African Journal of Biotechnology

Volume 15 Number 9, 2 March 2016

ISSN 1684-5315

Academic Journals

ABOUT AJB

The African Journal of Biotechnology (AJB) (ISSN 1684-5315) is published weekly (one volume per year) by Academic Journals.

African Journal of Biotechnology (AJB), a new broad-based journal, is an open access journal that was founded on two key tenets: To publish the most exciting research in all areas of applied biochemistry, industrial microbiology, molecular biology, genomics and proteomics, food and agricultural technologies, and metabolic engineering. Secondly, to provide the most rapid turn-around time possible for reviewing and publishing, and to disseminate the articles freely for teaching and reference purposes. All articles published in AJB are peer-reviewed.

Contact Us

Editorial Office: ajb@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJB

Submit manuscript online http://ms.academicjournals.me/

Editorial Board

African Journal of Biotechnology

Table of Content: Volume 15 Number 9 2 March, 2016

ARTICLES

Nutritional status, lifestyle and knowledge of predisposing factors on hyperlipedemia among outpatients in Enugu State University Teaching Hospital (ESUTH), Nigeria 284
Nwamarah, Joy Ugo, Otitoju, Olawale and Otitoju, Grace Taiwo O.

Protective effect of *Haloxylon salicornicum* on hepatic and renal functions of Wistar rats exposed to aluminium 293
Fatima Zohra TAHARI, Monya LABLACK, Nadia AÏT HAMADOUCHE, Zineb TAHARI and Abdelkader AOUES

Histopathological effects of Cyperdicot and vitamin E supplementation on selected organs of *Clarias gariepinus* (Burchell, 1822) reared in a tropical fish farm in Nigeria 303
Odo, G. E., Agwu, J. E., Madu, J., Ezea, C. O., Nnamonu, E. I. and Eneje, V. O.

Cellulose degradation capabilities of dung beetle, *Euoniticellus intermedius*, larva gut consortia 315
Munamato Mabhegedhe, Karl Rumbold and Monde Ntwasa

HPLC quantification of phenolic content and assessment of methanolic extract of *Antiaris Africana* for toxicological study 320
Omotayo B. Ilesanmi, Tolulope M. Olaleye, Afolabi C. Akinmoladun and Taiwo T. Alawode

Physical and chemical properties of the acid protease from *Onopordum acanthium*: Comparison between electrophoresis and HPLC of degradation casein profiles 331
Malika Benkahoul, Meriem Benchiheub, Ines Bellil, Douadi Khelifi and Aicha Mechakra Maza

academicJournals

Vol. 15(9), pp. 284-292, 2 March, 2016
DOI: 10.5897/AJB2015.14961
Article Number: 1AA69C857396
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Nutritional status, lifestyle and knowledge of predisposing factors on hyperlipedemia among out-patients in Enugu State University Teaching Hospital (ESUTH), Nigeria

Nwamarah, Joy Ugo[1]*, Otitoju, Olawale[2] and Otitoju, Grace Taiwo O.[1]

[1]Department of Home Science, Nutrition and Dietetics, University of Nigeria, Nsukka, Enugu, State, Nigeria.
[2]Department of Biochemistry, Federal University, Wukari, Taraba State, Nigeria.

Received 2 September, 2015; Accepted 30 October, 2015

The growing trend of hyperlipidemia in most developing countries has resulted largely from changes in lifestyle, diet and lack of adequate exercise which have led to decreased life expectancy and burden of cardiovascular diseases. A purposive sample size of 206 out-patients was selected for this study after obtaining their due consents. Out of these, 108 (52.84%) were female and 98 (47.6%) males. Validated structured questionnaires were administered to collect basic socio-economic data from the respondents. Information obtained include drinking habits, medical history, and dietary habits. Data were presented by using descriptive statistics: frequencies and percentages. The anthropometric variables (weight and height) were analysed by using mean and standard deviation tools. Body mass index (BMI) was determined using the weight and height measurements. About 39.8% of respondents were within the age bracket (29-39 years), 25.7% had family history of diabetes mellitus, 25.2% hypertension and 11.7% had hypercholesterolemia. More than half (66.5%) of the respondents consumed alcoholic drinks. About 65.1% of the alcoholics, drink 1-3 times weekly and 46.1% took alcohol 4-6 times weekly while 14.5% drink daily. Some respondents who consumed groundnut, banana with groundnut, biscuit, buns, bread, meat pies, cakes or chicken pies with malt, juice or soft drink were 63.5%. Less than half (40.2%) of the respondents were overweight and about 37.4% of them were obese. About 45.6% of the respondents had a fair knowledge of hyperlipidemia, 40.3% had poor knowledge while 14.1% had a good knowledge. This study showed a strong evidence of poor knowledge of hyperlipidemia among out-patients attending Enugu State University Teaching Hospital (ESUTH), which may have influenced their lifestyle, dietary habit and subsequently their nutritional/health status.

Key words: Hyperlipidemia, nutritional status, diet, diabetes, cardiovascular diseases.

INTRODUCTION

Overweight and obesity are increasing at an alarming rate globally. World Health Organization projected that by 2020, at least 50% of all death will occur due to coronary heart disease (CHD) in most countries (Ahaneku et al., 2014; Lopez, 2004; Lopez et al., 2006). Hyperlipidemia is considered as a major risk factor in CHD all over the world. The epidemic of hyperlipidemia in developing countries has resulted in large part, from the economic

Table 1. BMI classification.

WHO popular (kg/m^2)	BMI Co-morbidities	Risk of classification	Description of classification
Class weight	Thin	Less than 18.5	Low (but risk of other clinical problems)
Normal	Normal	18.5 – 24.9	----------
Underweight	-------	Greater than 25	----------
Pre-obese	Overweight	25 – 29.9	Increased
Obese class I	Obese	30.0 – 34.9	Moderate
Obese class II	Obese	35.0 – 39.9	Severe
Obese class III	Moderate	Greater than 40	Very severe

growth and associated socio demographic changes that have occurred over recent decades. During this period, changes in lifestyle and diet have led to an increase in life expectancy and a greatly increased burden of cardio-vascular disease and other chronic diseases (Reddy and Yusuf, 1998).

Hyperlipidemia is a well-known and major risk factor for ischemic heart disease, as elevated levels of triglycerides, total cholesterol, low density lipoprotein cholesterol (HDL-C) are documented risk factors for atherogenesis (Osuji et al., 2010). It has also been shown that serum total cholesterol levels are continuously correlated with CHD risk over a broad range of cholesterol values in various populations throughout the world (Osuji et al., 2010). It is also strongly associated with hypertension and plays a crucial role in the development of cardiovascular disease, which has become a leading cause of death in most developing countries such as Nigeria (Ahaneku et al., 2014).

There is low awareness programme on cardiovascular diseases among people in the developing communities. Most people are not well informed on the causes and mode of prevention of these diseases. Similarly, the increasing level of consumption of junk foods in many households might have predisposed them to this ailment. The objective of this study was to examine the nutritional status, lifestyle and knowledge of predisposing factors on hyperlidemia among adults in Enugu State University Teaching Hospital (ESUTH) out-patients department.

MATERIALS AND METHODS

This study was carried out in Enugu State Nigeria, at the Enugu State University Teaching Hospital (ESUTH). The study design was a cross-sectional survey. The study population consisted of men and women who attended Enugu State University Teaching Hospital Out-Patients Ward in the year 2014. A purposive sample size of 206 out-patients was selected for this study after obtaining their due consents. Out of these, 108 (52.84%) were female and 98(47.6%) males. The sample size was calculated using the modified formula by Aloysius (1998):

$Ns= (Z/E)^2 x\ P\ (1-P)$

Where Ns = Sample size; Z= standard Z score (1.96 using a confidence level of 95%); E= proportion of sample error in a given population (5% prevalence); P = estimated prevalence of hyperlipidemia 15% in Zaria (Akuyam et al., 2010).

The sample size calculated using the formula is 195.9, the sample size was increased to 206 samples to make room for dropouts or incorrectly filled questionnaire.

Data collection

Questionnaire was designed to provide information on the socioeconomic status, family and medical history and drinking habits among these out-patients. Knowledge questions were drafted in the questionnaire. Information was collected on diet history (food frequency) by interview. Five experts of the Department of Home Science, Nutrition and Dietetics validated the questionnaire for data collection.

Anthropometric assessment

Height and weight measurements were taken using standard procedures.

Classification of body mass index (BMI)

The respondents' body mass indexes were collated by measuring their weights and heights; thereafter BMI values were computed and compared (WHO, 2002) as shown in Table 1. Respective body mass indices (BMI) were calculated with the following formula: BMI = weight (kg)/height^2 (m^2).

Data analysis

This was expressed using descriptive statistics: frequencies and percentages. Anthropometric variables (weight and height) were analysed by using mean and standard deviation.

*Corresponding author. E-mail: joy.nwamarah@unn.edu.ng.

Table 2. Demography and socio-economic status of out-patients of Enugu State University Teaching Hospital (ESUTH), Nigeria.

Variable	Frequency	Percentage
Age(years)		
18-28	35	17.0
29-39	82	39.8
40-50	41	19.9
51-60	32	15.5
Above 60	16	7.8
Total	206	100.0
Sex		
Female	108	52.4
Male	98	47.6
Total	206	100.0
Marital status		
Single	61	29.6
Married	140	68.0
Widowed	05	2.4
Total	206	100.0
Educational attainment		
No formal education	08	3.9
Primary	12	5.8
Secondary	36	27.8
Tertiary	150	72.8
Total	206	100.0
Occupation		
Civil servant	104	50.5
Business	29	14.1
Student	30	14.6
Trading	08	3.9
Tailor	04	1.9
Other	31	15.0
Total	206	100.0

RESULTS

The results of the socio-economic status of the respondents are shown in Table 2. The result showed that less than half (39.8%) of the respondents were in the age range of 29-39 years, 19.9% were within 40-50years, 17.0% were within the 18-28years, 15.5% were within 51-60 years and 7.8% were above 60 years. However, 52.4% of the respondents were females. More than half (68.0%) of the respondents were married, 29.6% were single and 2.4% where widowed. Majority of the respondents (72.8%) had tertiary education, 17.5% had only secondary school education, 5.8% had only primary school education while 3.9% had no formal education.

Table 3 shows the consumption pattern of alcohol among ESUTH out-patients. The result shows that 66.5% of the respondents take alcoholic drinks while 33.5% claimed not to directly consume alcoholic drink. Table 4 shows the number of times the respondents consumed alcohol. Daily palm wine and stout consumers were 6.8 and 5.8%, respectively. While 17.0 and 12.1% palm wine drinkers, consumed it 1-3 and 4-6 times weekly, respectively. Spirit (gin) was consumed by 11.7% of the respondents, 4-6 times weekly. Beer was consumed by 14.6 and 2.96%, 1-3 and 4-6 times weekly, respectively.

Table 5 shows the family medical history of the respondents. Less than half (42.0%) of the respondents' family members had diabetes mellitus, 30.1% had

Table 3. Alcohol consumption pattern among ESUTH out-patients.

Variables	Frequency	Percentage
Do you take alcohol?		
Yes	137	66.5
No	69	33.5
Total	206	100.0
***If yes which ones?**		
Beer	40	19.4
Stout	81	39.3
Wine	26	12.6
Spirit/gin	24	11.7
Palm wine	74	35.9
Local gin	14	6.8

*n = multiple responses.

Table 4. Frequency of alcohol consumption among ESUTH out-patient respondents.

*Alcoholic drinks	Daily frequency (%)	4-6 times/week frequency (%)	1-3 times/week frequency (%)
Beer	4(1.9)	6(2.9)	30(14.6)
Stout	12 (5.8)	18(8.7)	51(24.8)
Wine	---------	8(3.9)	18(8.7)
Spirit	---------	24(11.7)	---------
Palm wine	14(6.8)	25(12.1)	35(17.0)
Local gin		14(6.8)	--------
	(14.5)	(46.1)	(65.1)

*n = Multiple responses.

Table 5. Family medical history of ESUTH out-patient respondents.

Variables	Frequency	Percentage
***Disease suffered by family members**		
Diabetes mellitus	92	42.0
Hypertension	66	30.1
Hypercholesterolemia	37	16.9
Obesity	24	11.0
***If yes who?**		
Father	78	37.5
Mother	58	27.9
Relatives	72	34.6
***Age when disease developed**		
≤35years	23	8.8
36-44years	57	21.8
45-54years	43	29.5
55-65years	41	21.2
>65years	33	12.6

Table 5. Contd.

***Diseases the Respondents had**		
Diabetes mellitus	57	25.7
Hypertension	56	25.2
Hypercholesterolemia	26	11.7
Obesity	83	37.4

*n = Multiple responses.

Table 6. Meal consumption pattern of the out-patient respondents.

Variable	Frequency	Percentage
No of times respondents eat/day		
Twice	27	13.2
3times	167	81.0
>3times	12	5.8
Total	206	100.0
Do skip meals		
Yes	27	13.2
No	179	86.8
Total	206	100.0
Snack consumption		
Yes	198	96.1
No	8	3.9
Total	206	100.0
***Types of snacks**		
Biscuits	83	40.3
Groundnuts	63	30.6
Banana with groundnuts	120	58.3
Combinations of meat pies or cakes or chicken pies with malt or soft drinks	64	31.1
Buns	53	25.7
Breads	57	27.7
Fruits	50	24.3
African salad (*abacha*)	39	18.9
Bambara groundnut (*okpa*)	74	35.9
Corn meal	39	18.9

*n = Multiple responses

hypertension, 16.9% had hypercholesterolemia and 11.0% had obesity. A greater percentage of respondents' family member (37.5%) that suffered these diseases was their fathers, 34.6% were their relatives and 27.9% were mothers. About 39.8% were within the age range of 29-39 years while only 8.8% was ≤ 35years. A greater percentage of the respondents (37.4%) were obese, 25.7% had diabetes mellitus and 25.2% had hypertension while 11.7% had hypercholesterolemia.

Table 6 shows the food consumption pattern of the respondents. Majority of them (81.0%) ate thrice daily, 13.1% ate twice, while 5.8% ate >3 times daily. More than three quarter (86.8%) of respondents did not skip meals while only 13.2% did skip meals. Nearly all the respondents (96.1%) took snacks with soft drinks.

Table 7 reveals the dietary pattern of the respondents. Majority (86.4%) make use of salad dressing as well as 67.4% as salad cream. Types of spread mainly by respondents were butter (34.5%) and margarine (29.0%). Types of oil used in cooking are mainly pure groundnut

Table 7. Dietary pattern of the respondents.

Variables	Frequency	Percentage
Use of salad dressing		
Yes	178	86.4
No	28	13.6
Total	**206**	**100.0**
If yes, which one?		
Salad cream	120	67.4
Mayonnaise	58	32.6
Total	**178**	**100.0**
Type of spread		
Butter	69	34.5
Honey	14	7.0
Margarine	58	29.0
Peanut butter	28	14.0
Mayonnaise	15	7.5
Others	22	8.0
Total	**200**	**100.0**
Types of oil used in cooking		
Pure groundnut oil	123	68.7
Olive oil	4	2.2
Bleached palm oil	6	3.4
Unbleached palm oil	24	13.4
Pure soybean oil	22	12.3
Total	**179**	**100.0**
Form of vegetable oil used in cooking		
Congealed on cold weather	81	39.3
Uncongealed on cold weather	51	24.8
Both above	74	35.0
Total	**206**	**100.0**
Number of times one consumed fried foods		
Not at all	11	5.3
Once daily	30	14.6
2-3 times daily	9	4.4
Once per week	39	18.9
2-3times weekly	117	56.8
Total	**206**	**100.0**
Do you reuse the same oil to fry again?		
Yes	125	60.7
No	81	39.3
Total	**206**	**100.0**
Snack consumption		
Yes	198	96.1
No	8	3.9
Total	**206**	**100**
Types of snacks		
Biscuit	83	13.8
Groundnut	63	10.4
Banana with Groundnut	120	19.9
Meat pies, cake, chicken pie with malt, juices or soft drinks	64	10.6
Buns	53	8.8

Table 7. Contd.

Breads	57	9.5
Fruits	50	8.3
African salad (Abacha)	39	6.5
Corn meal (Agidi).	74	12.3

*n = Multiple responses.

Table 8. Body mass index (BMI) of the respondents.

Body mass index (BMI)	Frequency	Percentage
Underweight	8	3.9
Normal	85	41.3
Overweight	83	40.2
Obesity I	11	5.3
Obesity II	16	7.8
Obesity III	3	1.5
Total	206	100.0

Table 9. Knowledge level on hyperlipidemia of respondents.

Knowledge level	Frequency	Percentage
Poor (0-39%)	83	40.3
Fair (40-69%)	94	45.6
Good (>69%)	29	14.1
Total	206	100.0

oil (68.7%) and unbleached palm oil (13.4%). Mostly (39.3%) used vegetable oil that congeals under cold weather while 35.0% used both congealed and uncongealed vegetable oil in cooking. Fried foods were consumed 2-3 times weekly by 56.8% while 5.3% only do not consume same. The reusage of same oil used for frying were by majority (60.7%). Snack consumption was by 96.1% respondents. Banana and groundnut were the snacks mostly (19.9%) consumed by respondents while 6.5% consumed African salad (abacha).

Table 8 shows the BMI of the respondents. Less than half (41.3%) were normal, 40.2% were overweight, 7.8% were obese (class II), 5.3% were obese (class I), 3.9% were underweight, while 1.5% were obese (class III). Figure 1 shows that females had higher prevalence (34.73 and 31.13%) for overweight and obesity, respectively.

In Table 9, the knowledge levels of the respondents on hyperlipidemia were examined. About 46% of respondents had a fair knowledge, 40.3% had poor knowledge and 14.1% had good knowledge of hyperlipidemia. Figure 1 revealed prevalence of 34.73% overweight and 31.13% obesity amongst the female than the male respondents. Figure 2 also shows the knowledge levels of the respondents according to their education attainment. More (19.53%) of the respondents that completed tertiary school had good knowledge while none of the respondents that had no formal education had good knowledge. The trend was similar for those who had poor knowledge.

DISCUSSION

Recent rise in the epidemiology of obesity and overweight among the black population gives a lot of concern for nutritionist and health care managers (de Onis et al., 2010; Adeboye et al., 2012). Although lots of factors including environmental and nutritional factors have been adduced to contribute to this prevalence (Adeboye et al., 2012; Steyn and McHiza, 2014), this study examined alcoholic intake and how it contributed to many health challenges among the general Nigeria population.

Majority of the respondents were below 51 years. This by implication explains that most of the subjects were in their middle age. This is similar to a work done by Skylar (2004) where it was shown that most of the respondents were within similar age group. Slightly more than half of the subjects were civil servants. Sedentary work has a predisposing factor on hyperlipidemia.

The study also revealed that a greater percentage (42.0%) of the respondents had family history of diabetes followed by hypertension. This is in agreement with earlier study by Njeleka et al. *(*2009) among adults in an urban east African setting where it was reported that cardiovascular disease risk factors, including hypertension, hyperlipidemia and diabetes mellitus were prevalent among family members. Gordon and Margaret (2002) in their work also reported that diabetes mellitus runs in certain families and it sometimes co-exist with other cardiovascular related diseases.

Alcohol abuse can certainly lead to obesity. It has been reported that alcohol intake may give an additional 1000 to 3000 calories per day apart from the calories gained from the diet (Traversy and Chapu, 2015). In this study, 66.5% of the respondents consumed alcoholic drink (stout and palm wine) and a few others took other brand of alcoholic drinks. Detailed breakdown showed that 11.6% of the respondents took alcohol daily and 36.7% took alcohol 4-6 times while 51.6% consumed alcohol 1-3

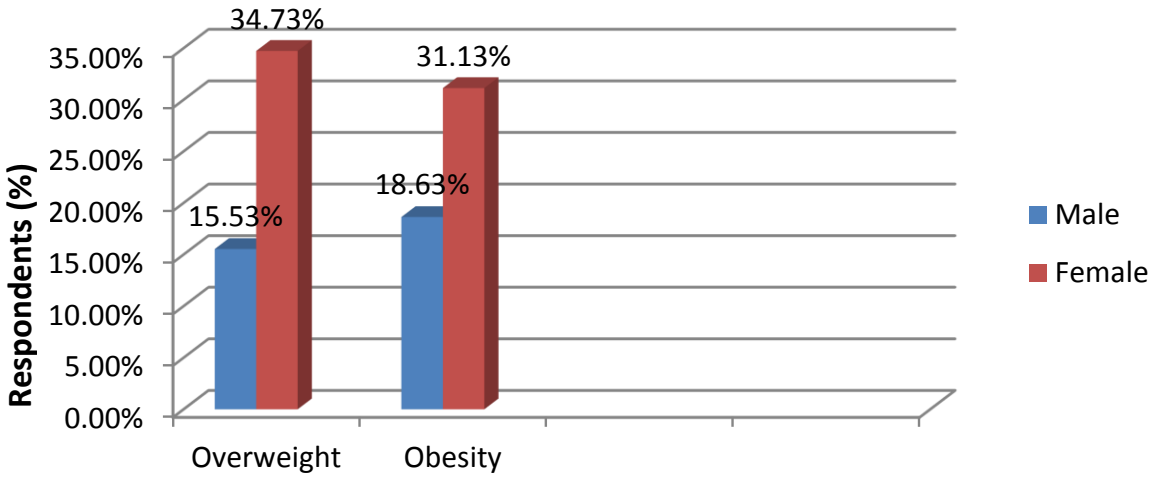

Figure 1. Prevalence of overweight and obesity according to sex.

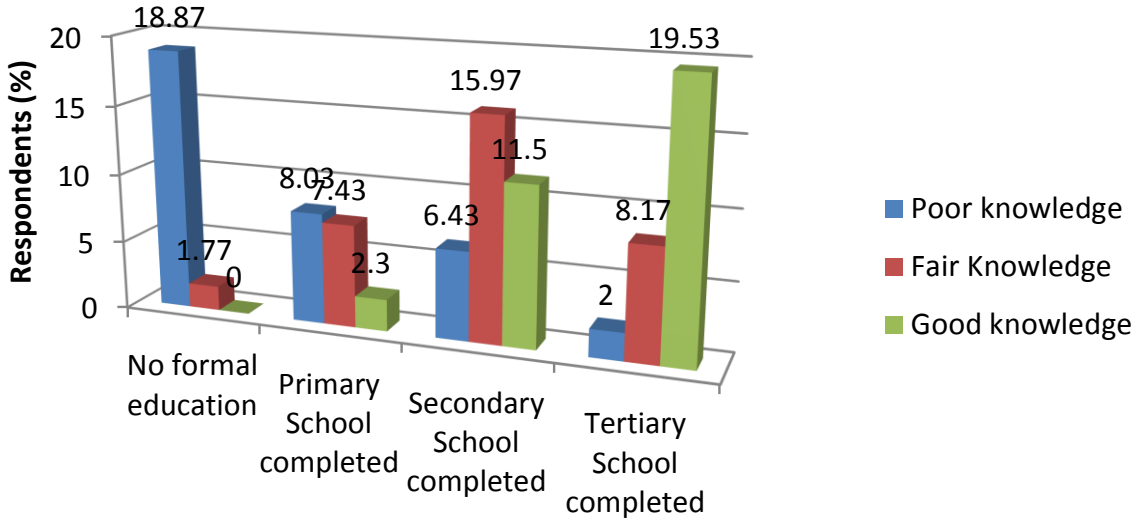

Figure 2. Knowledge level of respondents according to education attainment.

times weekly. This practice may lead to alcohol abuse which is a major risk factor of hyperlipidemia.

Nearly all the respondents (96.1%) consumed snacks. More than half (63.5%) of the respondents consumed biscuit, groundnut and banana, meat pies, cakes, chicken pies with malt juice or soft drink and buns. This practice should not be encouraged for it reflects the poor eating habits of the respondents. These foods are rich in saturated fat and trans fatty fats which may predispose them to hyperlipidemia in the near future. According to Fleming et al. (2013), there is a link between dietary pattern of the people and hyperlipidemia, they recommended from their work that reduction of saturated fat daily intake of 7% calories should be encouraged.

Less than half (40.2%) of the respondents were overweight and about 14% of them were obese. This is lower than the findings of Daousi et al. (2006) which showed that 86% of the studied subjects were obese. It is also lower than the findings of the study done in Oyo State, Nigeria by Fadupin et al. (2004) where it was found that 83.0% of the out-patients were either overweight or obese.

Some studies have reported a positive association between measure of overweight and obesity and components of lipid profile (Perry et al., 1997). According to Walker et al. (2001), the high proportion of overweight and obesity among respondents in their study may be attributed to socio-cultural factors, namely patterns of work activities, sedentary lifestyle and cultural standard of physical attractiveness in African countries. The higher

overweight and obesity in females than in males gave credence to the findings of Fadupin et al. (2004) where they found out that overweight and obesity were higher in females than males. In developing countries, changes in occupation type and socio-cultural factors that affect physical activity, particularly among women, are related to why there are more overweight and obese women in these countries when compared with men (Kanter and Caballero, 2012).

Poor knowledge was observed in 40 while 46% had fair knowledge of hyperlipidemia, this implies that there is low awareness of the risk factors of hyperlipidemia among the respondents. The poor knowledge was higher (18.87%) among those who had no formal education, good knowledge of hyperlipidemia was higher (19.53%) among those that had tertiary education. This shows that education of the respondents may have had a strong link to the knowledge acquisition of the respondents.

Conclusion

Although, majority of the respondents had fair knowledge of the relationship between hyperlipidemia, diabetes and cardiovascular diseases, the study still showed strong evidence and high prevalence of the risk factors of hyperlipidemia in ESUTH out-patients. This is reflected in their poor drinking habits, that is, alcohol abuse by out-patients. Majority of respondents were either obese or overweight and it was higher in females than male patients. A good number of the respondents had chronic diseases which is also a predisposing factor caused by hyperlipidemia.

Conflict of interests

The authors have not declare any conflict of interest.

REFERENCES

Adeboye B, Bermano G, Rolland C (2012). Obesity and its health impact in Africa: a systematic review. Cardiovasc. J. Afr. 23(9):512–521.

Ahaneku GI, Ahaneku JE, Osuji CU, Oguejiofor CO, Opara PC (2014). Lipid patterns, alcohol intake and BMI of adult Nigerians in a sub-urban slum in Enugu, Nigeria. Pan Afr. Med. J. 18:37.

Akuyam SA, Anaja PO, Isah HS, Aliyu IS, Yusuf R (2010). Lipid abnormalities: A case study of Ahmadu Bello University Teaching Hospital, Zaria, Nigeria. Ann. Niger. Med. 4(1):10-3.

Daousi C, Casson IF, Gill GV, MacFarlane IA, Wilding JPH, and Pinkney JH (2006). Prevalence of obesity in type 2 diabetes in secondary care: association with cardiovascular risk factors. Postgrad. Med. J. 82(966):280-284.

de Onis M, Blössner M, Borghi E (2010). Global prevalence and trends of overweight and obesity among preschool children. Am. J. Clin. Nutr. 92:1257-1264.

Fadupin G, Joseph E, Keshinro O (2004). Prevalence of obesity among type 2 diabetics in Nigeria a case study of patients in Ibadan, Oyo State, Nigeria. Afr. J. Med. Med. Sci. 33:381–384.

Fleming JA, Holligan S, Kris-Etherton PM (2013). Dietary Patterns that Decrease Cardiovascular Disease and Increase Longevity. J. Clin. Exp. Cardiolog. S6: 006.

Gordon M, Margaret K (2002). Perspectives on Nutrition. New York City, USA: McGraw-Hill companies, Inc.

Kanter R, Caballero B (2012). Global gender disparities in obesity: a review. Adv. Nutr. 3:491-498.

Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJL (2006). Measuring the Global Burden of Disease and Risk Factors, 1990–2001. In: Global Burden of Disease and Risk Factors (2006). (eds) AD Lopez, Mathers CD, Ezzati M, Jamison DT, Murray CJL. Washington (DC): World Bank.ISBN-10: 0-8213-6262-3ISBN-13: 978-0-8213-6262-4.

Lopez R (2004). Urban Sprawl and Risk for Being Overweight or Obese. Am. J. Public Health. 94(9):1574–1579.

Njeleka M, Mpembent R, Muhih A, Milgillche N, Speigelman D, Hertzmark E (2009). Gender-related differences in the prevalence of cardiovascular disease risk factors and their correlates in urban Tanzania. BMC Cardiovasc. Disorders 9:30.

Osuji CU, Nzerem BA, Meludu S, Dioka CE, Nwobodo E, Amilo GI (2010). The prevalence of overweight/obesity and dyslipidemia amongst a group of women attending "August" meeting. Niger. Med. J. 51:155-159.

Perry A, Applegate E, Allison M, Miller P, Signonle J (1997). Relation between the anthropomtric measures of the fat distribution and cardiovascular risk factors in overweight pre and post-menopausal women. Am. J. Clin. Nutr. 66 (4): 829-836.

Reddy K, Yusuf S (1998). Emerging epidermic of cardiovascular disease in developing countries. Circulation 97:596-601.

Skylar J (2004). Diabetes mellitus: pathogenesis and treatment strategies. J. Med. Chem. 47 (17):4113-4117.

Steyn NP, McHiza ZJ (2014). Obesity and the nutrition transition in Sub-Saharan Africa. Ann. N Y Acad. Sci. 1311:88-101.

Traversy G, Chapu JP (2015). Alcohol Consumption and Obesity: An Update. Curr. Obes. Rep. 4(1):122-130.

Walker A, Adam BF (2001). World pandemic of obesity: The situation in South African populations. Public Health 115 (6):368-372.

World Health Organization (WHO). (2002). The world health report. Geneva.

academicJournals

Vol. 15(9), pp. 293-302, 2 March, 2016
DOI: 10.5897/AJB2015.15037
Article Number: 1EF202457398
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Protective effect of *Haloxylon salicornicum* on hepatic and renal functions of Wistar rats exposed to aluminium

Fatima Zohra TAHARI[1]*, Monya LABLACK[1], Nadia AÏT HAMADOUCHE[1], Zineb TAHARI[2] and Abdelkader AOUES[1]

[1]Laboratoire de Biotoxicologie Expérimentale, Bio-dépollution, Phytoremédiation, Université Oran 1 - Ahmed Ben Bella, Algérie.
[2]Laboratoire de Biologie du Développement et de la Différenciation, Université Oran 1 - Ahmed Ben Bella, Algérie.

Received 11 October, 2015; Accepted 25 January, 2016

Aluminium is present in some manufactured medicines and foods. It is known that aluminium causes oxidative stress. Therefore, the present study was undertaken to determine the effectiveness of *Haloxylon salicornicum* extract in modulating aluminium chloride ($AlCl_3$) induced oxidative stress in rats. Male rats (40 to 50 g) were divided into four groups of six animals each. The experimental protocol was based on the administration of $AlCl_3$ (30 mg/kg/body weight) intraperitoneally (ip) every 5 days for 15 days. The groups treated with the plant receive daily dose of 0.05 g/kg/body weight. Increased level of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), urea, and creatinine in serum indicated hepatic and renal dysfunction. The variation of catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), and lipid peroxidation (thiobarbituric acid reactive substances, TBARS) were assessed. These parameters indicated the extent of oxidative damage in liver and kidney, thus confirming the histology results in liver and kidney. It was found that the consumption of *H. salicornicum* extract protects the liver and kidney against aluminium chloride toxicity. The aim of the present study was to evaluate the protective effect of the *H. salicornicum* extract on the damages caused by administration of aluminium chloride ($AlCl_3$) in young rats.

Key words: Aluminum chloride, Wistar rats, *Haloxylon salicornicum*, lipid peroxidation, superoxide dismutase (SOD), reduced glutathione, catalase.

INTRODUCTION

Aluminium is an abundant metallic element on the earth; it is used highly because of its excellent properties (Bai et al., 2012). Biological function of aluminium is not understood very well (Farina et al., 2002; Joshi et al., 2013). Aluminum presents 8% of all mineral components of earth. There is not a maximum level fixed for aluminium in food (AFSSAPS, 2011).

It can be used as a food additive and in materials in

*Corresponding author. E-mail: fatimazohratahari@yahoo.fr

contact with foodstuffs (AFSSAPS, 2011), used as water clarifying agent (coagulation, flocculation, etc.). It is found in water intended for human consumption with a maximum concentration of 0.2 mg/L (AFSSAPS, 2011). This metal is also used in the preparation of some drugs (Ochmanski and Barabasz, 2000; Turkez et al., 2010). In mammals, aluminum is accumulated in kidney, liver, heart, blood, bones, and brain (Sanchez-Iglesias et al., 2007; Gonzalez et al., 2009; Al-Kahtani, 2010). The accumulation of aluminum in liver generates significant lesions (Nikolov et al., 2010; Shati and Alamri, 2010). Kidney prevents the accumulation of aluminium in different organs, this by urinary excretion (Shirley et al., 2004; Stoehr et al., 2006). However, kidney can be very vulnerable to the nephrotoxic effects of aluminium (Mahieu et al., 2003; Stoehr et al., 2006).

Aluminium can produce free radicals in the body. It induces a toxic effect due to its ability to transfer electrons (Taus et al., 2013). These free radicals resulting from aluminum can affect cell integrity, producing the peroxidation of the lipids in the intracellular membranes, and the cross-linking with the macromolecules in the membranes (Taus et al., 2013). Aluminium is known to affect the permeability of cellular membranes, of sub-cellular organelles, the structure and functions of proteins and the structure of nucleic acids (Taus et al., 2013).

Plants have always play a major role in treatment of human and animal diseases. Medical plants are a therapeutic resource much used in the traditional population of the world specifically for health care (Kadham, 2008).

Haloxylon salicornicum is a desert plant which belongs to the family of Chenopodiaceae which includes 100 genus and 1200 species (Ferheen et al., 2005). Most of the species of this family are weedy and grow in unfertile areas of soil (Ferheen et al., 2005). *H. salicornicum* is a diffuse shrub with a lot of branches, woody stem, 25 to 60 cm tall. It is widely distributed in Northern Africa and Asia, in both temperate and tropical regions (Ashraf et al., 2012). This plant contains, among other chemical constituent such as sodium (28.48%), potassium (9.49%), carbonate (61.06%), alkaloid, saponin and phenol (Al-Saeed, 2002). This plant was used effectively in folk medicine as diuretic (Al-Saeed, 2002), anti-ulcer (Shahana et al., 1990), hypoglycemic and anti-microbial (Abbas et al., 2006). Ahmad and Eram (2011) have shown that this plant has a hepatoprotective effect.

The aim of the present study was to evaluate the protective effect of the *H. salicornicum* extract on the damages caused by administration of aluminium chloride ($AlCl_3$) in young rats.

MATERIALS AND METHODS

Plant

The plant was collected from May to April in El-bayadh region of Southern Algeria. Aerial parts of *H. salicornicum* were air-dried at room temperature for 30 min. The dried plants were collected and ground to fine powder.

Preparation of aqueous extract

The powdered aerial parts of *H. salicornicum* (10 g) were extracted with boiled water (100 ml) for 20 min. After this step, the decoction was filtered and was freeze dried. The yield of this extraction is 13.01%.

Animals

Wistar rats were used as an experimental model to evaluate the effect of aluminium on liver and kidney. Aluminium chloride ($AlCl_3$) was administered intraperitoneally to rats at a dose of 30 mg/kg/body weight, every five days for 15 days. The lyophilized extract of *H. salicornicum* is dissolved in distilled water. The plant is administered daily to the rats using a feeding tube at a dose of 0.05 g/kg/body weight. Wistar rats (40 to 50 g) were randomly distributed in four groups containing 6 rats each: group (control): control rats were given nothing; group (Al): animals were given three injections of $AlCl_3$ (30 mg/kg/body weight); group (Al+P): rats received three injections of $AlCl_3$ (30 mg/kg/body weight) in addition to the plant (0.05 g/kg/body weight); group (P): animals were given only the plant (0.05 g/kg/body weight).

Rats were sacrificed after 5 days of the last dose and blood was collected in hemolysis tube. Liver and kidney were rinsed with saline solution (0.9% NaCl). For evaluation of oxidative status, liver and kidney were homogenized in suitable buffers: in 0.1 M phosphate buffer (pH 7.1) for superoxide dismutase (SOD), CAT, and reduced glutathione (GSH) in 1.15% KCl for thiobarbituric acid reactive substances (TBARS). Some portions of liver and kidney were fixed in formalin for histological study.

Biochemical parameters

Aspartate aminotransferase (AST), alanine aminotransferase (ALP), lactate dehydrogenase (LDH), creatinine, and urea were measured using Chronolab kits (Spain). Biochemical parameters were estimated in serum.

Catalase activity (CAT)

CAT was assayed colorimetrically at 620 nm, expressed as µmoles of H_2O_2 consumed/min/mg/protein (Sinha, 1972). The reaction mixture of 1.5 ml contained 1.0 ml of 0.01 M pH 7.0 phosphate buffer, 0.1 ml of homogenate, and 0.4 ml of 2 M H_2O_2. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent, 5% potassium dichromate, and glacial acetic acid was mixed in 1:3 ratios.

Lipid peroxidation (TBARS)

Lipid peroxidation was assessed by measuring substances which react with TBARS (Yagi, 1976). 125 µl of sample, 50 µl of TBA, and 125 µl of trichloroacetic acid-butylated hydroxytoluene (TCA-BHT) were mixed. After stirring, incubation was done at 80°C (10 min) and centrifugation was done (100 *g* for 10 min). The supernatant was removed. 200 µl of the supernatant were mixed with 40 µl of HCl (0.6 M) and 160 µl of Tris-TBA (26 mM Tris, 120 mM thiobarbituric acid). After stirring, and incubation at 80°C (10 min), the optical density was measured at 530 nm. The quantity of

Table 1. Levels of ALT, AST, and LDH in serum.

Group	ALT (U/L)	AST (U/L)	LDH (U/L)
Control	5.32±1.02	21.25±3.71	200.92±20.00
Al	21.17±1.11***	101.57±71.02***	548.92±7.15***
Al +P	12.53±0.26*§§§	41.22±2.36**§§§	267.33±21.39*§§
P	5.12±0.17§§§	18.66±0.82*§§§	173.26±67.17§

*Comparison with the control group. §Comparison with Al group.

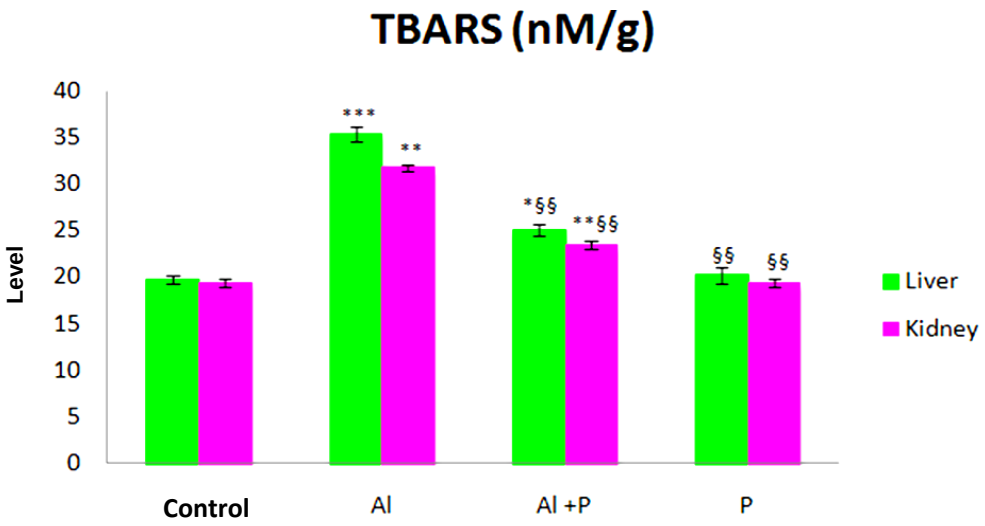

Figure 1. Levels of TBARS in liver and kidney. *Comparison with the control group. §Comparison with Al group.

TBARS was calculated using an extinction coefficient of 156 mM^{-1} cm^{-1}.

Reduced glutathione (GSH)

GSH was measured by the method of Ellman (1959). Samples were deproteinized with 5-5 'dithiobes (2-nitrobenzioc acid), and the optical density was measured at 412 nm.

Superoxide dismutase (SOD)

SOD was assessed using assay kit provided by Cayman (Chemical, USA).

Histological study

The sections were taken from liver and kidney and they were fixed in a formalin solution 1:10 to pH 7, dehydrated in acetone, clarified in xylene, and finally embedded in paraffin. Histological sections were cut at 3 µm using a microtome. The samples were stained with hematoxylin-eosin, and observed with an optical microscope.

Statistical analysis

The data were analyzed using Student's *t*-test. At $p < 0.05$, the results were considered significant. Values are expressed as the mean ± standard error.

RESULTS

Liver

The liver function is assessed by measuring of transaminases and LDH activities. AST, alanine aminotransferase (ALT), and LDH activities in the group (Al) were increased highly significantly ($p<0.001$) as compared to the group (control) (Table 1). A significant decrease of these activities is recorded in the group (Al+P) as compared to group (Al). The comparison between groups (control) and (P) shows that there was no significant difference except for AST (Table 1). Activities decreased highly significantly for ALT and AST ($p<0.001$), and significantly for LDH ($p<0.05$) in group (Al+P) in relation to the group (Al) (Table 1).

TBARS (Figure 1) and GSH (Figure 2) are higher in group (Al). The difference between groups (control) and (Al) is highly significant ($p<0.001$) for TBARS and very significant ($p<0.01$) for GSH. On the other hand, the comparison between groups (Al) and (Al+P) is very significant

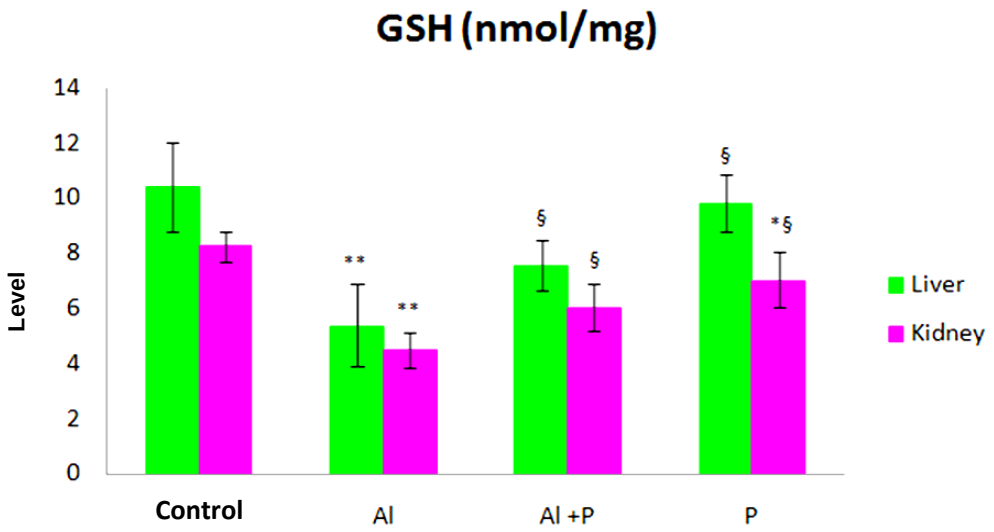

Figure 2. Levels of GSH in liver and kidney. *Comparison with the control group. §Comparison with Al group.

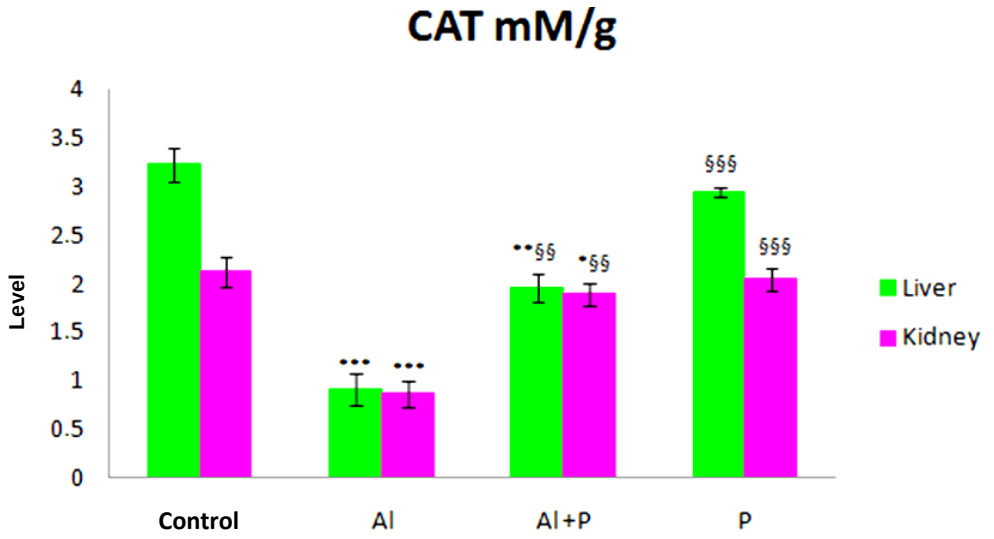

Figure 3. Levels of CAT in liver and kidney. *Comparison with the control group. §Comparison with Al group.

significant ($p<0.01$) for TBARS (Figure 1) and significant ($p<0.05$) for GSH. In group (P), TBARS and GSH (Figure 2) decreased.

The CAT (Figure 3) and SOD (Figure 4) activities are decreased in group (Al). The difference between groups (control) and (Al) is highly significant ($p<0.001$) for CAT and very significant ($p<0.01$) for SOD. The comparison between groups (Al) and (Al+P) is very significant ($p<0.01$) for CAT (Figure 3) and significant ($p<0.05$) for SOD (Figure 4).

Figure 5a and d presents regular histological structure of the liver tissue of control rats with a granular cytoplasm, central core, and the open sinusoidal spaces of hepatocytes. Exposure to aluminium (Figure 5b) causes a loss of the arrangement of the hepatocytes with a degeneration of membrane, nucleus, and cytoplasm. Histological observations of liver of rats treated with *H. salicornicum* illustrate an improvement in some areas and a normal sinusoidal space (Figure 5c).

Kidney

Creatinine and urea were examined to verify the renal

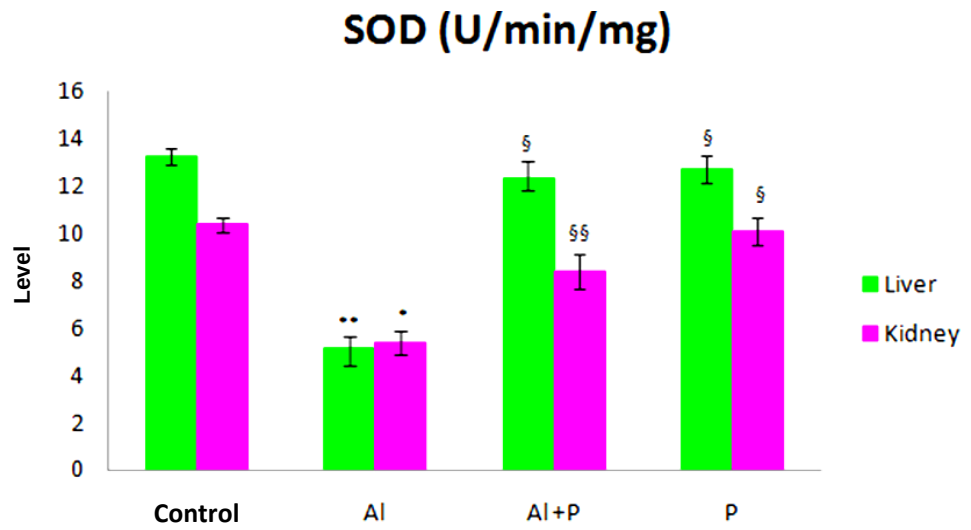

Figure 4. Levels of SOD in liver and kidney. *Comparison with the control group. §Comparison with Al group.

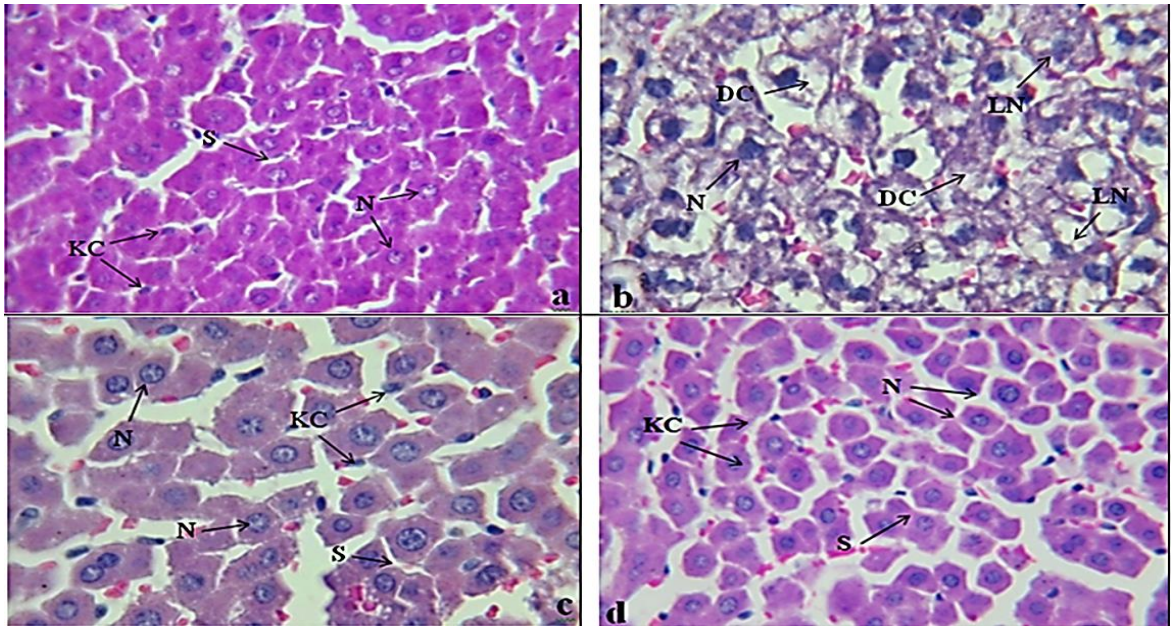

Figure 5. Optic microscopy sections of liver of Wistar rats stained using the haematoxylin-eosin method (400×). **(a)** Control. **(b)** Al. **(c)** Al+P. **(d)** P. DC, Degeneration in cytoplasm; KC, kupffer cells; LN, loss of nucleus; N, nuclei; S, sinusoidal spaces.

Table 2. Levels of creatinine and urea in serum.

Group	Creatinine (mg/L)	Urea (g/L)
Control	2.90±0.53	0.21±0.01
Al	6.30±0.26***	0.61±0.01***
Al +P	5.27±0.21*§§	0.51±0.02**§§§
P	3.17±0.06§§	0.26±0.03§§

*Comparison with the control group. §Comparison with Al group.

function. These parameters increased highly significantly (*p<0.001*) in rats treated with aluminum (Al) relative to control) (Table 2). The consumption of plant leads a remarkable improvement (Table 2). The comparison between groups (Al) and (Al+P) shows that creatinine decreased very significantly (*p<0.01*) and urea decreased highly significantly (*p<0.001*).

The difference between groups (control) and (P) is not

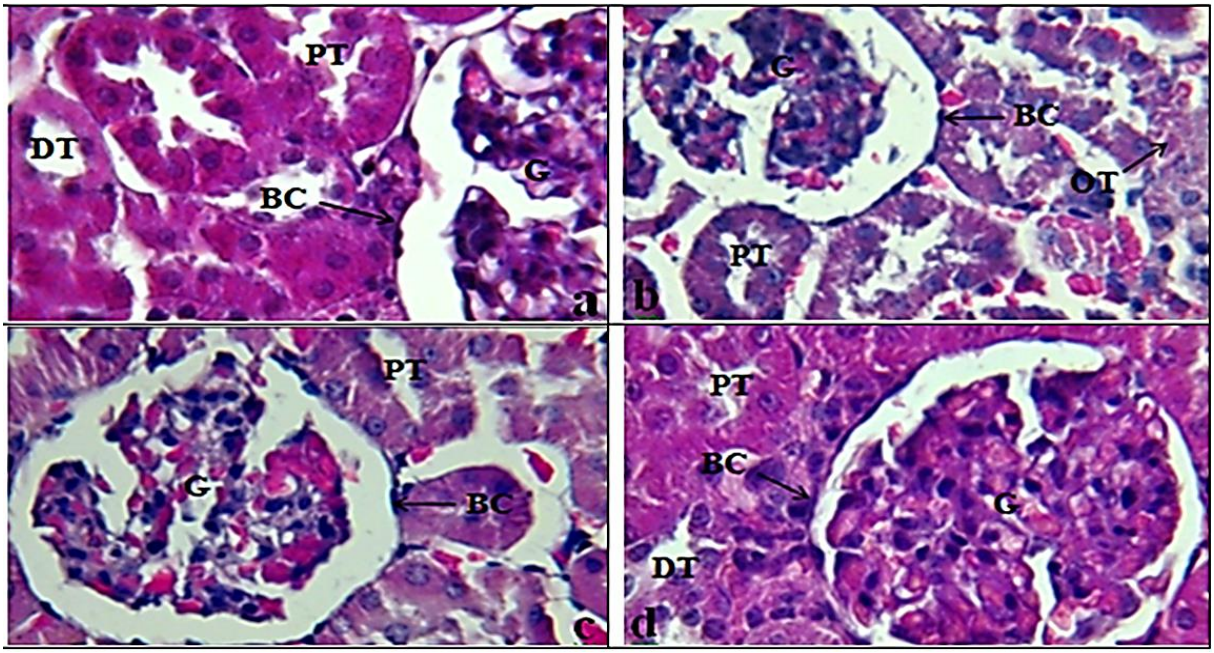

Figure 6. Optic microscopy sections of kidney (cortex) of Wistar rats stained using the haematoxylin-eosin method (400×). ×). **(a)** Control. **(b)** Al. **(c)** Al+P. **(d)** P. G, Glomerulus; DC, degeneration in cytoplasm; DT, distal tubules; PT, proximal tubules; OT, obstruction in renal tubules; LN, loss of nucleus; N, nuclei.

significant; however, the comparison between groups (Al) and (P) parameters decreased very significantly ($p<0.01$). The difference between groups (control) and (Al) is very significant ($p<0.01$) for TBARS (Figure 1) and significant ($p<0.05$) for GSH (Figure 2). The comparison between groups (Al) and (Al+P) is very significant ($p<0.01$) for TBARS (Figure 1) and significant ($p<0.05$) for GSH (Figure 2).

The comparison between groups (control) and (Al) is highly significant ($p<0.001$) for CAT (Figure 3) and significant ($p<0.05$) for SOD (Figure 4). The difference between groups (Al) and (Al+P) is very significant ($p<0.01$) for CAT and significant ($p<0.05$) for SOD. The consumption of plant only did not cause changes.

Figure 6a shows a normal histological structure of kidney cortex of control rats, Bowman capsules with an appropriate form, containing regular glomeruli and normal proximal and distal tubules with basal prominent nuclei (Figure 6a). Exposure to aluminium shows a kidney cortex with Bowman's capsule dilated, atrophic and destroyed glomerulus, tubular obstruction, and degeneration of tubular cells with pyknotic nuclei (Figure 6b). In group (Al+P), glomerulus adorned the greatest and not atrophied tubules are partially damaged. Despite these changes, the architecture of the renal cortex remained intact than that observed in the group treated with aluminum (Figure 6c). Kidney of the group treated with plant showed a normal histological structure (Figure 6d).

Kidney medulla of control rats shows that collecting tubules had a regular shape. The tubular cells are distinct and well defined (Figure 7a). Treatment with aluminium caused degeneration of tubular cells and loss of nuclei (Figure 7b). The group (Al+P) presents a degeneration of cytoplasm and tubular cells, however, the administration of the plant had induced a remarkable improvement in this tissue (Figure 7c). Kidney medulla of group treated with the plant shows a normal histological structure (Figure 7d).

DISCUSSION

The liver is a vital organ that is responsible for many of the processes that keep us alive. It drives a variety of metabolic substance and synthesizes a large number of enzyme (Bai et al., 2012). Hepatocytes are easily disintegrated by a variety of factors and harmful products (Bai et al., 2012). Exposure to high doses of aluminium may give an accumulation of this metal in the liver and cause alterations of the hepatic function (Nikolov et al., 2010). Degeneration, inflammation, and necrosis caused by hepatocyte damage can lead to an increase in the permeability of cell membranes. While AST and ALT are released into the blood through the cell membrane and their concentrations in the blood increases, ALT and AST are indicators of liver damage (Chinoy and Memon, 2001; El-Demerdash, 2004; Yeh et al., 2009; Shati and Alamri, 2010; Bai et al., 2012). In our study, the activities of AST and ALT in serum significantly increased in rats intoxicated with aluminium compared to controls. It was a

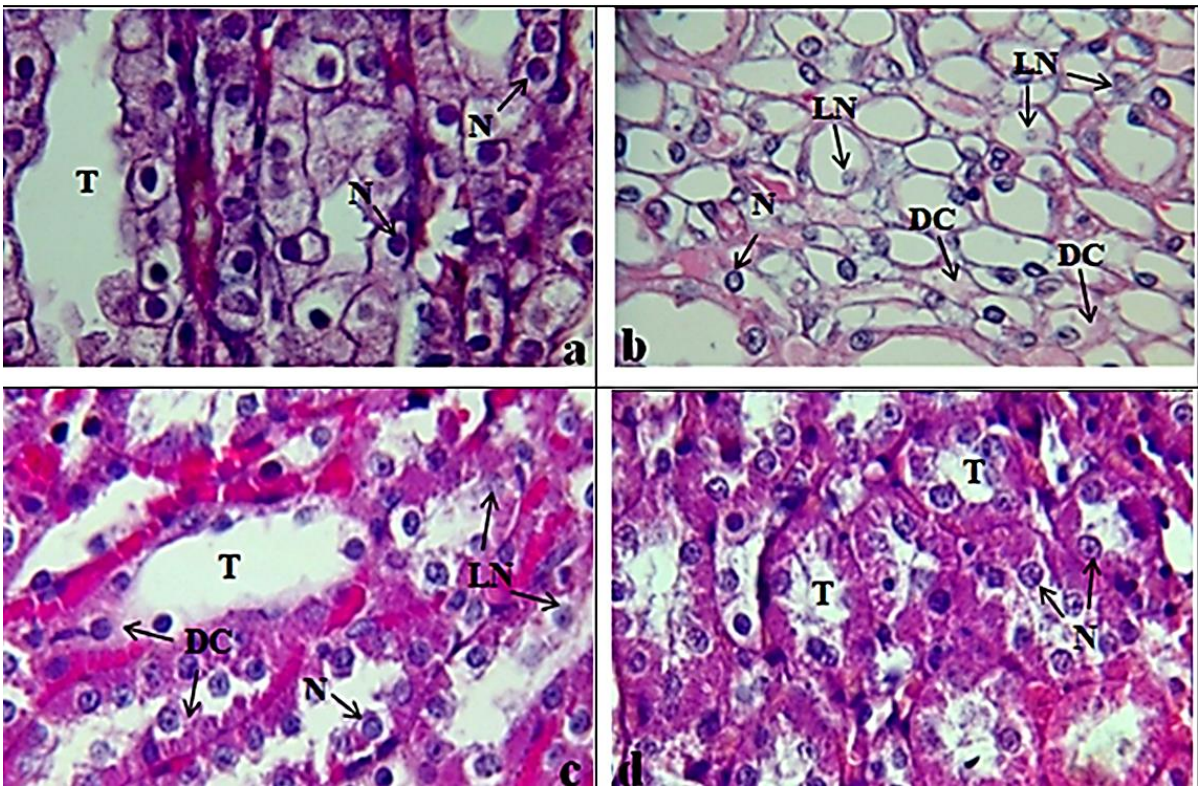

Figure 7. Optic microscopy sections of kidney (medulla) of Wistar rats stained using the haematoxylin-eosin method (400×). **(a)** Control. (**b)** Al. **(c)** Al+P; (**d)** P. DC, Degeneration in cytoplasm; T, tubules; LN, loss of nucleus; N, nuclei.

sign of deterioration of liver function, these results were compatible with results of Zhuo et al. (2007), Ma et al. (2008), Yeh et al. (2009), Bai et al. (2012), Bhadauria (2012), Denen et al. (2015), and Kalaiselvi et al. (2015).

In group (Al+P), enzyme activities of AST and ALT in serum significantly decreased; therefore, the liver damage induced by aluminium can be improved by the plant.

LDH is an enzyme that is used to evaluate tissue damage of the affected organ (Anane and Creppy, 2001; Bhatti et al., 2014). Serum LDH is a biomarker of liver tissue lesions (Suzuki et al., 1995) and kidney (Karadeniz Cerit et al., 2013). Cell necrosis leads to increase LDH in tissue and serum (Türkez et al., 2011). LDH release in the blood is an indicator of cell death and disintegration of the cell membrane (Lindell et al., 1996). LDH is a marker of aluminium toxicity (Anan and Creppy, 2001). In this study, serum LDH was very high in rats treated with aluminium; it increased significantly in rats intoxicated with aluminium as compared to controls. The results of this study are consistent with results of Chinoy and Memon (2001), El-Demerdash (2004), and Türkez et al. (2011).

Exposure to aluminium causes changes in renal function (El-Demerdash, 2004; Şener et al., 2007; Renugadevi and Prabu, 2010; Vijayaprakash et al., 2013). The assessment of harmful effects of aluminium is realized by measuring of biochemical parameters (Kowalczyk et al., 2004). Creatinine and urea are significant indicators of renal function (Al-Attar and Al-Taisan, 2010; Yakubu and Musa, 2012). According to the results of this study, the comparison between rats intoxicated with aluminium and control indicate a significant increase in both parameters. Yeh et al. (2009) reported that the plasma creatinine level is elevated in rats that received diets containing aluminium as compared to controls. According to Soudani et al. (2010), the increases of creatinine and urea concentrations indicate that the glomeruli and tubules are damaged.

Histology of organs is realized in order to verify the proper functioning of these organs. Microscopic observations in the control group (Figures 3a, 4a, and 5a) are similar to that of group (P) (Figures 3d, 4d, and 5d), respectively. These groups showed a normal structure of the liver and kidney, against group (Al) having abnormalities in the liver and kidney (Figure 3b, 4b, and 5b). Treatment with *H. salicornicum* induced an improvement in histology of liver and kidney (Figure 3c, 4c, and 5c). Therefore, *H. salicornicum* was found to reshuffle the damage led by aluminium.

Bhadauria (2012) cited that aluminium exposure caused loss of cord arrangement of hepatocytes and sinusoidal spaces with degenerated plasma membranes and nuclei; for central vein filled with debris, severe necrosis was also seen. Buraimoh et al. (2012) mentioned that in their

study, there were distorted sinusoids and congested central vein of the liver of the aluminium treated rats. Tehrani et al. (2013) reported that exposed animals showed cell damage in the liver. Al-Qayim and Mashi (2014) reported that light microscopic examination of kidney tissues sections revealed the negative and deleterious effects of aluminium chloride. Rats that received aluminium chloride showed infiltration of inflammatory cells and congestion of blood vessels. In spite of the material deposed in the lumen of tubules, there was enlargement of epithelial cells lining urinary tubules leading to occlusion of some urinary tubules and stenosis in others. Joshi et al. (2015) reported that aluminium induced kidney damage was illustrated not only by a significant alteration in serum metabolites, but also an altered histological feature in the kidney tissue reminiscent of some known diseases.

Accumulation of aluminum in organism can cause lesion directly or indirectly (Bai et al., 2012). It has been reported that aluminium exposure can increase lipid peroxidation rates (Anand et al., 2012). Lipid peroxidation is a chemical mechanism capable of disrupting the structure and the function of the biological membranes that occurs as a result of free radical attack on lipids (Yeh et al., 2009).

In this study, the rate of TBARS in liver and kidney of rats intoxicated is significantly increased as compared to the control rats. It was found out that the consumption of *H. salicornicum* induces a decrease in TBARS. From these results, it was shown that *H. salicornicum* has a potential protective effect against oxidative stress induced by aluminium. El-Demerdash (2004) reported that administration of 34 mg $AlCl_3$/kg body weight induced free radicals and increased TBARS level in liver. Yeh et al. (2009) reported that the level of TBARS of the plasma and liver was increased with the increasing dose of aluminium. Mahieu et al. (2009) administered to male rats 0.57 mg aluminium per 100 g of body weight; they mentioned that their results indicated a significant increase of TBARS level of kidney in Al-treated rats when compared with the control animals. Joshi et al. (2013) administered 100 mg/kg of aluminium by gavage to young and adult rats; they reported a significant increase the lipid peroxidation in rats intoxicated versus control in liver and kidney.

The oxidant (reactive oxygen species [ROS] and nitrogen species [RNS]) and antioxidants are balanced in healthy individuals (Joshi et al., 2013). If the balance is disrupted due to over production of ROS, oxidative stress may occur, which influences oxidative damage to organs (Joshi et al., 2013). GSH is an important non-protein thiol present in the animal cells (Özkaya et al., 2010). It is a key component of the cellular defense cascade against injury caused by ROS (Hsu et al., 2000). The level of GSH in tissues serves as an indicator of oxidative stress (Hsu et al., 2000). Some antioxidant enzymes, such as SOD and CAT, play a principal role in antioxidation and elimination of ROS (Jihen et al., 2009). SOD catalyzes the dismutation of O_2^- to H_2O_2 which is decomposed by CAT. However, H_2O_2 may react with O_2 to generate OH°, which is the initiator of the lipoperoxidation. The hydroxyl radical is highly toxic, because there is no specific enzyme system able to degrade it (Vamecq et al., 2004). According to our results, the comparison between groups (control) and (Al) demonstrate that the levels of GSH, SOD, and CAT decreased after exposure to aluminium. However, *H. salicornicum* consumption causes an increase in GSH levels, SOD, and CAT when compared with intoxicated rats. Based on the results of this study, it was concluded that *H. salicornicum* reshuffles oxidative stress caused by aluminium in liver and kidney.

These results are in good accordance with those obtained by Özkaya et al. (2010), Bai et al. (2012), and Joshi et al. (2013). Newairy et al. (2009) revealed that the level of TBARS was increased and the activities of GST, SOD, and CAT were decreased in liver, kidney, and brain of rat treated with 34 mg/kg body weight $AlCl_3$ daily for 70 days. Yeh et al. (2009) mentioned that aluminium depleted GSH stores and reduced CAT activities.

Conclusion

Oxidative stress results from an imbalance between prooxidants and antioxidants. Biochemical indicators were measured and the redox status in liver and kidney was evaluated to check aluminium effect in both organs. From our results, exposure to aluminum release oxidative stress and a dysfunction in liver and kidney. Histological study confirmed the results. The administration of plant *H. salicornicum* induced improvement in all parameters studied. It was concluded that the plant reshuffle the harmful effects of aluminium.

Conflict of Interests

The authors have not declared any conflicts of interest.

REFERENCES

Abbas B, Al-Saeed M, Othman R (2006). Value of anti-microbial of *Haloxylon salicornicum*. J. Babylon Univ.14:3.

AFSSAPS : Agence française de sécurité sanitaire des produits de santé (2011). Évaluation du risque lié à l'utilisation de l'aluminium dans les produits cosmétiques. Rapport d'expertise.

Ahmad M, Eram S (2011). Hepatoprotective studies on *Haloxylon Salicornicum*: a plant from Cholistan desert. Pak. J. Pharm. Sci. 24 (3):377-382.

Al-Attar AM, Al-Taisan WA (2010). Preventive effects of black seed (Nigella sativa) extract on Sprague Dawley rats exposed to diazinon. Aust. J. Basic Appl. Sci. 4:957-968.

Al-Kahtani MA (2010). Renal damage mediated by oxidative stress in mice treated with aluminum chloride: Protective effect of Taurine. J. Biol. Sci. 10(7):584-595.

Al-Qayim MAJ, Mashi S (2014). Renal effects of propolis and malic acid in Aluminum exposed male rats. App. Sci. Rep. 5(1):26-30.

Al-Saeed AH (2002). Study the effect of some extract of *Haloxylon* sp. on blood glucose level in normal and hyperglycemic rabbits induced by alloxan. M.Sc. thesis, College Science, University of Basrah.

Anand R, Kumari P, Kaushal A, Bal A, Wani WY, Sunkaria A, Dua R, Singh S, Bhalla A, Gill KD (2012). Effect of acute aluminum phosphide exposure on rats: a biochemical and histological correlation. Toxicol. Lett. 215(1):62-69.

Anane R, Creppy EE (2001). Lipid peroxidation as pathway of aluminium cytotoxicity in human skin fibroblast cultures: prevention by superoxide dismutase+catalase and vitamins E and C. Hum. Exp. Toxicol. 20:477-481.

Ashraf MA, Karamat M, Shahnaz K, Abdul W, Ismail Y (2012). Study of chemical and mineral constituents of *Haloxylon salicornicum* collected from Cholistan Desert, Bahawalpur, Pakistan. Wlfenia J. 19(10):306-327.

Bai CS, Wang F, Zhao HS, Li YF (2012). Effects of Subchronic Aluminum Exposure on Liver Function in Rats. J. Northeast Agric. Univ. 19(2):62-65.

Bhadauria M (2012). Combined treatment of HEDTA and propolis prevents aluminum induced toxicity in rats. Food Chem. Toxicol. 50:2487-2495. Bhatti GK, Sidhu IPS, Saini NK, Puar SK, Singh G, Bhatti JS (2014). Ameliorative role of melatonin against cypermethrin induced hepatotoxicity and impaired antioxidant defense system in Wistar. IOSR J. Environ. Sci. Toxicol. Food Technol. 8(1):39-48.

Buraimoh AA, Ojo SA, Hambolu JO, Adebisi SS (2012). Effects of aluminium chloride exposure on the histology of the liver of adult wistar rats. IOSR J. Pharm. 2(3):525-533.

Chinoy NJ, Memon MR (2001). Beneficial effects of some vitamins and calcium on fluoride and aluminium toxicity on gastrocnemius muscle and liver of male mice. Fluoride 34:21-33.

Denen A, Samuel OO, Joseph ET, Egesie UG, Ejike DE (2015). Effects of ethanol extract of Garcinia Kola on biochemical markers of liver function of Wister rats. Int. J. Pharm. Sci. Invent. 4(5):5- 8.

El-Demerdash FM (2004). Antioxidant effect of vitamin E and selenium on lipid peroxidation, enzymes activities and biochemical parameter in rats exposed to aluminium. J. Trace Elem. Med. Biol. 18:113-122.

Ellman GL (1959). Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82:70-77.

Farina M, Lara FS, Brandao R, Jacques R, Rocha JBT (2002). Effects of aluminum sulfate on erythropoiesis in rats. Toxicol. Lett. 132:131-139.

Ferheen S, Ahmed E, Afza N, Malik A, Shah MR, Nawaz SA, Choudhary MI (2005). *Haloxylines* A and B, Antifungal and Cholinesterase Inhibiting Piperidine Alkaloids from *Haloxylon salicornicum*. Chem. Pharm. Bull. 53(5):570-572.

Gonzalez MA, Bernal CA, Mahieu S, Carrillo MC (2009). The interaction between the chronic exposure to Aluminium and liver regeneration on bile flow and organic anion transport in rats. Biol. Trace Elem. Res. 127:164-176.

Hsu CH, Han BC, Liu MY, Yeh CY, Casida JE (2000). Phosphine-induced oxidative damage in rats: attenuation by melatonin. Free Radic. Biol. Med. 28(4):636-642.

Jihen EH, Imed M, Fatima H, Abdelhamid K (2009). Protective effects of selenium (Se) and zinc (Zn) on cadmium (Cd) toxicity in the liver of the rat: effects on the oxidative stress. Ecotox. Environ. Saf. 72:1559-1564.

Joshi D K, Tripathi S, Mishra S, Choudhary M, Rai R, Mahdi AA (2015). Docosahexenoic acid prevents aluminum induced metabonomic changes in rat urine: A Proton nuclear magnetic resonance study. Scholars. Acad. J. Biosci. 3(3):248-253.

Joshi DK, Choudhary M, Tripathi S, Singh Negi MP, Mahdi AA (2013). Age dependent relative risk of aluminum toxicity: levels of metals and enzymic and non enzymic antioxidants status in liver, kidney and brain of aluminum treated young and old rats. Int. J. Biol. Pharm. Res. 4(3):176-185

Kadham MA (2008). Some pharmacological and toxicological investigations of *Haloxylon salicornicum* in rabbits. Coll. Vet. Med. 7(2):25-32.

Kalaiselvi A, Aadhinath Reddy G, Ramalingam V (2015). Ameliorating Effect of Ginger Extract (*Zingiber officinale* Roscoe) on Liver Marker Enzymes, Lipid Profile in Aluminium chloride Induced Male Rats. Int. J. Pharm. Sci. Drug Res. 7(1):52-58.

Karadeniz Cerit K, Karakoyun B, Yűksel M, Őzkan N, Çetinel Ş, Dağli ET, Yeğen BÇ, Tuğtepe H (2013). The antifibrotic drug halofuginone reduces ischemia/reperfusion-induced oxidative renal damage in rats. J. Pediatr. Urol. 9:174-183.

Kowalczyk E, Kopff A, Kędziora J, Błaszczyk J, Kopff M, Niedworok J, Fijałkowski P (2004). Effect of long-term aluminium chloride intoxication on selected biochemical parameters and oxidative-antioxidative balance in experimental animals. Pol. J. Environ. Stud. 13(1):41-43.

Lindell SL, Hansen T, Rankin M, Danielewicz R, Belzer FO, Southard JH (1996). Donor nutritional status - A determinant of liver preservation injury. Transplantation 61:239-247.

Ma YJ, Xu JG, Lin JY (2008). Effects of anetholtrithione on hepatic lipid peroxidation induced by aluminum in rats. Ind. Health Occup. Dis. 34(6):325-328.

Mahieu S, Contini MDC. Gonzalez M, Miller NC (2009). Melatonin reduces oxidative damage induced by aluminum in rat kidney. Toxicol. Lett. 190:9-15.

Mahieu SN, Gionotti M, Millen N, Elias MM (2003). Effect of chronic accumulation of aluminum in renal functions, cortical renal oxidative stress and cortical renal organic transport in rats. Arch. Toxicol. 77:605-612.

Newairy AS, Salama AF, Hussien HM, Yousef MI (2009). Propolis alleviates aluminum-induced lipid peroxidaion and biochemical parameters in male rats. Food Chem. Toxicol. 47(6):1093-1098.

Nikolov IG, Joki N, Vicca S, Patey N, Auchere D, Benchitrit J, Flinois JP, Ziol M, Beaune P, Drueke TB, Lacour B (2010). Tissue accumulation of lanthanum as compared to aluminum in rats with chronic renal failure-possible harmful effects after long-term exposure. Nephron Exp. Nephrol. 115:112-121.

Ochmanski W, Barabasz W (2000). Aluminum-occurrence and toxicity for organisms. Przegl. Lek. 57:665-668.

Özkaya A, Çelik S, Yüce A, Şahin Z, Yilmaz Ö (2010). The Effects of ellagic acid on some biochemical parameters in the liver of rats against oxidative stress induced by aluminum. Kafkas Univ. Vet. Fak Derg 16(2):263-268.

Renugadevi J, Prabu SM (2010). Quercetin protects against oxidative stress-related renal dysfunction by cadmium in rats. Exp. Toxicol. Pathol. 62:471-481.

Sanchez-Iglesias S, Soto-Otero R, Iglesias-Gonzalez J, Barciela-Alonso MC, Bermejo-Barrera P, Mendez-Alvare E (2007). Analysis of brain regional distribution of aluminium in rats via oral and intraperitoneal administration. J. Trace Elem. Med. Biol. 21:31-34.

Şener G, Sehirli Ö, Tozan A, Velioğlu-Övünç A, Gedik N, Omurtag GZ (2007). Ginkgo biloba extract protects against mercury (II)-induced oxidative tissue damage in rats. Food Chem. Toxicol. 45:543-550.

Shahana MM, Mirhom YW, Generah AA, Aboutabi EA, Amer HA (1990). Study into wild Egyptian plants potential medicinal activity. Ninth communication: hypoglycemic activity of some selected plants in normal fasting and alloxanised rats. Arch. Exp. Vet. Med. 44(3):389-394.

Shati AA, Alamri SA (2010). Role of saffron (Crocus sativus L.) and honey syrup on aluminum-induced hepatotoxicity, Saudi. Med. J. 31:1106-1113.

Shirley DG, Walter MF, Walter SJ, Thewles A, Lote CJ (2004). Renal aluminum handling in rats: A micropuncture assessment. Clin. Sci. 107:159-165.

Sinha AK (1972). Colorimetric assay of catalase. Anal. Biochem. 47:389-394.

Soudani N, Sefi M, Amara IB, Boudawara T, Zeghal N (2010). Protective effects of selenium (Se) on chromium (VI) induced nephrotoxicity in adult rats. Ecotox. Environ. Saf. 73:671-678.

Stoehr G, Leubbers K, Wilhelm M, Hoelzer J, Ohmann C (2006). Aluminum load in ICU patients during stress ulcer prophylaxis. Eur. J. Int. Med.17:561-566.

Suzuki KT, Kano S, Misawa S, Aoki Y (1995). Copper-metabolism leading to and following acute hepatitis in lec rats. Toxicology 97:81-92.

Taus N, Farraj M, Tănase S, Mironescu A, Boicu M, Necula V, Taus L (2013). Aluminum - a chemical neurotoxic agent. Bulletin of the Transilvania University of Braşov Series VI. Med. Sci. 2(55):1-8.

Tehrani H, Halvaie Z, Shadnia S, Soltaninejad K, Abdollahi M (2013). Protective effects of N-acetylcysteine on aluminum phosphide-induced oxidative stress in acute human poisoning. Clin. Toxicol. (Phila). 51(1):23-28.

Türkez H, Geyikoğlu F, Çolak S (2011). The protective effect of boric acid on aluminum-induced hepatotoxicity and genotoxicity in rats. Turk. J. Biol. 35:293-301.
Turkez H, Yousef MI, Geyikoglu F (2010). Propolis prevents aluminum-induced genetic and hepatic damages in rat liver. Food Chem. Toxicol. 48: 2741-2746.
Vamecq J, Vallée L, Storme L, Gelé P, Bordet R (2004). Les acteurs immédiats du stress oxydant, La lettre du pharmacologue. 18:16-22.
Vijayaprakash S, Langeswaran K, Kumar SG, Revathy R, Balasubramanian MP (2013). Nephro-protective significance of kaempferol on mercuric chloride induced toxicity in Wistar albino rats. Biomed. Aging Pathol. 3:119-124.
Yagi K (1976). A simple fluorometric assay for lipoperoxide in blood plasma. Biochem. Med. 15:212-216.
Yakubu MT, Musa IF (2012). Liver and Kidney Functional Indices of Pregnant Rats Following the Administration of the Crude Alkaloids from Senna alata (Linn. Roxb) Leaves. Iran. J. Toxicol. 6(16):615-625.
Yeh YH, Lee YT, Hsieh HS, Hwang DF (2009). Effect of taurine on toxicity of aluminum in rats. Eur. J. Clin. Nutr. Metabol. 4:187-192.
Zhuo JH, Liu P, Feng GC, Pingguo C (2007). Effect of aluminum chelating agent on liver zymogram and on essential elements of rats induced by aluminum. Chin. J. Public Health 23(3):316-317.

academicJournals

Vol. 15(9), pp. 303-314, 2 March, 2016
DOI: 10.5897/AJB2015.14801
Article Number: 0A6CB0457400
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Histopathological effects of Cyperdicot and vitamin E supplementation on selected organs of *Clarias gariepinus* (Burchell, 1822) reared in a tropical fish farm in Nigeria

Odo, G. E.[1]*, Agwu, J. E.[1], Madu, J.[1], Ezea, C. O.[2], Nnamonu, E. I.[1] and Eneje, V. O.[3]

[1]Department of Zoology and Environmental Biology, University of Nigeria, Nsukka, Enugu, Nigeria.
[2]Federal University of Technology, Owerri, Imo State, Nigeria.
[3]Department of Applied Biology and Biotechnology, Enugu State University of Science and Technology, Enugu, Nigeria.

Received 15 June, 2015; Accepted 29 December, 2015

This study conducted in 2014 investigated the histopathological effects of Cyperdicot and vitamin E supplementation on some selected organs in juveniles of *Clarias gariepinus*. Fish were exposed to 0. 0.08 and 0.16 mg/L Cyperdicot and vitamin E. Fish were divided into six groups: control, 0.80 mg/L; Cyperdicot, 0.16 mg/L; Cyperdicot, vitamin E, vitamin E + 0.08 mg/L Cyperdicot, and vitamin E + 0.16 m/L Cyperdicot insecticide. There was significant relation between temperature, pH, and dissolved oxygen with Cyperdicot concentration. The LC_{50} value based on probit analysis was found to be 0.08 mg/L for 96 h. Samples were taken at fixed times for histopathological studies. The fish exhibited behavioural and dermatological changes. Vitamin E + 0.08 mg/L Cyperdicot and vitamin E + 0.16 mg/L Cyperdicot treated fish showed abnormalities in their behaviour. Gills, liver, and kidneys of the 0.08 mg/L Cyperdicot treated group also showed several histopathological changes during the experimental periods. The organs of the fish treated with vitamin E + 0.16 mg/L Cyperdicot induced histopathological changes. The toxic effect of Cyperdicot is clear on the behavioural and histopathological aspects of the fish gills, liver, and kidney tissues, while vitamin E had no amelioration effects on them.

Key words: Cyperdicot, vitamin E, *Clarias gariepinus*, pesticide, sub lethal toxicity.

INTRODUCTION

The application of environmental toxicological studies on non-mammalian vertebrates is rapidly expanding, and for aquatic system, fish have become the indicators for the evaluation of the effects of noxious compounds (Ervnest, 2007). Pesticides occupy a unique position among many chemicals which are encountered daily by man. Cyperdicot is an agrochemical pesticide formulated with dimethoate and cypermetrin. It is a broad-spectrum

*Corresponding author. E-mail: gregory.odo@unn.edu.ng.

pesticide widely used throughout the world for agriculture and domestic purposes (Ali et al., 2009). Pesticides at high concentrations are known to reduce the survival, growth, and reproduction of fish and produce many visible effects on fish (Rahman et al., 2002). Peebua et al. (2007) recorded that, the 96-h LC_{50} values of Cyperdicot freshwater fish rainbow trout and fat head minnows were 15 and 0.8 µg/L, respectively.

A great proportion of acute poisoning cases are caused by exposure to pesticides, especially organophosphate (OP) compounds. The primary mechanism of action of OP pesticides is based on inhibition of the acetylcholinesterase (AChE) enzyme (Eto, 1979). Once AChE has been inactivated, acetylcholine (Ach) accumulates throughout the nervous system, resulting in overstimulation of muscarinic and nicotinic receptors. Like all organophosphate insecticides, Cyperdicot acts on the nervous system as inhibitor of AchE, an enzyme that hydrolyzes acetylcholine (Ach). Ach is a molecule that is involved in the transmission of nervous signals from nerves to nerves and between neurons in the brain (Jungera, 2014). Due to Cyperdicot toxicity, the Environmental Protection Agency (EPA) has classified it as restricted use pesticide (RUP) that warrants special handling (ATSDR, 2005). Large amounts of pesticides find their ways into water bodies due to repeated application for the control of pests. The indiscriminate use of pesticides, careless handling, accidental spillage or discharge into natural water ways have harmful effects on fish population and other aquatic organisms and may contribute to long-term effects (Nwani et al., 2013b)

Histopathological studies help to establish casual relations between exposure to contaminants and various biological responses and have proven to be a sensitive tool used in detecting direct effects of chemical compounds within target organs of laboratory experiments (Altinok and Capkin, 2007; Boran et al., 2012). A study of histopathology provides very important and useful data concerning changes in cellular or sub-cellular structure of an organ much earlier than external notification. One of the advantages of using histopathological biomarkers in environmental studies is that it allows the examination of target organs and the alterations found in these organs are easier to identify than functional ones (Fanta et al., 2003). These alterations serve as warning signs of damage to the wellbeing of the organism.

Histopathological biomarkers in the gills may be valuable as indicators of the general health of the fish and mirror effects of exposure to a variety of anthropogenic pollutants (Wiieyaratne and Pathiratne, 2006). The histopathological effects on the gills of freshwater fish showed necrosis, abnormalities to gill lamellae, extensive fusions of secondary lamellae, and a thick coat of mucus on the gill filaments upon exposure to Cyperdicot (Rao et al., 2005). In addition, hyperplasia of the gill filaments, edema separation of primary gill lamellae, haemorrhage in the blood vessel, clubbing, fusion of adjacent filaments, and hyperplasia in the secondary lamellae in freshwater *Clarias gariepinus* exposed to Cyperdicot were also observed.

The kidney, for instance, is a target of toxic chemicals, which can disrupt its functions, and cause temporary or permanent derangement of homeostasis. Several authors recorded histopathological changes in the kidney and liver of freshwater fish, *Puntius conchoniu* and *Channa punctatus* exposed to organophosphate insecticides, such as diazinon, monocrotophos, dimethoate, and elsan, respectively (Miller, 2002). Todd and Van Leeuwen (2002) reported that pesticides present in aquatic environments can affect aquatic organisms in different ways which include toxic effects at both lethal and sub-lethal concentration which may change their growth rate, development, reproduction, histopathology, biochemistry, physiology, and behaviour. The most histopathological changes in the kidney and liver of freshwater catfish *(Heteropneutes fossilis)* and zebra fish *(Danio rerio)* exposed to insecticide Cyperdicot were shrunken glomeruli, dilated lumina of the renal tubules, vacuolated blood cells in the glomerular tuft, caryolysis, and widespread vacuolar degeneration of the hepatocytes (Scheil et al., 2009).

Clarias species are mostly freshwater fish which are distributed throughout African and Asian lakes, swamps, and rivers. *Clarias* can be obtained throughout the year in Nigerian rivers and are anadromous (Akinwole and Faturoti, 2007; Adewolu et al., 2008). The fish is in high demand because of its flesh and good taste. To meet the ever increasing demand, *C. gariepinus* is the fish of choice in Nigerian aquaculture. There has been paucity of information on the effect of Cyperdicot on freshwater cat fish in Nigeria aquatic ecosystem. Hence, this work determined the half-lethal concentration (96 h LC_{50}) of the insecticide to juveniles of *C. gariepinus* in order to evaluate the effect of its sub-lethal toxicity on some selected organs (gills, liver and kidneys) of the fish, and the effect of vitamin E supplementation against the different concentrations of Cyperdicot pesticide were also evaluated.

MATERIALS AND METHODS

Experimental fish

Three hundred African catfish, *C. gariepinus* juvenile (mean weight 150 ± 5.20 g, length = 35.00 ± 2.50 cm) were obtained from Sacen Fish Farm, Enugu, Nigeria and transported in a 300 L capacity plastic tank to the Fisheries Wet Laboratory, Department of Zoology and Environmental Biology, University of Nigeria Nsukka. The fish specimens were treated with 0.05% potassium permanganate to avoid possible dermal infections. They were acclimatized for 20 days in a 1000 L plastic tank during which they were fed 3% body weight (BW) in divided rations twice daily (7.00 am and 7.00 pm) with locally laboratory prepared pelleted diet containing 35% crude protein (Eyo et al., 2013). The feeding was terminated 24 h prior to the range finding and toxicity test to avoid interference of faeces

(Reish and Oshida, 1987). The ethical guidelines of the Animal Care Committee (UNN-EGACC, protocol no. 0430/2013) of the University of Nigeria, Nsukka were strictly followed.

Pesticide

Cyperdicot is composed of cypermethrin and dimethoate. Cypermethrin is an insecticide in the synthetic pyrethroid family, first marketed in 1977. The primary manufacturers in the U.S. are Zeneca Inc., FMC Corp., and American Cyanamid Co. Common brand names are Demon, Cymbush, Ammo, and Cynoff. Dimethoate, first marketed in 2001 by FAO, is an organ phosphorus and systemic pesticide with stomach and cholinesterase inhibition actions. The trade names are Danadim, Rogo, and Roxion. The primary manufacturers in Denmark and Italy are Cheminouta.

Diets

The basal control diet (diet C) was formulated according to Kim et al. (2003) from practical ingredients to satisfy all known nutrient requirements of *C. gariepinus* with adequate levels of vitamin E, a-tochopheryl acetate in diet (a-TA).

Vitamin E

Vitamin E is composed of a compound that includes tocopherols and tocotrienols. The molecules that contribute a-tocopherols activity are four tocopherols and four tocotrienols, identified by alpha (a), beta (β), gama (y), and delta (ð). The brand names are Aqua-E, Aquasol E, Aqueous Vitamin E and E-400 clear (Packer et al., 2001).

Toxicity test

Determination of the LC_{50} concentration

A toxicity assay to determine the 96 h LC_{50} values of Cyperdicot was conducted with a definitive test in a semi-static system in the laboratory following standard methods (APHA, AWWA, WPCF, 2005). A range-finding tests (5, 4, 3.5, 3, and 2.5 mg^{-1}) was carried out to determine the concentrations of the test solution for the definitive test. The test solution was changed on every alternate day to counter-balance the decreasing pesticide concentrations. To prevent oxygen depletion, experimental tanks were continuously oxygenated using an air pump. Dead fish were immediately removed to avoid possible deterioration of the water quality. Behavioural changes in fin and opercular movements, equilibrium status, swimming rate, air gulping, and skin coloration during the test period were observed. In the definitive test, a set of 10 fish specimens was randomly exposed to Cyperdicot at 5, 4, 3.5, 3, and 2.5 mg^{-1} concentration. Another set of 10 fish specimens was simultaneously maintained in tap water, without test chemical, and considered as control. The experiment was set in triplicate to obtain LC_{50} values of the test chemical under a photoperiod of 12 h light and 12 h dark.

Determination of sub-lethal concentrations

The 96 h LC_{50} value of Cyperdicot on *C. gariepinus* was determined to be 0.80 mg/L. Based on this value, two sub-lethal concentrations of 0.08 and 0.16 mg/L corresponding to 1/20 and 1/10th of the 96 h LC_{50} of the pesticide, respectively, were prepared by serial dilution of the stock solution and were used for *in vivo* exposure. A total of 90 fish from the acclimatized batch were used during the *in vivo* experiment. The fish were randomly divided into three groups of 30 fish without regard to sex. Fish in the first treatment group were exposed to tap water and served as control, while those in the second and third groups were treated with 0.08 and 0.16 mg/L of Cyperdicot, respectively. The exposure lasted for a period of 4 weeks during which the fish were fed daily with small quantity of food approximately 1% of total body weight about an hour before the test solution was renewed to avoid catabolism and subsequent mortality.

Morphometric data

The condition factor (CF) and hepatosomatic index (HSI) of the fish were calculated after White and Fietcher (1985) as follows:

$$\text{Condition factor (CF)} = \text{Bodyweight (g)}/\text{Standard length (cm)}^{3} \times 100$$

$$\text{Hepatosomatic index (HIS)} = \text{Liver weight (g)}/\text{Bodyweight (g)} \times 100$$

Experimental design

The 96 h LC_{50} of Cyperdicot insecticide to juveniles of *C. gariepinus* was determined to be 0.80 mg/L. In the sub lethal toxicity test, fish were exposed to 0.08 mg/L (1/10 LC_{50}) and 0.16 mg/L (1/5 LC_{50}) Cyperdicot and/or vitamin E and as a result, six concentrations were established and used for the definitive test for four weeks: (i) Group 1: Control; (ii) Group 2: 0.08 mg/L Cyperdicot; (iii) Group 3: 0.16 mg/L Cyperdicot; (IV) Group 4: vitamin E; (v) Group 5: vitamin E + 0.08 mg/L Cyperdicot; (vi) Group 6: vitamin E + 0.16 mg/L Cyperdicot.

Fish were collected at the end of the 1st, 2nd, 3rd and 4th week for behavioural and histopathological studies. The cumulative mortality was recorded throughout the period of study and the fish were examined to determine the cause of their death. The immediate behavioural changes of the fish were recorded before death.

Histopathological examinations

On 1, 7, 14, 21 and 28 days, one fish from each replicate treatment group and control was sacrificed after anesthetizing with tricaine methanesulfonate (MS 222) to minimize stress. The fish were dissected and gill, kidney, and liver tissues were removed, preserved in 10% phosphate buffered formalin for 24 h, dehydrated by a series of upgraded ethanol solution, embedded in paraffin, and sectioned at 5 µm thick. A total of three tissue sections of gill, kidney, and liver each per fish for each replicate concentration were routinely processed and stained with Hematoxylin and Eosin (H&E) and examined by light microscopy according to Bancroft and Gamble (2002). Photomicrographs were then taken and the fish of the control groups were compared with that of exposed groups under the guidance of a pathologist.

Statistical analysis

Mean values were analyzed for significant differences ($p \leq 0.5$) using the analysis of variance (ANOVA). Differences between means were partitioned using the Duncan new multiple range test. The mean physicochemical parameters of the test concentrations of Cyperdicot on *C. gariepinus* were observed. Condition factor and hepatosomatic indices were calculated. The Statistical Package for Social Sciences (SPSS), version 17, was used for all analysis. The probity value was determined from the probity model developed by Finney (1971).

Table 1. Mean physicochemical parameters of the test concentrations (Cyperdicot) on *C. gariepinus*.

Parameter	pH	Temperature (°C)	Cond (µM/cm)	DO (mgl^{-1})	Alk (mg/L)	Th (mg/L)
Control	7.0±0.1[a]	26.0±0.7[a]	67.0±0.1[a]	6.3± 0.	24.2±0.1	6.2±0.2
0.08 mg/L Cyperdicot	6.9±0.2[a]	27.0±0.1[b]	66.4±0.2[b]	5.4±0.1	23.9±0.8	6.0±0.1
0.16 mg/L Cyperdicot	6.7±0.1[b]	27.2±0.1[b]	65.3±0.3[b]	5.3±0.6	23.6±0.2	5.8±0.3
Vitamin E	6.6±0.2 [c]	27.4±0.3[c]	63.8±0.6[c]	5.2±0.1	22.8±0.9	5.6±0.2
0.08 mg/L Cyperdicot + Vitamin E	6.3±0.3[c]	27.5±0.1[c]	62.1±0.2d[e]	4.9±0.1	22.6±0.5	5.4±0.6
0.16 mg/L Cyperdicot + Vitamin E	63±0.0.1[e]	27.0±0.3[d]	59.2 ±0.2[e]	4.7±0.2	22.1±0.3	4.9 ±0.4

Cond = conductivity, micro-siemens per centimeter (μscm^{-1}); DO = dissolved oxygen (mgl^{-1}); milligram per litre; Alk = total alkalinity = milligram per litre =mgl^{-1}; Th = total hardness = milligram per litre = mgl^{-1}.

RESULTS

The mean physicochemical parameters of the test concentration (Cyperdicot) on *C. gariepinus* are shown in Table 1.

Toxicity and behavioural responses

No adverse behavioural changes or any mortality were recorded in the control fish and vitamin E throughout the period of the bioassay (Table 2). The behaviour of the control fishes and their colour were normal. Symptoms of toxicosis observed in the fish behaviour with Cyperdicot include lack of balance, agitated or erratic swimming, air gulping, restlessness, sudden quick movement, excessive secretion of mucus, and swimming on the back. The juveniles of *C. gariepinus* exposed to sub-lethal concentrations (0.08 and 0.16 mg/L) of Cyperdicot, respectively exhibited fast behavioural changes even at the low concentration. Fish showed a colour fading and retardation in opercular movement. They also lost their feeding appetite. An increase in skin mucus secretion and its bioaccumulation on the gills were also observed. Vitamin E treated fish were behaviourally normal and had good appetite as the control group. Those treated with vitamin E + Cyperdicot showed abnormalities in their behaviour similar to that of the fish treated with Cyperdicot.

Mortality

The percentage mortality increased with increase in toxicant concentration. Catfish juveniles were exposed to 0.08, 0.16, vitamin E + 0.08 and vitamin E+ 0.16 had 6.67, 13.33, 9.71, and 13.33% mortalities, respectively (Table 3). The first 24 h LC_{50} at 95% confidence limit was estimated as 1100.78 mg/L. The percentage mortality at second day increased with the toxicant concentration. Catfish juveniles exposed to 0.08, 0.16, vitamin E + 0.08 and vitamin E + 0.16 mg/L had 13.33, 13.33, 9.16, 6.01, and 5.96% mortalities, respectively (Table 3). The second day LC_{50} at 95% confidence limit for toxicant concentration was estimated as 416.67 mg/L. The percentage mortality at third day increased with the toxicant concentration. Catfish juveniles exposed to 0.08, 0.16, vitamin E + 0.08 and vitamin E + 0.16 mg/L had 16.51, 26.67, 33.33, 19.61, and 24.39% mortalities, respectively (Table 3). The third day LC_{50} at 95% confidence limit for toxicant concentration was estimated at 181.76 mg/L. The percentage mortality at the fourth day increased with the toxicant concentration. Catfish juveniles exposed to 0.08, 0.16, vitamin E + 0.08 and vitamin E + 0.16 mg/L had 6.67, 13.33, 10.11 and 13.05% mortalities, respectively (Table 3). The four days LC_{50} at 95% confidence limit for toxicant concentration was estimated at 152.02 mg/L.

Morphometric results

Condition factor (CF)

Condition factor is an index of growth rate. The condition factor (mg/cm^3) of *C gariepinus* of all treated groups did not significantly change after the end of the 1st, 2nd and 4th week as compared to the control group. Condition factor recorded at the end of the 3rd week significantly decreased in the 0.16 mg/L Cyperdicot and vitamin E and 0.16 mg/L Cyperdicot treated groups, respectively compared to the control group (Table 4). The results by the three-way ANOVA revealed that, Cyperdicot concentrations and time significantly affected the CF. Vitamin E did not significantly affect the CF, while the interaction between vitamin E and time significantly ($p < 0.001$) affected the CF.

Hepatosomatic index (HSI)

The hepatosomatic index (%) of juveniles of *C. gariepinus* recorded at the end of 1st week significantly ($p < 0.05$) decreased to 0.23 ± 0.035% in 0.16 mg/L

Table 2. Behavioural and dermatological changes of *C. gariepinus* juveniles exposed to various concentrations of Cyperdicot.

Parameter	Toxicity test																							
	Exposure time (h)																							
	24						48						72						96					
	Concentration (mg/L)																							
	0	0.08	0.16	Vit. E	0.08 + Vit. E	0.16 + Vit. E	0	0.08	0.16	Vit. E	Vit. E + 0.08	Vit. E + 0.16	0	0.08	0.16	Vit. E	Vit. E + 0.08	Vit. E + 0.16	0	0.08	0.16	Vit. E	Vit. E + 0.08	Vit. E + 0.16
Behavioural changes																								
Loss of reflex	-	+	+	-	+	+	-	++	++	-	++	++	-	++	++	-	++	++	-	+++	+++	-	+++	+++
Air gulping	-	+	+	-	+	+	-	++	++	-	++	++	-	++	++	-	++	++	-	+++	+++	-	+++	+++
Erratic swimming	-	-	+	-	-	+	-	-	+	-	-	+	-	+	++	-	+	++	-	++	++	-	++	+++
Dermatological changes																								
Discoloration	-	+	+	-	+	+	-	++	++	-	++	++	-	++	++	-	++	++	-	+++	+++	-	+++	+++
Haemorrhage	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-	++	++	-	++	++
	Sub-lethal test																							
Exposure time (week)	24						48						72						96					
Behavioural changes																								
Loss of reflex	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
Air gulping	-	+	+	-	+	+	-	-	+	-	+	+	-	+	+	-	+	+	-	+	++	-	++	+++
Erratic swimming	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	++	++
Dermatological changes																								
Discoloration	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	++	+	-	++	++	-	++	+++
Haemorrhage	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

-No significant, +low severity, ++moderate severity, +++high severity.

Cyperdicot treated group compared to 0.27 ± 0.028) of the control group. At the end of the 3rd week, a significant decrease (0.208 ± 0.015%) of the control group was also recorded (Table 5). The results of the three-way ANOVA revealed that the concentrations significantly ($p < 0.001$) affected the HSI. The interactions between Cyperdicot concentrations and time and between vitamin E and time significantly ($p < 0.01$) affected the HSI. The interaction between Cyperdicot concentrations, vitamin E, and time significantly ($p < 0.05$) affected HSI.

Histopathological observations of the gills, kidney and liver in *C. gariepinus* exposed to Cyperdicot

The histopathological changes observed in *C. gariepinus* during the experiment are presented in Tables 5 and Figures 1 and 2. No alterations were observed in the gills, kidney and liver of the control. The severity and frequency of gill, kidney and liver lesions were found to be more pronounced in fish exposed to the highest concentration of Cyperdicot. Histopathological alterations were concentrations and duration dependent.

DISCUSSION

Acute and chronic toxicity tests are mostly used to

Table 3. Percentage mortality of *C. gariepinus* juveniles exposed to various concentrations of Cyperdicot.

Toxicant concentration (mg/L)	Percentage mortality				
	24	48	72	96	Cumulative mortality (%)
0	0.00	0.00	0.00	0.00	0.00
0.08	6.67	13.33	20.00	6.67	46.67
0.16	13.33	13.33	26.67	13.33	66.67
Vitamin E	9.71	9.16	16.51	10.11	45.49
Vitamin E+0.08	6.09	6.01	19.61	6.21	41.79
Vitamin E+0.16	12.81	5.96	24.39	13.05	41.79
LC_{50} (mg/L)	1200.78	426.67	191.76	163.02	60.21
Log (concentration)	3.08	2.63	2.28	2.21	-

Table 4. Condition factor (FC) (g/cm^3) of juveniles of *C. gariepinus* daily exposed to various concentrations of Cyperdicot (0, 0.08 mg/L and 0.16 mg/L) and/or vitamin E (0.450 kg^{-1} dry weight diet).

Treatment	Time intervals			
	1st week	2nd week	3rd week	4th week
Control	0.433±0.10	0.441±0.03	0.455±0.04	1.464±0.03
0.08 mg/L Cyperdicot	0.631 ±0.05	0.31±0.03	0.334±0.035	1.278±0.06
0.16 mg/L Cyperdicot	0.769±0.04	0.39±0.05	0.268±0.04*	1.362±0.05
Vitamin E	0.441±0.08	0.449±0.09	0.525±0.06	1.568±0.05
0.08 mg/L Cyperdicot + Vitamin E	0.495±0.06	0.442±0.05	0.425±0.03	1.455±0.10
0.16 mg/L Cyperdicot + Vitamin E	0.434±0.05	0.352±0.06	0.287±0.02*	1.31±0.03

assess the toxicity of chemicals on non-target organisms (Santos et al., 2010). 96 h LC_{50} is one of the most important parameters for evaluating the toxic effects of pollutants (Nwani et al., 2015). In the present study, the 96 h LC_{50} values of Cyperdicot for the African catfish, *C. gariepinus* was found to be 0.80 mg/L. In general, toxicity of chemicals to aquatic organisms has been reported to be affected by temperature, pH, dissolved oxygen, size and age, strain of species, water quality, concentration and formulation of test chemicals (Nwani et al., 2010; Boran et al., 2012; Rauf and Arain, 2013). The magnitude of toxic effects of pesticides also depends on the length and weight, corporal surface/body weight ratio and breathing rate (Murty, 1986). Oh et al. (1991) also reported that varied inhibition of acetylcholinesterase, detoxicification and absorption are factors causing the selective toxicity of pesticides for various species of fish.

Clinical symptoms observed during acute exposure of *C. gariepinus* to Cyperdicot in the present study are consistent with the findings of other authors and may indicate Cyperdicot-induced suppressed activity acetylcholineesterase. The abnormal behavior exhibited by fish in the experimental groups, such as abnormal swimming behavior are due to inhibition of AChE activity leading to accumulation of acetylcholine in choligenic synpases, thus causing hyper stimulation. Muralidharan (2012) reported that hyperactivity of fish exposed to pollutants could be attributed to impaired gill function, and the secretion of increased amount of mucus to coat the body and gills may be an attempt to produce relief from the irritating pollutant. The secretion of copious mucus by fish could also be a defense mechanism to neutralize the effect of pesticide which gradually covers the body, gills and buccal cavity. Repeated opening and closing of the mouth and opercula covering accompanied by partially extended fin as observed in the present study could be due to clearance of the accumulated mucus debris in the gill region for proper breathing. Similar behavioral changes In *C. gariepinus* exposed to atrazine and chlorpyrifos (Nwani et al., 2013a) and *Cirrhinus mrigala* exposed to diazinon (Rauf and Arain, 2013) have been reported.

Histopathological lesions observed in gill tissues of *C. gariepinus* exposed to Cyperdicot in the present study are similar to reports in *Cyprinus carpio* (Muralidharan, 2014). Similar pathological lesions in the gill architecture were observed in *Orochromis niloticus* exposed to dimethoate (Elezaby et al., 2001), *Puntius gonionotus* exposed to paraquat (Cengiz and Unlu, 2006), *Oncorhynchus mykiss* exposed to fungicide captan (Boran et al., 2012) and in *Gobiocypris rarus* (Yang et al., 2010), *Gnathonemus petersii* (Alazemi et al., 2012) and

Table 5. Summarized histopathological effects in the kidney, gill and liver of *C. gareipinus* administered exposed to Cyperdicot and the control.

Concentration (mg/kg)	Liver						
	Duration (Days)	Lymphocytic infiltration	Cell rupture	Congested central vein	Glycogen vacuoltion	Pyknotic necrosis	Severe of infiltration of leukocytes
Control	7	0	0	0	0	0	0
0.08	7	1	3	2	0	0	0
0.16	7	0	1	1	0	0	0
Vit E	7	0	0	0	0	0	0
Vit E+0.08	7	0	0	0	0	0	0
Vit E +0.16	7						
Control	14	0	0	0	0	0	0
0.08	14	0	0	1	0	2	0
0.16	14	0	1	2	0	0	0
Vit E	14	3	0	0	0	0	0
Vit E+0.08	14	0	0	0	0	0	0
Vit E +0.16	14						
Control	28	0	0	0	0	0	0
0.08	28	0	3	3	0	0	0
0.16	28	0	0	0	0	0	0
Vit E	28	0	0	0	0	0	3
Vit E+0.08	28	0	0	0	0	0	0
Vit E 0.16	28	0	0	0	0	0	0

Concentration (mg/kg)	Gill						
	Duration (Days)	Necrotic tubules	Tubule disintegration/ degeneration	Cystic spaces	Proliferation of polymorphonuclear cells	Vacuolation	Hematopoetic tissue
Control	7	0	0	0	0	0	0
0.08	7	1	3	2	0	0	0
0.16	7	0	1	1	0	0	1
Vit E	7	0	0	0	0	0	0
Vit E+0.08	7	0	0	0	0	0	0
Vit E +0.16	7						
Control	14	0	0	0	0	0	0
0.08	14	0	0	1	0	2	0

Table 5. Contd.

0.16	14	2	1	2	0	0	1
Vit E	14	0	0	0	0	0	0
Vit E+0.08	14	0	0	0	0	0	0
Vit E +0.16	14						
Control	28	0	0	0	0	0	0
0.08	28	0	3	3	0	0	0
0.16	28	0	0	0	2	0	0
Vit E	28	0	0	0	0	0	0
Vit E+0.08	28	0	0	0	0	0	0
Vit E 0.16	28	0	0	0	0	0	0

Concentration (mg/kg)	Kidney						
	Duration (Days)	Congestion of bloodspaces	Hypertrophy	Epithelia lifting	Oedema	Obliteration of lamellaearchitecture	Necrosis of epithelia cells/damage of gills
Control	7	0	0	0	0	0	0
0.08	7	3	1	1	0	1	1
0.16	7	0	0	2	0	0	1
Vit E	7	0	2	0	0	0	0
Vit E+0.08	7	0	0	1	0	0	0
Vit E +0.16	7		0	1	0	0	0
Control	14	0	0	0	0	0	0
0.08	14	1	0	1	0	2	1
0.16	14	0	1	2	0	0	0
Vit E	14	0	0	0	0	0	0
Vit E+0.08	14	0	0	0	0	0	0
Vit E +0.16	14	0	0	0	0	0	0
Control	28	0	0	0	0	0	0
0.08	28	0	3	3	0	2	0
0.16	28	0	0	0	0	0	0
Vit E	28	0	0	0	0	0	0
Vit E+0.08	28	1	0	0	0	0	0
Vit E 0.16	28	0	0	0	0	0	0

Lesions were scored based on their severity (0 = none, 1 = mild, 2 = moderate, 3 = strong).

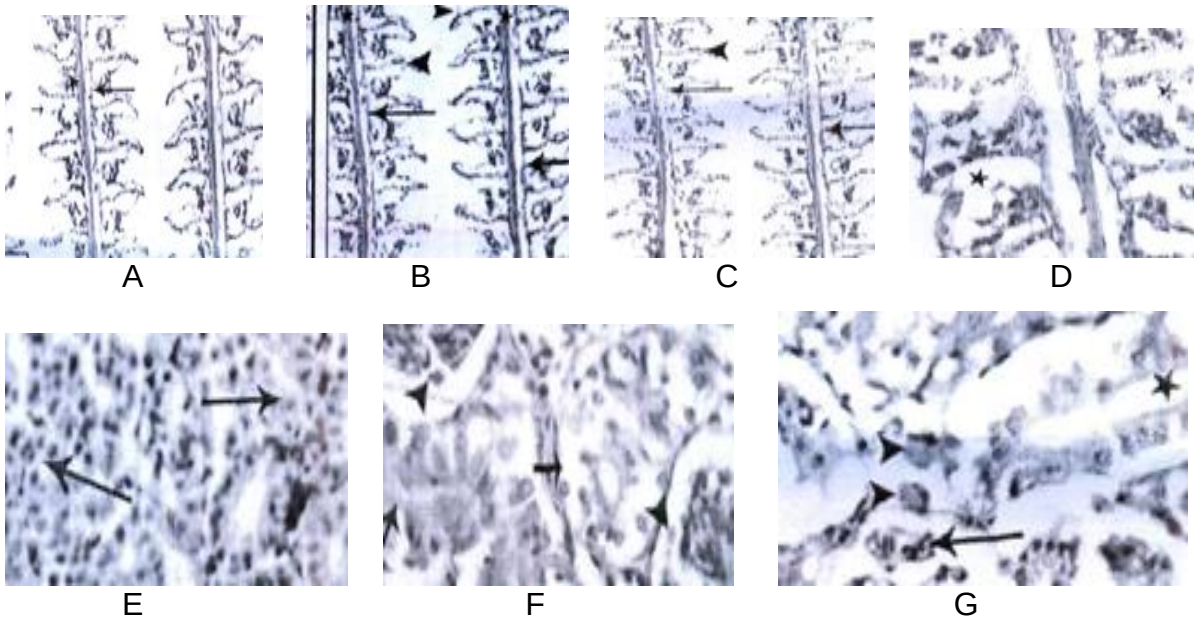

Figure 1. (A) Photomicrography of gill section of control group of juveniles' *C. gariepinus* showed no significant lesion ×400. (B) The 0.16 mg/L Cyperdioct administration on gill showed: a. Epithelia hyperplasia, lamellar aneurysm. b. Leucocytes infiltration and hyalinization of adductor muscles. c. Deformation of the cartilage, necrosis of epithelial cells, completes destruction of the gills, obliteration of lamellae architecture. (x400). (C) The Vitamin E and 0.08 mg/L Cyperdicot showed congestion of entire lamellae and epithelia hyperplasia, vacuolar degeneration pillar cells infiltration and Synechiae x400. (D) The 0.16 mg/L Cyperdicot, 1.70 mg/L hyperplasia and aneurysm, oedema intraepithelial oedema, fused epithelia hyperplasia. (E) Kidney observations: Control showed normal architecture and renal tubule x400. (F) The 0.08 mg/L Cyperdicot. a) Swelling of epithelial cells of renal tubule with diluted lumen and occlusion lumen and fragmentation of glomeruli. b) Complete destruction of tubule architecture, fragmentation of glomerulus brownish pigments x 400. (G) The 0.08 mg/L Cyperdicot. a) Swelling of epithelial cells of renal tubule with diluted lumen and occlusion lumen and fragmentation of glomeruli. b) Complete destruction of tubule architecture, fragmentation of glomerulus brownish pigments ×400.

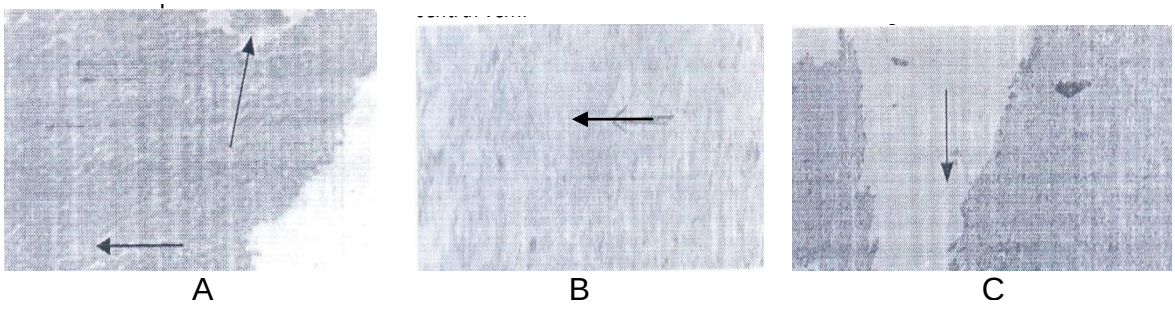

Figure 2. (A) Liver of *C. gariepinus* ×400. The 4 week exposed to 0.08 mg/L Cyperdicot showing glycogen vacuolation. (B) Liver *C.gariepinus* of ×400. The 4 week exposed to Vitamin E + 0.08 mg/L. Cyperdicot showing severe infiltration of leukocytes (arrow) pyknotic (N) and vacuoles(L). (C) Liver of *C.gariepinus* x400. The 4 week exposed to 0.16 mg/L + Vitamin E Cyperdicot showing diffused hepatic necrosis.

C. carpio (Blahova et al., 2014) exposed to atrazine. Epithelial hypertrophy could be as a result of epithelial detachment as stated by Machado and Fanta (2003). Epithelial lifting increases the distance through which the toxicant reaches the blood stream thereby causing impaired oxygen uptake (Kumar et al., 2010); it could result in dysfunction or even non-functional gills and eventually suffocate the fish. Lamella fusion could be a protective mechanism as it reduces the amount of vulnerable gill surface area. According to Olurin et al. (2006), these pathological changes may be a reaction to toxicant intake or an adaptive response to prevent the

entry of the pollutants through the gill surface and probably increase capillary permeability.

Histopathologically, this was due to proliferation of mucus cells and epithelial hyperplasia of gills. This could be as a result of coating the body so as to reduce contact with the toxic environment and get relief from the pollutant irritation (Al-Ghanim et al., 2008).

The liver of the exposed fish had slightly vacuolated cells showing evidence of fatty degeneration. Necrosis of some portions of the liver tissue observed resulted from the excessive work required by the fish to get rid of the toxicant from its body during the process of detoxification by the liver. The degenerative changes in the hepatocytes of *C. gariepinus* were found to increase as the investigation progresses into weeks. In Sri Lanka, gills of *Rasbora caverii* collected from canals near rice fields, covering pesticide application periods during rice cultivation season showed also similar changes (De Silva and Samayawardhena, 2002; Wijeyaratne and Pathiratne, 2006) with juvenile guppics (*Poecilla reticulate* Peters) exposed to sub-lethal concentration of Cypercicot (Rao et al., 2005; Kunjamma et al., 2008).

The different concentrations of Cyperdicot used in this study under different exposure periods showed different degrees of pathological changes. Similar results were recorded in the freshwater fish (*P. gonionotus, Gambusia affinis* and *Corydoras paleatus*) exposed to pesticides Paraquat and Dimethoate (Elezaby et al., 2001; Cengiz and Unlu, 2003; Fanta et al., 2003; Jiraungkoorskul et al., 2003).

These vacuolar degenerating of glomerular tuft, shrinkage of some glomeruli and dilatation of others, increased Bowman's capsule space, cloudy swelling of some epithelial tubules and dilatation of tubules lumens and obstruction observed are in agreement with the changes in the kidney of freshwater fish *(Piaractus mesopotamicus)* exposed to organophosphate insecticide (Mataqueiro et al., 2009). The shrinkage in renal corpuscles clearly indicated that treated fish adopt some other routes of nitrogen excretion while the dilation of the renal corpuscles may be due to an increase in the filtration rate and consequently in urine volume, which may be a mechanism used by fish to overcome the toxic effect of the pesticide (Roy and Bhattacharya, 2006). The decreases in the tubular lumen may be due to the cloudy swelling of the epithelial cells of the renal tubules, which could be a reversible change; also, the dilation in the tubules lumen may be due to the marked decrease in the length of the epithelial cells as a result of epithelial tubules degeneration. In the present study, the recognized homogenous eosinophilic deposits within tubular lumen could be attributed to the protein leakage into the filtrate due to the glomerular disease (Roberts, 2001).

Cyperdicot used in the present research caused some histopathological changes in the kidney tissues and the vitamin E was not able to prevent these changes. The insecticide Cyperdicot caused kidney damage, and a combination of vitamins E and C reduced partially this damage. On a relative basis, Cyperdicot appears to be capable of producing a wider spectrum of significant histopathology impairments in fish with even sub-lethal concentrations and should be categorized as an important pollutant of the aquatic environment (Oncu et al., 2002).

The morphometric study included condition factor and hepatosomatic indices. The condition factor (mg/cm^3) of *C. gariepinus* of all treated groups showed very minor change; significant decrease in the condition factor of 0.16 mg/L Cyperdicot at the end of the 3rd week when compared with the control group and vitamin E could not prevent this decrease. The significant lower condition factor was recorded by Teh et al. (2005) in *Pogonichthys macrolepidotus* exposed to sub lethal concentrations of diazinon. The few changes in condition factor in the present experimental periods could be attributed to this factor. This factor could not be enough sensitive biomarker to measure the environmental stress in natural environments (Wiieyaratne and Pathiratne, 2006). The recorded non-significant effect of vitamin E on the condition factor throughout the experimental periods was also recorded in the freshwater fish rainbow trout (*O. mykiss*) (Al-Juary et al., 2006). This indicated that the addition of a-tocopherol to the diet did not significantly alter the palatability of the diet, its nutrient content, and the caloric values (Al-Juary et al., 2006). Tissue somatic indices, such as the hepatosomatic index are general measurement of the overall condition of fish or growth status of a specific tissue (West, 1990). The minor changes were recorded in the present study in the hepatosomatic index of fish treated with Cyperdicot. A significant decrease in the hepatosomatic index of fish treated with the high sub lethal concentration of Cyperdicot was recorded at the end of the 1st and 3rd weeks. It could be concluded from the present study that the toxic effect of Cyperdicot on fish is clear on their behavioural and histopathological aspects of gills, liver and kidney tissues while vitamin E has no amelioration effects on these parameters.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Adewolu MA, Adeniji CA, Adejobi AB (2008). Feed utilization, growth and survival of *Clarias gariepinus* fingerlings cultured under different photoperiods. J. Aquacul. 283:64-67.

ATSDR (Agency for Toxic Substances and Diseases Registry) (2005). Toxicologyic Information about Insecticides Used for Eradicating Mosquitoes (West Nile Virus Control). Department of Health and Human Services: ATSDR. Available at: http:www.astdr.cdc.gov/consultations/west _nile _ virus/fenthion.html.

Akinwole AO, Faturoti EO (2007). Biological performance of African

catfish (*Clarias gariepinus*) cultured in recirculation system in Ibadan. Aquac. Eng. 36:18-23.
Alazemi BM, Lewis JW Andrews EB (2012). Gill damage in the freshwater fish Gna thonemus petersii (family: Mormyradae) exposed to selected pollutants: an ultrastructural study. Environ. Technol. 17(2):225-238.
Al-Ghanim H, Al-Balawi A, Al-Akel F, Al-Misned Z, Ahmad H (2008). Ethological response and haematological and biochemical profiles of carp *(Cyprinus carpio)* exposed to trichlorfon. J. Food Agric. Environ. 6:473-479.
Ali D, Nagpurea N, Kumar S, Kumar, B. Kushwahaa B, LakraaW (2009). Assessment Of genotoxic and mutagenic effects of chlorpyrifos in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Food Chem. Toxicol. 47:650-656.
Al-Juary AA, Al-Awward KH , Al-Torahi FA (2006). The effect of a-tocopherol on cholesterol and lipoproteins levels in rats. Pak. J. Nutr. 5:337-346.
Altinok I, Capkin E (2007). Histopathology of rainbow trout exposed to sublethal concentration of methiocarb, a carbmate pesticide, to rainbow trout. Toxicol. Pathol. 35:405-410.
APHA, AWWA, WPCF (2005). Standard Methods for the Examination of Water and Waste Water 21st, 401 Ed. American Public Health Associations, Washington, DC.
Bancroft J, Gamble M (2002). Theory and Practice of Histology Techniques. 5th (ed), Churchill Livingstone, London.
Blahova J, Modra H, Sevcikova M, Marsalek P, Zelinickova L, Skoric M, Svobodova Z (2014). Evaluation of biochemical, haematological and histopathological responses and recovery ability of common carp Cyprinus carpio) after acute exposure to atrazine herbicide. Biomed Res. Int. 2014:980948.
Boran H, Capkin E, Altinok I, Terzi E (2012). Assesement of acute toxicity and histopathology of the fungicide captain in rainbow trout. Exp. Toxicol. Pathol. 64:175-179.
Cengiz E, Unlu E (2003). Hisopathology of gills in mosquito fish, *Gambusia affinits* after long-term exposure to sub-lethal concentrations of Malathion. J. Environ. Sci. Health B 38:581-589.
Cengiz El, Unlu E (2006). Sublethal effects of commercial deltamethrin on the structure of the gills, liver and gut tissue of mosquito fish, Gambusia affinis: a microscopic study. Environ. Toxicol. Pharmacol. 21:246-253.
De Silva PM, Samayawardhena LA (2002). Low concentrations of lorsban in water result in far reaching behavioral and histological effects in early stages in guppy. Ecotoxicol. Environ. Saf. 53:248-254.
Elezaby M, El-Serafy S, Heckmann R, Sharf-Eldeen K, Seddek M (2001). Effect of some toxicants on the fresh water fish *Oreochromis niloticus.* J. Egypt Ger. Soc. Zool. 36:407-417.
Ervnest HA (2007). Text book of modern Toxicology (3rded).John Willy and sons, Inc. Hoboken, New Jersey ISBN 0-471-26508-X557.
Eto M (1979). Organophosphorous pesticides: Organic and Biological Chemistry. Boca Raton (FL): CRC Press.
Eyo JE, Levi CA, Asogwa CN, Odii EC, Chukwuka CO, Ivoke N, Onoja US, Onyeke CC (2013) .Toxicity and effect of Carica papaya seed aqueous extract on liver biomarkers of *Clarias gariepinus*. Int. J. Indig. Med. Plants 46:1301-1307.
Fanta E, Rios F, Romao S, Vianna A, Freiberger S (2003). Histopathology of the fish *Corydoras paleatus* contaminated with sub-lethal levels of organophosphorus in water and food. Ecotoxicol. Environ. Saf. 54:119-130
Finney DJ (1971). Probit analysis, 3rd edition Cambridge University Press. Cambridge. P 20.
Jiraungkoorskul W, Upatham E, Kruatrachue M, Sahaplong S, Vichasri-Grams S, Pokethitryook P (2003). Biochemical and histopathological effects of glyphosate herbicide on Nile tilapia (*Oreochromisniloticus).* Environ. Toxicol. 18(4):260-267.
Jungera P (2014). Fenthion safety summary for veterinary use of dogs, cats, cattles, sheep, goats and swine. Toxicity, poisoning, intoxication, overdose, antidote. Parasitipedia.net. Accessed 18th February 2015.
Kim K, Wang X, Choi S, Park G, Koo J, Bai S (2003). No synergistic effects by the dietary Supplementation of ascorbic acid, a-tocopherl acetate and selenium on the growth performance and challenge test of *Edwardsiellatarda*in fingerling Nile tilapia, *Oreochromis niloticus L.* Aquac. Res. 34:1053-1058.
Kumar M, Prasad MR, Srivastva K, Tripathi S, Srivastva AK (2010). Branchial histopathological study of catfish Heteropneustes fossilis following exposure to purified neem extract, Azadirachtin. World J. Zool. 5(4):239-243.
Kunjamma A, Philip B, Bhanu S, JoseJ (2008). Histopathological effects on *Oreochromis mossambicus*(Tilapia) exposed to chlorpyrifos. J. Environ. Res. Dev. 2:553-559
Machado RM, Fanta E (2003). Effects of the organophorous methyl parathion on the branchial epithelium of a freshwater fish *Melynniseoosevelti.* Braz. Arch. Biol. Technol. 46:361-372.
Mataqueiro M, Nakaghi S, de Souza J, da Cruz J, de Oliveira G, Urbinati E (2009). Histopathological changes in the gill, liver and kidney of pacu *(Piaratus mesopotamicus,* exposed to various concentrations of trichlorfon. J. Appl. Ichthyol. 25:124-127.
Miller DS (2002). Xenobiotic export pumps, endothelin signaling and tubular nephrotoxicantsa case of molecular hijacking. J. Biochem. Mol. Toxicol. 23:121-127.
Muralidharan L (2012). Haemato-Biochemical alterations induced by chronic exposure to fenthion in Cyprinus carpio. Trends Fish. Res. 1(3):19-25.
Muralidharan L (2014). Histopathological studies on carp (Cyprinus carpio) exposed to fenthion. Int. J. Adv. Res. 2:11-26.
Murty AS (1986). Toxicity of pesticides to Fish. CRC Press Inc Boca Raton.
Nwani CD, Ama UI, Okoh F,Oji UO, Ogbonyealu RC, Agha A (2013a). Acute toxicity of the chloroacetanilide herbicide butachlor and its effect on behavior of the freshwater fish Tilipia zili. Afr. J. Biotechnol. 12:499-503.
Nwani CD, Ekweme hi, Ejere VC, Onyeke CC, Chukwuka CO, Onas PS, Nwadinigwe AO (2015). Physiological effects of paraquat in juvenile African Catfish Clarias gariepinus (Burchel 1822). J. Coast. Life Med. 3(1):35-43.
Nwani CD, Ivoko N, Ugwu DO, Atama C, Onyishi GC, Echi PC, Ogbonna SA (2013b). Investigation on acute toxicity and behavioural changes in a freshwater catfish, Clarias gariepinus Burchell, 1822), exposed to organophosphorous pesticide, Termifos. Pak. J. Zool. 45(4):959-965.
Nwani CD, Lakra WS, Nagpure NS, Kumar, R, Kushwaha B, Srivastava SK (2010). Mutagenic and genotoxic effects on carbosulphan in freshwater fish in Chanaa Punctatus (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Food Chem. Toxicol. 48:202-208.
Oh HS, Lee SK, Kim YH, Roh JK (1991).Mechanism of selective toxicity of diainon to killifish (Oryzias latipes) and loach (Misgurmus anguillicaudatus).Aquat. Toxicol. Risk. Assess. 14:343-353.
Olurin K, Olojo E, Mbaka G, Akindele A (2006). Histopathological responses to gill and liver tissues of Clarias gariepinus fingerlings to the herbicide, glyphosate. Afr. J. Biotechnol. 5:2480-2487.
Oncu M, Gultekin F, Karaz E, Altunas I, Delibas N (2002). Nephrotoxicity in rats induced by chlorpryfos-ethyl and ameliorating effects of antioxidants. Hum. Exp. Toxicol. 21:223-230.
Packer L, Weber SU, Rerbach G, Weber R (2001).Molecular aspects of a-tocotrienol action and cell signaling. J. Nutr..131(2):369-446.
Peebua P, Kosiyachinda P, Pokethitiyook M, Kruatrachue M (2007). Evaluation of alachlor herbicide impacts on Nile tilapia *(Oreochromisniloticus)* using biochemical biomarkers. Bull. Environ. Contam. Toxicol.78:138-141.
Rahman MZ, Hossain Z, Mellah MFR, Ahmed GU (2002). Effect of Diazinon 60EC on *Anabustestudinus*, *Channapunctatus* and *Barbadesgomonotus*. NAGA: The ICLARM Quarterly 25:8-11.
Rao J, Begum G, Pallela R, Usman PR (2005). Changes in behavior and brain acetycholinesterase activity in mosquito fish, *Gambusia affinis*in Response to the sub-lethal exposure to chlorpyrifos. Int. J. Environ. Res. Public Health 2:478-483.
Rauf A, Arain N (2013).Acute toxicity of diazinon and its effects on haematological parameters in the Indian carp, *Cirrhinus mrigala* (Hamilton).Turk. J. Vet. Anim. Sci. 37:535-540.
Reish DJ, Oshida RS (1987). Manual of methods in aquatic environment research, Part 10. Short-term static bioassay.FAO Fish. Tech. Paper 247, 62.

Roberts RJ (2001). Fish Pathology 3rd (Ed). W.B. Sauners. New York.
Roy S, Bhattacharya S (2006). Arsenic-induced histopathology and synthesis of Stress proteins in liver and kidney of *Channa puntatus*. Ecotoxicol. Environ. Saf. 65(2):218-229.
Santos LH, Araujo AN, Fachini A, Pena A, Deleure-Matos C, Montenegro MCB (2010). Ecotixicogical aspects related to the presence of pharmaceuticals in the aquatic environment. J. Hazard Mat. 175:45-95.
Scheil Vl, Zurn A, Kohler H, Triebskorn R (2009). Embryo development, stress protein (Hsp70) responses, and histopathology in Zebrafish (Daniorerio) following exposure to nickel chloride, chlorpyrifos, and binary mixtures of them. Environ. Toxicol. 25:83-93.
Teh S, Hung S, The F, Deng D, Werner I (2005). Sublethal toxicity of orchard storm water runoff in Sacramento splittail (*Pogonichthys macrolepidotus*) larvae. Mar. Environ. Res. 59(3):203-216.
Todd NE, Van Leeuwen MV (2002). Effects of Sevin (Carbaryl Insecticide) on Early Life Stages of Zebrafish (*Daniorerio*). J. Ecotoxicol. Environ. Saf. 53:267-272.
West G (1990). Methods of assessing ovarian development in fishes-a review. Aust. J. Mar. Freshw. Res. 41(12):99-222.
White A, Fietcher TC (1985). Seasonal changes in serum glucose and condition of the Plaice, Pleuronectes platessa L. J. Fish Biol. 26:755-764.
Wiieyaratne W, Pathiratne A (2006). Acetyl cholinesterase inhibition and gill lesions in *Rasbora caverii*an indigenous fish inhabiting rice field associated water bodies in Sri Lanka. Ecotoxicology 1:609-619.
Yang I, Zha J, Li Z, Wang Z (2010).Atrazaine affects Kidney and adrenal hormones (AHS) related genes expressions of rare minnow (Gobiocypris rarus). Aquat.Toxicol. 97:204-211.

academicJournals

Vol. 15(9), pp. 315-319, 2 March, 2016
DOI: 10.5897/AJB2015.14915
Article Number: FF87FD557402
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Cellulose degradation capabilities of dung beetle, *Euoniticellus intermedius,* larva gut consortia

Munamato Mabhegedhe[1]*, Karl Rumbold[2] and Monde Ntwasa[2]

[1]Gary Magadzire School of Agriculture and Natural Sciences, Great Zimbabwe University, Off Great Zimbabwe Road, P. O. Box 1235, Masvingo, Zimbabwe.
[2]School of Molecular and Cell Biology, Faculty of Science, University of the Witwatersrand, 1 Jan Smuts Avenue, Braamfontein 2000, Johannesburg, South Africa .

Received 14 August, 2015; Accepted 10 December, 2015

This study assessed the capabilities of the dung beetle, *Euoniticellus intermedius* (Coleoptera: Scarabaeida), larva gut consortia in degrading cellulose that can serve as glucose source for biofuels production. A total of 144 live dung beetles were randomly collected from a dairy farm and bred in a temperature controlled insect rearing room. On reaching the late second to third instar stage, dung beetle larvae were harvested, dissected and the gut micro-flora were cultured in medium containing cellulose as sole carbon source. Microbial growth (total protein concentration) and cellulose degradation activity (reducing sugars concentration) in the cellulose cultures were monitored successively for 15 days. Statistical analysis showed that there was significant microbial growth, but no significant increase in reducing sugar levels. Despite the lack of increase in reducing sugar levels, it was concluded that the dung beetle larva gut has micro-flora with cellulose degrading capabilities that allowed it to grow and survive in the cellulose minimal medium.

Key words: Dung beetle, *Euoniticellus intermedius*, cellulose, biofuels.

INTRODUCTION

The global energy demand has increased more than twenty fold in the last century (Jegannathan et al., 2009). Previously, abundant and cheaply accessible, fossil fuels have undoubtedly played a big role in the industrialization of most of the developed states seen today and they are still an integral part of today's energy mix. However, due to their non-renewable nature, fossil fuel reserves have been dwindling at an alarming rate, at a crucial time when the world' energy needs have also been on a constant rise (Adsul et al., 2011; Jegannathan et al., 2009). In addition, the damaging environmental effects such as global warming and climate change, associated with fossil fuel use have also compounded the current challenges attributed to the world's heavy reliance on fossil fuels (Adsul et al., 2011; Jegannathan et al., 2009). To counter the consequences associated with rampant fossil fuel use, massive investments in renewable and sustainable energy resources have been recently noted

*Corresponding author. E-mail: mmabhegedhe@gzu.ac.zw, mmabhegedhe@gmail.com, mmabhegedhe@yahoo.co.uk. Tel: +263 775 295641.

with sustainable bio-fuels research also given top priority.

Historically, transportation bio-fuels like bio-ethanol have primarily been derived from food sources such as sugar cane and corn (Balat and Balat, 2009; Yuan et al., 2008), a situation that has sparked a lot of controversy on food versus fuel debate. This has consequently led to more research being focused towards the production of second generation bio-fuels, such as cellulose-derived bio-ethanol. Despite huge financial investment and concerted research efforts in this area, and the abundance of cellulose raw materials, production of second generation bio-ethanol at an industrial scale is still hampered by unreasonably high operating costs due to the recalcitrant nature of cellulose, which ultimately makes its production economically unfeasible (Margeot et al., 2009; Ruane et al., 2010).

Many organisms (cattle, rabbits, buffalo, elephants and insects) have been reported to have the capability to degrade cellulose matter through the use of symbiotic micro-organisms and their own cellulose degrading enzymes (Inoue et al., 2005; Feng et al., 2007; Duan et al., 2009; Wang et al., 2009). Scarab beetles have been reported as one of such organisms since they can effectively utilize various cellulose plant matter and animal waste as energy sources (Huang et al., 2010). Dung beetles belong to the family Scarabaeidae and order Coleoptera, which is regarded as the largest order in the insect kingdom. Scarab beetles are commonly found in various environmental niches where decaying plant and/or animal wastes form a high proportion of the available biomass (Huang et al., 2010). This is because scarabaeids are reportedly herbivorous or saprophagous and many of their species feed on plant roots, decaying organic matter and animal waste of very low nutritional value (Zhang and Jackson, 2008). As such, scarab beetles play an important role in the ecological control of dung, decaying wood and plant matter (Koyama et al., 2003).

Even today, a lot of research is still being undertaken in a bid to find the elusive solution and consequently break the financial barriers which are currently hindering any progress in the industrial production of the second generation bio-fuels. In this study, the biology of *E. intermedius*, a dung beetle that spends its life cycle in cow dung was studied. The dung beetle larvae were selected in this study because they eat the actual dung, whereas adult dung beetles only survived on the dung juices (Khanyile et al., 2008). Consequently, it is expected that the larval stage of this organism possesses biological capability for efficient processing of dung. This research seeks to contribute to the growing list of possible biological routes of cellulose degradation by investigating the cellulose degrading capabilities of the symbiotic micro-flora naturally residing in the gut of the dung beetle, *Euoniticellus intermedius* (Coleoptera: Scarabaeida), larvae, and their potential application and contribution to the sustainable production of cellulose-derived bio-fuels.

MATERIALS AND METHODS

Dung beetle collection

In this study, a total of 144 dung beetles that were randomly collected from a dairy farm about 10 km South-West of Johannesburg, South Africa were used. The insects were collected between August and September, 2010 and bred at a temperature (28°C) controlled dung beetle rearing room. According to Edwards (1991), this is regarded as the best time to collect the dung beetles in Johannesburg, due to the high rainfall patterns, which favour high reproduction and survival rates. The adult dung beetles were collected from deep inside or the surface of the soil beneath the dung pats.

Dung beetle rearing

Initially, 144 beetles were sorted into 17 males and 127 females. Males were identified by the presence of a horn on the head, a feature that is absent in the females. In population numbers, the females were found to be more than 6 times numerous than the males. It is also known that a single male can mate with more than one female. Therefore, instead of using 3 pairs of dung beetles in equal male and female proportion, two males were mixed with four females in a breeding container. Breeding was done in plastic containers measuring 160 × 130 × 130 mm. The containers were half-filled with thoroughly sieved and compacted slightly moist soil. Fresh cattle dung collected from the same farm as the dung beetles was used as the dung beetle feed and was stored in small 1 kg plastic packets at -20°C. On every third day (72 h), one packet of dung was added to each container containing the breeding pairs. The soil component provided the beetles with tunnel burrowing space to make the brood balls.

Once a week, the breeding containers were sieved to remove any brood balls present. An average of 20 to 30 brood balls per week was harvested from each plastic container with dung beetle breeding pairs. The brood balls from different containers were then pooled together and placed in a separate plastic container, half-filled with moist sand, measuring 400 × 300 × 200 mm and then labeled according to the date of collection. A wet sponge was placed on top of the sand to maintain the desired moisture levels.

Larvae developed inside the brood ball and their nourishment came from the dung contained therein. Unlike the adult dung beetles which feed on the fluid component of the dung, the larvae feed by ingesting whole dung particles (Edwards, 1991). To propagate the dung beetle culture, larvae were initially allowed to grow for 4 to 5 weeks developing into adults. Newly, emerged beetles were collected and kept in single-sex plastic containers for 2 weeks to allow them to develop into sexual maturity (Lailvaux et al., 2005; Pomfret and Knell, 2006).

Dung beetle larvae dissection

Larvae were allowed to develop for approximately 2 to 3 weeks before harvesting them for dissection. This allowed them to grow into late second or third instars.

A modified dung beetle dissection protocol similar to the one described by Lemke et al. (2003) was used to perform the dissection. The steel/metal dissection equipment was autoclaved at 121°C for 15 min. The preparation dish was immersed in 70% ethanol and left overnight (approximately 12 hours) to minimize microbial contamination. Insect ringer solution was prepared

according to the protocol described by Hayashi and Kamimura (2001) and autoclaved for 15 min at 121°C. Larvae dissection was performed in a sterile preparation dish under a dissecting microscope, in a laminar flow cabinet. The larvae were first anesthetized by exposing them to a nitrogen, hydrogen and carbon dioxide (71/7/22 vol/vol respectively) (Afrox grade) gas mixture for 15 min. They were then fixed onto the preparation dish with steel pins, with the larvae laid on its sides, and sterile insect ringer solution added. The cuticle was cut along the side lines and the ventral integument, circular muscles and trachea were then carefully removed. After decapitating a larva, a circular cut was made on the anus to allow careful retrieval of the intestinal tract. The guts were then stored in 1.5 ml tubes at -70°C.

Culturing of dung beetle larvae gut micro-flora

Media preparation

A basal cellulose medium for gut micro-flora cultivation was prepared using cellulose (Whatman filter paper) as the sole carbon source, according to a modified method described by Lemke et al. (2003). To the basal media preparation, 1 ml of trace element solution (SL11) and 1 ml of selenium tungstate solution were added and then adjusted to pH 8 using a 0.1 M sodium phosphate buffer before autoclaving.

Media inoculation

Larval guts weighing 1 g were homogenized in 9 ml of phosphate buffered saline (pH 7.4). The homogenate (1 ml) was then inoculated into 9 ml of cellulose media (in the place of Whatman filter paper, 0.405 g of cellulose microcrystalline was used during pre-culturing experiments). The inoculations were done in triplicate. The pre-cultures were incubated aerobically at 30°C for 48 h in the dark, shaking at a speed of 80 revolutions/min on a rotary shaker. Microbial growth was visually observed by the degree of turbidity of cultures after 24 and 48 h of incubation period.

Inoculation of pre-culture samples (10 ml) into the main cultures (90 ml) was done in triplicate after 48 h of pre-culturing. The main cultures were incubated aerobically at 30°C in the dark, shaking at 80 rpm for 15 days. Samples for microbial growth determination and cellulose degradation activity were aseptically taken on a daily basis, 3 ml per culture sample and kept at -70°C until analysis.

Microbial growth determination

In order to monitor microbial growth, the time (number of days) required to achieve maximal microbial growth was determined as a function of total protein concentration, using the Bradford protein assay procedure (Bradford, 1976). This assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions play a crucial role in stabilizing the anionic form of the dye, resulting in a visible colour change (Bradford, 1976; Stoscheck, 1990).

Samples (Day 0 to Day 15) for microbial growth determination were taken daily in triplicate (one sample from each main culture) and pooled together to make a composite sample. Due to the poor microbial growth in the culture samples, the Bradford micro-assay method was used to plot the standard curve.

Total protein concentration determination in the test-samples was done by adding test-samples to the Bradford working reagent in a glass spectrophotometer cuvette and incubating the mixture for 5 min at room temperature. Volume adjustments were done on the samples to get absorbance values of between 0.1 and 0.9. The absorbance values of the test-samples were then measured using a spectrophotometer at 595 nm wavelength, as mentioned earlier. The total protein concentrations of the samples were determined by comparing the samples' absorbance values against the plotted Bradford micro-assay standard curve. Any volume adjustments performed on the samples were catered for during calculations of total protein concentration. Measurements were done in quartet for every composite daily culture sample. Total protein concentration values were regarded as directly proportional to microbial growth.

Cellulose degradation

Cellulose degradation activity was determined by measuring the concentration of released reducing sugars in the culture samples using a modified dinitrosalicyclic acid (DNS) assay described by Adney and Baker (1996).

To measure the reducing sugar concentration in the cellulose culture samples, 0.5 ml samples were added to test tubes containing 1 ml citrate buffer and 3 ml of DNS solution added, as described earlier. The mixture was boiled for 5 to 10 min, allowed to cool. Absorbance of the test-samples was then measured at 540 nm without diluting them. Reducing sugar concentration was determined by comparing the absorbance values of test-samples against the plotted glucose standard curve. The volume adjustments made for the standard curve were taken into consideration during the test-sample glucose/reducing sugar concentration calculations. Measurements were done in quartet for every composite culture sample.

RESULTS

Microbial growth

Microbial growth was observed during the course of 15 days increasing from a total protein concentration of 15.1955 µg/ml on Day 1 to 25.34475 µg/ml on Day 15. This increase was shown to be significant at 5% level using the statistical analysis equation $y = 15.319e^{0.0262x}$ and R^2 value of 0.5395 as shown in Figure 1. Statistical analyses were done using Microsoft Excel (2010).

Cellulose degradation

The reducing sugar concentrations in culture samples remained relatively constant at 2.9 mg/ml from Day 0 to Day 15, with an exception of Day 5 where a concentration of 3.1 mg/ml was observed (Figure 2).

DISCUSSION

The observed increase in microbial growth is attributed to the ability of the dung beetle larva gut consortia to successfully degrade cellulose and use the by-products for its metabolic activities essential for growth and survival. This is in agreement with previous research that has shown that different insects have gut microbial consortia capable of digesting cellulose material into many different metabolites (Lemke et al., 2003; Dillion

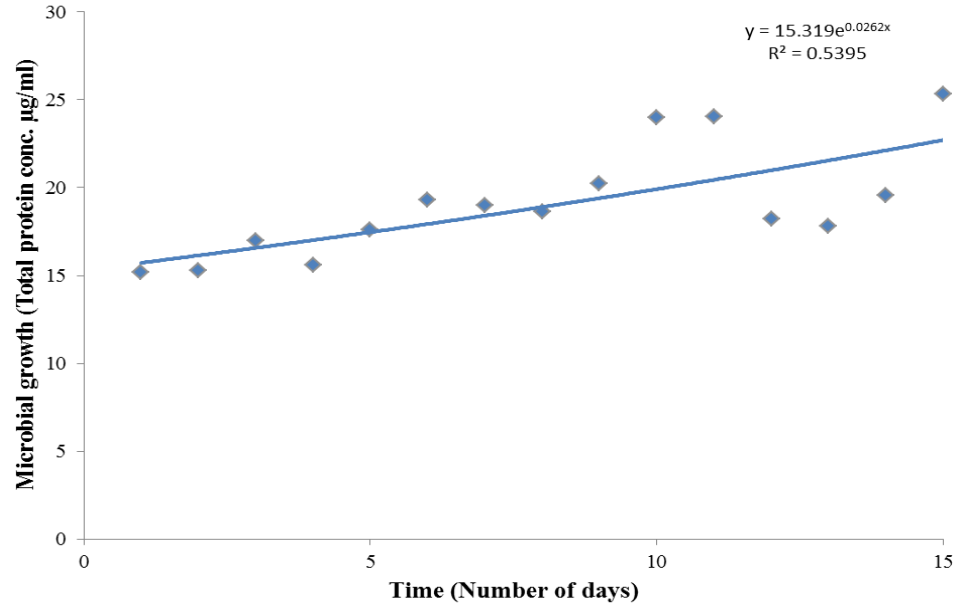

Figure 1. Microbial growth of cellulose media culture.

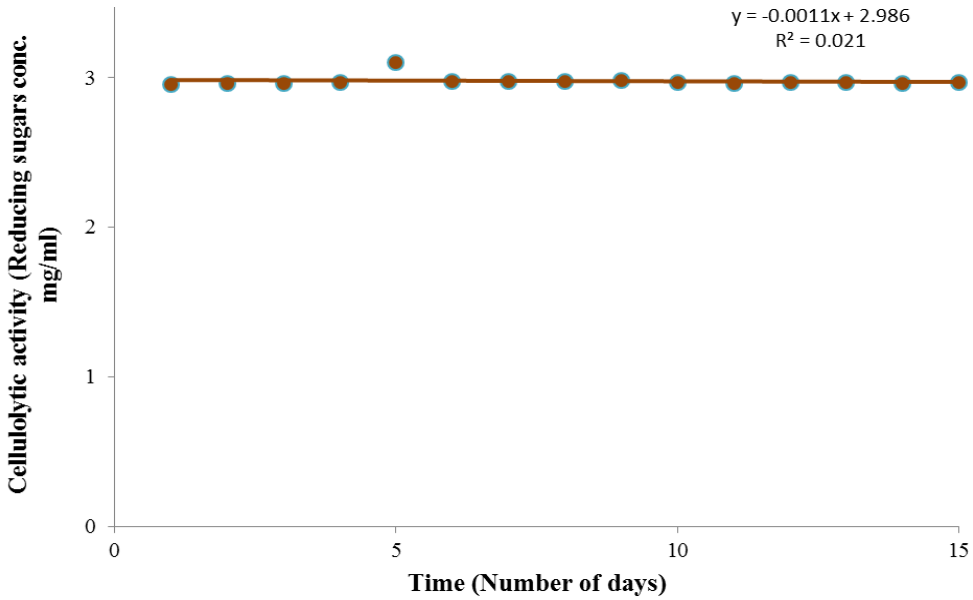

Figure 2. Cellulose degradation activity observed in cellulose media culture samples.

and Dillion, 2004; Egert et al., 2005). Instead of sharp increase in growth normally associated with microbial cultures, the observed steady growth pattern can be explained by the disruptive effect of cellulose medium culturing (by only selecting cellulose degrading micro-flora and excluding non-cellulose degrading organisms) to the inherent community structure and relationships which naturally exists in the dung beetle larva gut. According to Streit and Schmitz (2004), many culture-dependent approaches currently used to explore the diversity and potential of microbial communities are biased, because of the limitations of cultivation methods. In addition to this, in the dung beetle larva digestive tract, different types of micro-organisms work synergistically, by performing different roles which collectively contribute to the survival and well-being of the whole microbial community (Streit and Schmitz, 2004).

Previous research work has shown that the gut of

scarab beetle larvae comprises a rich population of xylan and pectin degrading micro-organisms (Egert et al., 2005; Zhang and Jackson, 2008). The lack of increase in reducing sugar concentration in the cultures is attributed to the ability of the larva gut micro-flora to immediately utilize the produced reducing sugars as a carbon source, in addition to cellulose matter. As mentioned previously, in the digestive tract of the dung beetle larva, different types of micro-organisms work synergistically by performing different roles (these may include cellulose degradation and other hydrolytic roles), which collectively contribute to the survival and well-being of the whole microbial community (Huang et al., 2010). This can mean that any reducing sugars produced during the cellulose degradation process were immediately utilized by another sub-section of the cultured gut micro-flora before they could be accounted for using the DNS assay.

Conclusion

The cellulose degradation capability of the cultured dung beetle larva gut micro-flora was successfully demonstrated by the ability of the gut micro-flora to grow in exclusive cellulose medium. Despite the observed insignificant increase in reducing sugars, it can be concluded that the mere ability of the gut micro-flora to significantly grow and survive on cellulose media containing only filter paper as the sole carbon source should confirm that there is indeed a sub-set of gut micro-flora from *E. intermedius* that can degrade cellulose matter.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

The authors would like to thank Mr. Lazarus Chapungu for his help in editing this manuscript. Special thanks go to Mr. Phanankosi Moyo for laying the foundation upon which this study was established and for taking time from his busy schedule to review the manuscript. This research was funded by the University of the Witwatersrand Postgraduate Merit Award (PMA), the Bradlow Scholarship and the African Union Commission (Mwalimu Nyerere African Union Postgraduate Scholarship).

REFERENCES

Adney B, Baker J (1996). Measurement of cellulase activities. Laboratory Analytical Procedures (LAP). Technical Report NREL/TP-510-42628. January 2008.

Adsul MG, Singhvi MS, Gaikaiwari SA, Gokhale DV (2011). Development of biocatalysts for production of commodity chemicals from lignocellulosic biomass. Bioresour. Technol. 102:43042-4312.

Balat M, Balat H (2009). Recent trends in global production and utilization of bio-ethanol fuel. Appl. Energy. 86:2273-2282.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Dillion RJ, Dillion VM (2004). The gut bacteria of insects: Non-pathogenic interactions. Annu. Rev. Entomol. 49:71-92.

Duan C, Xian L, Zhao G, Feng Y, Pang H, Bai X, Tang J, Ma Q, Feng J (2009). Isolation and partial characterization of novel genes encoding acidic cellulases from metagenomes of buffalo rumens. J. Appl. Microbiol. 107:245-256.

Edwards PB (1991). Seasonal variation in the dung of African grazing mammals, and its consequences for coprophagous insects. Funct. Ecol. 5:617-628.

Egert M, Stingl U, Bruun LD, Pommerenke B, Brune A, Friedrich MW (2005). Structure and topology of microbial communities in the major gut compartments of *Melolontha melolontha* larvae (Coleoptera: Scarabaeidae). Appl. Environ. Microbiol. 71:4556-4566.

Feng Y, Duan C, Pang H, Mo X, Wu C, Yu Y, Hu Y, Wei J, Tang J, Feng J (2007). Cloning and identification of novel cellulase genes from uncultured microorganisms in rabbit cecum and characterization of the expressed cellulases. Appl. Microbiol. Biotechnol. 75:319-328.

Hayashi F, Kamimura Y (2001). The potential for incorporation of male derived proteins into developing eggs in the leafhopper Bothrogonia ferruginea. J. Insect Physiol. 48:153-159.

Huang S, Zhang H, Marshall S, Jackson TA (2010). The scarab gut: A potential bioreactor for bio-fuel production. Insect Sci. 17:175-183.

Inoue T, Moriya S, Ohkuma M, Kudo T (2005). Molecular cloning and characterization of a cellulase gene from a symbiotic protist of the lower termite, *Coptotermes formosanus*. Gene 349:67-75.

Jegannathan KR, Chan E, Ravindra P (2009). Harnessing biofuels: A global Renaissance in energy production? Renew. Sustain. Energy Rev. 13: 2163-2168.

Khanyile L M, Hull R, Ntwasa M (2008). Dung beetle database: Comparison with other invertebrate transcriptomes. Bioinformation 3 (4):159-161.

Koyama M, Iwata R, Yamane A, Katase T, Ueda S (2003). Nutrient intake in the third instar larvae of *Anomala cuprea* and *Protaetia orientalis submarmorea* (Coleoptera: Scarabaeidae) from a mixture of cow dung and wood chips: Results from stable isotope analyses of nitrogen and carbon. Appl. Entomol. Zool. 38:305-311.

Lailvaux SP, Hathway J, Pomfret J, Knell RJ (2005). Horn size predicts physical performance in the beetle *Euoniticellus intermedius* (Coleoptera: Scarabaeidae). Funct. Ecol. 19:632-639.

Lemke T, Stingl U, Egert M, Friedrich MW, Brune A (2003). Physicochemical conditions and microbial activities in the highly alkaline gut of the humus feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Appl. Environ. Microbiol. 69:6650-6658

Margeot A, Hahn-Hagerdal B, Edlund M, Slade R, Monot F (2009). New improvements for lignocellulosic ethanol. Curr. Opin. Biotechnol. 20:372-380.

Pomfret JC, Knell RJ (2006). Sexual selection and horn allometry in the dung beetle *Euoniticellus intermedius*. Anim. Behav. 71:567-576.

Ruane J, Sonnino A, Agostini A (2010). Bioenergy and the potential contribution of agricultural biotechnologies in developing countries. Biomass Bioenergy 34:1427-1439.

Stoscheck CM (1990). Quantitation of Protein. Methods Enzymol. 182:50-69.

Streit WR, Schmitz RA (2004). Metagenomics – the key to the uncultured microbes. Curr. Opin. Microbiol. 7:492-498.

Wang F, Li F, Chen G, Liu W (2009). Isolation and characterization of novel cellulase genes from uncultured microorganisms in different environmental niches. Microbiol. Res. 164:650-657.

Yuan JS, Tiller KH, Al-Ahmad H, Stewart NR, Stewart Jr. CN (2008). Plants to power: bioenergy to fuel the future. Trends Plant Sci. 13:421-429.

Zhang HY, Jackson TA (2008). Autochthonous bacterial flora indicated by PCR-DGGE of 16S rRNA gene fragments from the alimentary tract of *Costelytra zealandica* (Coleoptera: Scarabaeidae). J. Appl. Microbiol. 105:1277-1285.

academicJournals

Vol. 15(9), pp. 320-330, 2 March, 2016
DOI: 10.5897/AJB2015.14825
Article Number: 36363D657404
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

HPLC quantification of phenolic content and assessment of methanolic extract of *Antiaris africana* for toxicological study

Omotayo B. Ilesanmi[1,2]*, Tolulope M. Olaleye[1], Afolabi C. Akinmoladun[1] and Taiwo T. Alawode[3]

[1]Department of Biochemistry, School of Sciences, Federal University of Technology Akure, Ondo state, Nigeria.
[2]Department of Biological Sciences, Faculty of Science, Federal University Otuoke, Bayelsa stae.
[3]Department of Chemical Science, Faculty of Science, Federal University Otuoke, Bayelsa State.

Received 29 June, 2015; Accepted 17 December, 2015

The study was aimed at evaluating the toxicological and antioxidant activities of *Antiaris africana* Engl. (family Moraceae), that is used in Nigeria and other West Africa countries as a panacea for the treatment of several ailments. The methanolic extract of *A. africana* (MEA) obtained was analysed for antioxidant activities *in vitro* and screened for various phytochemicals present. Phenolic and flavonoid contents were determined followed with high performance liquid chromatography -diode-array detection (HPLC-DAD) fingerprinting of phenolic content. Furthermore, the sub-acute toxicity of MEA was determined via oral administration of varying doses for 14 consecutive days (0, 50, 100, 200 and 400 mg/kg) in rats. After oral administration for 14 consecutive days in male rats, the toxicity effect was assayed by determining aspartate aminotransferase (AST) and alanine aminotransferase (ALT) for hepatic function; urea and creatinine for renal function; creatinine kinase (CK) for cardiac function; and lipid profile. HPLC results showed that the major phenolics present are quercetin, rutin, caffeic acid, garlic acid and quercetin. MEA was able to scavenge diphenyl picryl hydrazyl, hydroxyl and nitric oxide radicals and prevent lipid peroxidation induced by ferrous sulphate at all concentration tested. The toxicology investigation showed that at low doses, *A. africana* is non-toxic, while at high doses; it is moderately toxic to the animals. In conclusion, *A. Africana* is generally non-toxic; however, care must be taken in administration at higher doses.

Key words: Toxicology, HPLC, phytochemicals, *Antiaris africana.*

INTRODUCTION

Herbal medicine is gaining ground as the treatment of choice in the western world (Dey and De, 2015). Most countries in Africa and other developing countries rely on its usage for their primary healthcare (Eisenberg et al., 1998; World Health Organization, 2008). Nigeria has been planning on integrating herbal medicine as a degree program into the tertiary institution curriculum (Vanguard, 2014). This development is not surprising, looking at the

*Corresponding author. E-mail: ilesanmi.omotayo@gmail.com. Tel: +2348062320794.

fact that traditional medicine has played an important role in disease treatment since the emergence of man's evolution (Ahmada et al., 2013; Pankaj et al., 2009). Most drugs have their origin from natural sources, either they are directly isolated from plants or the isolated compound is modified to improve its efficacy (Fu et al., 2013). Most natural compounds are majorly classified as alkaloids, steroids, tannins, phenolic compounds, flavonoids and saponin (Bishnu et al., 2009). *Antiaris africana* is a plant found in various parts of Nigeria and West Africa. It is commonly called Ooro, Oriro or ako Iroko in the South West part of Nigeria; Farin Loko in the North; and Ojianwu in the South East. The plant is a large tree usually about 15 to 20 m high, but it can grow sometimes up to 40 m, and has white latex and alternate dissymmetric leaves (Berg et al., 1985; Berhaut, 1979), with heavy flat crown and blotchy grey and white bark. The flowers are small with a greenish white color that produces a red velvety fruits (Gill, 1992). It has a wide usage both in industry (timber making) and traditional medicine. Various part of the plant such as leaves, stems and barks are ethnobotanically used in the treatment of various diseases such as rheumatic and respiratory infection (Gill, 1992; Mann et al., 2003), epilepsy, lumbargo, skin irritant, purgative, chest pain (Okogun et al., 1976), syphilis (Berhaut, 1979), throat infection, leprosy, cancer (Kuete et al., 2009), and nervous disorders in the northern part of Nigeria (Moronkola and Faruq, 2013). However, the report on the industrial usage has overshadowed its ethno pharmacological usage as more works are published on it as compared to the latter. This has lead to dearth of report on the scientific rationale behind its ethnopharmarcological usage. Therefore, this experiment was conducted to give scientific insight to its traditional usage.

MATERIALS AND METHODS

Chemicals

Quercetin, rutin, ascorbic acid, tannic acid, Folin-Ciocalteu reagent, sodium carbonate, aluminum chloride, potassium acetate, deoxyribose, 1,1, diphenyl – 2,2- picryl hydrazyl (DPPH), were obtained from Sigma (Chemical Co, St. Louis, MO, USA). ALT, AST, creatinine (CREA), urea, CK, total cholesterol, triglyceride and high density lipoprotein kits were from Randox. All other reagents were of analytical grade. All UV–Vis measurements were recorded on a Shimadzu UV–1800.

Plant collection and preparation of extract

Leaves of *A. africana* were collected at Forest Research Institute of Nigeria (FRIN) garden and authenticated at the herbarium of Botany Department, University of Ibadan, Nigeria by Mr. Esimakhair (voucher number 070613M). The leaves were air dried and pulverized. The powdered sample (1.23 kg) was macerated in 5 L of 80% methanol for 72 h and then filtered. The filtrate was concentrated and then lyophilized to obtain the 81.1 g methanol extract of *A. africana* (MEA) used for the study. This was stored in an amber bottle and refrigerated.

Phytochemical screening

Extracts were phytochemically screened for the presence of alkaloids, saponins, tannins, phlobatannins, anthraquinones, flavonoids, steroids, and terpenoids using standard laboratory procedures (Sofowora, 1993; Trease and Evans, 1985).

Estimation of total phenolic content

The total phenolic content of the extract was estimated according to a modified procedure of Singleton et al. (1999). Briefly, deionised water (0.5 ml) and 125 µl of Folin–Colcalteu reagent were added to 125 µl of MEA dissolved in distilled water. The mixture was allowed to stand for 6 min before adding 1.25 ml of 7% (w/v) Na_2CO_3 solution. The reaction mixture was then allowed to stand for additional 90 min before taking the absorbance at 760 nm against the blank. The tannic acid standard curve was prepared by adding 125 µl of tannic acid dissolved in distilled water (2, 4, 8 and 10 µg/ml final concentrations) in lieu of extract. The amount of total phenolics was expressed as tannic acid equivalents (TAE, mg tannic acid/g sample) through the calibration curve of tannic acid.

Estimation total flavonoid content

Flavonoid content was estimated using the aluminum chloride colorimetric method (Chang et al., 2002). The plant extract in methanol (1 g/ml) was mixed with 0.1 ml of 10% aluminum chloride (w/v), 0.1 ml of 1 M potassium acetate and 2.8 ml distilled water. The mixture was allowed to stand at room temperature for 30 min. The absorbance of the reaction mixture was read at 415 nm. Results were expressed as mg/g quercetin equivalent (QE).

Quantification of compounds in extract by HPLC-DAD

HPLC fingerprinting and reverse phase chromatographic analyses were carried out under gradient conditions using agilent eclipse plus C_{18} column (4.6 x 150 mm) packed with 5 µm diameter particles. Quantification of phenols involved a mobile phase made up of water containing 2% acetic acid (A) and methanol (B). The composition of B was varied starting from the more polar (5% of B) until 2 min and increased gradually till 100% of B as described by the method of Sabir et al. (2012), with slight modification. All chromatography operations were carried out at ambient temperature and in triplicate. High performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software.

Determination of reducing power

Varying amounts of the extract (10 to 800 µg) in 1 ml of distilled water were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of 10% TCA was added to the mixture which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$ and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Nitric oxide radical scavenging assay

10-400 µg of MEA was added in the test tubes to 1 ml of sodium nitroprusside solution (25 mM) and the tubes incubated at 37°C for 2 h. An aliquot (0.5 ml) of the incubation solution was removed and diluted with 0.3 ml of Griess reagent (1% sulphanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed was immediately read at 570 nm against distilled water as blank with catechin (50 µg) used as standard. Results were expressed as percentage radical scavenging activity (RSA).

Potential to inhibit deoxyribose degradation (deoxyribose assay)

200-1000 µg of sample in 100 µl of distilled water was added to a solution containing 200 µl KH_2PO_4 – KOH (100 mM), 200 ul deoxyribose (15 mM), 200 µl $FeCl_3$ (500 µM) and 100 µl EDTA (1 mM) in a test tube and allowed to mix. The reaction was initiated by addition of 100 µl H_2O_2 (10 mM) and 100 µl ascorbic acid (1 mM). The reaction mixture was incubated at 37°C for 1 h. At the end of the incubation period, 1 ml of 1% w/v TBA was added to each mixture followed by the addition of 1 ml of 2.8% w/v TCA. The solution was heated in a water bath at 80°C for 20 min to develop the pink colored MDA-$(TBA)_2$ adduct. After cooling, the solution was centrifuged and the absorbance of the supernatant measured at 532 nm against distilled water as blank. Results were expressed as the percentage inhibition of deoxyribose degradation.

Inhibition of Fe^{2+}/ascorbate – induced lipid peroxidation

Liver homogenate from rat was prepared by removing liver immediately after sacrifice. The liver was rinsed in ice-cold 1.15% KCl to remove blood stain, blotted and weighed. The weighed tissue was then homogenized in four volumes of ice-cold 0.1 M phosphate buffer; pH 7.4. The reaction mixture containing 0.1 ml of liver homogenate in 30 mM tris buffer, 0.16 mM ferrous ammonium sulphate, 0.06 mM ascorbic acid and different amount of the extract (10-1000 µg), was incubated for 1 h at 37°C. The resulting thiobarbituric reactive species (TBARS) was measured by the method of Varshney and Kale (1990). An aliquot (0.4 ml) of the reaction mixture was mixed with 1.6 ml of 0.15 M tris-KCl buffer to which 0.5 ml of 30% TCA was added. Then 0.5 ml of 0.75% TBA was added and placed in a water bath for 30 min at 85°C, after which it was cooled in ice and centrifuged at room temperature for 3 min at 3000 *g*. The absorbance of the clear supernatant was measured against reference blank of distilled water at 532 nm.

Animal handling and treatment

Animals

Albino rats (Wistar strain) aged four weeks were obtained from the animal house of the Lagos University Teaching Hospital and fed with commercially available standard pelleted feed and water *ad libitum* throughout the period of experiment. All animal experimental protocols conformed to the international guide for the care and use of laboratory animals (National Research Council, 2011).

Rats were randomly divided into five groups with six animals per group. Group I (control) received normal saline (0.9% NaCl) orally. Groups II, III, IV and V received MEA (50, 100, 200 and 400 mg/kg/day, respectively) orally for 14 consecutive days. Twenty four hours after the last administration, blood sample was collected via cervical dislocation for the evaluation of markers of oxidative stress and hepatic, renal and cardiac functions.

There were no observable physical changes in animals administered MEA as compared with the control throughout the duration of the experiment.

ASSAY

Serum collections

At the end of 14-day period, blood was obtained via cardiac puncture under light chlorohydate anaesthesia. Blood was collected in serum bottles. The blood was centrifuged at 4000 rpm at 4°C for 10 min to obtain the serum, which was stored at -20°C until analysis for biochemical parameters.

Biochemical estimations

The activities of albumin, AST, ALT, ALP, CK, CREA, urea, triglyceride (TG), total cholesterol (TC), high density lipoprotein (HDL) and low density lipoprotein (LDL) were estimated using assay kits from Randox Laboratories Ltd., UK according to the instructions of the manufacturer.

Statistical analysis

Results are expressed as mean ± standard deviation. Differences between groups were determined by one-way analysis of variance (ANOVA) using SPSS software package for windows. Post hoc testing was performed for inter-group comparisons using the least significant difference (LSD) test and p-value < 0.05 was considered significant.

RESULTS

HPLC analysis

HPLC fingerprinting of *A. Africana* extract revealed the presence of the garlic acid (t_R = 12.40 min; peak 1), catechin (t_R = 16.35 min; peak 2), chlorogenic acid (t_R = 23.08 min; peak 3), caffeic acid (t_R = 25.39 min; peak 4), ellagic acid (t_R = 30.79 min; peak 5), epigallocatechin (t_R = 33.56 min; peak 6), rutin (t_R = 39.18 min; peak 7), isoquercitrin (t_R = 43.97 min; peak 8), quercitrin (t_R = 47.73 min; peak 9), quercetin (t_R = 52.03 min; peak 10) and kaempferol (t_R = 64.15 min; peak 11) (Figure 1 and Table 3).

Phytochemical screening revealed the presence of saponin, tannin, phlobatannin, flavonoid and terpenoid in the methanol extract of *A. africana* (MEA) leaves. Phytochemical screening also showed that alkaloid is absent in the MEA (Table 1) while quantification of total phenolic content TPC was 243.71±13.18 mg TAE/g extract and total flavonoids was 155.85±9.28 mg QE/g extract (Table 2). HPLC DAD reveals the presence of gallic acid, catechin, chlorogenic acid, caffeic acid, ellagic acid, epigallocatechin, rutin, isoquercitrin, quercetin and kaempferol. Caffeic acid is the most abundant phenolic compound (35.97 ± 0.02 mg/g extract) and rutin is the most abundant flavonoid (30.37 ± 0.04 mg/g extract) (Table 3 and Figure 1).

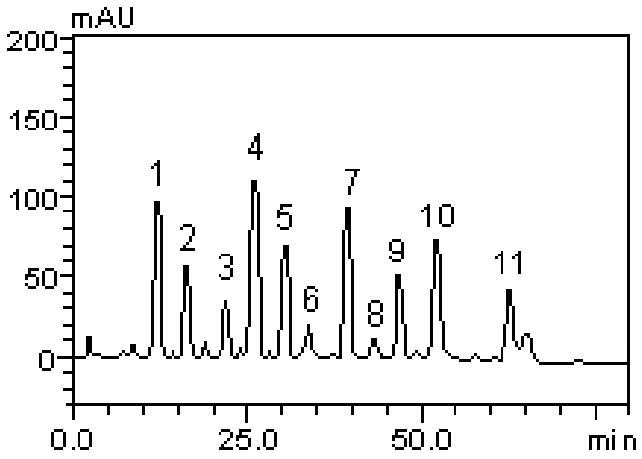

Figure 1. Representative high performance liquid chromatography profile of *Antiaris africana* extract: gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak 5), epigallocatechin (peak 6), rutin (peak 7), isoquercitrin (peak 8), quercitrin (peak 9), quercetin (peak 10) and kaempferol (peak 11). Chromatographic conditions are described in the methods section.

Table 1. Phytochemical constituents of *Antiaris africana.*

Phytochemical	Observation
Alkaloid	Absent
Saponin	Present
Tannin	Present
Phlobatannins	Present
Anthraquinone	Absent
Flavonoid	Present
Terpenoids	Present

The antioxidant activity of plants is generally attributed to the presence of phytochemicals present. Free radical scavenging and inhibition of TBARS are one of the important assays for the determination of antioxidant activity of plant extracts. Hence, in order to explore and understand these possible mechanisms, several antioxidant assays including NO, DPPH and OH radical scavenging assays were performed and evaluation of the antioxidant activities of the results confirmed that this plant has a broad range of antioxidant properties, including substantial inhibition of lipid peroxidation.

Analysis of the free radical scavenging of NO (Figure 2), DPPH (Figure 3) and OH (Figure 4) of the extracts revealed a concentration-dependent antiradical activity resulting in the conversion of the radicals to non- radical form. MEA significantly scavenge hydroxyl radical generation as observed in the percentage increase in prevention of deoxyribose degradation at all concentration. Also, MEA showed a concentration-dependent anti- DPPH radical scavenging ability. It thus appears that the extracts possess hydrogen donating ability and act as antioxidant. However, the scavenging ability of quercetin, a known antioxidant used as positive control was greater than that of the extracts.

The antioxidant activity was further analyzed by the TBARS method, which is used to quantify lipid peroxidation that corresponds to a cell membrane damage caused by oxidative stress. The Fe (II) induced stimulation in brain TBARS levels. The MEA at all tested concentration was able to prevent lipid peroxidation as observed in the reduction in the amount of TBARS formed (Figure 5), though it was not as potent as the reference drug (quercetin).

Reducing power ability

One of the key activities of antioxidant is their ability to donate electrons and thus reducing radicals to a less active species. This is measured by the reduction of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} formation can be monitored spectrophotometrically. At concentration 400 µg/ml, the methanolic extract (0.7013 ± 0.194) showed greater absorbance than ascorbic acid (0.6016 ± 0.338). Reducing power of the MEA is shown in Figure 6.

Renal function indices

Figure 7 shows the effect of MEA on renal function. All doses of MEA had no significant effect on urea level in the serum as compared to the control ($P<0.05$), with the urea level falling between 18.63 ± 0.4 and 28.0 3± 0.72 mg/dl, except 200 mg/kg urea level of 6.20 ± 1.51 mg/dl, while the creatinine level was significantly decreased in the serum as compared to the control ($P<0.05$) with the concentration falling between 0.46 ± 0.04 to 0.58 ± 0.06 mg/dl.

Hepatic and cardiac function

Figures 8 and 9 reveal the AST, ALT and CK activity respectively in animals administered MEA. There was no significant difference in the activity of AST at all doses as compared to the control, with the activity falling between 58.00 ± 2.8 to 84.20 ± 3.5U/I. The serum ALT activityvaries with each doses of extract administered when compared to the control. MEA at 50 and 100 mg/kg with 20.4 ± 0.19 and 14.8 ± 0.34 U/I, respectively was not significantly different from the control ($P<0.05$), while 200 and 400 mg/kg of MEA showed a significant elevation in the activity of ALT with 98.0 ± 0.41 and 112.8 ± 0.33, respectively as compared to the control ($P<0.05$).

Table 2. Effect of MEA on serum lipid profile.

Group	CHOL (mg/dl)	HDL (mg/dl)	TG (mg/dl)	LDL (mg/dl)
Control	50.28±4.93	24.86±4.58	18.65±2.44	21.67±3.71
50 mg/kg	41.94±6.43*	4.01±1.97*	17.45±3.12	34.44±6.12*
100 mg/kg	37.02±3.04*	5.34±1.93*	21.94±7.48*	27.67±3.24
200 mg/kg	30.22±1.76*	11.95±4.72*	26.12±2.70*	13.05±4.17*
400 mg/kg	23.78±4.80*	9.30±1.40*	29.00±1.92*	8.67±3.03*

Values are expressed as mean ± standard error of the mean. *Significantly different from control.

Table 3. Composition of *Antiaris africana* extract.

A. Africana compounds	Composition		LOD (µg/mL)	LOQ (µg/mL)
	mg/g	%		
Gallic acid	30.74 ± 0.03a	3.07	0.037	0.123
Catechin	16.11 ± 0.02b	1.61	0.028	0.092
Chlorogenic acid	8.36 ± 0.01c	0.83	0.016	0.054
Caffeic acid	35.97 ± 0.02d	3.59	0.012	0.039
Ellagic acid	22.08 ± 0.03e	2.20	0.035	0.115
Epigallocatechin	4.51 ± 0.01f	0.45	0.007	0.023
Rutin	30.37 ± 0.04a	3.03	0.021	0.070
Isoquercitrin*	2.14 ± 0.03g	0.21	-	-
Quercitrin*	15.46 ± 0.01b	1.54	-	-
Quercetin	26.71 ± 0.03e	2.67	0.009	0.029
Kaempferol	13.05 ± 0.02h	1.30	0.014	0.047

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Turkey test at $p < 0.05$.*Quantified was quercetin. LOD, Limit of detection; LOQ, Limit of quantification.

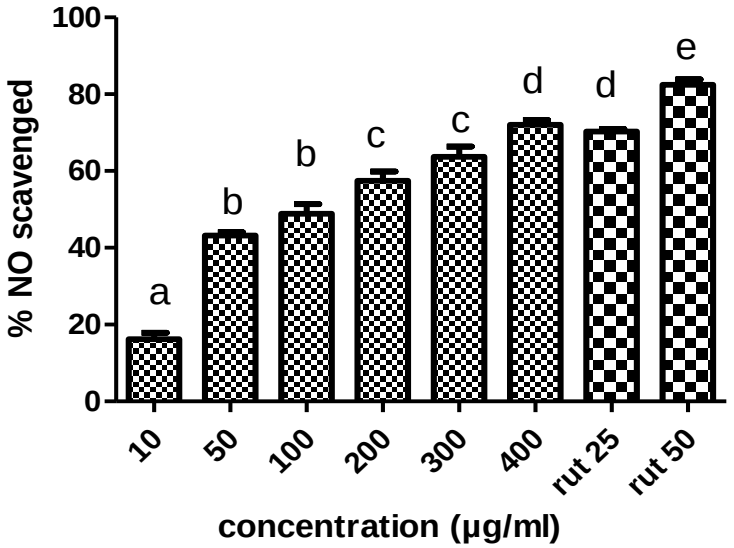

Figure 2. Nitric oxide (NO^{-}) radical scavenging activity (RSA) of methanolic extract of *Antiaris Africana* (MEA). Values represent the mean ± S.E.M. of the values of inhibition *in vitro*; n = 3 experiments in duplicate. $P < 0.05$ versus control. Values with similar alphabets are not significantly different.

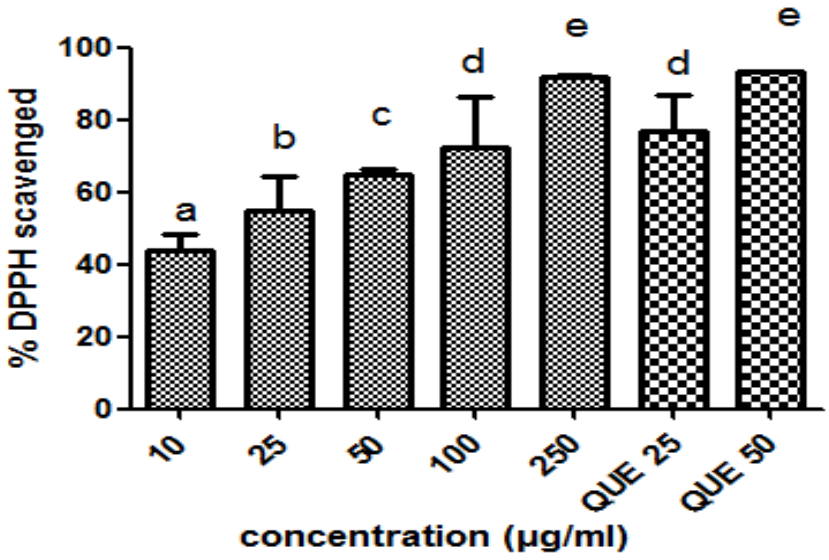

Figure 3. DPPH scavenging activity of MEA. Values represent the mean ± S.E.M. of the values of inhibition *in vitro*, n = 3 experiments in duplicate. P < 0.05 versus control. Values with similar alphabets are not significantly different.

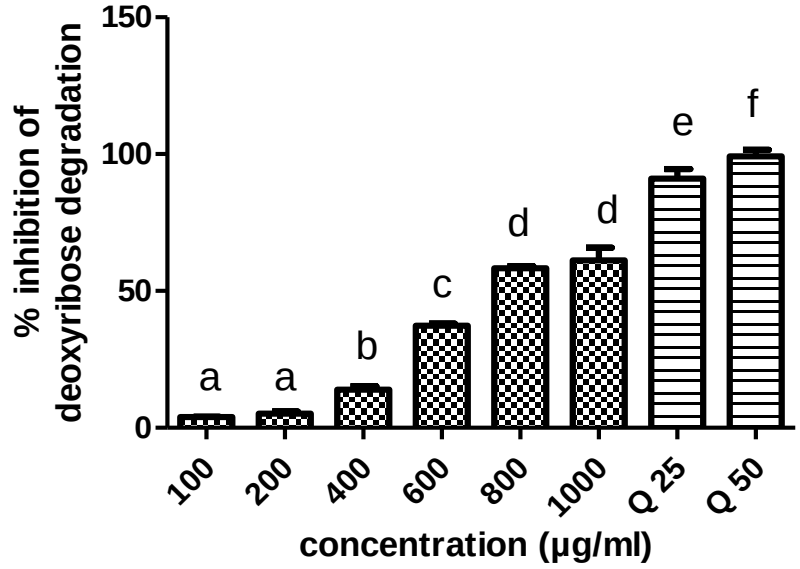

Figure 4. Inhibition of deoxyribose oxidation (hydroxyl radical scavenging activity) by methanolic extract of *Antiaris africana* (MEA). Values represent the mean ± S.E.M. of the values of inhibition *in vitro*; n = 3 experiments in duplicate. P < 0.05 versus control. Values with similar alphabets are not significantly different.

Administration of MEA at all doses generally had no significant effect on CK level, with the activity falling between 52.83 ± 2.88 and 107.83 U/l (P<0.05).

Lipid profile

Table 2 summarizes the effect of varying doses of MEA on lipid profiles in animals. It shows that MEA was able to significantly decrease total cholesterol, triglyceride and LDL-c level in a dose dependent manner when compared to the control; while, a dose dependent elevation of HDL-c level when compared to the control was also observed.

DISCUSSION

The present study is designed to investigate the

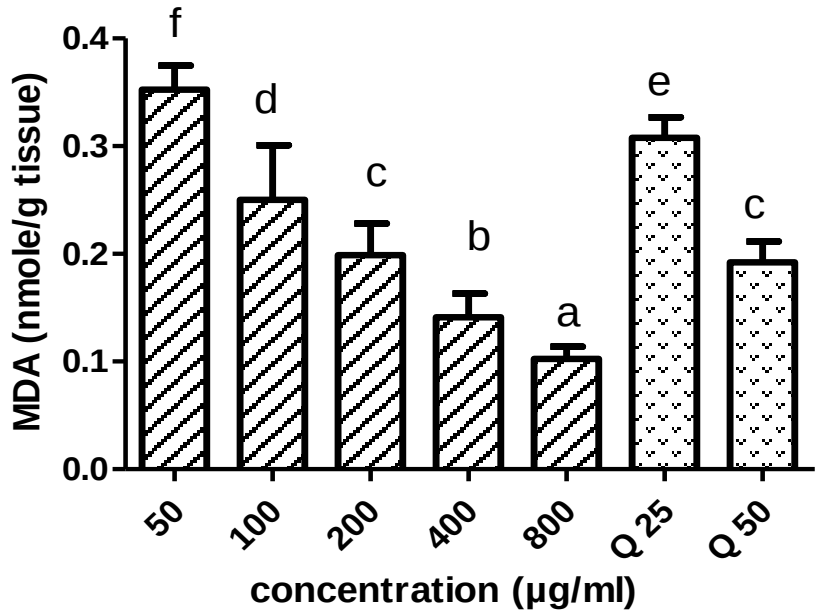

Figure 5. Effect of methanolic extract of *Antiaris Africana* (MEA) on $FeSO_4$ induced lipid peroxidation. Values represent the mean ± S.E.M. of the values of inhibition *in vitro*; n = 3 experiments in duplicate. P < 0.05 versus control. Values with similar alphabets are not significantly different.

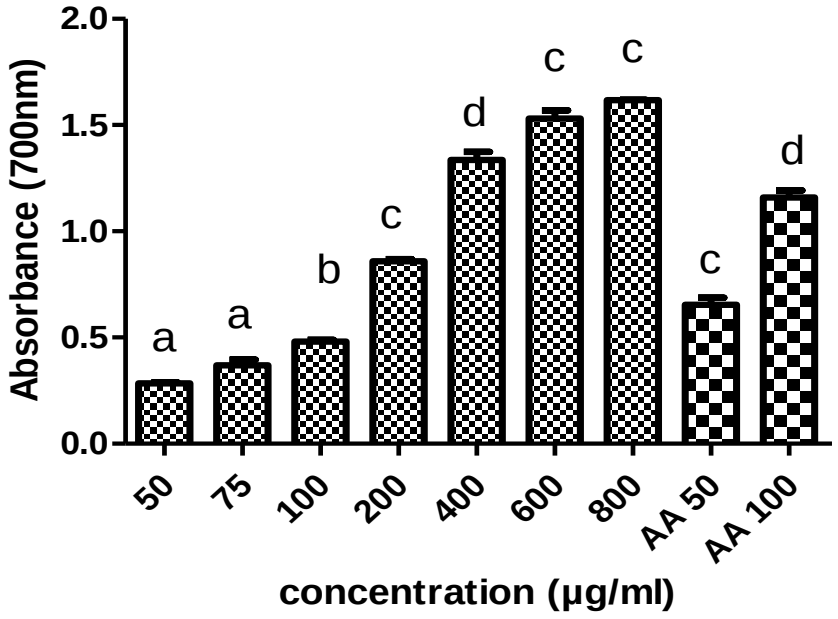

Figure 6. Reducing power of methanolic extract of *Antiaris africana* (MEA). Values represent the mean ± S.E.M. of the values of inhibition *in vitro*, n = 3 experiments in duplicate. P < 0.05 versus control. Values with similar alphabets are not significantly different.

phytochemical constituents present and the biochemical effect of oral administration of MEA on the biomarkers of organ toxicity. *A. africana* (AA) is a plant that is commonly used in Nigeria and other part of West African countries as a panacea in the treatment of several ailments (Kuete et al., 2009). The local usage of AA in

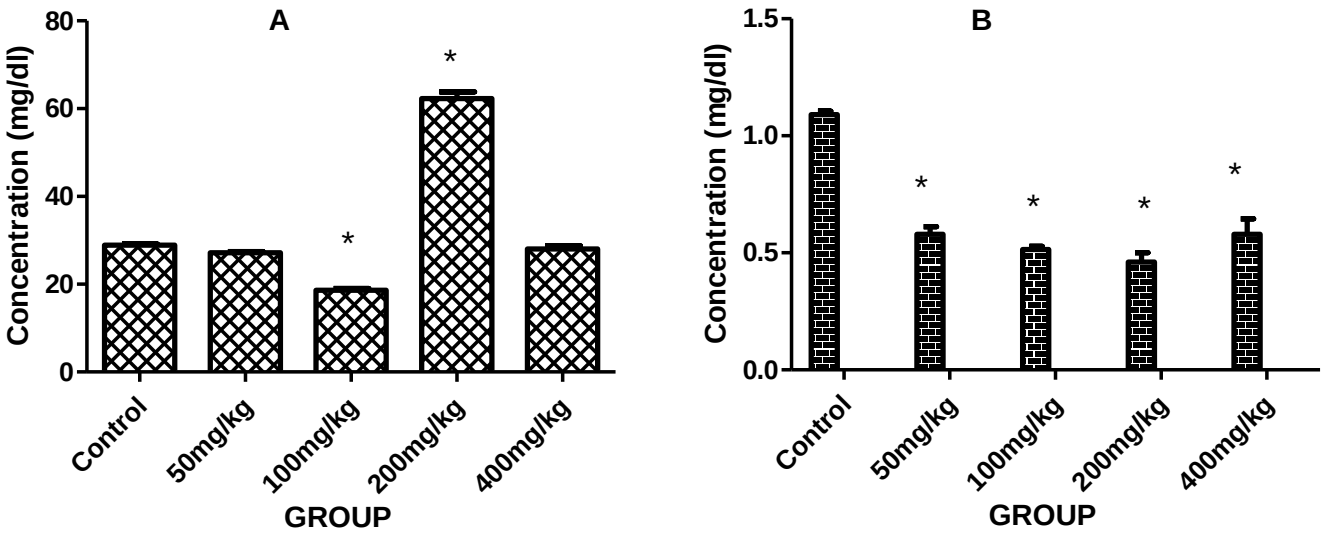

Figure 7. The effect of MEA on marker of renal toxicity A (Urea concentration); B (CREA concentration). Values are expressed as mean ± standard error of the mean. *Significantly different from control (P<0.05).

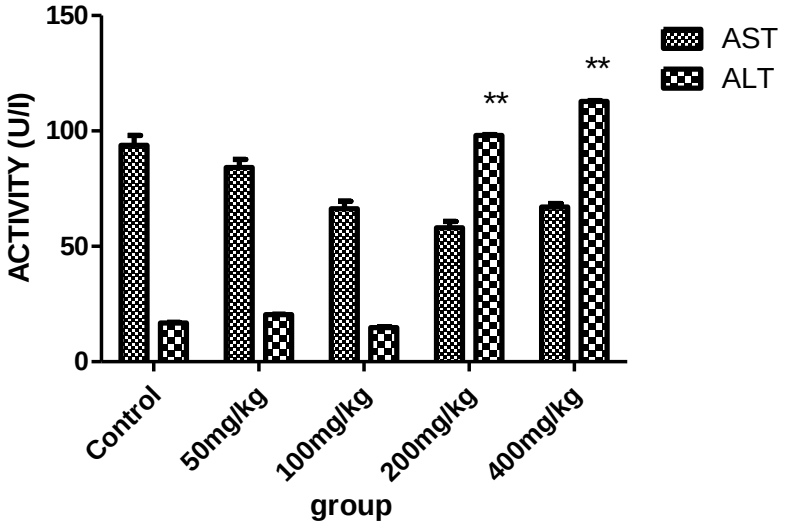

Figure 8. Effects of MEA on markers of hepatotoxic enzyme. Values are expressed as mean ± standard error of the mean. *Significantly different from control (P<0.05).

the treatment of several ailments has been going on for ages past in different part of Nigeria, however there has been no scientific report on the phytochemicals present in it and a proper scientific report on its safety doses upon oral administration of the plant. Phytochemical components such as alkaloids, polyphenols, flavonoid, cardiac glycoside and saponin have been reported to be responsible for both pharmacological and toxic activities in plants (Akinmoladun et al., 2010; Aggarwal et al., 2006). This necessitated a preliminary investigation to identify the various phytochemicals present, assess its antioxidant potentials *in vitro* and possible toxicities of AA. Biomarkers of toxicity include ALT and AST for cytotoxicity and disturbance in hepatic function; urea and creatinine for disturbance in renal function; creatinine kinase is for cardiac function and lipid profile as a link to cardiotoxicity, diabetes and obesity.

Phytochemical screening revealed the presence of saponins, tannins, phlobatannins, flavonoids and terpenoids. Phenolic compounds are not found in animals, majorly synthesized by plants, they are secondary metabolites derived from the shikimate-phenylpropanoids-flavonoids pathways. Flavonoids, one of the largest groups of polyphenols have been reported to be of health

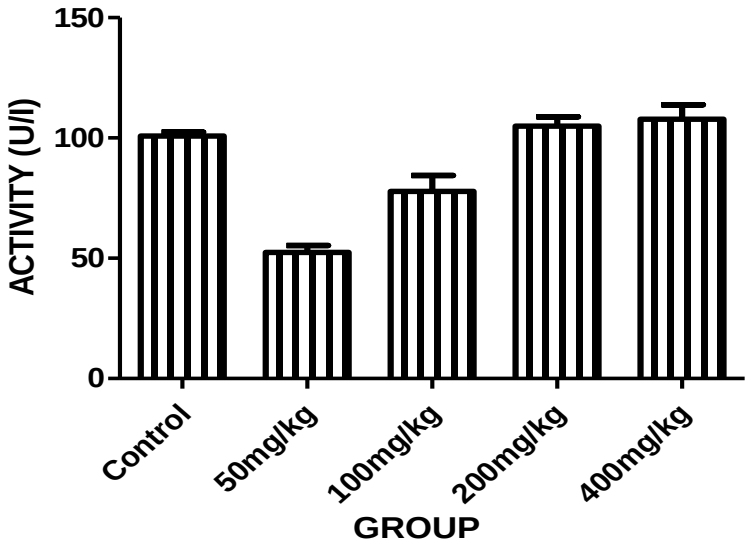

Figure 9. Effect of MEA on creatinine kinase activity. Values are expressed as mean ± standard error of the mean. *Significantly different from control ($P<0.05$).

benefit to human. Several reports has shown that flavonoid possess antioxidative, anti-inflammatory, antimicrobial, cardioprotective and neuroprotective effects (Petti and Scully, 2009; Asensi et al., 2011; Taamalli et al 2013) anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, anti-thrombotic, cardioprotective and vasodilatory effects (Petti and Scully, 2009; Asensi et al., 2011). These metabolites are said to be useful to plant itself but can be toxic to animals, including man. Saponin is a known anti-nutritional phytochemicals that possess the potential to reduce the uptake of certain nutrients including cholesterol and glucose at the gut through intralumenal physicochemical interaction or other yet unidentified activity (Price et al., 1987), suggesting a possible uses in the treatment of diabetes and cardiovascular related diseases; while, tannin, phlobatannin flavonoid and terpenoids are polyphenolic compounds that have been reported to be responsible for most biological activity in plants (Al-Sereiti et al., 1999). Attack of lipid and its derivatives by radicals is one of the mechanisms by which oxidative stress take place, leading to both cell membrane damage and cytotoxicity. Thus the ability of plant extract to inhibit lipid peroxidation is regarded as a key indicator of antioxidant activities. Our data shows that MEA inhibit lipid peroxidation in a concentration dependent manner using brain homogenate. Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity (Babu et al., 2001). The ability of extracts to quench hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and they seem to be good scavengers of active oxygen species, thus terminating or reducing the rate of reaction. MEA scavenged the generated hydroxyl radicals as observed in the prevention of 2-deoxy-2-ribose degradation in a concentration dependent manner. The antioxidant potential of plant extracts can also be determined by the ability of plant extract to reduce ferric ion to ferrous ion (Fe^{3+} to Fe^{2+}) by electron donation termed reducing power (RP). The amount of Fe^{2+} formed is now monitored spectrophotometrically (Ebrahimzadeh et al., 2008). Several researchers have reported a direct correlation between antioxidant activities and reducing power of certain plant extract (Akinmoladun et al., 2010; Koncic et al., 2010; Olaleye et al., 2010). MEA showed reducing power at all concentration.

In the safe dose study in rats given the MEA orally at doses ranging from 50-400 mg/kg, all the animals in each group showed no behavioural difference, though there was an initial response to the MEA on first administration which was normalized few hours later. One of the key indicators of adverse reaction to drugs and chemicals is alteration in body weight (Sellers et al., 2007; Raza et al., 2002; Teo et al., 2002; Tofovic and Jackson, 1999). Administration of MEA showed no significant change in body weight (not reported). Biological markers like endogenous enzymes have been shown and established to be organ-specific and can leak from a damaged or an injured organ (Kubavat and Asdaq, 2009). Hepatic function has been monitored by the evaluation of the levels of ALT and AST in conjunction with biochemical analytes such as cholesterol, creatine kinase and creatinine in the serum. The activity of plant extract to lower the serum level of AST and ALT is one of the mechanism of mediating hepatoprotective or

hepatotoxicity effect of plant extract (Adewusi and Afolayan, 2010). Any damage to the hepatic organ shows an increase in the serum level of AST and ALT (Olaleye et al., 2010; Ahsan et al., 2009; Fakurazi et al., 2008; Liu et al., 2006). From the experiment, there was varying observation in the serum level of AST and ALT as compared to the control; MEA showed no toxic effect on AST activity at all administered doses, while the effect on ALT activity showed that 50 and 100 mg/kg dosages were not toxic, however, administration of 200 and 400 mg/kg was toxic as observed in the significant elevation in serum ALT activity. AST is a general marker of toxicity, thus its leakage is not only dependent on hepatic disturbances, it can be implicated in other tisuues as well. However ALT is a major biomarker of hepatic cytotoxicity (James et al., 1993), thus its increased activity at 200 and 400 mg/kg is a sign of toxicity of MEA at high dosages. One of the risk factors implicated in the occurrence of coronary disease is high level of cholesterol (hypercholesterolemia). Recently, it has been reported that the onset of cardiovascular events can be well controlled after reducing the serum LDL-cholesterol level using several therapeutic agents (Cheng et al., 2004). MEA caused significant decrease in the serum levels of triglycerides, total cholesterol, LDL cholesterol, but increased HDL-cholesterol. One of the mechanism of drugs used in the treatment of cardiovascular disease is in their ability to lower lipid level in the blood (Nofer et al., 2001). Thus, it can be deduced that MEA possess hypolipidemic potential and can be a potential drug for treatment of cardiovascular related diseases. This might be linked to a probable role of MEA in lipid metabolism and clearance from the body. The effect of different plant species on lipid lowering has been reported as a key factor in their medicinal use (Kono et al., 1992; Naidu and Thippeswamy, 2002; Devi and Sharma, 2004). Polyphenol is one of the most abundant phytochemical present in plants; it is abundant in various part of the plants, such as the leaves, roots, stems and seeds. One of the key features of the therapeutic uses of polyphenol is based on its antioxidant properties, anti-inflammatory, antitumor and antimicrobial activities (Xu et al., 2012; Wang et al., 2009). Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups. They are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants, with more than 8,000 phenolic structures currently known, ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins (Dai and Mumper, 2010; Harborne and Williams, 2000).

The main classes of polyphenols, based on their structure, are phenolic acids, flavonoids, stilbenes, and lignans. Flavonoids account for approximately two-thirds of the phenolics in our diet (Sathishkumar et al., 2008) and one of the most abundant polyphenols known, it can be classified into six major groups; flavonols, flavones, flavanones, flavan-3-ols, anthocyanins and isoflavones. In support of this,our results shows that flavonoid is the most abundant polyphenols present in *A. africana*. Polyphenols arise from the common intermediate phenylalanine, or a precursor, shikimic acid. Flavonoids are the group of polyphenols most studied and more than 4,000 have been identified and categorized into six subclasses: flavonols, flavones, flavanones, flavan-3-ols, anthocyanins and isoflavones. The interest in flavonoids arises from studies that have shown that consumption of food rich in it protects against many chronic diseases such as liver damage, chronic kidney injury, cancer, cardiovascular diseases and neurodegenerative diseases. This protective property is attributed to their antioxidant, anti-inflammatory and metal chelating abilities (Dai and Mumper, 2010). A variety of phenolic compounds, in addition to flavonoids, are found in fruit, vegetables and many herbs. Phenolic compounds (such as caffeic, ellagic and ferulic acids, sesamol and vanillin) have been reported to have an inhibitory on atherosclerosis disease (Decker, 1995). HPLC fingerprinting reveals the presence of quercetin, rutin, isoquercetirin, caffeic acid and gallic acid. The presence of these chemical constituents in this plant, is an indication that the plant, if properly screened, could yield drugs of pharmaceutical significance.

Conclusion

The oral administration of MEA for 14 consecutive days at doses ranging from 50-400 mg/kg showed no death among the animals. In addition, no significant effect was observed in the animal behavior and activities throughout the duration of administration, also no adverse effect was observed in the organ and body weights of the animals. From the biochemical parameters, it can be deduced that MEA is safe at low doses and significantly reduced cholesterol level as evident from significant reduction in LDLc parameters.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Adewusi EA, Afolayan AJ (2010). A review of natural products with hepatoprotective activity. J. Med. Plants Res. 4(13):1318-1334.

Aggarwal BB, Ichikawa H, Garodia P, Weerasinghe P, Sethi G, Bhatt ID, Pandey MK, Shishodia S, Nair MG (2006). From traditional ayurvedic medicine to modern medicine: identification of therapeutic targets for suppression of inflammation and cancer. Expert Opin. Ther. Targets 10:87-118.

Ahmada M, Chung PL, Gabriel AA, Nur NI, Hashima MA, Hora SY, Lee FA, Mun FY (2013). Safety assessment of standardised methanol extract of Cinnamomum burmannii. Phytomedicine 20:1124-1130.

Ahsan MR, Islam KM, Bulbul IJ (2009). Hepatoprotective activity of Methanol Extract of some medicinal plants against carbon tetrachloride-induced hepatotoxicity in rats. Eur. J. Sci. Res.

37(2):302-310.
Akinmoladun AC, Obuotor EM, Farombi EO (2010). Evaluation of Antioxidant and Free Radical Scavenging Capacities of Some Nigerian Indigenous Medicinal Plants. J. Med. Food 13(2):444-451.
Al-Sereiti MR, Abu-Amer KM, Sen P (1999). Pharmacology of rosemary (*Rosmarinus officinalis* Linn.) and its therapeutic potentials. Indian J. Exp. Biol. 37:124-130.
Asensi M, Ortega A, Mena S, Feddi F, Estrela JM (2011). Natural polyphenols in cancer therapy. Crit. Rev. Clin. Lab. Sci 48:197-216.
Babu BH, Shylesh BS, Padikkala J (2001). Antioxidant and hepatoprotective effect of *Alanthus icicifocus*. Fitoterapia 2:272-277.
Berg CC, Hijman MEE, Weerdenburg JCA (1985). Flore du Cameroun: Moracées. Paris: Muséum National d'Histoire Naturelle, Laboratoire de Phanérogamie.
Berhaut J (1979). Flore Illustree du Senegal: Dicotyledones, Tome VI: Linacées à Nymphéacées. Dakar: Ministère du Développement Rural et de l'Hydraulique.
Bishnu J, Sunil L, Anuja S (2009). Antibacterial property of different medicinalplants: Ocimum sanctum, Cinnamomum zeylanicum, Xanthoxylum armatum andOriganum majorana. Kathmandu Univ. J. Sci. Eng. Technol. 5(1):143-150.
Dai J, Mumper RJ (2010). Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. Molecules 15:7313-7352.
Decker EA (1995). The role of phenolics, conjugated linoleic acid, carnosine, and pyrroloquinoline quinone as nonessential dietary antioxidants. Nutr. Rev. 53:49-56.
Devi R, Sharma DK (2004). Hypolipidemic effect of different extracts of *Clerodendron colebrookianum* Walp in normal and high-fat diet fed rats. J. Ethnopharmacol. 90:63-68.
Dey A, De JN (2015). Neuroprotective therapeutics from botanicals and phytochemicals against Huntington's disease and related neurodegenerative disorders. J. Herb. Med. 5:1-19.
Ebrahimzadeh MA, Hosseinimehr SJ, Hamidinia A, Jafar M (2008). Antioxidant and free radical scavenging activity of *Feijoa sallowiana* fruits peel and leaves. Pharmacologyonline 1:7-14.
Eisenberg DM, Davis RB, Ettner SL, Appel S, Wilkey S, Van Rompay M, Kessler RC (1998). Trends in alternative medicine use in the United States, 1990–1997: results of a follow-up national survey. JAMA 280(18):1569-1575.
Fakurazi S, Hairuszah I, Nanthini U (2008). *Moringa oleifera* Lam. prevents acetaminophen induced liver injury through restoration of glutathione level. Food Chem. Toxicol. 46(8):2611-2615.
Fu J, Dai L, Lin Z, Lu H (2013). *Houttuynia cordata* Thunb: A Review of Phytochemistry and Pharmacology and Quality Control. Chin. Med. 4:101-123.
Gill LS (1992). Ethnomedical uses of plants in Nigeria. University of Benin Press, Benin City, Nigeria, 12pp.
Harborne JB, Williams CA (1992). Advances in flavonoid research since 1992. Phytochemistry 55:481-485.
James RC, Harbison RD, Roberts SM (1993). Phenylpropanolamine Potentiation of Acetaminophen-Induced Hepatotoxicity: Evidence for a Glutathione-Dependent Mechanism. Toxicol Appl Pharmacol. 118(2):159-68.
Koncic MZ, Kremer D, Gruz J, Strnad M, Bisevac G, Kosalec I (2010). Antioxidant and antimicrobial properties of *Moltkia petraea* (tratt.)griseb. flower, leaf and stem infusions. Food Chem. Toxicol. 48:1537-1542.
Kono S, Shinchi K, Ikeda N, Yanai F, Imanishi K (1992). Green tea consumption and serum lipid profiles: a cross-sectional study in northern Kyushu, Japan. Prev. Med. 21:526-531.
Kubavat JB, Asdaq SM (2009). Role of *Sida cordifolia* L. leaves on biochemical and antioxidant profile during myocardial injury. J. Ethnopharmacol. 124:162-165.
Kuete V, Vouffo B, Mbaveng AT, Vouffo EY, Siagat RM, Dongo E (2009). Evaluation of *Antiaris africana* methanol extract and compounds for antioxidant and antitumor activities. Pharm. Biol. 47(11):1042-1049.
Liu CT, Chuang PT, Wu CY, Weng YM, Wenlung C, Tseng CY (2006). Antioxidative and *in vitro* hepatoprotective activity of *Bupleurum kaoi* leaf infusion. Phytother. Res. 20(11):1003-1008.
Mann A, Gbate M, Nda-Umar A (2003). Medicinal and Economic Plants of Nupeland. Jube-Evans Books and Publications, ISBN 978-33921-9-0, Bida, Niger State, Nigeria, 276 p.
Moronkola DO, Faruq UZ (2013). Chemical Constituents of the Leaf, Stem-bark and Root Essential oils of *Antiaris Africana.* J. Appl. Pharm. Sci. 3(6):168-171.
Naidu KA, Thippeswamy NB (2002). Inhibition of human low density lipoprotein oxidation by active principles from spices. Mol. Cell. Biochem. 229:19-23.
Nofer JR, Kehrel B, Fobker M, Levkau B, Assmann G, von Eckardstein A (2002). HDL and arteriosclerosis: beyond reverse cholesterol transport. Atherosclerosis 161:1-16.
Okogun IJ, Spiff AI, Ekong DEU (1976). Triterpenoids and betaines from the latex and bark of Antiaris africana. Phytochemistry 15:826-827
Olaleye MT, Akinmoladun AC, Ogunboye AA, Akindahunsi AA (2010). Antioxidant activity and hepatoprotective property of leaf extracts of Boerhaavia diffusa Linn against acetaminophen-induced liver damage in rats. Food Chem. Toxicol. 48:2200-2205.
Pankaj G, Abhishek C, Kaushik P (2009). Laboratory evaluation of crude extractsof Cinnamomum tamala for potential antibacterial activity. Electronic J. Biol. 5(4):75-79.
Price KR, Johnson TI, Fenwick GR (1987). The chemical and biological significance of saponins in foods and feeding stuff. Crit. Rev. Food Sci. Nutr. 26:22-48.
Raza M, Al-Shabanah OA, El-Hadiyah TM, Al-Majed AA (2002). Effect of prolonged vigabatrin treatment on hematological and biochemical parameters in plasma, liver and kidney of Swiss albino mice. Sci. Pharm. 70:135-145.
Sabir SM, Ahmad SD, Hamid A, Khan MQ, Athayde ML, Santos DB, Boligon AA, Rocha JBT (2012). Antioxidant and hepatoprotective activity of ethanolic extract of leaves of Solidago microglossa containing polyphenolic compounds. Food Chem. 131(3):741-747.
Sathishkumar T, Baskar R, Shanmugam S, Rajasekaran P, Sadasivam S, Manikandan V (2008). Optimization of flavonoids extraction from the leaves of *Tabernaemontana heyneana Wall.* using L16 Orthogonal design. Nat. Sci. 6(3):10-21.
Sellers RS, Morton D, Michael B, Roome N, Johnson JK, Yano BL, Perry R, Schafer K (2007). Society of Toxicologic Pathology position paper: organ weight recommendations for toxicology studies. Toxicol. Pathol. 35:751-755.
Sofowora A (1993). Medicinal plants and Traditional medicine in Africa. Spectrum Books Ltd, Ibadan.
Teo S, Stirling D, Thomas S, Hoberman A, Kiorpes A, Khetani V (2002). A 90-day oral gavage toxicity study of Δ-methylphenidate and Δ,l-methylphenidate in Sprague–Dawley rats. Toxicology 179:183-196.
Tofovic SP, Jackson EK (1999). Effects of long-term caffeine consumption on renal function in spontaneously hypertensive heart failure prone rats. J. Cardiovasc. Pharmacol. 33:360-366.
Vanguard (2014). Available at http://www.vanguardngr.com/2014/04/minister-mauks-study-herbal-medicine-nigerian-universities/
Varshney R, Kale RK (1990). Effect of calmodulin antagonist on radiation induced lipid peroxidation in microsomes. Int. J. Rad. Biol. 58:733-743.
World Health Organization (2008). Traditional Medicine (accessed 02.09.09).
Xu T, Li D, Jiang D (2012). Targeting Cell Signaling and Apoptotic Pathways by Luteolin: Cardioprotective Role in Rat Cardiomyocytes Following Ischemia/Reperfusion. Nutrients 4(12):2008-2019.

academicJournals

Vol. 15(9), pp. 331-340, 2 March, 2016
DOI: 10.5897/AJB2015.14810
Article Number: 27AEB7D57408
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Physical and chemical properties of the acid protease from *Onopordum acanthium*: Comparison between electrophoresis and HPLC of degradation casein profiles

Malika Benkahoul[1], Meriem Benchiheub[1], Ines Bellil[2], Douadi Khelifi[2] and Aicha Mechakra Maza[1]*

[1]Laboratory of Biology and Environment, Faculty of Nature and Life Sciences, Constantine 1 University, Constantine, Algeria.
[2]Laboratory of Genetics Biochemistry and Plant Biotechnologies, Faculty of Nature and Life Sciences, Constantine 1 University, Constantine, Algeria.

Received 19 June, 2015; Accepted 30 October, 2015

A protease was extracted by grinding, precipitation and gel filtration from *Onopordum acanthium* flowers. The physicochemical study of the enzyme showed an optimum pH of 4, a temperature of 40°C and kinetic parameters of 12.25 mM^{-1} for K_M and 1329.6 UmL^{-1} for V_{max}. The inhibition by pepstatin indicated that it is an aspartyl-protease (APs). Zymogram showed that the protease has a monomeric structure and a molecular mass (MM) of 45 kDa. The hydrolysis of α, β and κ- and whole casein by the protease was evaluated using electrophoresis and HPLC; the profiles showed many similarities between the vegetal protease action and that of industrial chymosin. So, the properties of the protease studied and the quality of its action showed its effectiveness and relevance of its use as a milk clotting enzyme which leads to a better use of extract of flowers *O. acanthium* as a locally substitute for rennet.

Key words: Aspartic protease, *Onopordum acanthium*, purification, characterization, casein hydrolysis.

INTRODUCTION

Proteases are one of the industrially most important enzymes. They modify the chemical, physical and biological properties of proteins and, account for approximately 60% of all enzyme sales because of their varied applications in food, pharmaceutical and a number of other industries (Ikasari and Mitchell., 1996). Proteolysis is the principal set of biochemical changes during ripening of most cheeses. The demand for alternative sources of milk coagulants, to replace the expensive and limited natural rennet supplies, has increased (Esteves et al., 2001). In Portugal and bordering regions of Spain, crude aqueous extracts of thistle flowers (*Cynara*

*Corresponding author. E-mail: aicha.mechakra@umc.edu.dz. Tel: +213 554045501.

cardunculus, Cynara humilis and *Cynara scolymus*), have been used for centuries as coagulant of milk for the artisanal cheese making (Reis et al., 2000). The extracts of the flowers of *Cynara* species have been claimed to be effective as rennet (Silva and Malcata, 2000; Chazarra et al., 2007). The flower extracts from C. *cardunculus* possess two aspartic proteases, A and B cardosins, which are chymosin- and pepsin-like in activity and specificity, respectively (Heimgartner et al., 1990; Silva et al., 2006). All commercial enzymes used in milk coagulation are aspartic proteinases (E.C. 3.4.23); they are most active at acidic pH, specifically inhibited by pepstatin A and contain two aspartic residues for catalytic activity (Davies, 1990). In most studies of plant enzymes, other aspects take generally precedence over their characterization.

The main objectives of the present work were to characterize the physical and chemical properties of aspartic protease obtained from flowers of *Onopordum acanthium* (family *Asteraceae*) and to determine its milk coagulating ability by analysis of the products of casein hydrolysis with electrophoresis and reverse phase high performance liquid chromatography (RP-HPLC) techniques.

MATERIALS AND METHODS

Plant material

O. acanthium L., a flowering plant belonging to the *Asteraceae* family, was collected from roadsides in Constantine, Algeria, during flowering season (May). Flowers were dried in a desiccator using the $CaCl_2$.

Enzyme extraction and purification

The dried flowers were ground in a mortar under liquid nitrogen, solubilized in citrate/sodium 0.05 M buffer pH 5.5, stirred for 30 min and cleared by centrifugation at 15 000 *g* for 20 min at 4°C. The supernatant obtained was the crude extract.

Ammonium sulfate fractionation

Crude extract was precipitated between 30 and 80% saturation of $(NH_4)_2SO_4$. The precipitate obtained was centrifuged at 10000 *g* for 30 min at 4°C, suspended in 0.05 M citrate/sodium buffer pH 5.5 then dialyzed overnight against the same buffer. The salt concentration providing the highest protease recovery was chosen for further purification.

Size-exclusion chromatography

The concentrated enzyme dialyzed was subjected to gel filtration on a Sephadex G-100 column (60 cm × 1.5 cm) pre-equilibrated with 50 mM citrate/sodium buffer pH 5.5. Enzyme fractions of 2 ml were eluted at 12 ml/h flow rate with the same buffer and were analyzed for protease activity, milk-clotting activity and protein content. Active enzyme fractions were pooled, concentrated by lyophilization and used for molecular weight determination and for further assays.

Protein and proteolytic activity assays

Proteins content was assessed with the Lowry method (1951), using bovine serum albumin as the standard (BSA, sigma chemical). The proteolytic activity was measured according to Mechakra et al. (1999) method using casein as substrate (2% in 0.02 M citrate/sodium), the reaction solution containing 2.5 ml of casein plus 1.5 ml citrate/sodium buffer (pH 5.5). The reaction was started by adding 1 ml of the enzyme solution; the assay mixture was incubated at 40°C in water bath for 60 min, 5 ml of the 4% (w/v) trichloroacetic acid was added to stop the reaction. Blanks were prepared by adding TCA to the enzyme, then adding the substrate. The precipitates formed were removed by filtration through Whatman No. 1 filter paper. 2.5 ml of 2% of Na_2CO_3 dissolved in 0.1 N NaOH was added to 1 ml of the above clear filtrate then incubated at room temperature for 10 min then adding 0.25 ml of Folin-Ciocalteu reagent diluted to 50%. This was further incubated for 30 min at room temperature for colour development. The optical density at 750 nm expresses activity. One unit of protease activity was defined as the enzyme quantity which liberates 1 µg/mL of tyrosine per hour under assay conditions.

Milk clotting activity

The milk clotting activity (MCA) of the plant protease was determined as described by Berridge (1945). One milk clotting unit is defined as the amount of enzyme present in 1 ml of extract clotting 10 ml substrate in 100 s at 30°C according to the calculation:

$$RS = 10 \times V / Tc \times v$$

Where, RS = rennet strength, V one volume of milk (ml), v one volume of rennet (ml) and t the clotting time in seconds.

Characterization of protease

Optimum temperature and thermal stability

The protease activity was measured using casein as a substrate at different temperatures ranging from 20 to 80°C at pH 5.5. Heat stability and half-life time of the purified protease were estimated by measuring the residual activity after incubation at 60°C for 10 to 60 min. The non-heated enzyme was used as 100% control. The experimental half-life for the characterized protease is the time at which loss of activity reached 50%; ($t_{1/2}$= ln 2/k), with k is the constant of inactivation enzyme.

Determination of optimum pH

The optimum pH was determined by measuring the effect of pH between 2.5 to 6.5 on the proteolytic activity using denatured hemoglobin as substrate instead of the casein which precipitates at acid pH.

Kinetic parameters determination

The kinetic parameters (V_{max} and K_M) of the pure protease were calculated from the graphical representations of the effect of substrate concentration on the activity according to the Michaelis and Lineweaver-Burk method.

Effect of inhibitors

Effects of pepstatin-A (1 mM and 10 mM), EDTA (10 mM) and iodoacetamide (10 mM) on the protease activity have been examined. The enzyme extract was incubated with each compound for 2 h at 20 °C. The residual activities were measured at pH 5.5 using casein as substrate. Activities were compared with the enzyme activity in absence of any inhibitor (100%).

Determination of the molecular mass

The molecular mass of the purified protease was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% (w/v) acrylamide, according to Laemmli (1970). Zymogram was made to confirm proteolytic activity of the bands, according to the Westergaard's method (Westergaard et al., 1980) with minor modifications. The activity was detected by incubating the gel in 2% (w/v) casein in citrate/sodium 0.05 M buffer pH 5.5 for 1 h at 40°C. The gel was then washed three times in water and stained with Coomassie Brilliant Blue R-250. The development of a clear colorless area on the blue background of the gel indicated the presence of protease activity.

Enzymatic hydrolysis of caseins and electrophoresis

When a potential rennet substitute is studied, it is important to evaluate the degradation patterns of the caseins because of their effect on the yield, consistency, and flavor of the final cheese (Fox, 1989). The action of the *O. acanthium* protease was tested on whole commercial α, β and κ-casein. Caseinates (Sigma Aldrich Co.) were dissolved to a final concentration of 1% (w/v) in 100 mM sodium phosphate buffer (pH 6.5) containing 0.1% sodium azide (w/v) to prevent protein degradation by adventitious microflora. The reactions were started by addition of 450 µl of each substrate to 45 µl of commercial rennet and extract flower enzyme at 40°C. The reactions were stopped by addition of 500 µl of 5% TCA (w/v) at 30, 60, 90 and 120 min. The samples were left to precipitate overnight at 4°C, and then centrifuged at 10,000 *g* for 15 min. For the electrophoretic analysis, the precipitates were dissolved in 450 µl of 62.5 mM Tris–HCl buffer pH 6.8 containing 2% SDS (w/v), 0.5% 2-mercaptoethanol (v/v), 0.02‰ (w/v) bromophenol blue and 10% glycerol (w/v). The mixture was vortexed four times for 30 s, then heated at 100°C for 5 min. Controls containing Na-caseinate and NaN_3 at the same concentrations but without addition of enzyme was also sampled. Samples were subjected to denaturing electrophoresis (SDS PAGE) in glycine as described by Laemmli (1970) at 4°C using a constant voltage (90 to 100 V) and a staining solution containing 0.1% (w/v) Coomassie Brilliant Blue R-250 in ethanol.

Enzymatic hydrolysis of caseins and RP-HPLC

Hydrolysis and RP-HPLC analyses were carried out according to Gallagher et al. (1994) with some modifications. Hydrolysis of caseins (Sigma Aldrich Co.) at 1% in 50 mM sodium citrate buffer pH 5.5 was realized at 40°C by addition of 0.1 mL of enzyme solution to 0.9 mL of substrate. Enzyme solutions (*O. acanthium* extract and commercial rennet enzyme) were standardized to equal milk-clotting activity by the method of Arima et al. (1970). The reaction was stopped by heating at boiling temperature for 6 min. An aliquot was filtered through 0.45 µm filters prior to RP-HPLC analyses to remove any particulate protein material in the hydrolysates. For RP-HPLC analyses, a Jasco PU-2089 HPLC Pump was fitted with a TRACER EXTRASIL ODS2 C18 5 µm reversed phase column (4.6 to 250 mm) and a UV detector at a wavelength of 220 nm. Each sample (20 µL) was injected and eluted with 0.06% trifluoroacetic acid (TFA)/HPLC grade water as a mobile phase, at a flow rate of 0.7 mL/min. The concentration of the mobile phase modifier (0.056% TFA/HPLC grade methanol) was increased linearly from 0 to 91%.

RESULTS AND DISCUSSION

Purification of protease

Table 1 shows the steps of purification of protease: Ammonium sulphate precipitated (30% saturation) and dialyzed, and partially purified protease was further purified by the size-exclusion chromatography. The extent of purification was up to 26.65-fold with 30% recovery (specific activity 5145.51 U/mg), having high specific caseinolytic activity desirable for rennet substitutes. These results are higher than those reported about microbial acid proteases as from *Synergistes* sp. and *Aspergillus niger* that were purified with 15.8 and 9.14 fold-purification and 2.4 and 20.4% recovery successively (Ganesh Kumar et al., 2008; Fazouane-Naimi et al., 2010). Effectively, the same protocol was followed in both work compared with ours, except for the concentration of ammonium sulfate used during the precipitation (80% instead of 30%). Indeed, a study conducted by Phanuphong et al. (2010) on the extraction and separation of proteases from papaya peels showed the highest protease activity recovery and the highest purification fold when concentration of $(NH_4)_2SO_4$, decreased. Narayan et al. (2008) reported that the increased concentration of $(NH_4)_2SO_4$, decreased the degree of purification significantly.

Milk clotting activity

The ability of crude extract and purified enzyme from *O. acanthium* flowers for milk clotting was measured compared with the commercial rennet at different concentrations (Table 3).

According to the results, the milk clotting activity obtained with the commercial rennet increases with the concentration of the enzyme as reported by the literature (Chitipinityol and Crabbe, 1998). For the crude extract (193,07U/mg total protein), the clotting activity was $46.3x10^{-4}$ corresponding at 6 h of clotting time. This value is better compared to that achieved for the crude extract from *C. calcitrapa* seeds (180 U/mg total protein) which noted $37.03x10^{-4}$ of clotting activity so 7.5 h of clotting time (Matos Salvador et al., 2006).

On the other hand, the purified enzyme showed higher milk clotting activity than commercial rennet. Similar data was noted by Hashim et al. (2011) using enzyme fractions of ginger rhizomes and by Su et al. (2009) which also reported that ginger proteases could be a choice for cheese making as well as a milk-clotting enzyme source.

These results confirm the effectiveness of *O. acanthium*

Table 1. Purification data of acid protease from *O. acanthium* flowers.

Step of purification	Volume (ml)	Total activity (U)	Protein (mg)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude extract	55	28681.01	148.55	193.07	1	100
(NH4)2SO4 30%	5	16456.32	42.27	389.31	2.01	57.37
dialyse	6.5	23226.92	35.55	656.45	3.4	80.98
Sephadex G100	16	8541.55	1.66	5145.51	26.65	29.78

proteases in the coagulation of milk as many others plant proteases as the fig tree latex (*ficus carica*) and thistles extracts (*Cynara cardunculus* L. and *Cynara scolymus*) used for a long time in Algeria in the traditional cheesemaking preparations (Androuet, 2002).

The high milk coagulating activity associated with its ability to hydrolyze casein showed that the *O. acanthium* purified enzyme could be useful in the dairy industry for milk coagulation and for the enhancement of cheese ripening process in order to save time and costs of storage for maturation of cheeses.

Temperature optima and thermal stability

Figure 1a shows that the purified acid protease acted at an optimum temperature of 40°C; it loosed 35% of activity after 60 min of incubation at 50°C and it was completely inactivated at 70°C as observed by Sushil Kumar et al. (2005) and Sumantha et al. (2006) for proteases from *Rhizopus oryzae* and seeds of *Centaurea Calcitrapa*, respectively. A progressive reduction in rennet coagulation times as temperature increases from 20 to 40°C has been reported by Najera et al. (2003). Figure 1b shows the thermal stability profiles at 60°C. Loss of activity of 50% is observed after 20 min of incubation and of 90% after 60 min. The *O. acanthium* protease is more stable than microbial like acid protease from *Mucor sp* which retained only 13% of activity at 60°C after 30 min incubation time (Fernandez-Lahor et al., 1999). The experimental half-life for the characterized protease was 19 min at 60°C (Figure 1c) indicated the better stability than the acid protease from *Penicillium expansum* which noted 17 min at 50°C (Umar Dahot, 2001).

Determination of the optimal pH

The effect of pH on the activity of purified protease was determined with denatured hemoglobin over the pH range of 2.5 to 6.5 using citrate sodium buffer (0.05 M). The maximum reaction product was at pH 4 (Figure 2), as observed for other aspartic proteinases such as calf, pig and lamb chymosins (Foltmann and Szecsi, 1998). This is similar to that of aspartyl proteases from *Silybum Marianum* (Vairo-Cavalli et al., 2005) and flowers of *C. calcitrapa* (Domingos et al., 1998) while higher peak activity in Australian cardoon (*Centaurea Cardunculus*) was reached at pH 6.0 (Chen et al., 2003), but in this case casein (not hemoglobin) was used as substrate crude extracts of dried flowers of artichoke obtained at different pH values tested for their clotting activity showed a maximum activity at pH of around 4. Extraction pH 4 was therefore used for rennet preparation (Chazarra et al., 2007).

Kinetic parameters determination (V_{max} and K_M)

The rate of aspartic protease catalyzed reactions was obtained at different concentrations of casein as substrate. A plot was drawn between the rates of acid protease catalyzed reaction (V) versus the casein concentration (S), varying substrate concentration gave an hyperbolic response (Figure 3a). The same data was obtained by Sushil Kumar et al. (2005). V_{max} and K_M evaluated from Lineweaver and Burk plot (Figure 3b) were 2.34 g/L and 1329.6 UmL^{-1}, respectively, with R^2 of 0.91. The low K_M and high V_{max} values inferred that the high affinity and efficient catalytic role of the enzyme.

Effect of inhibitors

The inhibitors (pepstatin A, iodoacetamide and EDTA) were tested to identify the active site groups of the studied enzyme (Table 2). The total inhibition in the presence of 1 mM of pepstatin A indicates that the protease belongs to the class of aspartic proteases (acid proteases), while the little inhibition by iodoacetamide (2%) and EDTA (2.5%) shows that the enzyme is not a cysteyl protease or a metalloprotease. Similar results were observed for acid protease from latex of *Ficus racemosa* which has been inhibited at 100% by pepstatin A (1 mM) and no affected with EDTA (residual activity of 99%) (Devaraj et al., 2008). Brutti et al. (2012) showed 99.5% of inhibition of acid protease from the same plant by the pepstatin- A and Benchiheb et al. (2013) obtained similar data concerning the inhibition of the acid protease from *Scolymus maculatus* flowers.

Determination of the molecular weight of *O. acanthium* protease

The SDS-PAGE and the zymogram analysis of *O.*

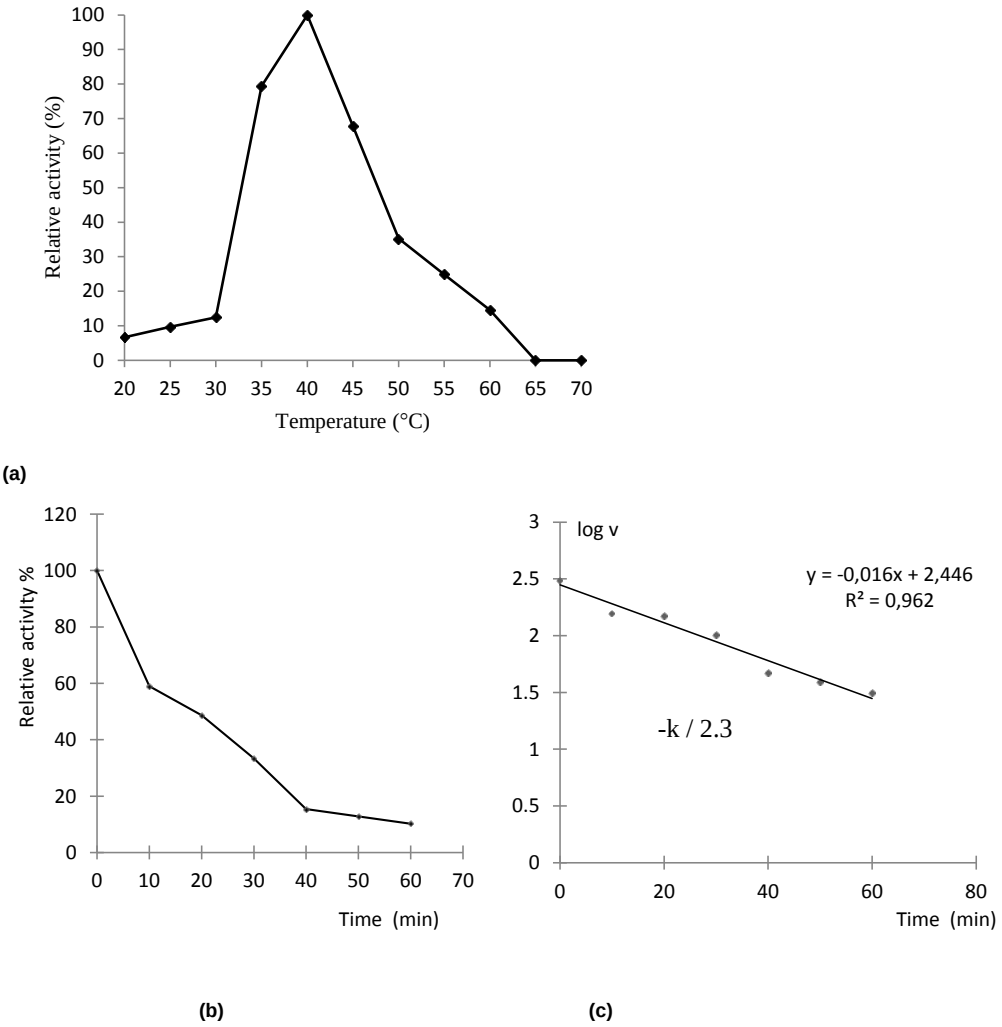

Figure 1. Effect of temperature (a) Activity in the temperature range of 20-70°C (b) Stability of the purified acid protease at 60°C (c) Half-life of acid protease at 60°C.

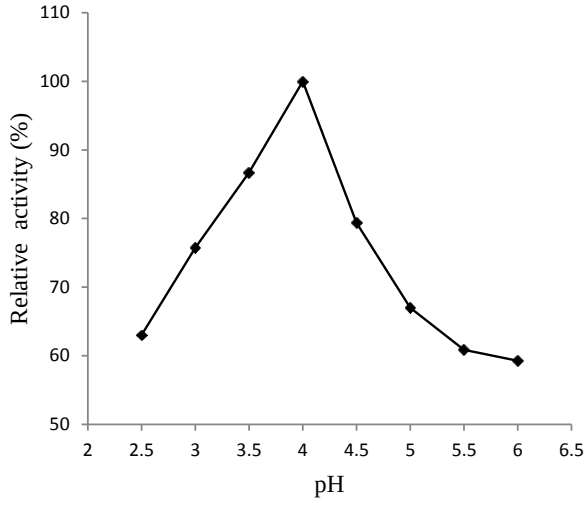

Figure 2. Effect of pH on protease activity purified from *Onopordum acanthium*.

acanthium protease obtained after molecular filtration showed a single band of proteolytic activity on the gel, so suggesting the monomeric nature of enzyme (Figure 4). The marker calibration curve allowed calculating a molecular weight of 45 kDa as obtained by Brutti et al. (2012). Many others aspartyl proteases have similar MM to our results; both for those obtained from plant sources and those from fungal sources. This is the case for the acid protease of *S. maculatus* Benchiheb et al. (2013) and of moulds Nouani et al. (2009) and Fazouane-Naimi et al. (2010).

Hydrolysis profile of caseins by *O. acanthium* protease

Electrophoresis profile of casein degradation

The extent of degradation of major caseins and the

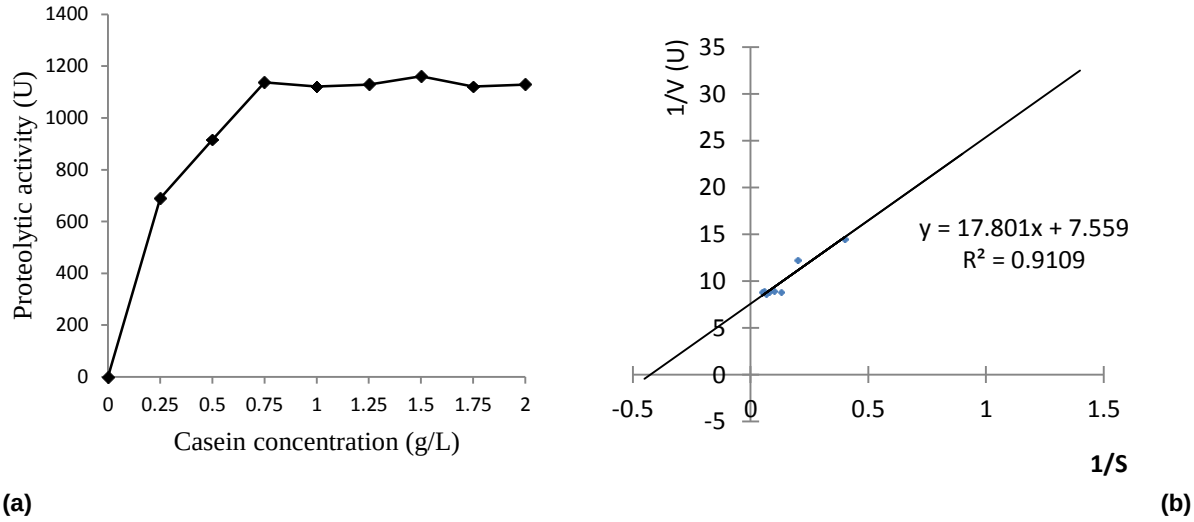

Figure 3. Kinetic parameters determination. (a) Michaelis-Menten plot V vs. (S); (b) Lineweaver-Burk plot 1/V vs. 1/(S).

Table 2. Effect of inhibitors on *Onopordum acanthium* acid protease.

Inhibitors	Concentrations (mM)	Residual activity (%)
None	-	100
EDTA	10	97.43
Pepstatin- A	1	0
Pepstatin- A	10	0
Iodoacetamide	10	98%

Table 3. Milk-clotting activity of *O. acanthium* crude extract and purified protease and of commercial rennet.

Enzyme	Concentration (mg /ml)	Milk-cloting activity
O. acanthium crude extract	2.7	$46.3x10^{-4}$
O. acanthium purified protease	0.10	$139\ x10^{-4}$
Commercial rennet	2	$10,73x10^{-3}$
Commercial rennet	4	$200x10^{-3}$
Commercial rennet	6	$220x10^{-3}$
Commercial rennet	8	$224x10^{-3}$
Commercial rennet	10	$330x10^{-3}$

hydrolysis products by protease from *O. acanthium* and by commercial rennet are presented in Figure 5. The κ -casein was the fraction with highest mobility than β-casein and α-casein as observed by Pardo and Natalucci (2001). After incubation with enzyme extract, casein bands tended to disappear; with higher mobilities appeared after 30 min, showing the formation of casein fractions with lower molecular mass. As can be seen in Figure 5, the content of casein components decreased in both types of enzymes. After 120 min, the degradation of κ-casein was lower with commercial rennet than those obtained with plant protease. Otherwise, both enzymes showed a similar behavior on α-casein, but the β-casein disappeared immediately in the case of *Onopordum* protease, and not completely degraded at 120 min in the case of the commercial rennet. The comparable data was obtained by many others such as Brutti et al. (2012); Vairo-Cavalli et al. (2005); Chazarra et al. (2007) and Egito et al. (2007).

Electrophoresis profile of κ casein hydrolysis

After 30 min of incubation, the similar profile of the

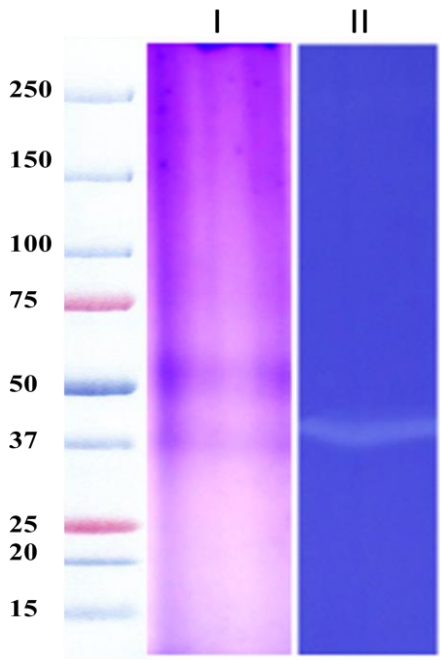

Figure 4: SDS-PAGE gel electrophoresis of the purified *Onopordum acanthium* protease. Lane I: purified protease; Lane II: zymogram of protease with casein as substrate.

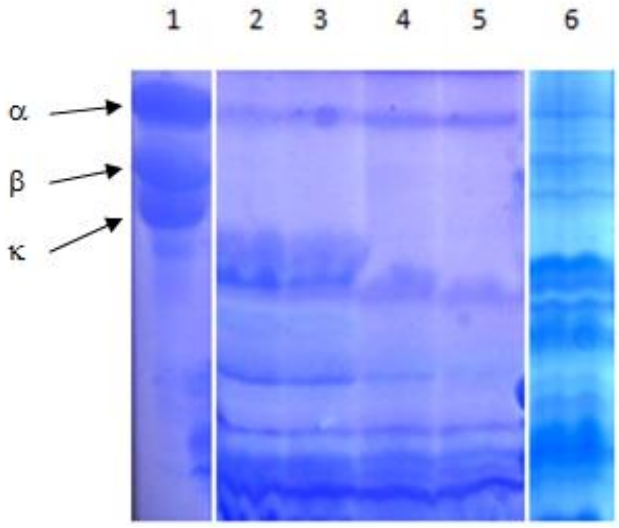

Figure 5. Degradation of of α, β and κ-casein by *Onopordum acanthium* protease and commercial rennet. Lane 1: intact caseins; Lanes 2-5: caseins hydrolysis by *O. acanthium* protease for 30, 60, 90 and 120 min; Lane 6: caseins hydrolysis by commercial rennet for 120 min.

degradation of κ-casein was observed by *O. acanthium* proteases and by commercial rennet (Figure 6), the band of κ-casein disappeared immediately in the cases 2 and

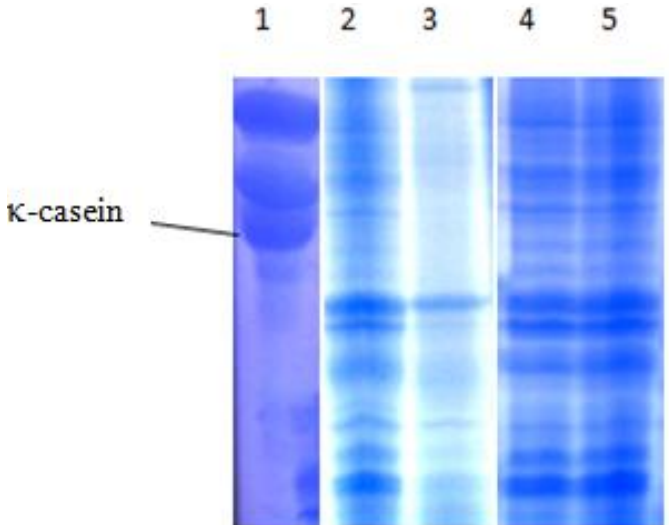

Figure 6. Degradation of κ casein by *Onopordum acanthium* protease and commercial rennet. Lane 1: intact casein; Lanes 2- 3: casein hydrolysis by *Onopordum acanthium* protease for 30min and 120 min; Lanes 4- 5: casein hydrolysis by commercial rennet for 30min and 120 min.

4. However, after 120 min, the degradation of k-casein was lower by commercial rennet than with *Onopordum* protease, so that confirm the last result obtained with degradation of different caseins (Figure 5). Both enzymes present the similar profile of degradation of the κ-casein, but with a faster migration of the plant protease activity. Similar electrophoretic patterns was obtained with the cynarases A, B and C extracted from *C. scolymus* and also, with calf rennet (Chazarra et al., 2007).

RP-HPLC profiles

The potential of hydrolysis of the casein fractions are important to characterize the viability of an enzyme's industrial application. The chromatograms of peptides from intact caseins and from the hydrolysates formed by the action of the commercial rennet and enzymatic extract from *O. acanthium* are shown successively in Figures 7 and 8. The order of hydrolysis obtained with the plant enzyme was show in the following: κ-CN, β-CN, and α-CN; it corroborates the data obtained with electrophoresis. The κ-CN and β-CN components disappeared in 30 min of hydrolysis whereas α-CN was still present after 24 h (Figure 8d, 8e and 8f). This order of hydrolysis was similar to the order obtained by commercial rennet (Figures 8a, 8b and 8c). The pattern of peptide fragments formed by the action of the enzymatic extract from O. acanthium flowers and by commercial rennet is not identical but present similarities especially within the range 0 to 15 min. The three main casein components, α-CN, β-CN and κ-CN, were more

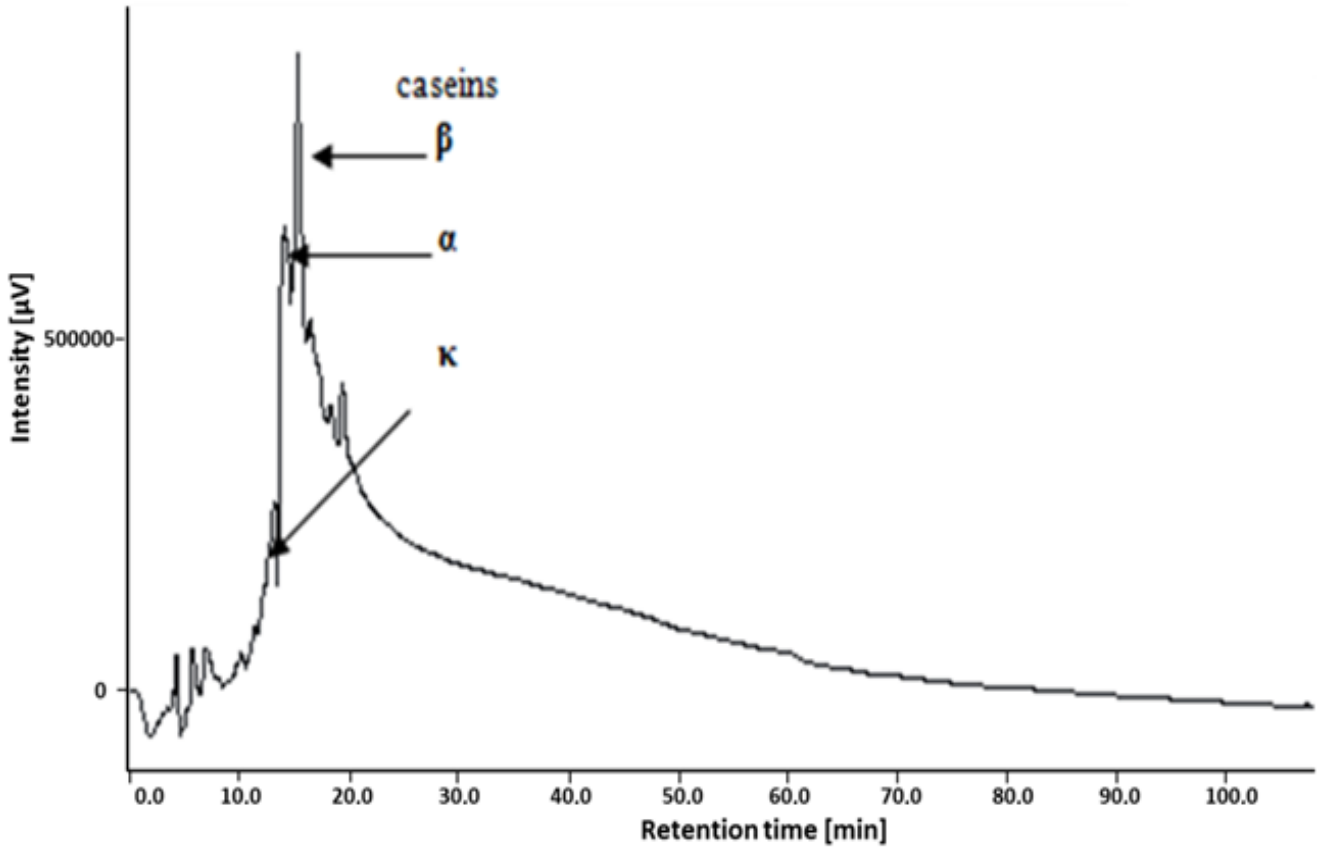

Figure 7. Reversed-phase high performance liquid chromatography (C18 column) of intact caseins (α, β and κ-casein).

sensitive to the action of the *O. acanthium* protease than toward the action of the commercial rennet (Figure 8). The same behavior was observed by Egito et al. (2007) where *Albizia* and sunflower seeds protein extracts were more proteolytic activity than chymosin. The three casein fractions were also degraded by the protease extracted from *Thermomucor indicae-seudaticae* N31 and the *Rhizomucor miehei* (Merheb-Dini et al., 2010). In contrast, the plant rennet from *C. cardunculus* was less proteolytic β-CN and α-CN than the animal rennet (Sousa and Malcata, 1997).

The study of the hydrolysis of caseins by *O. acanthium* acid protease by both methods, electrophoresis and HPLC, indicated the efficiency of the enzyme action on casein and their specificity on kappa-fraction. This action showed an hydrolysis of phe105-met106 responsible of the casein micelles aggregation that provokes the formation of calcium paracaseinate, which results in the coagulation of milk, as produced by commercial rennet (Gaucheron, 2008).

Conclusion

Acid protease from *O. acanthuim* flowers was purified up to 26.65 fold with 29.78% yield. The enzyme shows an optimal activity at pH 4 and an optimum temperature of 40°C. The molecular weight was estimated to 45 kDa and the K_M and V_M were calculated to be 2.34 g/L and 1329.6 UmL^{-1}, respectively. This protease has a capability to hydrolyze caseins and shows the same behavior as commercial rennet. The biochemical properties of the plant enzyme such as the low thermostability and the peptide profile encourage future cheese production experiments to check its potential as a plant rennin. Besides of the involvement of APs in fundamental processes, this protease enzyme is very interesting because of its ability to clot milk. It could be useful in the dairy industry as a rennet substitute. The peptide profile obtained with the enzymatic extract encourages further study of the plant enzymes. The *O. acanthium* protease represents a source of locally-available milk-clotting protease at low cost. Its use will enhance the artisanal production of traditional cheeses in Algeria.

Conflict of interests

The authors did not declare any conflict of interest.

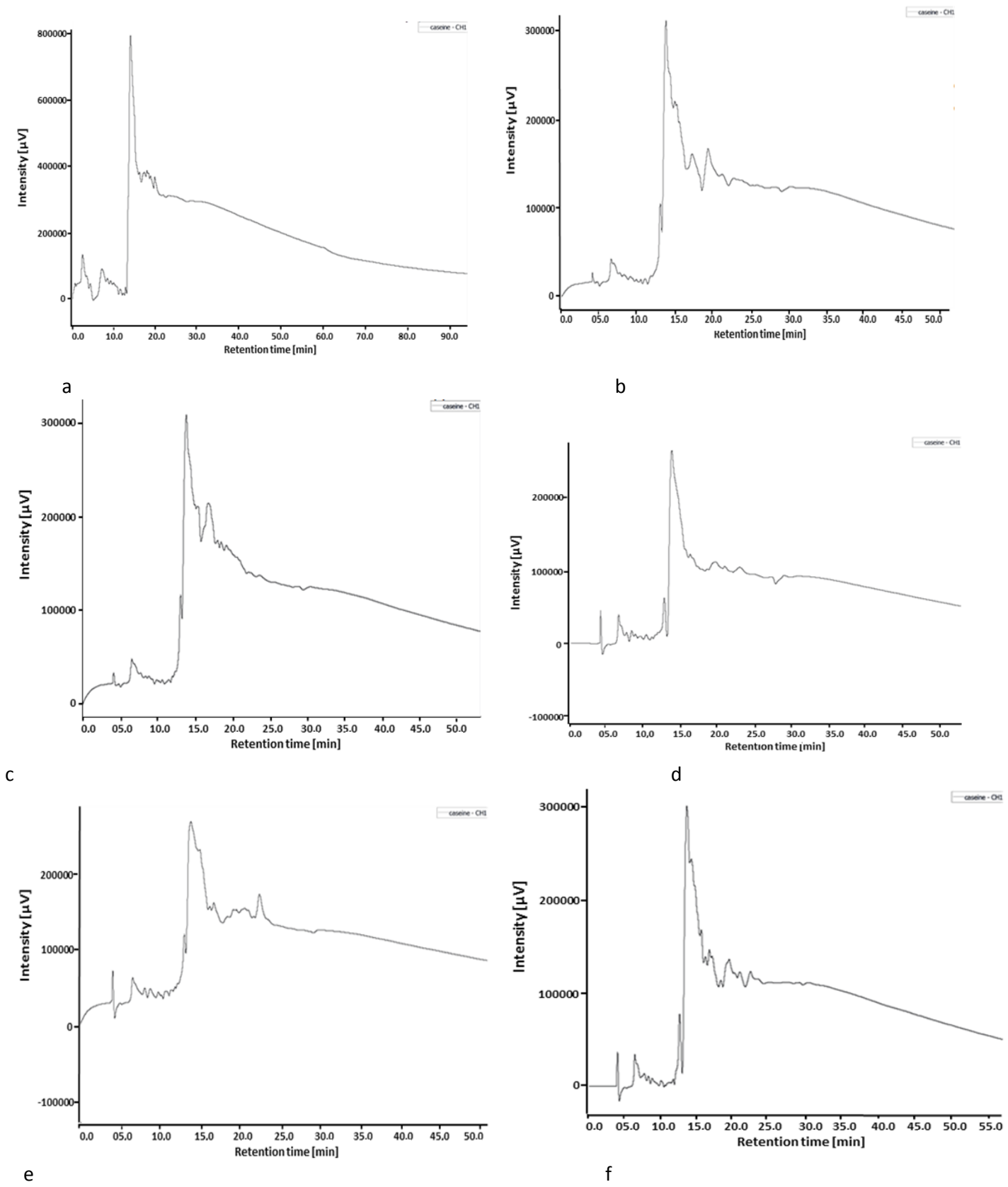

Figure 8. Reversed-phase high performance liquid chromatography (C18 column) of casein hydrolysates generated by *Onopordum acanthium* flowers protease extract and commercial rennet at pH 6.5, 40°C for different times. Commercial rennet: (a) 30 min, (b) 120 min (c) 24 ; Plant protease: (d) 30 min, (e) 120 min (f) 24 h.

REFERENCES

Androuet P (2002). Le dictionnaire des fromages du monde., Ed. Le Cherche Midi, Amazon. Collection Beaux Livres ISBN : 978-2-7491-0040-1

Arima K, Yu J, Iwasaki S (1970). Milk clotting enzyme from *Mucor pusillus* var.lindt. Methods Enzymol. 19:446-459.

Berridge NJ (1945). The purification and crystallisation of rennin. Biochemistry 39:179-186.

Brutti CB, Marcelo FP, Nestor OC, Claudia LN (2012). *Onopordum Acanthium* (Asteraceae) flowers coagulating agent for cheese making. Food Sci. Technol. 45:172-179.

Chazarra S, Sidrach L, Lo´pez-Molina D, Jose´ Neptuno Rodrı´guez-Lo´pez (2007). Characterization of the milk-clotting properties of extracts from artichoke (*Cynara scolymus, L*.) flowers. Int. Dairy J. 17:1393-1400.

Chen S, Zhao J, Agboola S (2003). Isolation and partial characterization of rennet-like proteases from Australian cardoon (Cynara cardunculus L.). J. Agric. Food Chem. 51:3127-3134.

Chitipinityol S, Crabbe MJC (1998). Chymosin and aspartic proteinases. Food Chem. 61 395-418.

Davies DR (1990). The structure and function of the aspartic proteinases. Annu. Rev. Biophys. Chem. 19:189-215.

Devaraj KB, Latitha R, Gowda A, Prakash V (2008). An anusual thermostable aspartic protease from the latex of *ficus racemosa.* Phytochemistry 69:647-660.

Domingos A, Xue Z T, Guruprasad K, Clemente A, Blundell T, Pais M S (1998). An aspartic proteinase from flowers of *Centaure calcitrapa.* Purification, caracterization, molecular cloning and modelling of its three-dimentional structure. Advs Exp. Med. Biol. 436:465-472.

Egito AS, Girardet JM, Laguna LE, Poirson C, Mollé D, Miclo L, Humbert G, Gaillard JL (2007). Milk-clotting activity of enzyme extracts from sunflower and albizia seeds and specific hydrolysis of bovine k-casein. Int. Dairy J. 17:816-825.

Esteve CLC, Lucey JA, Pires EMV (2001). Mathematical modelling of the formation of rennet-induced gels by plant coagulants and chymosin. J. Dairy Res. 68:499-510.

Fazouane-Naimi F, Mechakra A, Abdellaoui R, Nouani A, Saadatou M, Alzouma A, Gais S (2010). Characterization and cheese- making properties of rennet-like enzyme produced by a local algerian isolate of *Aspergillus niger*. Food Biotechnol. 24:1-12.

Fernandez-Lahore HM, Auday RM, Fraile ER, Biscoglio de Jimenez M, Bonino Pirpignani L, Machalinski C, Cascone O (1999). Purification and characterization of an acid proteinase from mesophilic *Mucor sp*. solid-state cultures. J. Peptide Res. 53:599-605.

Foltmann B, Szecsi PB (1998). Chymosin. In: *Handbook* of Proteolytic Enzymes (Eds AJ Barrett, D Rawlings and JF Woessner) London: Academic Press. pp. 815-819.

Fox PF (1989). Proteolysis during cheese manufacture and ripening. J. Dairy Sci. 72:1379-1400.

Gallagher J, Kanekanian AD, Evans EP (1994). Hydrolysis of casein: A comparative study of two proteases and their peptide maps. Int. J. Food Sci. Technol. 29:279-285.

Ganesh Kumar A, Nagesh N, Prabhakar TG, Sekaran G (2008). Purification of extracellular acid protease and analysis of fermentation metabolites by *Synergistes sp*. utilizing proteinaceous solid waste from tanneries. Bioresour. Technol. 99:2364-2372.

Gaucheron F (2008). Fondements physico-chimiques de la technologie laitière. In : Croguennec T. ed Tec & Doc, Lavoisier pp. 95-98.

Hashim MM, Mingsheng D, Iqbal MF, Xiaohong C (2011). Ginger rhizome as a potential source of milk coagulating cysteine protease. Phytochemistry 72:458–464.

Heimgartner U, Pietrzak M, Geertsen R, Brodelius P, Silva Figueiredo A C, Pais MS (1990). Purification and partial characterization of milk clotting proteases from flowers of *Cynara cardunculus*. Phytochemistry 29:1405-1410.

Ikasari L, Mitchell D A (1996). Leaching and characterization of Rhizopus oligosporus acid protease from solid-state fermentation. Enzyme Microbiol. Technol. 19:171-175.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275

Matos Salvador S, Novo C, Domingos A (2006). Evaluation of the presence of aspartic protease from *Centaurea calcitrapa* during seed germination. Enzyme Microb. Technol. 38:893-898

Mechakra A, Auberget B, Remeuf F, Lenoir J (1999). Optimisation d'un milieu de culture pour la production d'enzymes protéolytiques acides par *Penicillium camemberti.* Sc. Aliments. 19:663-675.

Merheb-Dini C, Gomes E, Boscolo M, da Silva R (2010). Production and characterization of a milk-clotting protease in the crude enzymatic extract from the newly isolated *Thermomucor indicae-seudaticae* N31. Food Chem. 120:87-93.

Najera AI, Renobales M, Barron LJR(2003). Effects of pH, temperature, CaCl2 and nzyme concentrations on the rennet-clotting properties of milk: A multifactorial study. Food Chem. 80:345-352.

Narayan AV, Madhusudhan MC, Raghavarao KSMS (2008). Extraction and purification of Ipomoea peroxidase employing three-phase partitioning. Appl. Biochem. Biotechnol. 151:263-272.

Nouani A, Belhamiche N, Slamani R, Fazouane F, Belbraouet S, Bellal MM (2009). Purification et caractérisation électrophoretique d'une protéase coagulant le lait de *Mucor pusillus*: Comparaison de Méthodes. Euro. J. Sci. Res. 35:512-521.

Pardo MF, Natalucci CL (2002). Electrophoretic analysis (Tricine–SDS-PAGE) of bovine caseins. Acta Farm. Bonaerense 21:57-60.

Phanuphong C, Punyawatt P, Saroat R (2010). Extraction and three-phase partitioning behavior of proteases from papaya peels. Process Biochem. 45:1172-1175.

Reis PM, Lourenço PL, Domingos A, Clemente AF, Pai MS, Malcata FX (2000). Applicability of extracts from *Centaurea calcitrapa* in ripening of bovine cheese. Int.. Dairy J. 10:775-780.

Silva SV, Malcata FX (2000). Action of cardosin A from *Cynara humilis* on ovine and caprine caseinates. J. Dairy Res. 67:449-457.

Silva SV, Pihlanto A, Malcata FX (2006). Bioactive peptides in ovine and caprine cheeselike systems prepared with proteases from *Cynara cardunculus*. J. Dairy Sci. 89:3336-3344.

Sousa MJ, Malcata FX (1997). Comparison of plant and animal rennets in terms of microbiological, chemical, and proteolysis characteristics of ovine cheese. J. Agric. Food Chem. 45:74-81.

Su HP, Huang MJ, Wang HT (2009). Characterization of ginger proteases and their potential as a rennin replacement. J. Sci. Food Agric. 89:1178-1185.

Sumantha A, Larroche C, Pandey A (2006). Microbiology and industrial biotechnology of Food-Grade proteases: A perspective. Food Technol. Biotechnol. 44:211-220.

Sushil Kumar S, Sharma NS, Saharan MR, Singh R (2005). Extracellular acid protease from *Rhizopus oryzae*: purification and characterization. Process Biochem. 40:1701-1705.

Umar Dahot M (2001). Purification and some properties of Acid protease from *Penicillium Expansum*. Pak. J. Appl. Sci. 1:405-408.

Vairo-Cavalli C, Claver S, Priolo N, Natalucc CL (2005). Extraction and partial characterization of a coagulant preparation from *Silybum Marianum* flowers. Its action on bovine caseinate. J. Dairy Res. 72:335-343.

Westergaard J, Hackbarth C, Treuhaft M, Roberts R (1980). Detection of proteinases in electrophoretograms of complex mixtures. J. Immunol. Methods 34:167-175.

African Journal of Biotechnology

Related Journals Published by Academic Journals

Biotechnology and Molecular Biology Reviews
African Journal of Microbiology Research
African Journal of Biochemistry Research
African Journal of Environmental Science and Technology
African Journal of Food Science
African Journal of Plant Science
Journal of Bioinformatics and Sequence Analysis
International Journal of Biodiversity and Conservation

academicJournals