glaciated soils [5]. However, edaphic conditions and soil moisture can impinge on the fecundity of animals [10]. This underpins our assumptions that jack-rabbits could be associated with vegetation and soils that are most like fresh cleared land converted to cultivation (see [11] for discussion). In addition, [12] and [13] say that moisture, soil elements, and chemistry impinge on the distribution of fauna. The moisture levels, chemistry, and soil elements create different environments where various species of vegetation grows and further, the vegetation determines which fauna will be in the area. The different soils also play a factor in the distribution of the vegetation which then in turn, affects the fauna. However, soil texture has not been analyzed in areas where jackrabbits are located.

Studies have been put forth regarding black-tailed (*Lepus californicus*) and white-tailed jack-rabbit nutrition and preferred vegetation (e.g. [14-18]). The study completed by [18] identified white-tailed jackrabbits coming into Canadian city limits to forage on spike plants (*Cordyline australis*) during the winter. As [18] point out, eating the spike plant could also indicate a lack of sufficient supply of jackrabbit preferred vegetation, or perhaps lack of sufficient nutrition for the jackrabbit. Further, [17] indicated that late winter forage quality may impinge on prenatal mortality for the first litter of white-tailed jackrabbits.

Other studies have highlighted the body condition of the white-tailed jackrabbit. For example, [19] conducted South Dakota state-wide research on the white-tailed jackrabbit and indi-cated seasonal changes of kidney fat index (KFI) on the jackrabbits and [4] also found kidney disease in white-tailed jackrabbits in Iowa. One role of the kidneys is to regulate glucose homeo-stasis [20].

Given that the metabolic rate of white tailed jackrabbits is higher in the winter [21], it is impor-tant to investigate the amount of carbohydrates available to them, especially as the jackrabbits prepare for their first litter of leverets in the spring. To date, the edaphic and vegetation parameters have not been studied with the white-tailed jackrabbit, especially in South Dakota and this is what we report here. There are specific areas in South Dakota where jackrabbit populations are present and areas where they are not located at all, but could be present. We report the areas where jackrabbits are present.

2. MATERIAL AND METHODS

Vegetation and soils were collected and analyzed from fall 2013 to the winter 2015 and then further analysis such as soil texture and carbohydrate content of vegetation collected were carried out. Collections were taken where jack-rabbits have eaten the plant or direct known presence (by observer or presence of fecal pellets). Soil depth collection was between 0-13 cm deep. Soil collec-tion was dependent upon the weather and how firm the ground was to collect the soil. GPS locations were taken with a Garmin Oregon 550. Additional tests on soil and vegetation were completed with a “Chemical Composition of soil kit” and A LaMotte® Plant Tissue Test kit was used to determine the presence or absence of nitrogen, phosphorus, and potassium in plant stems (see Appendix 1 for data). Single factor and two-way ANOVA in Excel was used for statistical analysis as there were more than two study areas and more than two means to compare. Two-way ANOVA was used to determine any significance between the three sites and four seasons. In addition, these areas were similar to each other in that they all had areas where jackrabbits were inhabited.

2.1. Soil parameters

Soil pH was measured with an Accumet Basic AB15. In addition, soil texture was analyzed by a LaMotte® soil texture unit. The procedures were followed with standard procedure.

2.2. Carbohydrate Assays

Vegetation stems and internodes were analyzed with Glucose and Fructose assay kits provided by Sigma Aldrich with a ThermoScientific Genesys 10S UV-VIS spectrophotometer. The procedures were followed according to the manual provided within the kits.

2.3. Study locations

Butte County, South Dakota: the soils there are Grummit clay (GrE), Manvel silty clay loam (McB), and Enning-Minnequa silty clay loams (PMd). This was a recreational area which contains species of wheatgrass (*Agropyron spp.*) clover (*Trifolium spp.*), sagebrush (*Artemisia spp.*), and smooth bromegrass (*Bromus spp.*). This is a grassland area with little shrubs and forbs.

Hughes County, South Dakota: the soil types are Highmore-Eakin silt loams and Onita silt loams. Some of the most predominant vegetation includes species of wheatgrass, ragweed (*Ambrosia spp.*), and alfalfa (*Medicago spp.*). This was an agricultural area.

Bennett County, South Dakota: the soil types are Keith-Rosebud silt loams and Oglala-Canyon loams. This was an agricultural area which contains species of wheatgrass and turf grasses.

3. RESULTS

3.1. Soil pH and Texture

Soil pH averaged to 7.36 between Butte, Hughes, and Bennett Counties. For sand content of soils ($\bar{X}$ = 37.4% SD=18.4) (Fig. 1), for silt, $\bar{X}$= 13.3% SD=11.4) (Fig. 2), and for clay soils, $\bar{X}$ = 48.5%, SD=26.6) (Fig. 3). Single factor ANOVA revealed no significant difference in soil texture between sites where jackrabbits inhabit (F=0.00015, p<0.001).

3.2. Glucose concentration

Single factor ANOVA revealed no significant difference in glucose concentrations between sites where jackrabbits inhabit (F=0.2817, p<0.001).Two way ANOVA also showed no significant difference in glucose concentrations between sites (F=0.2170, p<0.001) where jackrabbits are and season: fall, winter, spring, summer (F=0.3107, p<0.001) (Fig. 4). See Table 1 for data.

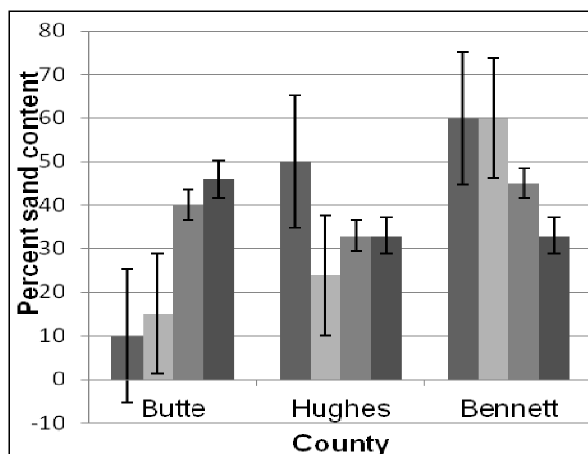

Figure 1. Percent sand content in Butte, Hughes, and Bennett Counties with standard error. For each county, there are four bars which represent winter, spring, summer, and fall left to right.

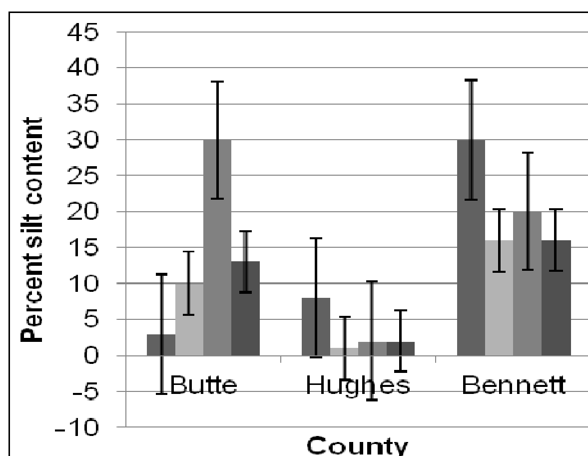

Figure 2. Percent silt content in Butte, Hughes, and Bennett Counties with standard error. For each county, there are four bars which represent winter, spring, summer, and fall left to right.

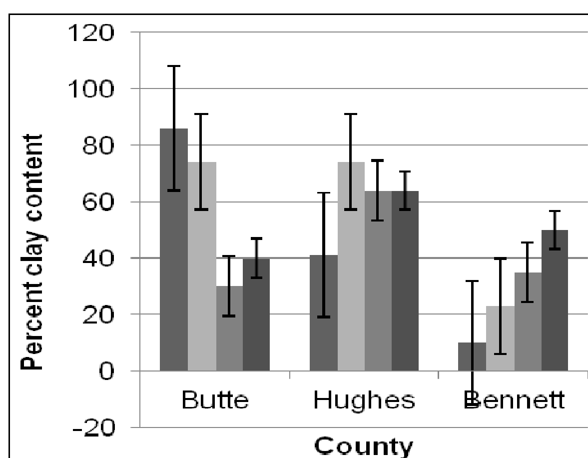

Figure 3. Percent clay content in Butte, Hughes, and Bennett Counties with standard error. For each county, there are four bars which represent winter, spring, summer, and fall left to right.

Table 1. Glucose concentrations between Butte, Hughes, and Bennett Counties.

Glucose						
Butte (mg)					**Hughes (mg)**	**Bennett (mg)**
	GrE	McB	PMd	PMd*		
Winter	0.0533	0.0489	0.6702	NA	0.8510	NA
Spring	0.3874	0.4149	0.3010	NA	0.0806	0.0170
Summer	0.7125	0.3982	0.1803	0.5661	0.0535	0.5431
Fall	0.4022	0.4174	0.3119	0.1304	NA	0.3923

*denotes second sample at PMd.

Table 2. Fructose concentrations between Butte, Hughes, and Bennett Counties.

Fructose						
Butte (mg)					**Hughes (mg)**	**Bennett (mg)**
	GrE	McB	PMd	PMd*		
Winter	1.378	0.4083	0.6908	NA	0.1719	NA
Spring	0.9578	0.8535	0.4851	NA	0.6631	0.4359
Summer	1.903	0.4236	0.3622	0.2885	0.4476	0.7846
Fall	0.2517	0.1720	0.1720	0.2517	NA	0.2271

*denotes second sample at PMd.

3.3. Fructose concentration

Single factor ANOVA revealed no significant difference in fructose concentrations between sites where jackrabbits inhabit (F=0.4047, p<0.001). Two way ANOVA also showed no significant difference in fructose concentrations between sites (F=0.5517, p<0.001) where jackrabbits are and season: fall, winter, spring, summer (F=2.089, p<0.001) (Fig. 4). See Table 2 for data.

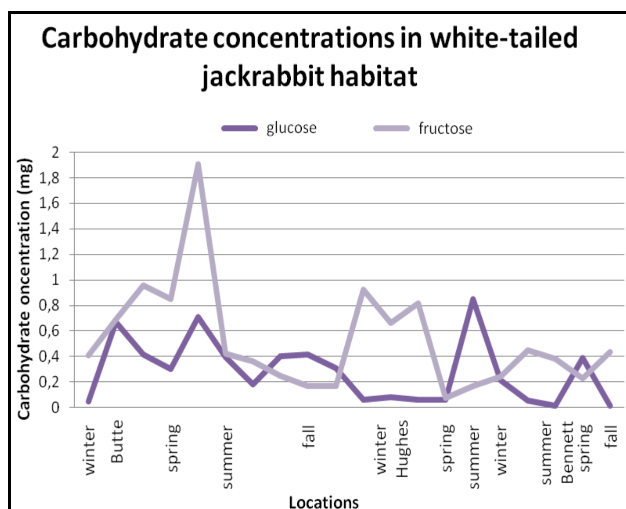

Figure 4. Glucose and fructose concentrations in white-tailed jackrabbit habitat.

4. DISCUSSION

One study had assembled a list of seasonal preferred foods for the white tailed jackrabbit [15]; however, the carbohydrate content of various forbs, grasses, and shrubs that the white-tailed jackrabbits eat at various seasons had not been attempted until this study. Our vegetation species identified where jackrabbits forage and inhabit were similar to the findings of [15]. These include a mixture of exotic and native grasses and shrubs (e.g. smooth brome, wheatgrass species, and sagebrush).

The results of this study also demonstrate the similarity of soil texture and glucose and fructose concentrations on the three white-tailed jackrabbit habitat sites in western South Dakota; however, for the majority of our plant species (except fall thickspike wheatgrass and winter ragweed), fructose was usually higher than glucose concentrations. This was also a similar result to [22] with higher fructose concentrations in vegetation species such as smooth brome grass. Since [8] describes the white-tailed jackrabbit in sagebrush habitat (also higher in fructose concentrations), could imply that jackrabbits in these study areas prefer higher fructose concentrations. Literature suggests that too much fructose may induce fatty liver and kidney disease [23]. Further, [24] discussed the relationship

between higher fructose diets and kidney fat accumulation in rats. However, this study was for rats and the dietary fructose concentration within jackrabbits vs. kidney fat has not yet been determined.

When it comes to peaks of carbohydrate concentrations, our results were comparable to [25], who found that carbohydrate concentrations peaked in the summer for brome grass. Further, [26] found that brome grass had a commendable metabolizable energy value. If brome grass had a metabolizable energy value and if the metabolic rate of white tailed jackrabbits is higher at lower temperatures in the winter [21], this could indicate a preferable dietary choice for the jackrabbit. Unfortunately, there is no data on the white-tailed jackrabbit that specifies a healthy carbohydrate load for seasons.

Between Butte, Hughes, and Bennett Counties, the white-tailed jackrabbits seemed to prefer clay soils. It is unknown as to why the jackrabbits would have preference with clay soils and lower silt concentrations. Clay soils are one of the most chemically active [27]. One possible explanation with clay soil association could be the nutrient availability as described by [28].

One study demonstrated that the amount of carbohydrates vary in rye grasses during different times of the day with fructose being at the highest concentration at noon [29], while [22] pointed out that the carbohydrates vary at different parts of the grasses and at different maturity stages. While our study analyzed the carbohydrate concentration during various seasons, future studies could focus on analyzing the carbohydrate concentrations of the grasses during various parts of the day with a focus of plant maturity. If one were to determine the time of day and season that jackrabbits foraged more heavily on that vegetation, one could determine if jackrabbits were optimizing their carbohydrate concentrations from the grasses.

Other studies have analyzed the relationship between organismal physiology and dietary preference. For example, [15] showed seasonal changes with uterine width and size of ovaries [15]. Further, that study showed that jackrabbits chose which vegetation they eat by season (e.g. the preference of shrubs such as Parry's rabbitbrush during the winter [15]). Further studies examining the relationships between physiology and biochemistry and diet are needed to help portray a better understanding of white-tailed jackrabbit population health.

5. CONCLUSION

This study quantitatively analyzed the amount of carbohydrates in grasses and soil texture in white-tailed jackrabbit habitats in central and western South Dakota. The results of this study suggest that the carbohydrate concentration (glucose and fructose) of grasses are low during the fall and winter when pre-natal nutrition for the first jackrabbit litter is important. The results of this study also showed the concentrations of glucose, fructose, and soil texture between all three counties were statistically significant ($p<0.001$). Jackrabbits were also found in areas with a higher clay concentration for soils. It would be beneficial to compare carbohydrate concentrations and soil texture analyses to other states that contain white-tailed jackrabbits and where they are not located to see if they are statistically significant. Further jackrabbit biochemical studies coupled with physiological research is needed to help portray a better understanding of white-tailed jackrabbit population health and population declines from the species.

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AUTHORS' CONTRIBUTION

KG: was the P.I. of the project, secured a grant, had the idea to test carbohydrate amounts in vegetation and test soil properties, developed the methodology.
KG, KI, AP, GB: obtained the data, tested the data, made analyses and wrote up. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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APPENDIX 1. Macronutrients from Butte, Hughes, and Martin Counties.

Butte													
	Macronutrient	Winter			Spring			Summer			Fall		
		GrE	McB	PMd	GrE	McB	PMd	GrE	McB	PMd	GrE	McB	PMd
Vegetation	Phosphorus	NA	NA	1	NA	1	1	1	1	1	1	1	1
	Nitrates	NA	NA	1	NA	1	1	0	0	1	0	0	1
	Potassium	NA	NA	1	NA	1	1	1	0	1	1	0	1
Soil	Carbonates	1	0	NA	NA	NA	1	0	1	1	0	1	1
	Nitrates	0	0	NA	NA	NA	0	0	0	0	0	0	0
	Sulfates	1	1	NA	NA	NA	1	0	0	1	0	0	0
	Ammonium	0	0	NA	NA	NA	0	0	0	0	0	0	0
	Phosphates	0	NA	NA	NA	NA	1	0	0	1	0	0	0
	Magnesium	1	NA	NA	NA	NA	0	0	0	0	0	0	0
	Calcium	1	1	NA	NA	NA	1	1	1	1	1	1	1
	Potassium	0	0	NA	NA	NA	0	1	0	0	1	0	0
	Iron	0	0	NA	NA	NA	0	1	0	0	1	0	0

Note: 0 denotes the lack of the nutrient and 1 denotes presence of the nutrient. “NA” denotes that these values were not examined.

Hughes					
	Macronutrient	Winter	Spring	Summer	Fall
Vegetation	Phosphorous	1	1	NA	1
	Nitrates	1	1	0	1
	Potassium	1	0	NA	0
Soil	Carbonates	0	NA	1	1
	Sulfates	0	NA	0	0
	Ammonium	0	NA	0	0
	Phosphates	1	NA	1	0
	Magnesium	1	NA	1	1
	Calcium	1	NA	1	1
	Potassium	1	NA	0	1
	Iron	1	NA	0	0

Note: 0 denotes the lack of the nutrient and 1 denotes presence of the nutrient. “NA” denotes that these values were not examined.

Martin					
	Macronutrient	**Winter**	**Spring**	**Summer**	**Fall**
Vegetation	Phosphorous	NA	NA	1	1
	Nitrates	NA	NA	1	1
	Potassium	NA	NA	0	0
Soil	Carbonates	NA	0	0	0
	Sulfates	NA	0	1	0
	Ammonium	NA	0	1	0
	Phosphates	NA	0	1	0
	Magnesium	NA	0	0	0
	Calcium	NA	1	1	1
	Potassium	NA	0	1	0
	Iron	NA	0	0	0

Note: 0 denotes the lack of the nutrient and 1 denotes presence of the nutrient. “NA” denotes that these values were not examined.

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Effects of crude plant extracts on wounded *Ricinus communis* plants

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ABSTRACT

The effects of mechanical wounding with or without crude extracts of neem (*Azadirachta indica*) and *Citrullus colocynthis* (CCT) supplementation on hydrogen peroxide (H_2O_2), lipid peroxidation (MDA), ascorbic acid (Asc A), phenolic compounds, and some enzymes activities in *Ricinus communis* plants were studied. In response to mechanical wounding *Ricinus* plants produced more ascorbic acid, MDA, free and bound phenolic components and to less extent H_2O_2. On the other hand, peroxidase, catalase and SOD activities were declined upon wounding as compared with unwounded plants. Neem and CCT crude extracts application, whether independently or in combination, counteracted in various degree the deleterious effects of wounding stress on the parameters tested. Effectively, their supplementation increased the antioxidant defense ability through enhancement of ascorbic acid, reduction of H_2O_2 and MDA intensities. In addition, crude extracts reduced membrane injury, increased phenolic components and improved wounded plants growth. The results clearly indicate that the protection by CCT and neem crude extracts may be mediated through the modulation of cellular antioxidant levels.

Keywords: Antioxidant; *Citrullus colocynthis*; Enzyme activities; Leakage; Neem; Phenolic compounds.

1. INTRODUCTION

Plants have evolved a highly sensitive and efficient system for monitoring changes in their environment. They can respond to physical damage by an increase in their general metabolism, including respiration rate, and this response appears to be in proportion to the severity of the damage [1]. Wounding is one of the many a biotic stresses that produce signals that migrate through cells into uninjured tissue and induces a number of physiological responses [2].

In fact, when plants are exposed to wound-causing agents they activate mechanisms directed to healing and further defense [3]. Most of the induced responses include the generation/release, perception and transduction of specific signals for the subsequent activation of wound-related defense genes [4].

Citrullus colocynthis (L.) (CCT) is one of the native plants of the Middle East countries used in traditional medicine and naturally adapted to arid environments and originally from tropical Asia and Africa. Watermelon (*Citrullus lanatus* var. *lanatus* (Thunb.) Matsum and Nakai) seed and root exudates inhibit germination and seedling growth of plants and growth of pathogenic fungi and bacteria. The results of Howard et al. [5] indicated that the testa of *Citrullus* contain at least two compounds that are

inhibitory to microorganisms and plants in bioassay. The plant extract may be acting as an antioxidant, which clears the reactive oxygen species (ROS) [6].

Azadirachta indica commonly known as neem is native of India and naturalized in most of tropical and subtropical countries are of great medicinal value and distributed widespread in the world [7]. The chemical constituents contain many biologically active compounds including alkaloids, flavonoids, triterpenoids, phenolic compounds, carotenoids, steroids and ketones, compounds that have a biological activity are salannin, volatile oils, meliantriol and nimbin. The antifungal, antibacterial, insecticidal and other versatile biological activities [8].

Previous studies have paid more attention to the abnormal variation of physiological-biochemical characteristic caused by the mechanical injuries, but the research on the adaptive mechanism itself is scarce. Also the effects of mechanical wounding, crude neem and CCT extracts as a bi-factorial combination have not been previously studied. Accordingly, the present investigation aims to study the effect of mechanical wounding, with or without the application of watermelon and neem extracts on oxidative defense ability of *Ricinus* plants. We also assayed *Ricinus* plants for the production of H_2O_2, ascorbic acid, lipid peroxidation and phenolic compounds in response to wounding. In addition, some aspects of a possible dual effect of mechanical wounding, neem and CCT crude extracts on some enzyme activities were also investigated.

2. MATERIAL AND METHODS

2.1. Plant material and experimental conditions

Castor bean (*Ricinus communis L.*) were grown in plastic pots containing 5 kg of air dry soil (sand/clay 1:2) in the experimental outdoor green house at Botany and Microbiology Department, the Faculty of Science, Assiut University (Egypt) under natural field conditions of temperature , humidity, light, and day/night regime. The plants (4 per pot) were watered with nutrient solution prepared according Down and Hellmers [9]. Plants were grown for three weeks in the soil, the water content of which was maintained at field capacity.

2.2. Mechanical wounding induced stress

The expanded leaves of castor bean plant were damaged by puncturing leaves on the plant [10]. For the puncture treatment, the leaf was punctured 0, 10, 20, and 30 times with a sterile needle of syringe (needle diameter was 0.1 cm). Wound treatment resulted in 10, 20 and 30 uniformly spaced perforations per leaf area in addition to unwounded leaves (0 pores).

2.3. Preparation of plant crude extracts

Neem (*Azadirachta indica* A. Juss) and Abujahl watermelon *(Citrullus colocynthis* L. Schrad) plants were collected from the natural sources of Assiut University and Wadi Natash in the south Eastern Desert of Egypt, respectively. The leaf and fruit of the two previous plants were collected freshly and shade dried at room temperature (22-25° C) in dark condition. After appropriate drying the dried leaves and fruits were cut into small pieces and coarsely powdered to a fine powder. A measure of fifty grams of dry powder of either leaves (neem) and fruits (*Citrullus*) were soaked in 1 liter of distilled water for 72 h at refrigerator temperature (4° C). The resulting solution was filtered through four layers of cheesecloth to remove debris, and centrifuged at 5,000×g for 30 min. The supernatant was then filtered through one layer of filter paper (Whatman No.1). The filtered solutions (stock solution) were held for a short time at 4° C until using. The extracts used for application were performed by diluting the stock solution (5% w/v) at concentration 0.25 g of the dried powders per 100 ml of distilled water [11].

2.4. Plant crude extracts application and experimental design

One set of the wounded plants (0, 10, 20 and 30 perforations) was foliar sprayed with *Citrullus* fruit aqueous extract solution (0.25%), the second set was foliar sprayed with neem (*Azadirachta indica* A. Juss) leaf aqueous extract solution (0.25%), the third set was foliar sprayed with a mixture of previously mentioned plant extracts (0.25%). Control (C) plants were sprayed with distilled water. Five pots were assigned at random to each treatment combination. A week after last foliar plant

extract applications, the plants were harvested and analyzed.

2.5. Membrane damage (electrolyte leakage %)

Electrolyte leakage % used to assess membrane permeability [12]. Leaf samples of one randomly chosen plant per replicate were taken from the youngest fully expanded leaf and cut into 1cm segments. Leaf samples were then placed in individual stopper vials containing 10 mL of distilled water after three washes with distilled water to remove surface contamination. These samples were incubated at room temperature (25 ºC) on a shaker (100 rpm) for 24 h. Electrical conductivity (EC) of bathing solution (EC1) was read after incubation using an electrical conductivity meter (JENWAY 4310). The same samples were then placed in an autoclave at 120 ºC for 20 min and the second reading (EC2) was determined after cooling solution to room temperature. The electrolyte leakage was calculated as EC1/ EC2 and expressed as percent.

2.6. Determination of malonydialdhyde (MDA)

The level of lipid peroxidation in plant tissues was measured by determination of MDA [13]. MDA content was determined with thiobarbituric acid (TBA) reaction. 0.2 g tissue sample was homogenized in 5 ml 0.1% TCA. The homogenate was centrifuged at 10,000×*g* for 5 min. 4 ml of 20% TCA containing 0.5% TBA was added to 1 ml aliquot of the supernatant. The mixture was heated at 95°C for 15 min and cooled immediately in an ice bath. The non-specific absorbance of the supernatant at 600 nm was subtracted from the maximum absorbance at 532 nm using Unico UV-2100 spectrophotometer. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.7. Determination of free and cell wall-bound phenolics

Free and cell wall-bound phenolics were determined according to Kofalvi and Nassuth [14]. A known weight (0.2 g) of the fresh samples of leaves was extracted with 50% cold methanol (v/v). Then 0.5 ml of the extraction was added to 0.5 ml Folin, Shaken allowed to stand for 3 min. Then one ml of saturated sodium carbonate was added to each tube followed by distilled water shaken and allowed to stand for 60 min. The optical density was determined at wave length of 725 nm using spectrophotometer (Unico UV-2100). Phenolic concentration in the extract was determined from standard curve prepared with gallic acid.

2.8. Hydrogen peroxide (H_2O_2) determination

H_2O_2 content of the shoots samples were colorimetrically measured as described by Jena and Choudhuri [15]. Fresh samples were extracted with cold acetone to determine H_2O_2 levels. An aliquot (1 mL) of the extracted solution was mixed with 0.1% titanium dioxide in 20% (v/v) H_2SO_4, and the mixture was then centrifuged at 6,000 g for 15 min. The intensity of the yellow color of the supernatant was measured at 415 nm (Unico UV-2100). The concentration of H_2O_2 was calculated from a standard curve plotted with known concentration of H_2O_2 and expressed as µmol g^{-1} FW.

2.9. Ascorbic acid determination

Ascorbic acid concentration was determined according to Mukherjee and Choudhury [16] by mixing 2 mol/L Folin-Ciocalteu reagent and 10% TCA with 20% tissue homogenate. The blue colour developed in the supernatant after centrifugation was read at 760 nm (Unico UV-2100) after 10 min. The amount of ascorbic acid was calculated from a standard curve of vitamin C using different concentrations of ascorbic acid.

2.10. Estimation of antioxidant enzymes

Enzyme activities in 0.5 g of fresh leaf tissues were ground to a fine powder in liquid nitrogen and then homogenized in 5 ml of 1.00 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA and 0.1 g PVP. The homogenate was centrifuged at 12000×g for 10 min at 4ºC, the supernatants were collected and used for enzyme assays. All enzyme activities were assayed at 25 ºC using a UV visible Unico UV-2100 spectrophotometer. The specific activity of the enzymes was

expressed as m mole mg^{-1} protein min^{-1}. Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by monitoring its ability to inhibit the photo-chemical reduction of NBT as described by Misra and Fridovich [17]. Catalase (EC 1.11.1.6) activity was assayed by following the consumption of H_2O_2 for 1 min Aebi [18]. Guaiacol peroxidase (POD, EC 1.11.1.7) activity was assayed by adding aliquot of the tissue extract (100 µl) to 3 ml of assay solution, consisting of 3 ml of reaction mixture containing 13 mM guaiacol, 5 mM H_2O_2 and 50 mM Na-phosphate (pH 6.5) as described by Tatiana et al. [19].

2.11. Statistical analysis

All data obtained have been subjected to one way analysis of variance (ANOVA) using the SPSS statistical package. For comparison of means, the Duncan's multiple range tests ($p < 0.05$) were used. Also, factorial ANOVA analysis using Ostle [20] method was performed. The relative effect of each single factor as well as their interactions in contributing to the total response was evaluated by calculation of the coefficient of determination (η^2). In such cases:

$$\eta 2 = \frac{\text{Sum of squares due to the factor}}{\text{Total sum of squares due to the treatment combination}}$$

3. RESULTS

3.1. Malonydialdhyde (MDA)

Wounding stress leads to an increase (up to 48 % at high wounding stress level) in the concentration of MDA (Table 1) as compared with unwounded plants. Application of crude extracts of CCT and neem resulted in great reduction in MDA concentration in wounded plants in comparing with the plants sprayed with distilled water.

3.2. Hydrogen peroxide (H_2O_2)

The results of Table 1 indicate that wounding stress-induced hydrogen peroxide accumulation were dependent upon wounding intensity. Mechanical wounding increased H_2O_2 concentration (Table 1) at higher wounding stress level (30 pore intensity). Contrarily, at low and moderate perforation levels (10 and 20 intensity) H_2O_2 concentration decreased slightly as compared with unwounded plants. Spraying with crude extracts of CCT and neem either independently or in combination resulted in an increase in H_2O_2 concentration at 10 and 20 pore intensity and marked decrease at high pore intensity level (30 pores) as well as in unwounded plants.

3.3. Membrane damage

Wounding leads to an increase in leakage from leaf membranes at all levels (Table 1). Neem crude extract either independently or in combination with CCT extracts reduced membrane damage in moderately and highly wounding stressed plants but increase the damage in unwounded and low wounded plants. Application of CCT extract separately, reduced membrane injury in wounded plants at all wounding stress levels but increased the damage in unwounded plants.

3.4. Ascorbic acid

Ascorbic acid concentrations (Table 2) were generally higher in wounding stressed plants than unwounded one. Wounded and unwounded plants receiving crude neem and CCT extracts had significantly higher ascorbic acid concentrations than the control (sprayed with distilled water). CCT extract was more effective than neem extracts in increasing concentration of ascorbic acid.

3.5. Free and bound phenolic contents

Free phenolic contents increased significantly in response to wounding stress at the three wounding stress level (Table 2). The same held true for bound phenolic at moderate and high wounding stress (20 and 30 pores intensity, respectively) but at low wounding stress (10 pores) the opposite response was true.

Treatment with neem crude extract decreased significantly the concentration of free phenolic in unwounded and high wounding levels and increased the concentration at low and moderate wounding stress (10 and 20 pores intensity, respectively).

Table 1. Effects of leaf wounding intensity stress (pores/leaf area) and *Citrullus colocynthis* L. (CCT), *Azadirachta indica* A. Juss (Neem) and their mixture (CCT +Neem) extracts of 0.25% (w/v) sprays on malondialdehyde (MDA), hydrogen peroxidase (H_2O_2) contents (µmol g^{-1} FW) and leaf membrane damage (as % of electrical conductivity) in *Ricinus communis* L. plants.

Parameter	Treatment				
	Wounding intensity stress	Control	CCT	Neem	CCT +Neem
(MDA)	00 Pores	51.510±0.089[a]	59.65±1.516[b]	46.960±1.653[a]	49.250±0.621[a]
	10 Pores	66.690±0.041[c]	54.05±1.182[b]	48.080±0.952[a]	52.160±2.940[ab]
	20 Pores	66.680±0.066[b]	52.43±2.649[a]	53.550±1.445[a]	58.500±4.160[ab]
	30 Pores	76.437±0.045[b]	53.31±0.122[a]	54.890±1.522[a]	49.250±1.305[a]
H_2O_2	00 Pores	0.1283±0.081[a]	0.057±0.0072[a]	0.062±0.0054[b]	0.098±0.00493[c]
	10 Pores	0.0380±0.004[a]	0.058±0.0037[b]	0.069±0.0045[b]	0.056±0.0024[b]
	20 Pores	0.0180±0.001[a]	0.049±0.0033[b]	0.053±0.0012[b]	0.051±0.0030[c]
	30 Pores	0.0400±0.001[a]	0.054±0.0025[b]	0.061±0.0022[c]	0.053±0.0009[b]
Membrane damage	00 Pores	14.070±0.407[a]	20.53±0.644[b]	14.64±0.910[a]	14.840±1.050[a]
	10 Pores	14.710±0.081[ab]	27.38±0.820[c]	10.96±1.080[a]	25.090±7.051[c]
	20 Pores	21.700±0.933[a]	19.12±0.517[a]	12.47±0.678[a]	12.810±1.100[a]
	30 Pores	23.410±0.592[b]	22.16±1.776[b]	13.75±1.307[a]	21.940±1.448[b]

Data are means of five replicates ± SD. Different letters denote significant differences between different treatments at $p \leq 0.05$ (Duncan's test).

Table 2. Effects of leaf wounding intensity stress (pores / leaf area) and *Citrullus colocynthis* L. (CCT), *Azadirachta indica* A. Juss (Neem) and their mixture (CCT+ Neem) extracts of 0.25% (w/v) sprays on contents of ascorbic acid (µmol g^{-1} FW), free and bound phenol (mg g^{-1} FW) in *Ricinus communis* L. plants.

Parameter	Treatment				
	Wounding intensity stress	Control	CCT	Neem	CCT + Neem
Ascorbic acid	00 Pores	0.868±0.0104[a]	0.956±0.0186[a]	1.063±0.1170[a]	1.725±0.0690[b]
	10 Pores	0.892±0.0159[a]	1.080±0.0806[ab]	1.122±0.0211[b]	1.019±0.0877[b]
	20 Pores	0.897±0.0425[a]	1.007±0.0060[ab]	1.743±0.0946[c]	1.145±0.0733[b]
	30 Pores	1.133±0.1675[a]	1.149±0.0275 [a]	1.755±0.1724[b]	1.203±0.0104[ab]
Free phenolics	00 Pores	2.820±0.196[ab]	2.530± 00.038[a]	2.710± 0.201[a]	3.530± 00.345[b]
	10 Pores	2.690±0.0904[a]	3.610±00.071[b]	2.300± 0.068[a]	2.590±00.254[a]
	20 Pores	3.050± 0.023[a]	3.040±00.395[a]	2.430± 0.162[a]	2.690±00.058[a]
	30 Pores	4.480± 0.206[b]	2.750±00.109[a]	2.630± 0.140[a]	2.600±00.095[a]
Bound phenolics	00 Pores	0.767± 0.067[a]	0.677±00.025[a]	0.772± 0.024[a]	0.677±00.150[a]
	10 Pores	0.701± 0.0153[a]	0.700±00.021[a]	0.810± 0.047[a]	0.755±00.044[a]
	20 Pores	0.910± 0.036[b]	1.087±00.063[b]	0.639± 0.019[a]	1.080±00.102[b]
	30 Pores	0.936± 0.0782[b]	1.190±00.058[c]	0.779±-0.042[a]	0.684±00.014[a]

Data are means of five replicates ± SD. Different letters denote significant differences between different treatments at $p \leq 0.05$ (Duncan's test).

Table 3. Effects of leaf wounding intensity stress (pores / leaf area) and *Citrullus colocynthis* L. (CCT), *Azadirachta indica* A. Juss (Neem) and their mixture (CCT +Neem) extracts of 0.25% (w/v) sprays on peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) activities (unit mg^{-1} protein) in leaves of *Ricinus communis* L. plants.

Parameter	Treatment				
	Wounding intensity stress	Control	CCT	Neem	CCT + Neem
POD	00 Pores	0.27554±0.0155[a]	0.22099±0.0109[a]	0.18507±0.0140[a]	0.43694±0.0525[b]
	10 Pores	0.20062±0.0414[a]	0.25874±0.0060[a]	0.13568±0.0020[a]	0.19597±0.0087[a]
	20 Pores	0.20425±0.0560[a]	0.18202±0.0010[a]	0.18114±0.0015[a]	0.21906±0.0101[a]
	30 Pores	0.11729±0.0011[a]	0.15316±0.0009[b]	0.10738±0.0065[a]	0.17536±0.0053[b]
SOD	00 Pores	0.0233±0.0040[b]	0.01310±0.0012[a]	0.0194±0.00001[ab]	0.0161±0.0012[ab]
	10 Pores	0.0137±0.0040[b]	0.0129±0.0041[a]	0.0227±0.0060[a]	0.0694±0.0182[b]
	20 Pores	0.0157±0.0029[a]	0.0338±0.0037[a]	0.023±0.00416[a]	0.0377±0.0088[b]
	30 Pores	0.0205±0.0013[a]	0.0158±0.0008[a]	0.0149±0.0011[a]	0.1234±0.0191[b]
CAT	00 Pores	0.6371±0.0823[a]	0.1334±0.0582[a]	0.1485±0.0799[a]	0.6200±0.2001[a]
	10 Pores	0.6236±0.1135[ab]	0.1380±0.0733[a]	0.6268±0.1954[ab]	0.8697±0.0490[b]
	20 Pores	0.5830±0.1076[b]	0.1826±0.0096[a]	0.1232±0.0530[a]	0.4010±0.1074[a]
	30 Pores	0.1489±0.0131[a]	0.1970±0.0676[a]	0.2137±0.0013[a]	0.1697±0.0174[a]

Data are means of five replicates ± SD. Different letters denote significant differences between different treatments at $p \leq 0.05$ (Duncan's test).

Table 4. Coefficient of determination (η2) values for the effects of Wounding, CCT, neem extracts and their interactions (Wound x CCT, Wound x Neem, CCT x Neem and Wound x CCT x Neem) on leaf membrane damage (as % of electrical conductivity), contents of malondialdehyde (MDA), hydrogen peroxidase (H_2O_2), ascorbic acid, free phenolics, bound phenolics, peroxidase, (POD) superoxide dismutase and (SOD) catalase activities in leaves of *Ricinus communis* L. plants.

Parameter	Treatment						
	Wounding	CCT	Neem	Wound x CCT	Wound x Neem	CCT x Neem	Wound x CCT x Neem
Membrane damage	0.145	0.234	0.234	0.04	0.281	0.008	0.058
MDA	0.112	0.300	0.085	0.049	0.199	0.146	0.109
H_2O_2	0.233	0.316	0.132	0.060	0.078	0.068	0.113
Ascorbic acid	0.085	0.359	0.007	0.08	0.242	0.045	0.182
Free phenolics	0.056	0.166	0.004	0.255	0.268	0.081	0.170
Bound phenolics	0.311	0.086	0.042	0.23	0.217	0.003	0.111
SOD	0.112	0.154	0.149	0.139	0.144	0.131	0.171
POD	0.453	0.001	0.133	0.09	0.046	0.113	0.164
Catalase	0.293	0.003	0.047	0.085	0.053	0.361	0.155

Application of the two extracts in combination decreased free phenolic in wounding stressed plants at all stress levels but increased the concentration in unwounded plants. Bound phenolic concentration were higher in low and moderate wound stressed plants receiving both extracts in combination, but the reverse held true in unwounded and highly stressed plants.

3.6. Superoxide dismutase (SOD), guaiacol peroxidase (POD) and catalase (CAT)

Wounding stress at all pore intensity significantly decreased the activities of peroxidase, catalase and SOD (Table 3). Castor bean plants showed great variations in their response to crude extracts treatments. CCT extract spraying resulted in significant decreases in catalase and SOD activities in both wounded and unwounded plants except at high wounding stress level. However, peroxidase activity increased in plants received CCT extract at low and high wounding stress level (10 and 30 pore intensity, respectively) but its activity decreased in unwounded and moderately wounded plants. Spraying with neem extract significantly decreased peroxidase and catalase activities in both wounded and unwounded plans as compared with plants sprayed with distilled water (highly wounding stressed plants were exceptions (30 pore for Catalase). SOD activity increased significantly in plants receiving neem extract at low and moderate wounding stress but decreased significantly in unwound plants as well as in high wounding stress levels.

Plants sprayed with both extracts in combination had higher peroxidase activity than those sprayed with distilled water. Wounded plants at all perforation intensities receiving both extracts in combination had higher SOD activity in comparing with control plants. Unwounded plants showed opposite trend in their response. Catalase activity increased in low and highly wounding-stressed plants received both extracts in combination but decreased in unwounded and moderately wounding-stressed plants.

The effects of single factors (wounding, CCT and neem), bi-factorial interactions (wounding × CCT, wounding × neem and CCT × neem) and three factorial (wounding × CCT × neem) interaction on the parameters tested were shown by analysis of variance to be statistically significant. Further statistical treatment of data by calculation of the coefficient of determination (η^2), which indicates the relative role of each factor on the total effect of treatment combination (Table 4), pointed to the following:

1. Wounding had dominant role in affecting lipid peroxidation, bound phenolic components, and POD activities.
2. CCT extract has a dominant effect on changes of MDA, Asc A and H_2O_2.
3. The role of neem and CCT was equal dominant in affecting membrane damage (η^2 0.234 for each).
4. Wounding × neem interaction had dominant role in affecting free phenolic components.
5. The role of CCT × neem interaction was dominant in changing catalase activities.
6. The three factorial interactions (wounding × CCT × neem) have dominant effect in SOD activities. The three factors and their interactions seem to have dual role in their subsidiary effect.

4. DISCUSSION

Wound signaling in plants is a complex process involving a whole array of molecules with regulatory activity [3]. In this study, wounding (e.g. perforation) increases phenolic metabolism in *Ricinus* leaf tissue with the production and accumulation of soluble phenolic compounds that subsequently react to produce wound-induced tissue browning. The synthesized phenolic correlated to an increase in antioxidant capacity [21]. The accumulation of phenolic compounds represents a major key factor in the inducible defense mechanisms of plants through the phenylpropanoid pathway [22]. Campos-Vargas et al. [23] found an initial response to wounding in lettuce is the de novo synthesis and increased activity of phenyl-alanine ammonialyase (PAL, EC 4.3.1.5). The soluble phenolic compounds produced by this enhanced phenylalanine ammonialyase (PAL) activity are sequestered in the vacuole and only participate in browning reactions when the disruption of membranes allows the substrates and enzymes to mix [23].

Supplying *Ricinus* plants with neem and CCT extracts reduce tissue browning could be due to the antioxidant component present in the extracts. CCT also contains flavonoids (such as quercetin, myricetin, and kaempferol [24], and two cucurbitacin glucosides [25]. The antioxidant property of these flavonoids was determined by the DPPH assay and showed significant antioxidant properties. Extracts from young flowers and leaves of neem have strong antioxidant potential.

Hydrogen peroxide (H_2O_2) generated in response to wounding was detected in wounded plants at high wounding stress level (30 pore intensity). The cumulative data suggest that systemic wound signals that induce H_2O_2 are widespread in the plant kingdom and that the response may be associated with the defense of plants against both herbivores and pathogens. The results of Man et al. [26] showed that the reactive oxygen species and membrane lipid peroxidation were all induced by the wounding stress treatments. They found that in wounded fruits, the antioxidant enzymes such as POD, SOD and some antioxidant substances were provoked immediately leaded by H_2O_2 production.

Neem and CCT extracts reduced H_2O_2 concentration at low and moderate wounding stress. The extracts may be acting as an antioxidant, which clears the reactive oxygen species [27]. This means that the protection by CCT may be mediated through the modulation of cellular antioxidant levels. On the other hand, at high wounding stress level the extracts caused additive increase in the H_2O_2 concentration. The elevated H_2O_2 levels at high wounding intensity may potentiate the plants' defense responses against invading pathogens, in which ROS play an important role [28].

Vitamin C (ascorbate, AsA) is the most abundant water-soluble antioxidant in plants. AsA content in plants varies in response to a biotic and biotic stresses, including wounding. The effects of wounding on AsA accumulation differ between species [29]. In the present investigation mechanical wounding decreased ascorbic acid concentrations in *Ricinus* plants. The majority of studies on wounding reported a decrease in AsA [29]. Supplying *Ricinus* plants with crude extracts had been shown to increase the levels of ascorbic acid. Ascorbate provides the first line of defense against damaging reactive oxygen species (ROS), and helps protect plant cells from many factors that induce oxidative stress, including wounding, ozone, high salinity, and pathogen attack [29].

Wounding at all intensity levels enhanced MDA accumulation in *Ricinus* plants. MDA can interact with DNA, causing strand breaks that in turn develop into chromosomal breaks. Treatments with neem and CCT extracts reduced MDA. Extract of CCT showed anti lipid peroxidation activity which significantly decreased the levels of MDA. This decrease in lipid peroxidation by CCT might be due to the scavenging of free radicals and ROS [27].

Antioxidant enzymes activities (peroxidase POD, catalase CAT and superoxide dismutase SOD) were inhibited in *Ricinus* plants in response to mechanical wounding, neem and CCT extracts application when used independently or as bi-factorial factors. However, when the three factors used in combination a marked increase in their activities was noticed. Shen et al. [30] reported that the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) were enhanced in wounded leaves.

Mechanical wounding injured leaf membrane of *Ricinus* plants and enhanced electrolyte leakage. Supplying crude extract reduced cell membrane damage could be due to their anti-oxidant abilities. A high level of antioxidant enzymes could decrease membrane lipid peroxidation levels and effectively induce plant defense responses [30].

Bi-factorial and tri-factorial interactions were mostly significant for the parameters tested as indicated by analysis of variance. These interactions between single factors could be modified or reversed their effect when used in combination. For example application of wounding stress, neem and CCT extracts independently decreased peroxidase, catalase and SOD activities. When used in combination the inhibitory effect was completely reversed and marked increase in the activities of these enzymes was reported. This means that the interaction between single factors (as in natural conditions) when used in combination could be modify or reverse their effects in certain cases (e.g. SOD and catalase activities and free phenolic components).

In conclusion, results of our study clearly indicate that *Ricinus communis* plants respond to wounding stress by changes in their biochemical processes through accumulation of MDA, ascorbic acid, free and bound phenolic components. *Citrullus colocynthis and A. indica* might be beneficial in attenuating the elevated biochemical parameters induced mechanical wounding damage. The results suggested the ability of the extracts to combat oxidative stress by quenching free radicals which reveals that, the attenuation due to their antioxidant property.

AUTHORS' CONTRIBUTION

Both authors made a significant contribution to experiment design, acquisition of data, analysis and preparing of the manuscript. The final manuscript has been read and approved by both authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Research Article

Enzyme producing capabilities of some extremophilic fungal strains isolated from different habitats of Wadi El-Natrun, Egypt. Part 1: Protease, lipase and phosphatase

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ABSTRACT

Thirty-nine isolates of the most commonly encountered fungal species from different extreme habitats of Wadi El-Natrun region were tested for their capabilities of producing protease, lipase and phosphatase enzymes. Most of these isolates had the capabilities of producing protease (84.2%), lipase (89.7%) and phosphatase (100%) but with different degrees. Thirty-five isolates showed high producing abilities of phosphatase (30 isolates), lipase (22) and protease (8) on different screening media. Some of these isolates were high producers for more than one enzyme, on one or more of the screening media. The present results reveal some very interesting strains having the high capabilities for producing more than one enzyme on more than one of the screening media.

Keywords: Extremophiles; Enzymes; Proteases; Lipases; Phosphatases; Wadi El-Natrun.

1. INTRODUCTION

Extremophilic microorganisms offer a multitude of actual or potential applications in various fields of biotechnology. Not only do many of them produce compounds of industrial interest such as enzymes, but they also possess useful physiological properties which can facilitate their exploitation for commercial purposes.

Alkaliphilic enzymes have attracted great attention as they are now available to industry such as proteases, cellulases, lipases, pullulanases [1, 2]. Detergent enzymes account for approximately 60% of total worldwide enzyme production. The main reason for selecting enzymes from alkaliphiles is their long term stability in detergent products, energy cost saving by lowering the washing temperatures, quicker and more reliable product, reduced effluent problems during the process, and stability in the presence of detergent additives such as bleach activators, softeners, bleaches and perfumes. Due to the unusual properties of these enzymes they are expected to fill the gap between biological and

chemical processes and have been greatly employed in laundry detergents.

Proteases are one of the most important groups of industrial enzymes and account for nearly 60% of the total enzyme sale [3], as alkaline protease accounts for at least 25% of the total enzyme sales [4]. The major uses of free proteases occur in dry cleaning, detergents (pepsin), brewing meat processing, leather and dairy industries, cheese making, production of digestive and certain medical treatments of inflammation and virulent wounds [5]. *Aspergillus* proteases have been also used in many fields, especially in food processing [6]. The protease-producing potential of the fungal isolates varied among the genera and even between isolates of the same species [7].

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) occur extensively in nature in animals, plants and microorganisms and catalyse the hydrolysis and synthesis of esters formed from glycerol and long chain fatty acids [8]. Lipases from fungi are found use in diverse range of industries like detergents, pharmaceuticals, beverages and dairy [9] which makes them commercially important enzymes. Lipases also are found use in health foods, chemicals and pharmaceuticals for transesterification and enantioselectivity [10]. Lipase enzymes from extremophiles are valuable objects of research of biotechnologists from the point of view of their widest capabilities of technological utilization.

Tarafdar et al. [11] found some fungal cultures isolated from Indian desert soils belonging to *Aspergillus, Penicillium, Acrophialophora* and *Alternaria* producing both acid and alkaline phosphatases in liquid medium. Acid phosphatases have been reported to occur in fungi, such as *Fusarium* [12], and *Aspergillus* [13, 14].

This work has been designed to highlight on the capabilities of the most common fungi isolated from the hypersaline habitat environment of Wadi El-Natrun of highly producing a wide range of enzymes including protease, lipase, phosphatase under acidic, alkaline or saline conditions.

2. MATERIALS AND METHODS

2.1. Fungal strains

Thirty-nine fungal strains representing 10 species commonly encountered from different sources (soil, mud, salt crusts, water and air) of hypersaline habitats in 8 lakes in Wadi El-Natrun region (refer to Gouda [15]), the Eastern desert of Egypt (Table 1) were screened for their abilities to produce 3 extracellular enzymes on solid media.

2.2. Protease production

The proteolytic ability was tested using casein hydrolysis medium [16]. The tested isolates were inoculated in the center of the casein agar plates and incubated at 28°C for 7 days. After incubation, complete degradation of milk protein was seen as clear zone around colonies. The diameter of the colony as well as the clear zone around the colony was measured (in cm). Protease index (PI) was calculated according to Ismail [17].

2.3. Lipase production

Lipolytic ability of fungal isolates was tested on the medium of Ullman and Blasins [18] with slight modification, in which tween 80 replaced Tween 20. This medium was considered as a control medium. The medium was sterilized by autoclaving at 121°C for 15 minutes. Tween 80 was autoclaved separately and added to the sterilized and cooled basal medium. The medium was dispensed aseptically in test tubes. Test tubes were inoculated on the surface of agar by a plug of 3 mm diameter and were incubated at 28°C for 10 days. The lipolytic producing ability was observed as a visible precipitate due to the formation of crystals of calcium salt of the oleic acid liberated by the enzyme. The depth of each visible precipitate (in cm) was measured.

2.4. Phosphatase production

The ability of fungal isolates to produce phosphatase enzyme was detected using phosphatase medium described by Gochenaur [19] to which diphosphophenolphthalein (filter-sterilized) was added. The agar plates were inoculated centrally and incubated at 28 °C for 5 days. After incubation one drop of 30% ammonium hydroxide solution was added in the lid of the inverted plate. Hydrolysis of diphosphophenolphthalein was considered positive if a deep fuchsin-coloured zone developed around the colony in the presence of ammonium hydroxide

vapours. The colour intensity was recorded as +++ for the highly producing isolates, ++ for the moderate producing ones and + for the weak producers and – for non- producers.

Table 1. Sources, lakes of Wadi El-Natrun and isolation media from which fungal species tested were recovered, and their deposition numbers at AUMC Culture Collection.

Species	AUMC	Source	Lake	Isolation medium
Alternaria alternata	5665	Soil	Al Gaar	Cz (pH 13)
	5666	Soil	El Zugm	Cz (10% NaCl)
	5667	Mud	Al Gaar	Cz (40% S)
	5668	Water	Khadra	Cz (pH 5)
Aspergillus flavus	5669	Soil	Umm Risha	Cz (pH 13)
	5670	Soil	Khadra	Cz (pH 4)
	5671	Soil	Hamra	Cz (40% S)
Aspergillus terreus	5672	Air	Rosetta	Cz
	5673	Mud	Khadra	Cz (pH 3)
	5674	Soil	El Zugm	Cz (pH 3)
	5675	Soil	Hamra	Cz (pH 3)
	5676	Water	Rosetta	Cz (pH 3)
	5677	Salts	Umm Risha	Cz (pH 13)
Chaetomium globosum	5678	Soil	El Zugm	Cz (pH 5)
	5679	Soil	Al Beida	Cz (pH 10)
Cladosporum cladosporioides	5680	Soil	Umm Risha	Cz (pH 13)
	5681	Soil	Umm Risha	Cz (pH 4)
	5682	Soil	Umm Risha	Cz (pH 13)
	5683	Soil	-	Cz (pH 4)
Cochliobolus australiensis	5694	Soil	Hamra	Cz (pH 4)
	5695	Soil	Hamra	Cz (pH 13)
	5696	Salts	Hamra	Cz (pH 13)
Emericella nidulans	5685	Soil	El Zugm	Cz (10% NaCl)
	5686	Soil	Umm Risha	Cz (pH 4)
	5687	Soil	Al Gaar	Cz (pH 13)
	5688	Soil	Al Beida	Cz (pH 4)
	5689	Water	Al Gaar	Cz (pH 13)
Fusarium solani	5690	Soil	Al Bida	Cz (pH 4)
	5691	Soil	Hamra	Cz (pH 13)
	5692	Soil	Umm Risha	Cz (10% NaCl)
	5693	Salts	Khadra	Cz (40% sucrose)
Myrothecium verrucaria	5697	Soil	Hamra	Cz (pH 4)
	5698	Soil	Khadra	Cz (pH 13)
	5699	Salts	Hamra	Cz (pH 10)
Pencillium chrysogenum	5700	Soil	AlGaar	Cz (pH 4)
	5701	Soil	Al Beida	Cz (pH 13)
	5702	Soil	Umm Risha	Cz (40% sucrose)
	5703	Water	-	Cz (pH 4)
	5704	Mud	Khadra	Cz (pH 5)

AUMC = Assiut University Mycological Center

To assess the effect of acidity, alkalinity or salinity on the production of the three extracellular enzymes, the control medium was adjusted at pH 4, pH 10 and pH 13 or supplemental with 10% NaCl (saline medium). The plates were then inoculated and incubated at 28° C.

3. RESULTS AND DISCUSSION

3.1. Enzymes production by most commonly encountered fungi from Wadi El-Natrun

Thirty-nine isolates related to 10 species of the most commonly encountered fungi from the different sources in 8 lakes of Wadi El-Natrun (Table 1) were screened for their abilities of producing protease, lipase and phosphatase enzymes on agar media adjusted at different pHs or supplemented with 10% NaCl.

3.1.1. Protease enzymes

Thirty-two, out of 38 isolates screened for their capability of producing protease, showed positive results on control medium (32 isolates), media adjusted at pH 4 (12) and pH 10 (2) or medium supplemented with 10% NaCl (6). It is worthed to mention that none of fungal strains tested on medium adjusted at pH 13 was protease positive. Only 7 of these positive isolates exhibited high production abilities on one or more of the screening media (Table 2). *Emericella nidulans* (2 isolates, AUMC 5686 and 5689) showed high proteolytic activity on control and medium adjusted to pH 4 while 5 isolates gave higher indexes on only one medium and these are: *Myrothecium verrucaria* (AUMC 5697 and 5699 on control and pH 4, respectively), *Cladosporum cladosporioides* (AUMC 5681 and 5682 both on control medium) and *Cochliobolus australiensis* (AUMC 5694 on 10% NaCl).

Moderate or low capability of protease production was proved by the remaining 25 strains (moderate of PI = 1.5 – 1.99, or low of PI < 1.5). From the results presented in Table 2 it is noted that 18 fungal isolates could produce protease only on control medium and these are: *Alternaria alternata* (1 strain), *A. terreus* (3), *Chatomium globosum* (1), *Cladosporum cladosporioides* (2), *Emericella nidulans* (1), *Fusarium solani* (2), *Cochliobolus australiensis* (1), *Myrothecium verrucaria* (2) and *Penicillium chrysogenum* (3). Moreover, six isolates belonging to *Alternaria alternate* (2), *A. terreus*, *A. flavus*, *Emericella nidulans* and *Fusarium solani* (one strain each) lacked the ability of producing protease on all media used.

Several types of proteolytic enzymes by fungi were reported, for example, *Aspergillus oryzae* [20], *A. flavus*, *A. niger* [7], *A. nidulans* [21], *Scopulariopsis brevicaulis* [22], species of *Fusarium* [23], *Geotrichum* [7], and *Candida* [24].

Abdel-Rahman et al. [25] found that *Aspergillus niger, Scopulariopsis brevicaulis, Humicola grisea*, *Oidiodendron flavum*, *A. fumigatus* and *Thermomyces lanuginosus* showed proteolytic activity, but *Penicillium chrysogenum* and *Zygorrhynchus vuillemini* lacked this activity.

The present results where some isolates produced proteases at different pH values (e. g. *E. nidulans*) are in agreement with those previously recorded from some species including *Aspergillus niger* [26] and *A. terreus* [27]. Acid proteases are synthesised by several species of *Mucor* [3, 28, 29]. The pepsin-like acid proteases are synthesized by *Rhizopus* species [30], *Aspergillus* species [31] and *Penicillium* species [32]. Also, Some species of *Penicillium* of acid-tolerant nature produce extracellular proteases [33]. On the other hand, proteases from *Saccharomyces cerevisiae* were highly active at pH 7 [34].

3.1.2. Lipase enzymes

Thirty-five, out of thirty-nine isolates tested were found to be positive for lipase production on control medium (32 isolates) or media adjusted at pH 4 (18), pH 10 (14) or pH 13 (8) or medium supplemented with 10% NaCl (27). Of these, 22 isolates showed high lipase production on one or more of the test media. Our finding reveals that with increase in pH value from 4 up to 13, the number of lipase positive isolates decreased and this is in agreement with the results of Abdel-Fattah and Hammad [35] who stated that increasing the pH range till 6, increased the activity of the tested isolates for lipase production and as the pH increased over 6, lipase production declined.

Table 2. Protease enzyme production on control, acidic, alkaline and salted media by tested fungi.

Species	AUMC	Control			pH 4			pH 10			pH 13			10% NaCl		
		CD	DCZ	PI	CD	DCZ	PI	CD	DCZ	PI	CD	DCZ	PI	CD	DCZ	PI
Alternaria alternata	5665	8.25	8.55	1.036	9	0	0	5	0	0	1.5	0	0	3.05	0	0
	5666	1	1.2	1.2	1.2	1.4	1.16	1	0	0	0.1	0	0	0.2	0	0
	5667	8	0	0	8	0	0	6	0	0	1	0	0	4.8	0	0
	5668	1.4	0	0	1	0	0	1.1	0	0	0.6	0	0	0.5	0	0
Aspergillus flavus	5669	6	6.2	1.03	8.2	0	0	8.1	0	0	2	0	0	2.1	2.3	1.09
	5670	5.7	6	1.05	5.6	5.9	1.03	7	0	0	2.3	0	0	3.9	0	0
	5671	7	0	0	6.9	0	0	6	0	0	3	0	0	4	0	0
Aspergillus terreus	5672	6	0	0	5.2	0	0	6.1	0	0	1.8	0	0	3.35	0	0
	5673	3.5	4.7	1.34	3.7	4.5	1.2	3.9	0	0	0.2	0	0	2	3.7	1.85
	5674	5.8	6	1	3.6	0	0	4	0	0	1.5	0	0	3.5	0	0
	5675	1.5	2.5	1.73	0.85	0	0	3	0	0	1	0	0	2.05	0	0
	5676	4.1	4.3	1.48	3.9	0	0	4.6	0	0	1.35	0	0	2	0	0
	5677	3.6	4	1.1	3.4	3.6	1	6	6	0	1.1	0	0	3	3.2	1.06
Chaetomium globosum	5678	4	4.3	1.075	3.7	3.9	1.05	6	0	0	1	0	0	2	0	0
	5679	5.15	5.5	1.067	4.3	0	0	5.55	0	0	0.1	0	0	2.1	0	0
Cladosporium cladosporioides	5680	3.6	4.5	1.25	3.1	0	0	2	2.2	1.1	0.2	0	0	2.3	0	0
	5681	1.4	5	3.57	0.6	0	0	2	0	0	0.25	0	0	0.65	0	0
	5682	1.1	2.5	2.27	1.25	0	0	3.9	0	0	0.1	0	0	1.05	0	0
	5683	2	3	1.5	3.1	4	1.29	3.6	0	0	0.1	0	0	2.1	0	0
Emericella nidulans	5685	3.95	5.25	1.33	3.1	4.05	1.3	4	4.2	1.05	1.5	0	0	3	0	0
	5686	1.65	3.7	2.24	1.1	3.3	3	1.1	0	0	0.75	0	0	2.45	0	0
	5687	4.25	5.05	1.18	1.9	0	0	5.1	0	0	0.7	0	0	1.5	0	0
	5688	6.1	0	0	6.35	0	0	7	0	0	2.25	0	0	3.55	0	0
	5689	2.05	4.6	2.24	1.4	3.2	2.28	2.4	0	0	0.8	0	0	2	0	0
Fusarium solani	5690	2.9	3.7	1.27	4	0	0	5	0	0	1	0	0	0.35	0	0
	5691	8.5	0	0	8.5	0	0	8	0	0	4.3	0	0	2	0	0
	5692	4.75	5.35	1.12	5.5	0	0	3	0	0	2	0	0	3	0	0
Cochliobolus australiensis	5694	5.9	6.75	1.144	8.5	0	0	6	0	0	3	0	0	0.5	1.5	3
	5695	8	8.2	1.025	8.5	0	0	7	0	0	1.5	0	0	1.5	1.7	1.133
	5696	5.65	6.75	1.19	8.35	0	0	6	0	0	1.6	0	0	1.35	0	0
Myrothecium verrucaria	5697	1.4	3	2.14	2.35	0	0	3	0	0	2.5	0	0	0.65	0	0
	5698	2.95	3.6	1.22	4.3	0	0	5.2	0	0	2	0	0	0.75	0	0
	5699	2.95	3.6	1.22	1.8	3.8	2.11	4	0	0	1.35	0	0	0.85	1.58	1.35
Penicillium chrysogenum	5700	3.4	4.4	1.3	3	0	0	4	0	0	1.1	0	0	4.4	0	0
	5701	3.4	3.6	1.05	3.7	0	0	5.2	0	0	0.8	0	0	2.7	0	0
	5702	3.5	3.9	1.11	3	3.4	1.13	4.6	0	0	1	0	0	3	0	0
	5703	4	5.1	1.27	4	0	0	5.7	0	0	0.5	0	0	3.5	0	0
	5704	3.65	4.65	1.3	3.05	4.2	1.37	4	0	0	1	0	0	3.5	0	0
Positive isolates (32)			32			12			2			0			6	
Negative isolates (6)			6			26			36			38			32	

*Control medium, and control medium adjusted at pH 4, pH 10, pH 13 or supplemental with 10% NaCl (saline medium); CD = Colony diameter (in cm), DCZ = Diameter of clear zone resulted from enzyme-degrading abilities (in cm), PI = Protease index calculated after Ismail [17].

Table 3. Lipase and phosphatase enzymes production on acidic, alkaline and salt media by the tested fungi.

Species	AUMC	Lipase					Phosphatase			
		Control	pH 4	pH 10	pH 13	10% NaCl	Control	pH 4	pH 10	10% Nacl
Alternaria alternata	5665	1.5	0	1.2	0	0.4	++	+++	+++	+++
	5666	0	0	0	0	0	+	-	-	-
	5667	0.9	2	0	0	0.6	+++	++	+++	+++
	5668	0	0	0	0	0	++	+++	+++	+
Aspergillus flavus	5669	2.1	0	0	0	0.3	++	++	+++	+++
	5670	2	0	0	0	0.8	+	+++	+++	+++
	5671	0	0	0	0	0.8	-	+++	++	++
Aspergillus terreus	5672	2	0	1.5	0	1.4	+++	+++	++	+++
	5673	1.4	0	0	0	1.1	++	-	+	++
	5674	2.2	1.5	0	0	1.4	-	+++	++	+++
	5675	1.2	1.2	0	0.7	0.6	+	+++	+	+++
	5676	3	2.3	3	1.1	2.2	+++	+++	-	+++
	5677	2.5	3	3	1	2	+	++	+	++
Cheatomium globosum	5678	1.3	1.3	1	0	0	+	++	+	++
	5679	1.1	1	0	0	0	+++	+++	-	+++
Cladosporium cladosporioides	5680	1.7	2.5	1	0	1.3	++	++	++	+++
	5681	1.5	0	0	0	1.1	+++	+++	+	-
	5682	2	0.5	0	0	1.5	++	-	-	+
	5683	1	0	1.2	0	0	-	+++	++	+++
Emericella nidulans	5685	1.7	0	0	0	1.3	+++	+	+	+++
	5686	0	0	0	0	0	++	+	-	+
	5687	0	2	2	0	1.5	+++	+++	+++	+++
	5688	2.1	2	0	0.7	0.5	+++	+++	-	++
	5689	1.8	0	1.3	0	0.7	+	-	+++	+++
Fusarium solani	5690	3	2.2	0	0	0	-	++	-	+
	5691	1.6	0	1.4	0.6	0	+	++	++	++
	5692	0.3	0	2.5	2	0.7	++	-	+++	++
	5693	2	0	0	1	0.6	+++	-	-	+
Cochliobolus australiensis	5694	0	0	0	0	0	+++	++	+++	++
	5695	2	2	0	0	0.6	++	-	+++	+++
	5696	1.8	2	0	0	0	+++	++	+++	+++
Myrothecium verrucoria	5697	2	0.6	0	0	0	+	+	-	-
	5698	1.3	0	1	0	1	+	+	+++	++
	5699	1.6	2.2	0	0	0	+++	+	+++	-
Penicillium chrysogenum	5700	2.1	1.3	0	0	0.7	++	+++	+++	+++
	5701	1.8	0	1.2	0	1.5	+	+	+	+
	5702	2.4	0	0	0.5	1.5	+	+++	++	+
	5703	0	2	0	0	0.7	+++	++	+++	++
	5704	2	0	1.7	0	0.5	+++	+++	-	+++
Positive isolates (35)		32	18	14	8	27	35	32	29	35
Negative isolates (4)		7	21	25	31	12	4	7	10	4

*Legends as in Table 2; lipase production was expressed as depth of visible precipitate (in cm: high producers ≥ 2 cm, low producers < 2 cm); +++ = high phosphatase producer, ++ = moderate producer, + = weak producer, - = non-producer.

Table 4. The highly producing isolates for cellulose, protease, lipase and/or phosphatase enzymes on different screening media.

Species	AUMC	Protease	Lipase	Phosphatase
Alternaria alternate	5665			pH 4, pH 10, 10% NaCl
	5667		pH 4	C, pH 10, 10% NaCl
	5668			pH 4, pH 10
Aspergillus flavus	5669		C	pH 10, 10% NaCl
	5670		C	pH 4, pH 10, 10% NaCl
	5671			pH 4
Aspergillus terreus	5672		C	C, pH 4, 10% NaCl
	5674		C	pH 4, 10% NaCl
	5675			pH 4, 10% NaCl
	5676		C, pH 4, pH 10, 10% NaCl	C, pH 4, 10% NaCl
	5677		C, pH 4, pH 10, 10% NaCl	
Cheatomium globosum	5679			C, pH4, 10% NaCl
Cladosporium cladosporioides	5680		pH4	10% NaCl
	5681	C		C, pH4
	5682	C	C	
	5683	C		pH 4, 10% NaCl
Emericella nidulans	5685			C, 10% NaCl
	5686	C, pH 4		
	5687		pH 4, pH 10	C, pH 4, pH 10, 10% NaCl
	5688		C, pH 4	C, pH 4
	5689	C, pH 4		pH 10, 10% NaCl
Fusarium solani	5690		C, pH 4	
	5692		pH 10, pH 13	pH 10
	5693		C	C
Cochliobolus australiensis	5694	10% NaCl		C, pH 10
	5695		C, pH 4	pH 10, 10% NaCl
	5696		pH 4	C, pH 10, 10% NaCl
Myrothecium verrucaria	5697	C	C	
	5698			pH 10
	5699	pH 4	pH 4	C, pH 10
Penicillium chrysogenum	5700		C	pH 4, pH 10, 10% NaCl
	5702		C	pH 4
	5703		pH 4	C, pH 10
	5704		C	C, pH 4, 10% NaCl

Screening media: C=Control medium, or medium adjusted at pH 4, pH 10, pH 13 or supplemented with 10% NaCl.

Aspergillus terreus (2 isolates, AUMC 5676 and 5677) showed high production on all test media except that adjusted at pH 13 while 3 isolates showed high lipase activity on both control medium and medium adjusted at pH 4 and these belong to *Emericella nidulans* (1 isolate, AUMC 5688), *Fusarium solani* (1, AUMC. 5690) and *Cochliobolus austaliensis* (1, AUMC 5695).

It is worthy to note that 10 isolates gave also high production on only one medium: 10 isolates on control medium, *Aspergillus flavus* (2, AUMC 5669 and 5670), *Aspergillus terreus* (2, AUMC 5672 and 5674), *Cladosporum cladosporioides* (AUMC 5682), *F. solani* (AUMC 5693), *Myrothecium verrucaria* (AUMC 5697), *Pencillium chrysogenum* (3, AUMC 5700, 5702 and 5704) and 5 isolates on medium adjusted at pH 4: *Alternaia alternata* AUMC 5667, *Cladosporum cladosporioides* (AUMC

5680), *Cochliobolus australiensis* (AUMC 5696), *Myrothecium verrucaria* (AUMC 5699) and *Penicillium chrysogenum* (AUMC 5703).

Ogundero [36] stated that *A. fumigatus* and *A. nidulans* were able to degrade vegetable oils and triglycerides. Moreover, Pabai et al. [37] reported that lipases from *Aspergillus* strains are active between pH 4 and 7 and at temperatures between 40 and 50 °C. Savitha et al. [38] found only 4 (three of *Aspergillus* and one of *Mucor*) out of 32 isolates were positive for alkaliphilic lipase. Magan et al. [39] stated that species of *Aspergillus, Eurotium* and *Penicillium* from rape seed could produce lipases over a range of water activities (aw) at both 15 and 25 °C on tributyrin agar and the highest producing fungi were *Aspergillus candidus, Aspergillus versicolor*, *Penicillium expansum* and *Penicillium hordei.*

Abdel-Fattah and Hammad [35] screened nine fungal species for lipase production and found that all are capable of production with the most potent species were *A. niger* followed by *A. terreus, A. fumigatus* and *Fusarium moniliforme* and the least productive ones were *Penicillium chrysogenum* and *Alternaria alternata.* Also, Gharamah et al. [24] who tested 30 fungal isolates from human keratitis cases and found that *Aspergillus flavus* (3 isolates), *Cochliobolus spicifer, Fusarium solani* and *F. verticillioides* (one strain each) showed high lipolytic activity.

Strains of *Fusarium, Aspergillus, Neurospora*, *Cladosporium,* Mucorales were screened for lipase production and all produced lipase with different lipolytic activities and the most active were Mucorales, *F. oxysporum, Aspergillus niger, A. flavus* [40, 41]. Commercial lipolytic enzymes for use in many industrial applications are produced from many species such as *Penicillium chrysogenum* [42], *Humicola lanuginosa* [43] and species of *Aspergillus* [44, 45].

3.1.3. Phosphatase enzymes

Phosphatases hydrolyze esters and anhydrides of phosphoric acid. Vincent et al. [46] classified these enzymes as: i) alkaline phosphatases, ii) high molecular mass acid phosphatases, iii) low molecular mass acid phosphatase, iv) purple acid phosphatases and v) protein phosphatases. Alkaline phosphatases are used in enzyme-linked immunoabsorbent assays (ELISA), nonisotopic probing and blotting and sequencing systems [47]. Industrial application of acid phosphatase is limited, but phytase, a type of acid phosphatase is used in animal feed [48]. These enzymes catalyze the release of phosphate from phytic acid, the major phosphorus storage form in cereal grains, legumes and oilseeds. Pigs and poultry lack phytase and excrete large amounts of phosphorus in the form of phytate into the environment, resulting in pollution by this element [49].

One isolate related to *Emericella nidulans* (AUMC 5687) showed high phosphatase production on all media used. Four isolates showed high production on the media of control, 10% NaCl and that adjusted at pH 4, and these are related to *Aspergillus terreus* (2, AUMC 5672 and 5676), *Chaetomium globosum* (AUMC 5679), and *Penicillium chrysogenum* (AUMC 5704). On the other hand, some other isolates were high phosphatase producers on control, medium adjusted at pH 10 and 10% NaCl medium *Alternaria alternata* AUMC 5667*, Cochliobolus australiensis* AUMC 5696 and *Penicillium chrysogenum* AUMC 5700, on control and medium adjusted at pH 4 (*Cladosporium cladosporioides* AUMC 5681 and *Emericella nidulans* AUMC 5688)*,* on control and medium adjusted at pH 10 (*Cochliobolus australiensis* AUMC 5694, *Myrothecium verrucaria* AUMC 5699 and *Penicillium chrysogenum* AUMC 5703), on medium adjusted at pH 4, pH 10 and 10% NaCl medium: (*Alternaria alternata* AUMC 5665 and *Aspergillus flavus* AUMC 5670), *P. chrysogenum* AUMC 5700 and other isolates showed high production capabilities on only one test medium: on control: (*Fusarium solani*, AUMC 5693), on medium adjusted at pH 10: (*Fusarium solani* AUMC 5692 and *Myrothecium verrucaria* AUMC 5698) and 2 isolates on medium adjusted at pH4 (*Aspergillus flavus* AUMC 5671 and *Penicillium chrysogenum* AUMC 5702). Acid and/or alkaline phosphatases have been reported to occur in fungi, such as species of *Aspergillus* [13, 14], *Fusarium* [12], *Neurospora* [50], *Acrophialophora* and *Alternaria* [11]. Phosphatase activity has been previously described in *Aspergillus nidulans* [51]. *Aspergillus terreus, A. niger, E. nidulans, Penicillium oxalicum* and *Neurospora crassa* were the most active in produ-

cing acid phosphatase [52, 53], while *Emericella nidulans* and *Aspergillus caespitosus* were the most efficient in producing alkaline phosphatase [54] whereas *P. chrysogenum* and *P. brevicompactum* were efficient in producing both acid and alkaline phosphatases [55].

4. CONCLUSION

The current results revealed that 35 strains showed high production abilities of either protease (8), lipase (22) or phosphatase (29) on different screening media. Also, some interesting isolates are high producers of more than one enzyme: *E. nidulans* AUMC 5689 of phosphatase and proteases, *E. nidulans* AUMC 5687 of lipase and phosphateses. Moreover, there are some interesting isolates such as those possessing the high capabilities on two or more of the screening media: for proteases *Emericella nidulans* AUMC 5686 and 5689 on control and acidic media, for lipase *Aspergillus terreus* AUMC 5676 and 5677 on four screening media (control, acidic, alkaline and NaCl media), for phosphatase *Alternaria alternata* AUMC 5665 and *Aspergillus flavus* AUMC 5670 on acidic, alkaline and 10% NaCl media, *Alternaria alternata* AUMC 5668 on acidic and alkaline media. Therefore**,** the present investigation represents a pioneer study of enzyme production by most common fungi recovered from different sources of Wadi El-Natrun which is a hypersaline and alkaline environment in Egypt. The results can be a prelude for a prosperous application of fungal biotechnology in the production of economically important enzymes by these fungi existing under stress conditions.

AUTHORS' CONTRIBUTION

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

TRANSPARENCY DECLARATION

The authors declare that they have no conflict of interest. Assiut University Mycological Centre (AUMC), Assiut University is acknowledged for the facilities provided to perform this work.

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Research Article

Enzyme producing capabilities of some extremophilic fungal strains isolated from different habitats of Wadi El-Natrun, Egypt. Part 2: Cellulase, xylanase and pectinase

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ABSTRACT

Forty isolates of the most commonly encountered fungal species from different extreme habitats of Wadi El-Natrun region were tested for their capabilities of producing cellulase, xylanase and pectinase enzymes. Most of these isolates had the capabilities of producing cellulase (95% of the isolates), but with different degrees; however only 3 out of 20 isolates tested were xylanolytic (15%) and one out of 39 was pectinolytic. Eleven strains showed high producing abilities of cellulase and only 2 of xylanase on different screening media. Of the high cellulase producers: some produced cellulase on one medium only e.g. the control medium (*Alternaria alternata*), medium adjusted at pH 4 (*Aspergillus terreus*, *Cladosporium cladosporioides*) or medium supplemented with 10% NaCl (*Emericella nidulans*, *Fusarium solani*, *Cochliobolus australiensis*). Others produced cellulase on the control, 10% NaCl and the acidic media (*Emericella nidulans*) or on control, 10% NaCl and alkaline media (*Cladosporium cladosporioides*). Some isolates produced cellulase on both the control, acidic, alkaline and NaCl media (*Emericella nidulans*). The highly xylanolytic activities were demonstrated only by *Emericella nidulan* isolates. Finally, the present results reveal some very interesting isolates having the high capabilities for producing more than one enzyme on more than one of the screening media.

Keywords: Extremophiles; Wadi El-Natrun; Enzymes; Cellulases; Xylanases; Pectinases.

1. INTRODUCTION

Extremophilic microorganisms offer a multitude of actual or potential applications in various fields of biotechnology. Not only do many of them produce compounds of industrial interest such as enzymes, but they also possess useful physiological properties which can facilitate their exploitation for commercial purposes.

Alkaliphilic enzymes have attracted great attention as they are now available to industry such

as proteases, cellulases, lipases, pullulanases [1, 2]. Detergent enzymes account for approximately 60% of total worldwide enzyme production. The main reason for selecting enzymes from alkaliphiles is their long term stability in detergent products, energy cost saving by lowering the washing temperatures, quicker and more reliable product, reduced effluent problems during the process, and stability in the presence of detergent additives such as bleach activators, softeners, bleaches and perfumes. Due to the unusual properties of these enzymes they are expected to fill the gap between biological and chemical processes and have been greatly employed in laundry detergents.

Cellulolytic enzymes play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by cellulolytic fungi, bacteria, actinomycetes and protozoa. In industry, these enzymes have found novel applications in the production of fermentable sugars and ethanol [3, 4], detergents and other chemicals [5]. They have been used in the pulp and paper industry, e. g., in the textile industry [6, 7], animal feed [8], and even in the food industry [9]. Enzymatic hydrolysis of cellulose requires a consortium of enzymes, including endo-β-1,4-glucanase (E.C.3.2.1.4), exo-β-1,4-glucanase (E.C.3.2.1.91) and β-glucosidase (E.C.3.2.1.21). There is a considerable interest in cellulases as biocatalysts. However, most of the well-studied cellulases show optimum activity at slightly acidic pH ranges (5.0-6.0) and at temperatures between 40 and 70ºC [10]. However, there is a very little information regarding the extremophilic cellulases. Extremophilic cellulases could be obtained by isolating extremophilic microorganisms where such unique properties of extremophilic cellulases may exist [11].

Xylanases are responsible for hydrolysis of xylan, a major hemicellulose of plant cell wall (second most abundant). Xylanases (E.C. 3.2.1.8) have potential applications in the food, feed, beverage and textile industries and in waste treatment. For example, in the food and beverage industry, the major uses of this enzyme are biopulping, biobleaching, clarifying and liquefying fruit and vegetable juices and wines and for extracting coffee and plant oils [12-14]. The amount of xylanases produced by fungi is comparatively higher than those from yeasts or bacteria [15].

Among the cell-wall degrading enzymes (WDEs), pectinases such as polygalacturonase (PG), pectin layase (PNL) and pectate layse (PEL) have been widely studied. Pectinases, which degrade the pectic component found in the middle lamella and primary cell wall of most plants, have been postulated to be involved in penetration and colonization of plants by phytopathogens. Various genera of microorganisms can produce pectinases of potential applications [16].

This work has been designed to highlight on the capabilities of the most common fungi isolated from the hypersaline habitat environment of Wadi El-Natrun of producing cellulase, pectinase and xylanase under acidic, alkaline or saline conditions.

2. MATERIALS AND METHODS

2.1. Fungal isolates

Forty fungal strains representing 10 species commonly encountered from different sources (soil, mud, salt crusts, water and air) of hypersaline habitats in 8 lakes in Wadi El-Natrun region (refer to Gouda [17]), the Eastern desert of Egypt (Table 1) were screened for their abilities to produce cellulase, xylanase and pectinase enzymes on solid media.

2.2. Cellulase production

Cellulase production was tested as described by Eggins and Pugh [18]. The pH was adjusted to 7 using acetate buffer and this medium was used as a control medium. After autoclaving, the medium was dispensed into plates that, after solidification, were inoculated with the tested fungal isolates and incubated at 28°C for 7 days. After incubation, cultures were flooded with a solution of chloro-iodide of zinc ($ZnCl_2$ 1% and iodine solution 1% in equal portions). Clear zone around colonies against deep blue colour at the periphery indicates hydrolysis of cellulose by the releasing C1 cellulase enzyme (exo-1,4 -β-glucanase). The diameter of the colony as well as the clear zone around the colony was measured (in cm). Cellulase index (CI) was calculated according to Ismail [19].

Table 1. Sources, lakes of Wadi El-Natrun and isolation media from which fungal species tested were recovered, and their deposition numbers at AUMC Culture Collection.

Species	AUMC	Source	Lake	Isolation medium
Alternaria alternata	5665	Soil	Al Gaar	Cz (pH 13)
	5666	Soil	El Zugm	Cz (10% NaCl)
	5667	Mud	Al Gaar	Cz (40% S)
	5668	Water	Khadra	Cz (pH 5)
Aspergillus flavus	5669	Soil	Umm Risha	Cz (pH 13)
	5670	Soil	Khadra	Cz (pH 4)
	5671	Soil	Hamra	Cz (40% S)
Aspergillus terreus	5672	Air	Rosetta	Cz
	5673	Mud	Khadra	Cz (pH 3)
	5674	Soil	El Zugm	Cz (pH 3)
	5675	Soil	Hamra	Cz (pH 3)
	5676	Water	Rosetta	Cz (pH 3)
	5677	Salts	Umm Risha	Cz (pH 13)
Chaetomium globosum	5678	Soil	El Zugm	Cz (pH 5)
	5679	Soil	Al Beida	Cz (pH 10)
Cladosporum cladosporioides	5680	Soil	Umm Risha	Cz (pH 13)
	5681	Soil	Umm Risha	Cz (pH 4)
	5682	Soil	Umm Risha	Cz (pH 13)
	5683	Soil	-	Cz (pH 4)
	5684	Soil	Al Gaar	Cz (40% S)
Cochliobolus australiensis	5694	Soil	Hamra	Cz (pH 4)
	5695	Soil	Hamra	Cz (pH 13)
	5696	Salts	Hamra	Cz (pH 13)
Emericella nidulans	5685	Soil	El Zugm	Cz (10% NaCl)
	5686	Soil	Umm Risha	Cz (pH 4)
	5687	Soil	Al Gaar	Cz (pH 13)
	5688	Soil	Al Beida	Cz (pH 4)
	5689	Water	Al Gaar	Cz (pH 13)
Fusarium solani	5690	Soil	Al Bida	Cz (pH 4)
	5691	Soil	Hamra	Cz (pH 13)
	5692	Soil	Umm Risha	Cz (10% NaCl)
	5693	Salts	Khadra	Cz (40% sucrose)
Myrothecium verrucaria	5697	Soil	Hamra	Cz (pH 4)
	5698	Soil	Khadra	CZ (pH 13)
	5699	Salts	Hamra	Cz (pH 10)
Pencillium chrysogenum	5700	Soil	AlGaar	Cz (pH 4)
	5701	Soil	Al Beida	Cz (pH 13)
	5702	Soil	Umm Risha	Cz (40% sucrose)
	5703	Water	-	Cz (pH 4)
	5704	Mud	Khadra	Cz (pH 5)

AUMC = Assiut University Mycological Center

2.3. Pectinase production

The method was carried out as described by Hankin et al. [20]. The two portions of the medium were adjusted to pH 7.0. After autoclaving at 121°C for 15 minutes, the two portions were mixed thoroughly. The medium was dispensed into 9 cm Petri dishes (20 ml per plate). The agar plates were then inoculated with different fungal isolates and allowed to grow at 28 °C for 7 days. Plates were flooded with 10% lead acetate and 6 N HCl. Appearance of clear zone around and beneath the colonies indicates the production of pectinase enzyme.

2.4. Xylanase production

Modified xylan agar medium of Nakamura et al. [21] with the following composition (g/l): xylan 5.0, peptone 5.0, yeast extract 5.0, K_2HPO_4 1.0, $MgSO_4 \cdot 7H_2O$ 0.2 and agar 20.0 was employed. Xylan agar plates were inoculated. After 7 days of incubation at 28 °C, areas of clear zones around the colonies were measured and the enzyme index was calculated.

To assess the effect of acidity, alkalinity or salinity on the production of these extracellular enzymes, the control medium was adjusted at pH 4, pH 10 and pH 13 or supplemental with 10% NaCl (saline medium). The plates were then inoculated and incubated at 28°C for 7 days.

3. RESULTS AND DISCUSSION

3.1. Enzymes production by most commonly encountered fungi from Wadi El-Natrun

Forty isolates related to 10 species of the most commonly encountered fungi from the different sources in 8 lakes of Wadi El-Natrun (Table 1) were screened for their abilities of producing cellulase, pectinase and xylanase enzymes on agar media adjusted at different pHs or supplemented with 10% NaCl.

3.1.1. Cellulase enzymes

Thirty-eight out of forty isolates tested for their capability of producing cellulase enzyme, showed positive results on control medium (25 isolates), media adjusted to pH 4 (23), pH 10 (30) and pH 13 (1) or medium supplemented with 10% NaCl (23).

From the results presented in Table 2, it is noted that some fungal isolates could produce cellulase on one medium but not on the others. Also some were of high production ability on one medium than on the other media. However, only two isolates belonging to *Fusarium solani* and *Cochliobolus australiensis* lacked the ability of producing cellulase on all media used.

Of the positive isolates, only 11 showed high production ability (CI ≥ 2) on one or more of the screening media. Two isolates of *Emericella nidulans* (AUMC 5686 and 5689) showed high production of cellulase on all media except that adjusted at pH 13 while the other 2 isolates related to *Emericella nidulans* (AUMC 5687) and *Cladosporum cladosporioides* (AUMC 5684) gave high yield on control, pH 4 and 10% NaCl media and control, pH 10 and 10% NaCl respectively. Seven isolates gave high cellulase production on only one medium and these are: *Alternaria alternata* AUMC 5666 (on control medium), *Aspergillus terreus* AUMC 5675 and *Cladosporum cladosporioides* AUMC 5681 and 5683 (on medium adjusted at pH 4), *Emericella nidulans* AUMC 5685, *Fusarium solani* AUMC 5691 and *Cochliobolus australiensis* AUMC 5694 (on medium supplemented with 10% NaCl).

The other 27 positive fungal strains for cellulase were either moderate producers (CI = 1.5-1.99) or low producers (CI ≥ 1.5). Our results also reveal that cellulase production was proved positive on pH 10 medium by 5 isolates (*Alternaria alternata*, AUMC 5665), *Aspergillus terreus* AUMC 5677, *Cladosporum cladosporioides* AUMC 5682, *Fusarium solani* AUMC 5692, *Myrothecium verrucaria* AUMC 5697), by only one isolate on pH 4 medium: (*Cladosporum cladosporioides* AUMC 5683) and by 3 isolates on 10% NaCl: (*Emericella nidulans* AUMC 5685), *Fusarium solani* AUMC 5690 and *Cochliobolus austaliensis* AUMC 5694). In this respect, the role of many fungal species e.g. *Trichoderma hamatum, T. harzianum and T. longibrachiatum, T. reesei, T. viride, T. koningii, Penicillium pinophilum, Phanerochaete chrysosporium*, *Fusarium solani, F. oxysporum, Talaromyces emersonii, Aspergillus niger*, *Rhizopus oryzae* and

species of *Acremonium, Chaetomium, Cladosporium, Geotrichum, Myrothecium* and *Paecilomyces* in the cellulose degradation process in various environments has been well-documented [22-25].

In agreement with our results where 6 fungal isolates showed high production abilities on medium adjusted to pH 4 compared to 3 isolates on medium adjusted to pH 10, the statement that pH range (4-6) is the most favourable hydrogen ion concentration for cellulolytic enzyme synthesis in several fungi such as *Aspergillus terreus* [26], *Trichoderma reesei* [27], and *T. longibrachiatum* [28].

Abdel-Hafez et al. [29] found that the strong cellulolytic isolates include *Aspergillus flavus, A. flavus* var. *columnaris, A. fumigatus, A. nidulans, A. niger, A. ochraceus, A. sydowii, A. tamarii, A. terreus, A. ustus, Botryotrichum piluliferum, Chaetomium globosum, Fusarium oxysporum, Myrothecium verrucaria, Paecilomyces variotii, Penicillium cyclopium, Syncephalastrum racemosum, Trichoderma koningii* and *Ulocladium consortiale*. Moreover, all 85 isolates from the tidal mud flats of Kuwait were able to produce cellulase enzymes [30]. Also, Dutta et al. [31] found that alkali-tolerant and thermostable cellulases were produced from an extremophilic filamentous fungus *Penicillium citrinum.*

3.1.2. Xylanase enzymes

Capabilities of 20 isolates of xylanase production were tested on the control medium supplemented with xylan. Of these isolates, only 3 were xylanalytic and all were related to *Emericella nidulans.* Two of these isolates gave high production (AUMC 5685 and 5688) (Table 3). However, a large proportion (98 out of 105 isolates) tested by Abdel-Sater and El-Said [32] could produce clear zones as a result of xylan utilization. Of these isolates, 29 showed strong xylanolytic activity (*A. flavus, A. niger, F. oxysporum, T. harzianum* and *T. viride*), 47 achieved moderate activity (*A. terreus, A. ustus, A. ochraceus*, *E. nidulans, P. variotii, P. chrysogenum, P. corylophilum* and *P. oxalicum*) and the remaining 22 positive isolates were found to be weak producers. In harmony with our results, *A. nidulans* was also reported as xylanase producer [33-35]. Also, it was stated that, when *A. nidulans* is grown on xylan as sole carbon source it produces a xylanolytic complex composed of at least three different endo-β-(1,4)-xylanases and one β-xylosidase [32]. Two of these enzymes are of interest in wine and bread production respectively [36, 37]. Several species of *Aspergillus* (*A. niger, A. ochraceus, A. oryzae, A. tamarii,* and *A. fumigatus*) have been also reported to produce xylanases [38]. Xylanases in acidic and alkaline media have been also been reported by many species of *Aspergillus, Penicillium, Aureobasidium*, *Talaromyces, Thermomyces* and *Trichoderma* [39-41]. Raghukumar et al. [42] found that several fungal isolates obtained from marine habitat showed alkaline xylanase activity, however the best xylanase-producing isolates were not obligate marine but facultative marine fungi (*A. niger* and *A. ustus*).

3.1.3. Pectinase enzymes

Out of 39 isolates tested for their abilities of producing pectinase on media adjusted at different pHs or supplemented with 10% NaCl, only one isolate (*Emericella nidulans* AUMC 5687) could produce pectinase on control medium (pH 7). Joshi et al. [16] stated that pectinase is produced by several fungi including *Aspergillus* sp., *Botrytis cinerea, Fusarium moniliforme, Rhizoctonia solani, Rhizopus stolonifer, Trichoderma* sp., *Neurospora crassa*, but *Aspergillus* is the major source. Boccas et al. [43] found that out of 248 fungal isolates from coffee plants and the soil samples from coffee plantations, 119 were able to produce pectolytic enzyme with 13 being high producers. All of these were related to either *Penicillium* (6 isolates) or *Aspergillus niger* (7 isolates).

4. CONCLUSION

The current results revealed that 11 strains showed high production abilities of cellulase and only 2 of xylanase on different screening media. There are some interesting isolates such as those possessing the high cellulase-producing capabilities on two or more of the screening media: *Emericella nidulans* AUMC 5686 and 5689, on control, acid, alkaline and 10% NaCl media and *E. nidulans* AUMC 5687 on the control, acidic and 10% NaCl media and *Cladosporium cladosporioides* AUMC 5684 on the control, alkaline and 10% NaCl media.

Table 2. Cellulase production on control, acidic, alkaline and salted media by tested fungi.

Species	AUMC	Control			pH 4			pH 10			pH 13			10% NaCl		
		CD	DCZ	CI	CD	DCZ	CI	CD	DCZ	CI	CD	DCZ	CI	CD	DCZ	CI
Alternaria alternata	5665	7.1	0	0	6.75	0	0	3.45	4.25	1.23	0.2	0	0	2.25	0	0
	5666	0.2	0.6	3	0.2	0	0	0.2	0	0	0.2	0	0	0.2	0	0
	5667	0.1	0	0	0.1	0	0	2.45	3.35	1.36	0.1	0	0	1.8	2.7	1.5
	5668	2.2	3	1.36	5	6.2	1.24	0.1	0	0	0.1	0	0	0.1	0	0
Aspergillus flavus	5669	7.1	8.05	1.133	5.5	7.6	1.38	6.1	7	1.14	0.1	0	0	6.45	7.55	1.17
	5670	6.2	7.5	1.2	6.8	7.5	1.1	5	5.6	1.12	NS	NS	NS	2.9	3.4	1.17
	5671	7.15	0	0	6	7.4	1.23	5.6	7	1.25	0.1	0	0	3.1	0	0
Aspergillus terreus	5672	4.5	6.5	1.45	3.5	4.8	1.37	3.55	5.6	1.58	0.2	0	0	2.2	3.2	1.45
	5673	3.5	4.7	1.34	3.7	4.5	1.21	4	4.4	1.1	0.2	0	0	2	3.7	1.85
	5674	5.5	7.5	1.36	6	7.4	1.23	5.2	6	1.15	0.1	0	0	2.5	3.5	1.4
	5675	3.5	4.9	1.4	0.9	2.2	2.45	3.2	3.8	1.19	0.1	0	0	1.85	0	0
	5676	3.5	5.1	1.46	2.2	3.6	1.64	5	8.5	1.7	0.2	0	0	1.7	2.7	1.59
	5677	4	0	0	1.3	0	0	4.6	5.65	1.22	0.1	0	0	1.1	0	0
Chaetomium globosum	5678	5	0	0	3	4.1	1.36	4.15	0	0	0.1	0	0	3	0	0
	5679	4.9	5.1	1.04	4.5	0	0	4	4.6	1.15	0.1	0	0	4.05	0	0
Cladosporium cladosporioides	5680	2.6	0	0	3	3.85	1.28	3	3.6	1.2	0.1	0	0	1.5	1.7	1.13
	5681	1.1	2.15	1.95	0.6	2.2	3.67	2.2	2.5	1.13	0.1	0	0	2.1	0	0
	5682	2	0	0	3.15	0	0	4	5.9	1.47	0.1	0	0	2	0	0
	5683	0.85	0	0	1.1	2.6	2.36	3	0	0	0.1	0	0	0.2	0	0
	5684	1.4	3.2	2.29	7.8	0	0	0.85	3.2	3.76	0.2	0	0	0.4	1.5	3.75
Emericella nidulans	5685	1.8	0	0	2	0	0	2.4	0	0	0.2	0	0	1	2	2
	5686	2.15	4.35	2.02	1.5	4.5	3	2.1	6.15	2.93	0.1	0	0	0.7	2.1	3
	5687	1.25	4.65	3.72	1.2	3.6	3	2.2	3.45	1.56	0.1	0	0	0.1	1.3	13
	5688	5	7.4	1.48	5.5	6.5	1.18	5	6.1	1.22	0.1	0	0	1.5	0	0
	5689	1.15	4.35	3.78	1.5	4.5	3	1.5	6.15	4.1	0.1	0	0	1	3.5	3.5
Fusarium solani	5690	0.3	0	0	0.2	0	0	1	0	0	0.3	0	0	1.1	1.4	1.27
	5691	3	3.7	1.23	3.5	3.8	1.08	3.7	3.95	1.06	0.1	0	0	0.1	0.3	3
	5692	1.1	0	0	0.25	0	0	2.2	2.8	1.27	0.1	0	0	2	0	0
	5693	6	0	0	5.75	0	0	5.9	0	0	0.1	0	0	2.2	0	0
Cochliobolus australiensis	5694	5.5	0	0	5.6	0	0	5.8	0	0	0.1	0	0	0.5	2.3	4.6
	5695	6.15	0	0	5.55	0	0	7.5	0	0	0.1	0	0	1.25	0	0
	5696	5.65	6.75	1.18	8.35	0	0	1.6	0	0	3.35	5	1.49	1.35	2.2	1.63
Myrothecium verrucaria	5697	3.7	0	0	2.5	0	0	3.2	6.25	1.95	0.8	0	0	0.1	0	0
	5698	3	4.5	1.5	3	0	0	4	6	1.5	0.2	0	0	1.1	0	0
	5699	2.6	3.6	1.38	3.4	0	0	3	3.6	1.2	0.1	0	0	0.1	0.2	2
Penicillium chrysogenum	5700	3.5	5.9	1.68	3.8	5.6	1.47	3.5	4.7	1.34	0.1	0	0	2.2	3.2	1.45
	5701	4	5.4	1.35	3.4	4.4	1.29	4	5.2	1.3	0.1	0	0	2.5	3.5	1.4
	5702	4	5.8	1.45	3.7	5.1	1.38	3.7	5	1.35	0.2	0	0	2.9	3.5	1.2
	5703	3.7	5.7	1.54	3.6	5	1.4	4.2	5.2	1.23	0.1	0	0	2.2	2.8	1.27
	5704	3	5.2	1.74	3.5	4.9	1.4	3.45	5.35	1.55	0.1	0	0	2.75	3.75	1.36
Positive isolates (38)			25			23			30			1			23	
Negative isolates (2)			15			17			10			39			17	

*Control medium, and control medium adjusted at pH 4, pH 10, pH 13 or supplemental with 10% NaCl (saline medium), CD = Colony diameter (in cm), DCZ = Diameter of clear zone resulted from enzyme-degrading abilities (in cm), CI = Cellulase index calculated after Ismail [19], NS = Not screened.

Table 3. The highly producing strains for cellulase and/or xylanase enzymes on different screening media.

Species	AUMC	Cellulase	Xylanase
Alternaria alternata	5666	C	
Aspergillus terreus	5675	pH 4	
Cladosporium cladosporioides	5681	pH 4	
	5683	pH 4	
	5684	C, pH 10, 10% NaCl	
Emericella nidulans	5685	10% NaCl	C
	5686	C, pH 4, pH 10, 10% NaCl	
	5687	C, pH 4, 10% NaCl	
	5688		C
	5689	C, pH 4, pH 10, 10% NaCl	
Fusarium solani	5691	10% NaCl	
Cochliobolus australiensis	5694	10% NaCl	

Screening media: C = control medium, or medium adjusted at pH 4, pH 10 or supplemented with 10% NaCl.

Therefore, the current investigation represents a pioneer study of enzyme production by most common fungi recovered from different sources of Wadi El-Natrun which is a hypersaline and alkaline environment in Egypt. The results can be a prelude for a prosperous application of fungal biotechnology in the production of economically important enzymes by these fungi existing under stress conditions.

AUTHORS' CONTRIBUTION

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

TRANSPARENCY DECLARATION

The authors declare that they have no conflict of interest. Assiut University Mycological Centre (AUMC), Assiut University is acknowledged for the facilities provided to perform this work.

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Research Article

Nutritive values of some edible forest tree seeds in Makurdi-Benue, Nigeria

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ABSTRACT

The seeds of *Adansonia digitata*, *Afzelia africana*, *Moringa oleifera*, *Prosopis africana* and *Terminalia catappa* in Makurdi, Nigeria were analyzed in water-soluble forms to determine their minerals (calcium, phosphorus, potassium, iron, magnesium and iodine) and vitamin (A, C and E) compositions in order to investigate the possible of using seeds for human consumption due to malnutrition in Nigeria. Atomic Absorption Spectrophotometer and Titration Method showed that the seeds were good source of minerals. Potassium (7233.50 ± 3.50 mg/100 g) was the highest from *Terminalia catappa* seed with the lowest (502.00 ± 1.00 mg/100 g) in *Moringa oleifera* seed; calcium (789.61 ± 0.01 mg/100 g) in *Terminalia catappa* seeds while 81.08 ± 0.05 mg/100 g was the lowest from *Adansonia digitata* seeds; magnesium (687.58 ± 0.01 mg/100 g) as the highest in *Terminalia catappa* seed while 3.59 ± 0.01 as the lowest in *Afzelia africana* seed and iron was determined with the highest content of 422.00 ± 1.00 mg/100 g in *Terminalia catappa* seeds while 3.49 ± 0.03 as the lowest in *Afzelia africana* seeds. Vitamin A and C evaluated from the seeds were found to be higher in *Adansonia digitata* seeds while *Prosopis africana* and *Terminalia catappa* seeds were low; vitamin E was high (2801.50 ± 1.50 mg/100 g) in *Moringa oleifera* seeds while *Afzelia africana* seeds contain the lowest vitamin with 0.74 ± 0.01 mg/100 g. The seeds are very good sources of mineral and vitamin nutrients for human body as useful dietary supplements and good health. There is the need to assess the available of the tree seeds and possible present of anti-nutrients.

Keywords: Dietary; Edible, Forest; Mineral; Vitamin; Seed.

1. INTRODUCTION

The negative effects of malnutrition in the developing countries (Nigeria) cannot be over emphasized; this consequence is mostly found in the rural communities and among the poor people dwelling in Urban, cities or towns. This can be attributed to mere ignorance of food trees around them. In the rural communities in Nigeria (Africa), most traditional diets often lack variety and consist of mainly staple foods with small amounts of other foods depending on season and availability; the staple foods provide the calories but are poor in

other nutrients like minerals, protein and vitamins [1]. The diets of Nigerians are mostly carbohydrate with little of protein, vitamins and minerals which contributes to low nutrition security of the rural dwellers.

Therefore, alternative nutrient sources (minerals and vitamins) are a major need in Nigeria and Africa at large. There are some forest tree seeds species that are high in minerals and vitamins and can be consume as food which could possibly reduced critical food shortage among the developing countries, if given adequate sensitization and research attention. Underutilized forest tree fruits could be used to meet world food security demands when properly processed for consumption; the trees food seeds rich in nutrients (such as minerals and vitamins) particularly legumes could effectively reduce the level of malnutrition [2]. These wild seeds could be good nutrient sources if integrated fully into human and animal nutrition.

In this regard, attention should be drawn to cheap sources of minerals and vitamins like *Terminalia catappa, Adansonia digitata, Moringa oleifera, Afzelia africana*, *Prosopis africana*, et cetera. Among other leguminous plants, some forest seeds have been proven to be edible, available, and affordable and contain most of the nutritional requirements in large proportion [3]. Some forest tree seeds or fruits are rich in minerals and vitamins particularly those of leguminous plants, which could reduce the level of malnutrition in most developing countries of Africa.

Forest tree seeds or fruits such as *Afzelia africana* seed is a better source of minerals and protein. The value of calcium (208 mg/kg) in afzelia seed was found to be higher than those of quinoa (86 mg/kg), African nutmeg (203.7 mg/kg) [4]. calcium accounts for about 75% of the weight of the mineral element present in the body; the deficiency of calcium can also affect the life of newly born baby where the body and the bone is very soft and this becomes hardened as the calcium intake increases, so the diet must supply a high amount of calcium for proper functions of the body; calcium is the principal contributor to bone formation [5]. This suggests that *Afzelia africana* seed is good as human food.

Iron is nutritionally important in human body system; it is highly needed during blood formation; the value of iron (10.8 mg/kg) is adequate for blood formulation. This value of iron in the sample was lower than those of spannish green olives (14.8 mg/kg) [6]; cat fish (15.5 mg/kg) but comparable with that of snake fish (10.6 mg/kg) [7, 8].

Baobab (*Adansonia digitata*) has nutritional properties that are important in body functioning [9]. According to Adubiaro et al. [10], the seed has very high content of protein 48.3% and moderately high content of carbohydrate 21.9%; the most abundant mineral in the seed flour is K 536 mg/100 g followed by Mg 352 mg/100 g. The least abundant are Zn (3.40 mg/100 g), Cu (4.26 mg/100 g) and Mn (5.23 mg/100 g). The levels of Na/K, Ca/P and Ca/Mg in the seed flour are desirable when compared with the recommended values. Other nutrients such as the crude fat in the seed shows that it will be a good source of fat which provides a major portion of man's energy; the crude protein of this seed 48.3% is higher than some tropical tree seeds, breadnut 19.25% [11], *Bombcopsis glabra* 16.56% [12] and locust bean 24.1% [13]. Adubiaro et al. [10] reported that the high protein content of baobab show that it would be useful as alternative source of protein in livestock feeding and also in man, especially in Nigeria where the scarcity and the cost of the conventionally used plant protein sources have nearly paralyzed most of the industries.

Terminalia catappa seed is edible and highly cherished by children. It is also used by many rural dwellers in southern Nigeria to fortify the local complimentary foods, which are usually low in protein, ailments [14]. The mineral element composition (mg/100 g) of the seed is phosphorus (10.0), sodium (5.0), potassium (350), iron (375), magnesium (26.4), and calcium (36.1) [15].

Prosopis africana seed as indehiscent pods are palatable to man and animals [16]. The seeds are fermented into a traditional condiment, okpehe which can serve as a low-cost source of nutrients (protein) especially for the rural people.

2. MATERIAL AND METHODS

2.1. Study area

The experiment was carried out in the Food Biochemistry laboratory of the Department of Food

Science Technology, University of Agriculture Makurdi (UAM); located between Longitude 8° 21' to 9° E and Latitude 7° 21' and 8° N in Benue State within the southern guinea savanna ecological zone [17, 18]. One important feature is the presence of the River Benue which divides the town into the Northern and Southern parts. The climate of the area is tropical sub-humid climate with high temperatures and high humidity; the average maximum and minimum daily temperature of 35°C and 21°C in wet season, as well as 37°C and 16°C in dry season [19]. Benue state has boundaries to the south with Enugu and Cross river States, to the East with Taraba state, North with Nasarawa state and West with Kogi state. The climate is characterized by two distinct seasons: rainy and dry seasons. The mean annual rainfall value is between 1200 mm to 1500 mm. The vegetation of the area has been described as Southern guinea savanna [20]. The major occupations of the people include; farming, civil service, trading and hunting; the major tribes found are Tivs, Idoma, Igede, etc.

2.2. Materials and methods

The minerals and vitamins of the selected seeds were determined using the standard procedures of the Association of Official Analytical Chemists [21], in the Food Biochemistry Laboratory in the Department of Food Science Technology, University of Agriculture Makurdi. Analytical reagent nitric acid, hydrochloric acid and standard iron solution were used.

2.3. Collection of seed samples

Matured seeds were obtained from *Wadata* Market in Makurdi and stored in a polythene bags to prevent moisture loss and possible contamination during transportation to the laboratory. The samples were identified at the Forestry general Laboratory, University of Agriculture Makurdi.

2.4. Sample preparation

The samples were prepared and used for minerals and vitamins analysis by washing with distilled water to removed all impurities and dried at room temperature to remove residual moisture; then placed in an oven and oven dried at 55°C for 24h. The dried seeds were grounded into powder using a mortar and pestle and then sieved through 20 inch mesh sieves and analyzed using the standard procedures of the Association of Official Analytical Chemists [22]. All analysis was done in triplicates.

2.5. Determination of mineral and vitamin contents

The mineral contents of samples were determined using Atomic Absorption Spectrophotometer, according to the procedure described by [21, 22]. One gram of each samples was digested by wet-digestion method using combination of percholic acid, nitric acid and sulphuric acid [23]; mixture in the ratio 8:2:1, respectively, and filtered. The digest so obtained was used for the various analyses. De-ionized water was used to avoid interferences from the minerals that may be contained in the water. The filtrate was made up to mark in a 5 ml volumetric flask. The filtered solution was loaded to an Atomic Absorption Spectrophotometer. The standard curve for each mineral was prepared from known standards and the mineral value of samples estimated against that of the standard curve. All the mineral elements were determined on spectrophotometer [21] using the filter corresponding to each mineral element. Vitamins A, C and E were analyzed in juices formed on 5 g sample and determined by filtration method described by [23] using Whatman filter paper. The filtrate was titrated hot (80-90°C) that persists for 30 seconds (using Titration method).

2.6. Statistical analysis of data

Data generated from all analyses were subjected to statistical analysis using SPSS version 21.0 through one way analysis of variance (ANOVA). Significant difference between samples was tested at P = 0.05 using LSD.

3. RESULTS

Composition of mineral elements and vitamins in selected edible forest seeds are presented in Tables 1 and 2.

Table 1. Mineral elements composition of some edible forest seeds.

Tree species	Minerals (mg/100 g)					
	Ca	K	Fe	P	Mg	I
Afzelia africana	322.00±1.00d	1351.00 ± 1.00b	3.49 ± 0.03a	9.48 ± 0.01a	3.59 ± 0.01a	134.04 ± 0.03e
Adansonia digitata	81.08 ± 0.05a	1381.70 ± 1.35c	6.94 ± 0.02b	112.55 ±0.35c	70.29 ± 0.03b	84.94 ± 0.01c
Moringa oleifera	245.73 ± 0.13c	502.00 ± 1.00a	37.08 ± 0.05d	468.00 ± 1.00e	218.20 ± 0.10c	59.05 ± 0.03a
Prosopis africana	101.60 ± 0.26b	1369.30 ±1.00d	26.06 ± 0.04c	201.50 ±0.20d	438.50 ± 0.20d	102.50 ± 0.20d
Terminalia catappa	789.61 ± 0.01e	7233.50 ± 3.50e	422.00 ±1.00e	15.12 ± 0.02b	687.58 ± 0.01e	82.44 ± 0.01e

Means on the same column with different superscript differ significantly (p<0.05).
Key: Ca - calcium; K - potassium; Fe - iron; P - phosphorus; Mg - magnesium; I - iodine.

Table 2. Vitamin composition in selected edible forest seeds.

Tree species	Vitamins (mg/100 g)		
	A	C	E
Afzelia africana	4.56 ± 0.03b	138.72 ± 0.52c	0.74 ± 0.01a
Adansonia digitata	322.50 ± 1.50d	522.50 ± 1.50e	429.00 ± 2.00d
Moringa oleifera	6.66 ± 0.01c	214.50 ± 6.50d	2801.50 ± 1.50c
Prosopis africana	0.07 ± 0.06a	46.90 ± 1.60b	51.15 ± 0.15c
Terminalia catappa	0.91 ± 0.00a	0.05 ± 0.00a	19.66 ± 0.2b

Means on the same column with different superscript differ significantly (p<0.05).

4. DISCUSSION

Minerals are important in the human diet because of their various functions in the body. They serve as cofactors for many physiologic and metabolic functions. Table 1, shows the mineral contents of the selected edible forest seeds differred significantly ($P < 0.05$) during the study. The calcium content of the selected seeds ranged from 81.08 ± 0.05 mg/100 g to 789.61 ± 0.01 mg/100 g. The calcium contend of *Adansonia digitata* seeds were found to be the least (81.08 ± 0.05 mg/100 g) while *Terminalia catappa was the highest with (*789.61 ± 0.01 mg/100 g). This value is high when compared to 2.17 mg/100 g obtained for cocoa bean [12], but low when compared to 900 mg/100 g obtained for Benni seed [24]. Calcium is an important mineral required for teeth formation, bone formation, muscle metabolism and neurological function [25]. The dietary allowance for calcium is 800 mg for 70 kg man [26]. This shows that almond can supplement other sources of dietary calcium since it is consumed mainly as snack [27]. Calcium deficiency in children leads to rickets, while in adults it may results in osteomalacia. As rickets is a common problem in the Africa, calcium intake is especially important for children who live in this part of Africa [28].

The potassium content of the selected edible forest seeds differed significantly ($P < 0.05$), with the lowest range content from 502.00 ± 1.00 mg/100 g for *Moringa oleifera* seeds which can also be recommended when compare to Maize with 0.93 content of potassium [29]. The highest potassium content was 7233.50 ± 3.50 mg/100 g for *Terminalia catappa,* recorded 350.00 mg/100 g of potassium in almond seed [15]. This value is high when compared to 330 mg/100g obtained for cocoa bean [12, 15]. The dietary allowance for potassium is (1875-5625 mg) for adults [26]. The seed could be recommended as source of dietary supplement for potassium.

Magnesium is an important mineral element whose deficiency is in connection with circulatory diseases such as heart disease. Without magnesium, many enzymes in the human body would function less efficiently and magnesium contributes to calcium and potassium metabolism and therefore essential for strengthening [25]. The highest magnesium content was found to be 687.58 ± 0.01 mg/100 g from *Terminalia catappa*. This value is high when compared to 520 mg/100 g obtained for cocoa bean [12]. The value is also high when compared to 300 mg/100 g reported for Benni seed

[24]; while *Afzelia africana* was the lowest with 3.59 ± 0.01 mg/100 g, even though it was found to be the lowest, yet it is high and recommended when compare to 0.13 obtained for *Zea mays* [29]. The human body contains 20-28 g of magnesium, more than half of which is stored in the bones. The element is an essential part of many enzyme systems and is also important in maintaining the electrical potential of nerve and muscle membranes [27].

The phosphorus content obtained from the selected seeds with the highest content was 468.00 ± 1.00 mg/100 g from *Moringa oleifera* and 9.48 ± 0.01 mg/100 g was the lowest phosphorus content from *Afzelia africana* seed. The dietary allowance for phosphorus is 800 mg/100 g [26]. Therefore *Moringa oleifera* seed can not be recommended as phosphorus supplements. Phosphorus a macro-nutrient comprises 22 percent of the total minerals of the body. Dietary deficiency of phosphorus is extremely unlikely as nearly all food content this mineral [27].

The value of 422.00 ± 1.00 mg/100 g was obtained as the highest Iron content among the selected edible forest seeds which was evaluated from *Terminalia catappa* seed. While *Afzelia africana* seed has the lowest content with 3.49 ± 0.03 mg/100 g. The iron content in *Terminalia catappa* seed is high when compared to 1.94 mg/100 g obtained for cocoa bean [12], when compared to 50 mg/100g obtained for Benni seeds [24]. The dietary allowance for iron is 10 g for 70 kg [26]. Therefore, *Terminalia catappa* seed could be recommended as a dietary supplement for people who need iron and those dwelling in the rural areas that live in poverty and even urban dwellers. This shows that these seeds are very rich in iron; and iron is very important for the formation of haemoglobin and normal functioning of the central nervous system [24].

The iodine content of the selected seeds was evaluated; *Moringa oleifera* seed was the lowest with iodine content of 59.05 ± 0.03 mg/100 g while *Afzelia africana* seed was the highest with 134.04 ± 0.03 mg/100 g. Therefore, *Afzelia africana* can be recommended as a dietary supplement in the developing countries especially for the purpose of food security.

The most abundant minerals among the mineral composition found in the selected edible forest seeds were potassium (7233.50 mg/100 g), calcium (789.61 mg/100 g), magnesium 687.58 (mg/100 g), phosphorus (468.00 mg/100 g), iodine (134.04) and iron (422.00 mg/100 g). The observation that potassium is the most abundant mineral element is consistent with the observation of [11], who reported potassium to be the most abundant mineral in Nigerian agricultural products. High amount of calcium, potassium and magnesium (as macroelements) may help to lower the blood pressure [31]. Several clinical studies have shown potassium, magnesium and calcium to be effective pressure lowering agents [32]; hence consumption of this seed flour may help achieve this purpose.

Table 2 shows vitamins A, C and E contents of the selected forest edible seeds differed significantly ($P < 0.05$). Vitamin A content determined from the selected seeds ranged from 0.07 ± 0.06 mg/100 g as the least content from *Prosopis africana* seed to 322.50 ± 1.50 mg/100 g as the highest content from *Adansonia digitata* seed; vitamin C content ranged from 0.05 ± 0.00 as the least evaluated *Terminalia catappa* seed and 522.50 ± 1.50 mg/100 g as the highest vitamin C content in *Adansonia digitata* seed. While *Afzelia africana* seed had the least vitamin E content of 0.74 ± 0.01 mg/100g and *Moringa oleifera* seed with the highest vitamin content of 2801.50 ± 1.50 mg/100 g. The vitamin A, C and E contents in these selected forest seeds are adequate to supplement other dietary sources. Vitamin A is a good treatment for people suffering form eye problem while deficiency of vitamin C leads to scurvy and gingivitis [26].

According to the results of the study, vitamins content of *Adansonia digitata* tree seed has a higher level of vitamin A and C among the selected forest seeds while *Moringa oleifera* tree seed has a higher level of vitamin E content when compared with other seeds under studied. This study is in accord with [33], who stated that *Adansonia digitata* seed contain essential nutrients for human good health and is important food; it is therefore a very dietary source of vitamins A and C for human body; also it would serve as useful dietary supplements.

5. CONCLUSION

The results of this study showed that the selected forest tree seeds evaluated have a high level

of mineral and vitamin contents and their availability in the forest indicates its potentials usefulness for human body especially in the developing countries which could reduce the level of malnutrition in most impoverished communities in Africa.

Recommendation: It is therefore recommend the encouraging production of the trees seeds for a more availability; also to promote their consumption by the population who usually uses these foods for their nutritive values. This study informs one only of the potential mineral and vitamin values of the selected forest tree seeds, the next step is to assess the availability of the tree seeds and possible present of anti-nutrients.

AUTHORS' CONTRIBUTION

All authors carried out the research. Report writing and revision was done by JHD, while EDK and SAD contributed financially, materially and impacted more idea and knowledge [reviewed the research work]. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare that they have no conflict of interest.

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Allellopathic effects of *Mesembryanthemum forsskalii* Hochst. ex Boiss. on seed germination and seedling growth of *Malva parviflora* L. and *Plantago ovata* Forssk.

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ABSTRACT

The present study focused on the allelopathic effects of the aqueous and methanol extracts of *Mesembryanthemum forsskalii* Hochst. ex Boiss. on germination and seedling growth of *Malva parviflora* and *Plantago ovata. M. forsskaliii* was collected from Al-Jouf area, Saudia Arabia. The dried shoot system of *M. fosskalii* was used to prepare water and methanol extracts with different concentrations (25, 50, 75 and 100%) and distilled water as the control. The results showed that the aqueous and methanol extracts of *M. forsskalii* contained phenolic compounds and flavonoids that might be embroiled as allelochemical agents. Petri-dish trial showed that the two extracts at all concentrations reduced total germination percentage. Pot experiment indicated variations in seedlings germination and growth between *M. parviflora* and *P. ovata* in response to aqueous and methanol extracts of *M. forsskalii*. On growth stage the shoot and root lengths were decreased probably due to the allelopathic effects of *M. forsskalii.* The fresh and dry weights of shoot were inhibited with increase in concentration of aqueous and methanol extracts. In *M. parviflora* and *P. ovata* the leaf area was decreased under all concentrations. The chlorophyll a, b and carotenoids (pigments) were decreased in *M. parviflora* and *P. ovata* for all concentrations of extracts, but methanol extract increased chl. a only in *M. parviflora* compared to control. Flavonoids, saponins, tannins, carbohydrates, glycosides and phenolic are the allelochemical compounds released from the *M. forsskalii* into aqueous and methanol extracts which inhibited germination and growth of the studied plants.

Keywords: Allelopathy; *Mesembrynthemum forsskalii*; *Plantago ovata*; *Malva parviflora;* Chlorophyll; Germination; Growth.

1. INTRODUCTION

Allelochemicals liberated as residues, exudates and leaches by many plants from leaves, stems, roots, fruits and seeds reported to interfere with growth of other plants [1]. These chemical products mainly affect plants at seed emergence and seedling levels [2]. The effects of allelopathic are combined to many plants species and can be spotted at any level of biological organization [3, 4]. Plants extract that is not decomposed was thought to contain secondary compounds with allelochemical activity or phytotoxic which cause growth inhibition [5]. Allelochemicals are think to be a mutual action of

several secondary metabolites including terpenoids [6], juglone [7], flavonoids [8] and phenolic compounds [9]. Some researchers have pointed that the inhibitory materials implicated in allelopthy are terpenoids and phenolic material [10, 11].

Mesembryanthemum forsskalii belonging to the family Aizoaceae and is considered as an important medicinal plant. It is an erect, annual herb bearing fleshy terete to subterete linear leaves widely distributed in the all Middle East Countries and Saudi Arabia [12]. Due to its highest content of protein, fat and carbohydrates, in Kingdom of Saudi Arabia people utilize the seeds of *M. forsskalii* as food and mix the powder of seeds with butter and prepare a traditional recipe known as pakilla [13]. Furthermore, this desert plant also has noticeable medicinal importance on liver enzymes and lipid profiles of streptozotocin - induced diabetic in Wistar rats [14, 15]. The flavonoids, tannins, saponin, phenolics and anthocyanins are the most active chemical constituents of *M. forsskalii* [16]. *M. forsskalii* has anti inflammatory and cardio-protective effects, cytotoxic, antioxidant and antimutagenic [17]. The objective of the present study was to assess the allelopathic effects of *Mesembrynathemum forsskalii* on germination and seedling growth of *Malva parviflora* and *Plantago ovata* under lab and greenhouse conditions. *Malva parviflora* and *Plantago ovata* are widely distributed weeds with the economic agricultural crops and the present study try to control these plants by allelopathic effects.

2. MATERIAL AND METHODS

M. forsskalii was selected for this study because it recognized to produce allelochemicals. Shoot system of *M. forsskalii* was collected from Al-Jouf area in Saudi Arabia. Samples of *M. forsskalii* were washed completely with distilled water and dried in the open air for 14 days. Then dried samples were ground into fine powder and stored dry until used. The seeds *of Malva parviflora* and *Plantago ovata* were collected at the end of the growing season 2015.

2.1. Water extract preparation

10 g of air dried of *M. forsskalii* shoot was soaked in 100 ml distilled water for 48 hours at room temperature. The filter paper (Whatman No.1) was used to filter this extract. The filtered solutions were caught in a refrigerator until experiment start. The filtered solution (10% w/v) was diluted befittingly with distilled water to produce the final concentrations of 25, 50, 75 and 100%. The distilled water was used as the control treatment to assessment possibility of seeds germination.

2.2. Methanol extract preparation

10 g of air dried *M. forsskalii* shoot was extracted by 100 ml methanol in soxhlet apparatus for 24 hours [18]. After the rotary evaporator, the residue was dissolved in 3 ml methanol and completed to 100 ml by distilled water. It was prepared 25, 50 and 100% concentrations.

The phenolic content of *M. forsskalii* was estimated in the methanolic and aqueous extracts. The solvent systems H_2O: HOAc (47: 3) and BAW (4: 1: 5) were used to achieved paper chromatography on Whattman No. 1. HPLC were analyzed the samples [19].

2.3. Experiment of germination

5% sodium hypochlorite solution was used for sterilized the seeds of *M. parviflora* and *P. ovata* for 10 minutes, swill through with deionized water several times. In this experiment, 25 seeds were placed in each petri dish on filter paper, supplied with 15.0 ml of extracts or distilled water. The petri dishes were kept in a growth chamber at the controlled temperature (25 ± 3˚C). The petri dishes were closed by paper parafilms to prevent evaporation and pollution for 10 days. When the radical extended through the seed coat, the seeds germination was considered. The germinated seeds number was counted for each petri dish [20].

2.4. Seedling growth

Seeds of *M. parviflora* and *P. ovata* were germinated in pots. In each pot of 14 cm diameter and 18 cm height was filled with fertile loamy soil up to ¾ the height of the pot. Each pot was supplied with 15 ml of *M. forsskalii* extracts (25, 50, 75 and 100% of water, and methanol) and control was

added to every day, in three replicates. Plant growth in controlled temperature (25 ± 3˚C) illumination (dark/light cycle: 14/10 h) and 80% humidity into a greenhouse of Botany and Microbiology Department, Faculty of Science, King Saud University was conducted. The shoot and root lengths were measured after 35 days of growth. Also, fresh and dry weights of shoot and root were measured. The leaf surface area was measured using portable area meter Model Li-3000. The content of pigments chlorophyll a, b and carotenoids were accomplished based on method of Stirban [21].

2.5. Statistical analysis

Each treatment was conducted in a complete randomized with three replicates. The data were subjected to analysis with one way ANOVA test. The results were presented as mean ± SD (Standard Deviation). The significant differences between treatments means were separated using LSD test ($p<0.05$).

3. RESULTS AND DISCUSSION

Analysis of aqueous and methanol extract of *Mesembryanthemum forsskalii* showed that, three galloylglucose and four flavonol glycosides in different concentrations were present (Table 1). In this respect, flavonoids may leach from shoots into the soil solution and inhibit seed germination and root elongation [22]. The flavonoids also show antagonistic properties with plant hormones Indol Acetic Acid (IAA), metabolism and ion uptake by the plants [23].

3.1. Seed germination

The effect of *M. forsskalii* extracts on the seed germination percent of *M. parviflora* and *P. ovata* (Table 2). Compared to the control, the effect of water and methanol extracts at the low concentrations of 25 and 50% have slightly inhibition on the seed germination of *P. ovata.*

Table 1. Phenolic content of aqueous and methanolic extract of *M. forsskalii.*

Constituents	Aqueous extract µg/ml	Methanol extract µg/ml
1-O-galloyl-β-glucopyranose	3.4	5.2
1,6 di-O-galloyl-β- glucopyranose	5.1	4.9
1,3,6 tri-O-galloyl-β- glucopyranose	6.2	7.1
Quercetin 3-O-rutinoside	8.1	10.5
Quercetin 3-O-glucosylgalactoside	6.3	7.8
Quercetin 3-O-galactoside	8.3	11.6
Quercetin 3-O-glucoside	8.4	12.5

Table 2. Germination percentage of the investigated plants.

Treatment	*Malva parviflora*		*Plantago ovata*	
	Germination %	LSD 0.05	Germination %	LSD 0.05
Control	90±14.1		100 ± 0	
Water 100%	23±15	67(*)	74 ± 0.01	0.16 (*)
Water 75%	24 ± 16.1	66(*)	81 ± 0.01	0.19 (*)
Water 50%	26± 5	63.5(*)	84 ± 0.02	0.18 (*)
Water 25%	28 ± 15	62.5(*)	94 ± 0.02	0.06 (*)
Methanol 100%	10 ± 4	80(*)	73 ± 0.01	0.26 (*)
Methanol 50%	20 ± 28.28	70(*)	87 ± 0.01	0.02 (*)
Methanol 25%	40 ± 31.62	50(*)	93 ± 0.01	0.03 (*)

* The mean difference is significant at the 0.05 level.
Mean of three replications in duplicates ± Standard deviation.

Table 3. Length of shoot at start and after 35 days of treatment of the investigated plants.

Treatment	*Malva parviflora*				*Plantago ovata*			
	Length of shoot at the start of experiment (cm)	**LSD 0.05**	**Length of shoot after 35 days of experiment (cm)**	**LSD 0.05**	**Length of shoot at the start of experiment (cm)**	**LSD 0.05**	**Length of shoot after 35 days of experiment (cm)**	**LSD 0.05**
Control	26.3 ± 5.5		29.3 ± 5.5		10.3 ± 0.6		21.7 ± 1.2	
Water 100%	16. 7 ± 1.5	4.3 (*)	17. 7 ± 2.5	4.0(*)	9.3 ± 0.5	0.00	14.3 ± 0.3	7.3 (*)
Water 75%	21.7 ± 7.4	4.6 (*)	22.2 ± 6.9	7.1 (*)	10.3 ± 0.6	1.0 (*)	13 ± 2.7	8. 7(*)
Water 50%	22± 4.3	9.7 (*)	24 ± 4.4	9.6 (*)	10.3 ± 0.5	0.00	10.3 ± 0.6	11.6(*)
Water 25%	22.3 ± 4.7	4.0 (*)	25.3 ± 2.5	5.3 (*)	10.6 ± 0.3	0.30	13.7 ± 0.2	8.0 (*)
Methanol 100%	22.3 ± 2. 9	3.3 (*)	24± 2.6	4.7(*)	9 ± 0.1	1.3 (*)	11.3 ± 0.5	10.4 (*)
Methanol 50%	23± 1.7	4.0 (*)	24. 7 ± 2. 9	5.3 (*)	9 ± 0.1	1.2(*)	12 ± 0.1	9. 7(*)
Methanol 25%	24 ± 4.6	2.3	26.3 ± 3.2	3.0 (*)	10.3 ± 0.57	0.00	12.3 ± 0.7	9. 4(*)

* The mean difference is significant at the 0.05 level. Mean of three replications in duplicates ± Standard deviation.

Table 4. Length of root after 35 days of treatment of the investigated plants.

Treatment	*Malva parviflora*		*Plantago ovata*	
	Length of root after 35 days of experiment (cm)	**LSD 0.05**	**Length of root after 35 days of experiment (cm)**	**LSD 0.05**
Control	11.3 ± 1.5		16.333 ± 0.6	
Water 100%	10.7± 0.6	1.3	15.1 ± 0.5	1.2 (*)
Water 75%	12.6 ± 0.8	1.6 (*)	20.7 ± 2.3	4.3 (*)
Water 50%	13 ± 1	1.7 (*)	17.6 ± 0.5	1.3(*)
Water 25%	13.3± 0.6	0.7 (*)	21 ± 0	4.6 (*)
Methanol 100%	3.3± 0.5	8.0 (*)	14.7 ± 0.5	1.6 (*)
Methanol 50%	7.3 ± 3.2	4.0 (*)	12.4 ± 0.5	3.6 (*)
Methanol 25%	9.7± 2.8	1.6 (*)	13.3 ± 0.8	3.0 (*)

* The mean difference is significant at the 0.05 level. Mean of three replications in duplicates ± Standard deviation.

While, at high concentrations of 75 and 100% it is noticed the highly inhibitory effect. But, the seed germination of *M. parviflora* showed significant inhibition at all concentrations and the inhibition increase with increasing the concentration.

From these results, it is cleared that, the water and methanol extracts contained allelochemicals of growth inhibiting and their effects dependent on the concentration of *M. forsskalii* extract.

Seyed et al. concluded that the impact of different extract concentration of *Artemisia annua* on the germination percent is related to control and the least was related to the highest concentration 100% of extract [24]. The inhibitory effect of the extracts increased with increasing extract concentration [25, 26]. The present studies were confirmed with these studies and also with Salama and Al-Rabiah [27]. They concluded that, the effects of allelopathic can cause both stimulatory and suppressive effects at lower and higher concentrations respectively.

3.2. Shoot and root lengths

Table 3 shows that aqueous and methanol extracts had inhibition effect on shoot lengths in *M. parviflora* and *P. ovata* while higher concentrations (75 and 100%) induced greater inhibition after 35 days of processing. The aqueous extract revealed that the slightly inhibition of root lengths of *M. parviflora* and *P. ovata* at 100% and stimulated root lengths at 25, 50 and 75% (Table 4). However,

at all concentration the methanol extract exhibited significance inhibition of root lengths and the inhibitory effect increase with increasing extract concentration in *M. parviflora* and *P. ovata*. Mahmood et al. [26] gained similar results and concluded that methanolic extract significantly inhibited root and shoot growth of *Solanum melongena*. The effect of allelopathic contents of *M. forsskalii* has been imputing to the production of several active chemical constituents including flavonoids, saponins, tannins, carbohydrates, glycosides and phenolic compounds [16].

3.3. Fresh and dry weights

Both fresh and dry weights of shoot of *M. parviflora* had the highest inhibitory which affected by aqueous extract (1.45 and 0.15 g respectively), meanwhile, the highest weights of fresh and dry weights were recorded at low concentrations of aqueous and methanol extracts (2.61 and 3.28 g fresh wt.), (0.43 and 1.2 g dry wt.) respectively (Table 5). In *P. ovata* it was establish that there was low inhibitory effect of aqueous and methanol extracts on the fresh and dry weights of the shoot compared to control (Table 5). Generally, the highest concentrations induce the effect of allelopathic for *M. parviflora* and *P. ovata* at all extracts. The shoots recorded the maximum fresh and dry weights in untreated control. The present study was confirmed with Salama and Al-Rabiah [27] that studied allelopathic effects of *Citrullus colocynthis* on *Vicia faba* and *Hordeum vulgare.* In all extracts the fresh and dry weights in *M. parviflora* and *P. ovata* were reduced significantly. These results were confirmed with those obtained by Djanaguiraman et al. [28], who notice that seedling dry matter of rice, sorghum and blackgram significantly reduced by leaf leachate of *E. globules* and highest inhibition was observed in highest concentration. Aqueous eucalyptus extract decreased fresh and dry weights of three wheat cultivars [29].

3.4. Leaf surface area

Table 6 shows that the aqueous extract of *M. forsskalii* on the leaf area had significant inhibition at both low and high concentration (7.4 and 8.5 cm^2 respectively) compared to control for *M. parviflora*. However, the methanol extract of *M. forsskalii* showed slightly inhibition at high concentration and stimulation at low concentration for *M. parviflora*. The effect of all extracts of *M. forsskalii* on *P. ovata* showed significant inhibition of leaf surface area at all concentrations. These results are in an agreement with Salama and Al-Rabiah [27]. They concluded that any secondary compound with allelochemical activity can cause both inhibitory and stimulatory effects.

3.5. Chlorophyll content

The effect of aqueous and methanol extracts of *M. forsskalii* on the content of chlorophyll (Chl. a, b and carotenoids) were differ greatly on *M. parviflora and P. ovata*. Aqueous extract (25%) significantly inhibited chlorophyll a, b and carotenoids (0.06, 0.06 and 0.05 mg/g) on *M. parviflora* compared to control (Table 7). High concentrations of aqueous and methanol extracts (100%) catalyzed chlorophyll a, b and carotenoids (0.79, 0.99 and 0.46 mg/g) for aqueous extract and (0.97, 0.75 and 0.61 mg/g) for methanol extract. In *M. parviflora* aqueous extract with different concentrations (25, 50 and 75%) significantly inhibited Chl. a, b and carotenoids. Methanol extracts with different concentrations significantly stimulated chlorophyll a, b and carotenoids. The highest stimulatory effect on Chl. a, b and carotenoids were found in 100% methanol concentration being (0.97, 0.75 and 0.61 mg/g respectively) as shown in table 7. All the extracts effect of *M. forsskalii* on *P. ovata* showed significant inhibition of chl. a, b and carotenoids at all concentration and the inhibitory effect increase with increasing extract concentration. The present results are supported by the finding of Salama and Al-Rabiah [27], Corsato et al. [30] and Glieessman [31], they declared that the effect of allelopathic chemicals is a natural interference in which the plant produces substances and metabolites that may benefit or harm other plants when released. Also, these results were confirmed with Abdel-Fattah et al. [32] who found that the effects of allelopathic chemicals can cause both stimulatory and suppressive effects at higher and lower concentrations respectively.

Table 5. Fresh and dry weights of shoot after 35 days of treatment of the investigated plants.

Treatment	*Malva parviflora.*				*Plantago ovata*			
	Fresh wt. (g)	**LSD 0.05**	**Dry wt. (g)**	**LSD 0.05**	**Fresh wt. (g)**	**LSD 0.05**	**Dry wt. (g)**	**LSD 0.05**
Control	34.12 ± 0.98		7.43 ± 1.2		3.4 ± 0		0.21 ± 0.06	
Water 100%	1.45 ± 0.76	32.6 (*)	0.15 ± 0.04	7.18 (*)	2.3 ± 0	0.06 (*)	0.13 ± 0.06	0.07 (*)
Water 75%	1.55 ± 0.76	32.7 (*)	0.32± 0.04	7.08 (*)	2.6 ± 0	0.11 (*)	0.14 ± 0.06	0.13 (*)
Water 50%	1.68 ± 0.96	32.5 (*)	0.35 ± 0.02	7.11 (*)	2.8 ± 0.06	0.11 (*)	0.15 ± 0.06	0.10 (*)
Water 25%	2.61 ± 0.01	33.2 (*)	0.43 ± 0	7.08 (*)	2.9 ± 0.06	0.32 (*)	0.16 ± 0.06	0.04 (*)
Methanol 100%	2.26 ± 0.33	31.2 (*)	0.4 ± 0.1	6.82. (*)	2.19 ± 0.01	0.03 (*)	0.08 ± 0	0.12(*)
Methanol 50%	2.84± 0.29	31.8 (*)	0.58 ± 0.01	7.03 (*)	2.21 ± 0.01	0.03 (*)	0.10 ± 0	0.11(*)
Methanol 25%	3.28 ± 0.8	30.8 (*)	1.2 ± 0.2	6.23 (*)	2.22 ± 0.07	0.04(*)	0.11 ± 0.06	0.09 (*)

* The mean difference is significant at the 0.05 level. Mean of three replications in duplicates ± Standard deviation.

Table 6. Leaf area of the investigated plants.

Treatment	*Malva parviflora*		*Plantago ovata*	
	Leaf area (cm^2)	**LSD 0.05**	**Leaf area (cm^2)**	**LSD 0.05**
Control	12.2 ± 3.5		3.2 ± 0.08	
Water 100%	7.4 ± 0.03	4.8 (*)	0.8 ± 0.54	2.3 (*)
Water 75%	7.5 ± 0.31	4.7 (*)	1.0 ± 0.29	2.1 (*)
Water 50%	7.9 ± 0.45	4.3 (*)	2.5 ± 0.08	0.58 (*)
Water 25%	8.5 ± 0.01	4.7 (*)	2.7 ± 0.21	0.44 (*)
Methanol 100%	11.4 ± 0.99	0.83	1.0 ± 0.06	1.8 (*)
Methanol 50%	12.6 ± 3.87	0.40	1.2 ± 0.24	2.0 (*)
Methanol 25%	14.3 ± 2.71	2.0(*)	1.3 ± 0.05	2.0 (*)

* The mean difference is significant at the 0.05 level. Mean of three replications in duplicates ± Standard deviation.

Table 7. Chl.a, b and Carotenoids of the investigated plants.

Treatment	*Malva parviflora*						*Plantago ovata*					
	Chl.a mg/g	**LSD 0.05**	**Chl.b mg/g**	**LSD 0.05**	**Carotenoid mg/g**	**LSD 0.05**	**Chl.a mg/g**	**LSD 0.05**	**Chl.b mg/g**	**LSD 0.05**	**Carotenoid mg/g**	**LSD 0.05**
Control	0.76 ± 0.1		0.34 ± 0.1		0.26 ± 0.2		1.32 ± 0.0		1.45 ± 0.0		0.42 ± 0.0	
Water 100%	0.79 ± 0.01	0.70 (*)	0.99 ± 0.1	0.13 (*)	0.46 ± 0.1	0.20 (*)	0.54 ± 0.0	0.53 (*)	0.69 ± 0.0	0.6 (*)	0.2 ± 0.0	0.02 (*)
Water 75%	0.27 ± 0.01	0.48 (*)	0.32 ± 0.1	0.65 (*)	0.26 ± 0.1	0.00	0.6 ± 0.0	0.7 (*)	0.84 ± 0.0	0.4 (*)	0.3 ± 0.0	0.09 (*)
Water 50%	0.58± 0.02	0.18 (*)	0.31 ± 0.1	0.09 (*)	0.2 ± 0.1	0.06 (*)	0.9 ± 0.0	0.4 (*)	1.1 ± 0.0	0.3 (*)	0.4 ± 0.0	0.04 (*)
Water 25%	0.06± 0.1	1.2 (*)	0.06 ± 0.1	0.26 (*)	0.05 ± 0.1	0.21 (*)	1.0 ± 0.0	0.3 (*)	1.2 ± 0.0	0.2 (*)	0.4 ± 0.0	0.0.0 (*)
Methanol 100%	0.97 ± 0.1	0.23(*)	0.75 ± 0.01	0.07 (*)	0.61 ± 0.01	0.07 (*)	0.4 ± 0.0	0.7 (*)	0.5 ± 0.0	1.0 (*)	0.2 ± 0.0	0.05 (*)
Methanol 50%	0.83± 0.1	0.23 (*)	0.66 ± 0.1	0.01 (*)	0.35± 0.01	0.08 (*)	0.6± 0.0	0.7 (*)	0.7 ± 0.0	1.2 (*)	0.3 ± 0.0	0.2 (*)
Methanol 25%	0.79 ± 0.1	0.20 (*)	0.49 ± 0.1	0.14 (*)	0.28± 0.01	0.35 (*)	0.9 ± 0.0	0.4 (*)	0.8 ± 0.0	1.1 (*)	0.4 ± 0.0	0.13 (*)

* The mean difference is significant at the 0.05 level. Mean of three replications in duplicates ± Standard deviation.

4. CONCLUSION

The present study has shown that *Mesembryanthemum forsskalii* Hochst. ex Boiss. contained allelochemical compounds in their tissues and released these into aqueous and methanol solutions. The present research revealed that both aqueous and methanol extracts of shoot showed inhibitory effects on seed germination of *M. parviflora and P. ovata*. Aqueous and methanol extracts had inhibition effect on shoot lengths in *M. parviflora and P. ovata* and the inhibitory effect increase with increasing extract concentration. The fresh and dry weights of *M. parviflora and P. ovata* showed inhibition at all concentration of aqueous and methanol extracts. In *M. parviflora and P. ovata* the leaf area was decreased under all concentrations. The chlorophyll a, b and carotenoids were decreased in *M. parviflora and P. ovata* for all concentrations of extracts.

The effects of allelopathic contents of *M. forsskalii* has been imputing to the production of several active chemical constituents (flavonoids, saponins, tannins, carbohydrates, glycosides and phenolic compounds).

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AUTHORS' CONTRIBUTION

HMHS: Conception and design; Acquisition of data; Writing, review and revision of the manuscript; Administrative, technical or material support. HMHS and MSAW: Development of methodology; Analysis and interpretation of data. The final manuscript has been read and approved by both authors.

TRANSPARENCY DECLARATION

The authors declare that they have no conflict of interest.

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Research Article

Protective role of supplemental vitamin E on brain acetylcholinesterase activities of rabbits fed diets contaminated with fumonisin B_1

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ABSTRACT

Forty-nine rabbits were used to evaluate the protective potential of an antioxidant, vitamin E, against the negative impact of fumonisin B_1 (FB_1) on acetylcholinesterase (AChE) activities in the brain regions. The animals were randomly assigned to a control diet without FB_1 and six diets containing different concentrations of dietary FB_1 or in combination with vitamin E (i.e. 2.5 mg FB_1, 5.0 mg FB_1, 7.5 mg FB_1, 2.5 mg FB_1 + 100 mg vitamin E, 5.0 mg FB_1 + 100 mg vitamin E, and 7.5 mg FB_1 + 100 mg vitamin E per kg of complete feed) (n = 7 rabbits/treatment group). After 10 weeks of feeding the respective diets, the animals were sacrificed; and the brains obtained were carefully dissected out. The AChE activities in the pons of rabbits exposed to diets containing > 5 mg FB_1/kg were significantly ($p < 0.05$) lower than those in other treatment groups. The AChE activities in the amygdala of rabbits exposed to diets contaminated with FB_1 were significantly ($p < 0.05$) lower than those in other treatment groups. There was significant ($p < 0.05$) reduction in the activities of AChE in the hippocampus and the medulla oblongata of the rabbits with increase contamination level of FB_1. The potential of vitamin E to combat the effect of FB_1 on the brain regional AChE and specific AChE activities reduced with increase in the dietary FB_1.

Keywords: Antioxidant; Brain; Fumonisin B_1; Mycotoxin; Neurotransmitter; Rabbit; Vitamin E.

1. INTRODUCTION

Exposure of humans and livestock to mycotoxin-contaminated food is particularly a serious problem in the tropics and its economic consequences are profound. Usually, crops contaminated with large amounts of mycotoxins are diverted into animal feeds, which pose a serious threat to the health and productivity of animals [1].

Fumonisin B_1 (FB_1), a mycotoxin produced by *Fusarium verticillioides* (= *F. moniliforme*) and other Fusarium species that grow on maize worldwide, has been documented to cause various pathophysiological responses in humans and animals. FB_1 acts as inhibitors of sphingolipid biosynthesis by inhibiting the enzyme sphingosine N-acetyltransferase [2] in most livestock species, including rabbits. FB_1 was found to be nephrotoxic and hepatotoxic in rabbits [3], and caused leuko-

encephalomalacia and haemorrhage in the brain of rabbit [4]. In addition, significant changes of water distribution in the brain and lung of embryos in pregnant rabbit does fed FB_1-contaminated diet were reported by Orova [5]. The effects of mycotoxins on the activities of brain neurotransmitters in animals have been documented [6, 7].

Acetylcholinesterase (AChE; EC 3.1.1.7) is one of the most efficient biological catalysts known and plays a key role in cholinergic neurotransmission by hydrolyzing the transmitter acetylcholine (ACh), thus terminating its action [8, 9]. ACh is implicated in brain plasticity and disease [10] and AChE is considered a key enzyme in detecting neurotoxicity. Swamy et al. [11] reported significantly reduced concentrations of dopamine (another neurotransmitter) in the hypothalamus and pons and concentrations of norepinephrine (another neurotransmitter) in the pons of brain of swine fed a blend of grains naturally contaminated with *Fusarium* mycotoxins. The study of brain enzyme activities such as of AChE is essential in detecting the neurotoxic effects of FB_1. Dietary concentration of $\geq$ 5.0 mg FB_1/kg reportedly impaired brain AChE activities in pigs in a 6-month study [7]. The neurotoxicological effects of *Fusarium* mycotoxins has been based on the reports indicating that these mycotoxins can reduce feed intake [11] and induce vomiting in pigs [12]. Although mechanisms responsible for feed refusal or reduced feed intake induced by *Fusarium* mycotoxins are not fully understood, it has been proposed that alterations in brain neurotransmitter concentrations represent one of the possible mechanisms [13].

In brain, high rate of lipid peroxidation (LPO) has been reported due to high susceptibility of brain to oxidative stress because of its high concentrations of phospholipids, which contain a large amount of polyunsaturated fatty acids, its high rate of oxygen utilization, and deficient in antioxidant defense system and a high content of transition metals like copper and iron [14]. Lipid peroxidation is the oxidative deterioration of the polyunsaturated lipids through the formation of hydroperoxides into short-chain aldehydes, ketones, and other oxygenated compounds considered to be responsible for the development of diseases in human and animals [15, 16].

Various nutritional strategies have been proposed to alleviate the adverse effects of mycotoxins. *In vitro* assays have demonstrated that mycotoxins may produce reactive oxygen species (ROS) generation that induces lipid peroxidation (LPO) and oxidative damage, apoptosis and necrosis via the mitochondrial pathway [17]. FB_1 induced generation of ROS in broiler chicken [18] and consequently lipid peroxidation. For this reason, the addition of natural or synthetic antioxidants is potentially efficacious in combating the impact of these mycotoxins on livestock because of the ability of these compounds to act as superoxide anion scavengers [19-22]. Vitamin E is known to be an effective lipid-soluble antioxidant and some studies have shown that it inhibits further free radical formation and mitigate their toxic effects [23]. However, there are only few data about the ameliorative effect of antioxidants on the negative impacts of some important mycotoxins, such as FB_1. The objectives of this study were, therefore, to investigate the effects of feeding diets containing blends of grains contaminated with FB_1 on the activities of brain regional AChE and to determine the efficacy of vitamin E in preventing the effects considering the fact that the vitamin is a potent antioxidant and neuroprotector.

2. MATERIAL AND METHODS

2.1. Production of FB_1 and experimental diet

Maize grits in 500 g quantities were placed into autoclavable polypropylene bags and soaked with 200 ml of distilled water for 2 h, then autoclaved for 1 h at 121°C and 120 kPa. The autoclaved maize grits were then cultured with a toxigenic strain of *F. verticillioides* (MRC 286) obtained from the Plant Pathology Laboratory of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria to produce FB_1 as described previously [24]. Samples of homogenously mixed maize grits were quantified in replicates for FB_1 and other common *Fusarium* mycotoxins including deoxynivalenol (DON), T-2 toxin, and zearalenone using quantitative CD-ELISA test kits (Neogen, Lansing, MI, USA) and reconfirmed by using HPLC analyses as described by Shephard et al. [25]. Uncultured and cultured maize grits were used to formulate seven diets consisting a Control diet

without FB_1 and six diets containing different concentrations of dietary FB_1 or in combination with vitamin E (2.5 mg FB_1, 5.0 mg FB_1, 7.5 mg FB_1, 2.5 mg FB_1 + 100 mg vitamin E, 5.0 mg FB_1 + 100 mg vitamin E, and 7.5 mg FB_1 + 100 mg vitamin E per kg of complete feed, respectively. The selected dose of 100 mg vitamin E per kg of complete feed was based on previous work [26]. The above mentioned diets were marked as A, B, C, D, E, F, and G, respectively. The diets provided ~15 % crude protein, 10 % crude fibre and 2600 kcal of digestible energy/kg (Table 1).

2.2. Experimental animals and management

Female rabbits were housed individually in wire-meshed in-door cages for a period of two weeks for physiological adjustment before the commencement of the feeding study. All the animals were fed daily. Kepromec Oral (Ivemectin®) manufactured by Kepro, B.V. of Holland with batch number 0649900 was administered through drinking water against potential ecto-parasite and endo-parasites for two days at recommended dosage by the manufacturer. Forty-nine clinically normal matured mixed-breed female rabbits weighing 1.65 to 2.0 kg body weight were obtained from a commercial rabbit farm. The animals were allocated to each of the seven experimental groups (n = 7 rabbits/treatment group) and were maintained on the experimental diets for 10 weeks at the Teaching and Research Farm of the Federal University of Technology, Akure, Nigeria. This study was approved by the local Institutional Animal Ethics Committee and was performed in accordance with "Guide for the care and use of Laboratory Animals" [27].

2.3. Determination of brain AChE activities and protein concentrations

At the end of the experiment, all the animals were stunned mechanically prior to bleeding, quickly decapitated and the brains immediately removed, freed of all adhering meninges and blood vessels. The brains obtained were dissected on ice-cold porcelain tile into the pons, cerebellum, amygdala, hippocampus, hypothalamus, cerebral cortex, mid-brain, and medulla oblongata.

Table 1. Gross composition (%) of the experimental diets.

Ingredients	Composition (%)
Maize[a]	40.68
Groundnut cake	11.78
Wheat offal	22.58
Palm kernel cake	19.63
Fish meal	1.96
Dicalcium phosphate	1.96
Salt	0.23
Methionine	0.10
Lysine	0.96
Minerals/vitamins premix	0.12
Total (kg)	**100.00**
Crude protein (%)	15.46
Crude fibre (%)	9.6
Digestible energy (kcal)	2597.96

[a] Varied proportion of *Fusarium*-cultured and non-cultured grains

Immediately after the brain samples from each animal were homogenized (1%, w/v) with a Potter-Elvehjem homogenizer in 0.1 m ice-cold phosphate buffer containing 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). The brain AChE activities (in µmol/g wet tissue per minute) and total protein concentrations were determined using colorimetric and Biuret methods by following the hydrolysis of acetylthiocholine according to the procedures of Ellmann et al. [28] and Reinhold [29] respectively. The AChE activity of each sample was divided by its total protein concentration to give the specific acetylcholinesterase (SAChE) activity in µmol/g protein/min.

2.4. Statistical analysis

The design used for the experiment is Completely Randomised Design (CRD). All the data obtained were subjected to statistical analysis using analysis of variance (ANOVA) procedure of SAS [30]. The significant treatment means were compared using the New Duncan Multiple Range test option of the same software and results giving $p < 0.05$ were considered significantly different.

3. RESULTS

The brain AChE activities of rabbits fed diets contaminated with varied levels of FB_1 with or without vitamin E are as shown in Table 2. The AChE activities in the pons of rabbits exposed to diets containing > 5 mg FB_1/kg were significantly ($p < 0.05$) lower than those on the control diet and those on vitamin E supplementation. The AChE activities in the amygdala of rabbits exposed to diets containing dietary FB_1/kg were significantly ($p < 0.05$) lower than those on other treatments, including the control, which were not different statistically from the AChE activities in the amygdala of those exposed to FB_1-containing diets supplemented with vitamin E. The AChE activities in the hypothalamus of rabbits fed the control diet were significantly ($p < 0.05$) higher than those fed diets containing FB_1, which were not different from those supplemented with vitamin E. There was significant ($p < 0.05$) reduction in the activities of AChE in the hippocampus and the medulla oblongata of the animals with increase in the dietary FB_1 and the potential of the vitamin to combat the effect of the toxin on the AChE activities also reduced with increase in the dietary FB_1. However, there was no significant effect of the mycotoxin on the AChE activities in the cerebellum and the cerebral cortex.

Specific acetylcholinesterase activities in the brain regions of the rabbits generally declined with increase in the dietary FB_1 and the potential of the vitamin to combat the effect of the toxin on the Specific AChE activities also reduced with increase in the dietary FB_1 (Table 3).

Table 2. Brain AChE activities (µmol/g tissue) of rabbits fed diets contaminated with FB_1 with or without vitamin E (Mean ± SEM).

	Diet (n = 7 rabbits/treatment)						
Brain region	**A**	**B**	**C**	**D**	**E**	**F**	**G**
Pons	6.55 ± 010^{a}	5.78 ± 0.02^{ab}	5.53 ± 0.37^{b}	5.69 ± 0.32^{b}	5.95 ± 0.31^{ab}	5.94 ± 0.28^{ab}	5.93 ± 0.12^{ab}
Cerebellum	4.49 ± 0.17	4.12 ± 0.30	3.69 ± 0.26	3.72 ± 0.43	4.68 ± 0.02	4.49 ± 0.20	4.11 ± 0.47
Amygdala	5.67 ± 0.02^{a}	4.72 ± 0.13^{b}	4.85 ± 0.36^{b}	4.63 ± 0.14^{b}	5.56 ± 0.15^{a}	5.57 ± 0.03^{a}	5.19 ± 0.35^{ab}
Hippocampus	5.81 ± 0.23^{a}	4.57 ± 0.05^{b}	4.45 ± 0.18^{b}	3.29 ± 0.05^{c}	4.84 ± 0.11^{b}	4.00 ± 0.44^{bc}	3.96 ± 0.49^{bc}
Hypothalamus	4.82 ± 0.41^{a}	3.60 ± 0.07^{c}	3.78 ± 0.02^{bc}	3.96 ± 0.13^{bc}	4.37 ± 0.01^{ab}	3.96 ± 0.34^{bc}	3.71 ± 0.22^{bc}
Cerebral cortex	3.86 ± 0.13	3.86 ± 0.27	3.74 ± 0.18	3.64 ± 0.09	3.87 ± 0.24	3.54 ± 0.22	3.35 ± 0.08
Midbrain	6.79 ± 0.02^{a}	4.92 ± 0.43^{b}	4.39 ± 0.08^{c}	4.19 ± 0.13^{cd}	5.75 ± 0.42^{b}	4.44 ± 0.13^{c}	3.58 ± 0.18^{d}
Medulla oblongata	5.19 ± 0.11^{a}	5.15 ± 0.03^{a}	4.93 ± 0.23^{b}	4.03 ± 0.36^{c}	4.93 ± 0.04^{ab}	4.33 ± 0.23^{bc}	4.21 ± 0.08^{c}

[a, b, c,d] Means on the same row with different superscripts differ significantly ($p < 0.05$)

Table 3. Specific brain AChE activities (µmol/g protein/min) of rabbits fed diets contaminated with FB_1 with or without vitamin E (Mean ± SEM).

	Diet (n = 7 rabbits/treatment)						
Brain region	**A**	**B**	**C**	**D**	**E**	**F**	**G**
Pons	4.88 ± 1.37	4.37 ± 0.27	4.40 ± 0.32	4.67 ± 0.48	3. 99 ± 0.85	4.98 ± 1.05	3.88 ± 0.76
Cerebellum	4.76 ± 1.56^{ab}	3.60 ± 0.31^{ab}	2.82 ± 0.22^{b}	2.79 ± 0.64^{b}	6.19 ± 1.72^{a}	3.58 ± 0.18^{ab}	2.36 ± 0.18^{b}
Amygdala	6.20 ± 1.48	3.14 ± 0.09	3.87 ± 0.86	3.72 ± 0.76	6.50 ± 0.44	6.54 ± 2.10	3.84 ±1.04
Hippocampus	3.76 ± 0.50^{ab}	2.81 ± 0.15^{ab}	3.72 ± 0.38^{ab}	2.63 ± 0.39^{ab}	4.77 ± 1.52^{a}	2.00 ± 0.23^{b}	3.73 ± 0.92^{ab}
Hypothalamus	3.40 ± 0.61	2.08 ± 0.40	2.82 ± 0.92	2.74 ± 0.58	3.55 ± 0.85	2.85 ±0.51	2.69 ± 0.81
Cerebral cortex	3.53 ± 0.14^{ab}	3.57 ± 0.62^{ab}	2.71 ± 0.57^{bc}	2.49 ± 0.15^{bc}	1.73 ± 0.28^{c}	3.51 ± 0.53^{ab}	4.27 ± 0.41^{a}
Midbrain	6.80 ± 0.45^{a}	3.12 ± 0.39^{c}	6.06 ± 0.46^{a}	4.01 ± 0.01^{bc}	5.66 ± 1.21^{ab}	3.48 ± 0.40^{c}	3.64 ± 0.69^{c}
Medulla oblongata	5.15 ± 0.94	4.89 ± 0.51	3.98 ± 0.50	3.57 ± 0.65	3.12 ± 0.29	3.29 ± 0.83	3.02 ± 0.90

[a, b, c] Means on the same row with different superscripts differ significantly ($p < 0.05$)

4. DISCUSSION

The brain's neurotransmitters are of vital importance to the physiological integrity and consequently, productivity of the animal [31] and their activities are usually under the control of several enzymes and multi-enzyme systems [32]. One of such enzymes is acetylcholinesterase (AChE; EC 3117) which participates in cholinergic neurotransmission. It breaks down acetylcholine which terminates the neurotransmission process [33, 34]. Acetylcholine (ACh) - a neurotransmitter at all synapses of the autonomic nervous system is involved in almost all body actions and organ functions [35]. Earlier report [7] that dietary FB_1 at concentrations of $\geq$ 5 mg/kg significantly altered AChE activities in the brain regions and pituitary gland of growing pigs agree with lower activities of AChE observed in brain regions of rabbits exposed to diets containing $\geq$ 5 mg FB_1/kg in this study. The results revealed that the animals exposed to feeds containing $\geq$ 5.0 mg FB_1/kg (Diets C and D) might have suffered significantly from hypofunctions of these brain regions. Tsakiris et al. [36] reported that AChE inhibition could affect ACh hydrolysis and its consequences in nervous system functions, including hormone production and release.

Since the occurence of FB_1 is harmful to animal and human health, considerable research has been directed at finding methods to prevent the negative impact of the mycotoxin. Antioxidants have been reported [37] to reduce the toxic effects of mycotoxins in animals as most of the mycotoxins provoke oxygen free radical formation. The dose-dependent decreases in AChE activities in the brain regions of rabbits exposed to diets containing $\geq$5 mg FB_1/kg in this study were reversed in animals fed FB_1-contaminated diets supplemented with vitamin E (i.e., diets E, F and G). This is an indication of the protective roles that the antioxidant could play on the adverse impacts of FB_1 on AChE activities in the brain of the animal. The mechanisms by which vitamin E might provide this protection include its function as an antioxidant and its roles in anti-inflammatory processes [38-42]. The antioxidant action of vitamin E involves first of all inactivation of ROS such as superoxide anion radical, hydroxyl radical, hydrogen peroxide and singlet oxygen [43]. In addition, vitamin E has been shown to enhance the restoration of cholinesterase activity [44]. All these properties may have contributed to the ability of vitamin E in mitigating FB_1 effect on AChE. The brain AChE activity which was markedly reduced by FB_1 ingestion, but restored by supplementation with vitamin E showed that vitamin E supplementation might be useful in maintaining brain AChE activity at the normal level in animals exposed to diets contaminated with FB_1.

The observation that the mid-brain was one of the most active regions of the brain in terms of AChE activities and the cerebral cortex region having the least AChE activity in pigs [7, 34] agreed with the current study in which the mid-brain and cerebral cortex regions had the highest and lowest AChE activities, respectively.

The results of the present study revealed that treatment of FB_1-exposed rabbits with vitamin E protects the brain against FB_1-induced inhibition of AChE activity. Vitamin E is an essential nutrient and its antioxidant and anti-inflammatory role is well established in the literature [38-42]. Moreover, it has been reported that vitamin E can partially restore the hypofunction of the cholinergic system in aging [45] and also has an effect on memory retention through the activation of this system [46]. Several studies have shown that vitamin E can also act effectively to prevent peroxidation in biological systems via the inhibition of free radical formation [47]. Neurotoxicity associated with FB_1 exposure may be the result of a series of small perturbations in brain metabolism, and, in particular, of oxidative stress. This observation could at least in part be explained by the fact that free radical production can decrease brain AChE activity [48].

4. CONCLUSION

The potential of the vitamin E to combat the effect of the toxin on the brain regional AChE and the specific AChE activities reduced with increase in the dietary FB_1. This study has shown that supplementation of FB_1-contaminated diets with vitamin E protects the brain against FB_1-induced inhibition of AChE activity.

AUTHORS' CONTRIBUTION

All authors contributed equally in conducting the

experiment, analysis and interpretation of data and manuscript preparation. The final manuscript has been read and approved by all authors.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest (both personal and institutional) regarding specific financial interests that are relevant to the research work reported in this manuscript.

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