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Journal of
Medicinal Plants Research

10 June 2018
ISSN 1996-0875
DOI: 10.5897/JMPR
www.academicjournals.org

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Vol. 12(17), pp. 209-216, 10 June, 2018
DOI: 10.5897/JMPR2018.6589
Article Number: F57D19C57338
ISSN 1996-0875

http://www.academicjournals.org/JMPR

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Journal of Medicinal Plants Research

Full Length Research Paper

Evaluation of *Procavia capensis* hyraceum used in traditional medicine for modulation of mutagen-induced genotoxicity

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Received 19 April, 2018; Accepted 22 May, 2018

Hyraceum (HM) is used in traditional medicine in Southern Africa. Three concentrations of HM (mg/ml in distilled water) (0.0156, 0.03125 and 0.0625) were assessed for cytotoxicity (CT), genotoxicity (GT) and modulation of cyclophosphamide (CP)- and ethyl methanesulfonate (EMS)-induced GT using the *Allium cepa* assay following 24 h treatment. CP (1.00 mg/ml) and EMS (0.0375 mg/ml) were not cytotoxic but genotoxic. HM (0.03125, 0.0625 mg/ml) and its mixtures with CP or EMS induced significant reduction ($p < 0.05$) of the mitotic index (MI) and were adjudged cytotoxic. HM alone and its mixtures with CP or EMS induced statistically significant genotoxicity ($p < 0.05$). Mixture of HM (0.016 mg/ml) with CP was not significantly more genotoxic than CP alone (ME 0.57). Each mixture of HM (0.03125, 0.0625 mg/ml) with CP was insignificantly less genotoxic than CP alone with modulatory effect (ME) of -0.14 and -0.01, respectively, which suggested no interaction between HM and CP. Mixtures of HM with EMS induced positive and significant (>2-fold) MEs and each mixture was significantly ($p < 0.05$) more genotoxic than HM or EMS alone which indicated a synergistic interaction. Sticky chromosomes, chromosome laggards, chromosome fragments, anaphase and telophase bridges, binucleate interphase cells were observed.

Key words: Cytotoxicity, cyclophosphamide, ethyl methanesulfonate (EMS), Genotoxicity; anti-genotoxicity.

INTRODUCTION

Since time immemorial, animals and animal products have been an important component of traditional medicine in practically every human culture in all parts of the world, used to treat and relieve a myriad of illnesses and diseases (Padmanabhan and Sujana, 2008). The importance of traditional medicine based on animals and animal products is well established and, in some cases, approved by pharmaceutical companies as it complements modern medical science (Debas et al., 2006; Jäger et al., 2005). The animal parts and products used in traditional medicine include the fresh manure of the dromedary (*Camelus dromedaries*) to alleviate arthritis (El-Kamali, 2000), and metabolic products such as urine and excreta (dung) separately or in combination with other herbs and minerals (Padmanabhan and Sujana, 2008) are used by tribes in Western Ghats of

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India to manage or cure about 21 diseases including tuberculosis, rheumatic and joints pain, asthma, piles, night blindness, paralysis, debility, impotence, rheumatic and muscle pain, paralysis, impotency, skin burns and rickets (Sinha and Sinha, 2001). Notwithstanding the reported value of animals, their products and their body parts in traditional medicine, studies on their therapeutic effects and, also their safety, have not received enough attention, when compared to plants (Solovan et al, 2004). The concoctions used in traditional medicine are usually crude extracts in water, alcohol, distillates or essential oil, which contain many secondary metabolites (SMs) from several structural groups and their activity is often due to synergistic interactions of SMs present (Eid et al., 2012; Mulyaningsih et al., 2010). The apparent broad-spectrum activity of concoctions used in traditional medicine has been ascribed to phenolic compounds and polysaccharides (Wink, 2015). At high concentrations, SMs change membrane fluidity and increase permeability. Therefore, many lipophilic SMs (especially those in essential oils) exhibit antimicrobial and cytotoxic activities (van Wyk and Wink, 2015).

Some SMs such as pyrrolizidine alkaloids and furanocoumarins are both lipophilic, aromatic and planar which allows them to intercalate or alkylate DNA, thereby causing mutations and even cancer and show substantial antibacterial, antifungal, antiviral and cytotoxic properties (van Wyk and Wink, 2015; Wink and Schimmer, 2010; Schmeller et al., 1997).

In Lesotho the fossilized excreta of *Procavia capensis* (rock hyrax) called *hyraceum* or *"moroto oa pela"* in Sesotho is used to treat respiratory infections, urinary tract or bladder infections, measles and non-communicable diseases such as *diabetes mellitus.* The hyraceum is also used in combination with other medicinal plant species to enhance its efficacy (Seleteng-Kose et al., 2015). The fossil is formed from the faeces and urine of *P. capensis* as the major components which accrete to form dark brown, resin-like masses to which plant material, pollen grains and other digestive remains are trapped. Fossilization occurs with time in arid regions. *P. capensis* inhabits shelters in rocky outcrops in a variety of biomes and feeds on a variety of grasses, shrubs, tree leaves, fruits and berries including the bark of trees (Olsen et al., 2007).

The only literature found on the biological activity of hyracei was that on its effect on the GABA-benzodiazepine receptor, which indicated that since the hyracei exhibited a high affinity for GABA-benzodiazepine they could be used to treat epilepsy, a non-communicable disease (Olsen et al., 2007). Other than that, no records were found on the scientific validation of traditional medicinal uses of hyraceum (van Wyk, 2008).

In view of complex nature of hyraceum, composed of animal metabolic waste and plant materials and its use to treat both communicable and non-communicable diseases, the present study was performed and involved the analyses of the methanolic extract of hyraceum for cytotoxic, genotoxic and modulation of mutagen-induced genotoxicity in the *Allium cepa* root tip. The *A. cepa* root tip assay was used in this study because plant genotoxicity assays are relatively inexpensive, fast, give reliable results and chemicals which cause chromosomal aberration (CA) in plant cells also produce CA in cultured animal cells that are frequently identical (Grant, 1978; Ma et al., 1994).

MATERIALS AND METHODS

Test organism

Onion (*A. cepa*) seeds of the variety, Texas Grano 502 P.R.R., a product of Sakata seeds, Lanseria 1748, Republic of South Africa were purchased from Maseru Garden Centre, Lesotho, Southern Africa.

Mutagens and chemicals

Cyclophosphamide (CP) and ethyl methanesulfonate (EMS) are products of Fluka (Biochemika, Germany). Methanol (absolute) is a product of Associated Chemical Enterprises (Pty) Ltd (Johannesburg, South Africa); hydrochloric acid glacial and acetic acid are products of UNILAB (Krugerdorp, South Africa); aceto-carmine stain was obtained from Carolina Biological Supply Company, Burlington, North Carolina, USA.

Procurement and processing of hyraceum

A sample of hyraceum was purchased at the main Maseru open air market. Maseru is the capital of Lesotho with the geographical coordinates of; latitude: 29° 19' 0.01" S and longitude: 27° 28' 59.99" E. The sample was fragmented and dried in a fanned oven (Labcon) at 35°C to a constant weight and brittle. The dried hyraceum was ground to a fine powder using an electric pulverizer (Kenwood), dissolved in water as solvent and assessed for cytotoxicity and genotoxicity in the *A. cepa* chromosome aberration assays.

Genotoxicity, cytotoxicity and modulatory effects of hyraceum crude extract using the *A. cepa* root tip chromosome aberration assay

The assays were conducted according to the methods of Asita et al. (2017) namely, preliminary dose selection assay to determine the concentrations of mutagens and hyraceum to use in the assays; cytotoxicity that is, mitotic index (MI), genotoxicity (GT) and the modulatory effect (ME) of hyraceum extract on mutagen-induced genotoxicity, including the root harvest, slide preparation and scoring of slides. In each assay, three root tips (triplicate) were assessed at each concentration.

On each of three slides (n = 3) per treatment, a total of 2000 cells, classified into interphase or dividing cell, that is, prophase (normal, N or aberrant, ABN), metaphase (N or ABN), anaphase (N or ABN) or telophase (N or ABN) were scored; that is, a total of 6000 cells each for the control and treatment groups. The aberrations observed were: Binucleate interphase (BN), nuclear bud (NB); micronuclei (MN), pyknotic interphase nuclei (PN), sticky chromosomes (S), C-metaphase (C-Mit), lagging chromosomes (L),

polyploidy (PP), anaphase and telophase bridges (A.B), chromosome fragment (F), multipolar anaphase and telophase (MP). For calculating the GT, only aberrant mitotic cells were considered.

Analysis of slide preparations

Cytotoxicity, as determined by the mitotic index (MI) was expressed as the number of dividing cells per 100 cells scored according to the formula:

MI = No. of dividing cells/Total No. of cells scored × 100 (1)

Genotoxicity (GT) was expressed as the number of aberrant mitotic cells (AMC) per 100 mitotic cells [that is, AMC + normal mitotic cells (NMC)].

Frequency of GT = AMC/(AMC + NMC) x 100 (2)

Modulatory effect (ME) of hyraceum extract on CP- or EMS-induced GT was calculated as:

ME = (B - C) - (A - C)/(A - C) (3)

where ME is the number of times of the mutagen-induced GT by which the GT of the mutagen was reduced (ME is negative) or increased (ME is positive) in mixtures with hyraceum extract; A is the GT induced by CP or EMS alone; B is the GT induced by each mixture of hyraceum extract and CP or EMS and C is the GT induced by tap water alone.

A positive (+) value of ME indicated that the mixture of mutagen and hyraceum extract was more genotoxic than the mutagen alone. If mixture is more genotoxic than both the mutagen alone and the genotoxic hyraceum extract alone then synergism is indicated. However, if the mixture is more genotoxic than the mutagen and also more or less genotoxic than the non-genotoxic hyraceum extract alone then mutagen potentiation is indicated.

A negative (-) value of ME indicated that the mixture of mutagen and hyraceum extract was less genotoxic than the mutagen alone. If mixture is less genotoxic than both the mutagen alone and the genotoxic hyraceum extract alone then antagonism is indicated. However, if the mixture is less genotoxic than the mutagen and also more or less genotoxic than the non-genotoxic hyraceum extract then antimutagenicity is indicated.

ME values, whether positive (increase) or negative (reduction) were considered to be significant when greater than 2.

Data analysis

Data were expressed as mean ± SD of three values. The mean value of each group of three slides per concentration of test agent was compared with that of the negative control group using student's t-test. P-values less than 0.05 ($p < 0.05$) were considered as indicative of significance.

RESULTS

Cytotoxicity (CT), genotoxicity (GT) and modulatory effects (ME) of hyraceum extract in the A. cepa root tip chromosome aberration assay

Photographs of the most representative pictures of normal mitotic cells and cells containing the different types of chromosome aberrations that were observed and scored are presented in Figure 1. The results of the CT, GT and modulatory effects (ME) of hyraceum extract in the *A. cepa* root tip chromosome aberration experiments with the HM extracts and the mutagens are presented in Table 1.

Cytotoxicity and genotoxicity analysis experiments with the hyraceum extracts, CP and EMS

(P + M)/(A + T) ratio

Examination of the (P + M)/(A + T) ratio in column 8 of Table 1 shows that none of the treatments, that is, CP, EMS, the three concentrations of hyraceum extract alone or mixtures of CP or EMS with each concentration of hyraceum induced a significant change in the (P + M)/(A + T) ratio, when compared with the water-treated negative control group ($p > 0.05$).

Cytotoxicity

Examination of the MI in column 9 of Table 1 shows that the concentration of CP (1.00 mg/ml) or EMS (0.0375 mg/ml) used was not toxic to the root meristem cells of *A. cepa* when compared to the water-treated negative control ($p > 0.05$). Of the three concentrations of HM used (0.016, 0.03125 and 0.0625 mg/ml) only the top two concentrations induced significant reduction of the MI compared to the water-treated negative control ($p > 0.05$) and were considered toxic to the root meristem cells. The mixture of CP (1.00 mg/ml) or EMS (0.0375 mg/ml) with each of the three individual concentrations of extract of HM induced significant reduction of the MI compared to the water-treated negative control ($p > 0.05$) and were considered toxic to the root meristem cells.

Genotoxicity

Examination of induction of GT in column 10 of Table 1 shows that the concentration of CP (100 mg/ml) or EMS (0.0375 mg/ml) used was genotoxic to the root meristem cells of *A. cepa* when compared to the water-treated negative control ($p < 0.05$). The three concentrations (0.016, 0.03125 and 0.0625 mg/ml) of HM used and their individual mixtures with CP (1.00 mg/ml) or EMS (0.0375 mg/ml) also induced statistically significant levels of aberrant mitotic cells ($p < 0.05$) and were therefore adjudged to be genotoxic to the root meristem cells.

Modulatory effect of HM on CP-induced genotoxicity

Examination of the ME in column 11 of Table 1 shows that the mixture of the lowest concentration (0.016 mg/ml) of HM with CP induced a positive but none-significant (<2-fold) value of ME of 0.57 which indicated that the mixture was more genotoxic than CP alone, but the

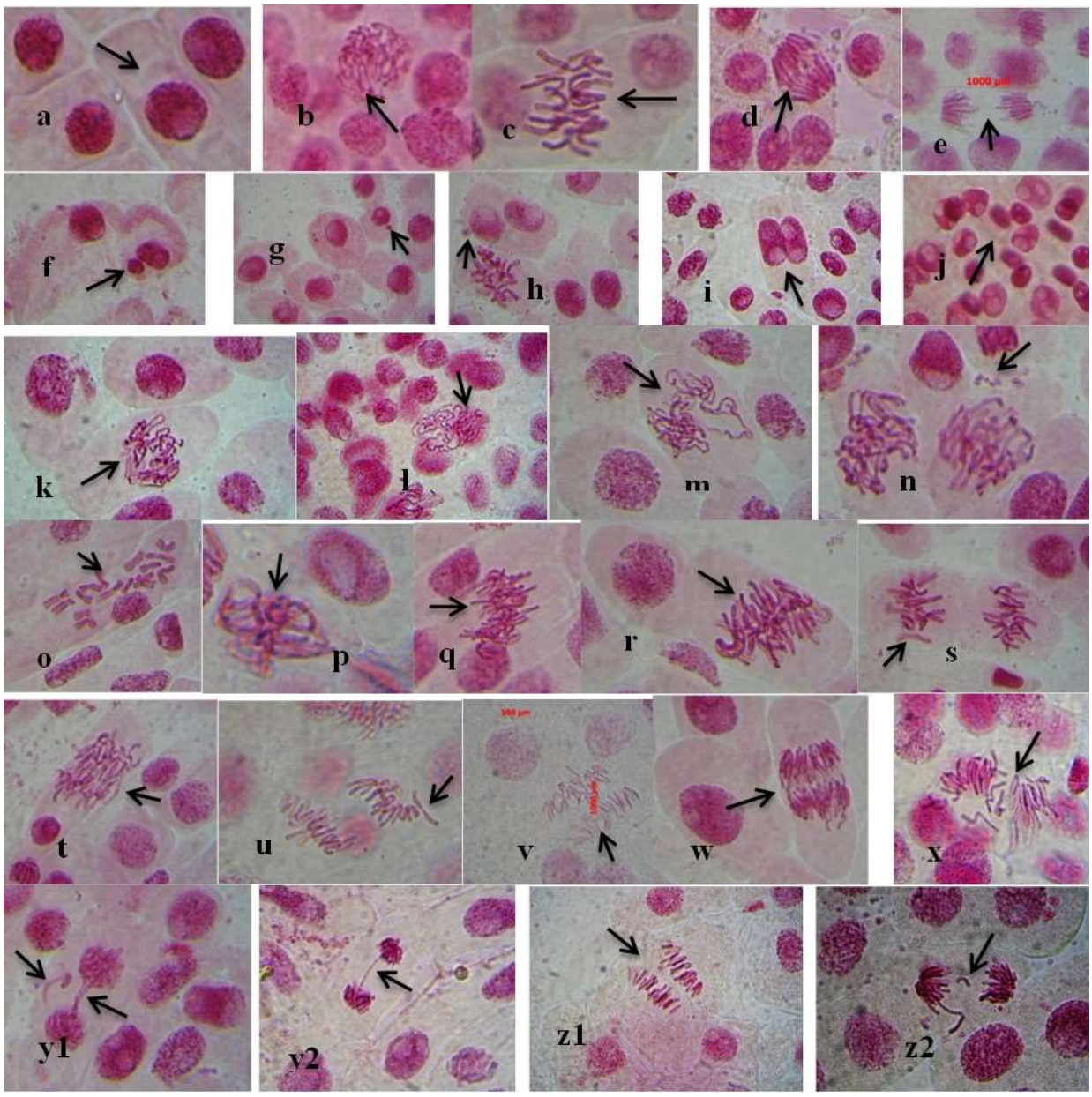

Figure 1. Photographs of cells of *Allium cepa* showing untreated cells in normal division stages and Chromosomal aberrations (arrowed) in cells treated with EMS, Cyclophosphamide, hyraceum (HM) extract or mixture of hyraceum (HM) extract with mutagen. (a) Normal interphase (b) Normal prophase (c) Normal metaphase (d) Normal anaphase (e) Normal telophase (f) Interphase nuclear bud (g,h) Interphase with micronucleus (MN) (i) Interphase cell with binucleus (BN) (j) Interphase with pyknotic nucleus (k,l,m) Prophase with chromosome stickiness (n) Prophase with chromosome stickiness and loss (o) C-Metaphase (p) Metaphase with chromosome stickiness (q, r) Polyploid metaphase (s) Metaphase with chromosomal loss or dislocation (t) Anaphase with chromosome stickiness (u) Anaphase with chromosome loss (v) Multipolar Anaphase (w) Polyploid anaphase (x) Anaphase with chromosome stickiness, displaced poles and scattered chromosomes (y1) Telophase with chromosome loss (shorter arrow), and chromosome bridge (y2) Telophase with bridge (z1) Multipolar telophase (z2) Telophase with lagging chromosome (L). Magnification is 1000 X.

increase was not significant. The mixture of the middle (0.03125 mg/ml) or the highest (0.0625 mg/ml) concentration of HM with CP induced a negative but none-significant (<2-fold) value of ME of -0.14 and -0.01 respectively which indicated that each mixture was less genotoxic than CP alone, but the decrease was not significant in each case. Therefore, neither synergistic (CP plus lowest concentration) nor antagonistic or anti-genotoxic (CP mixture with either the middle or highest concentration) interaction could be inferred.

Table 1. Cytotoxicity and genotoxicity of hyraceum (HM), EMS and CP to meristem cells of onion root tip and the modulatory effects (ME) of HM on EMS- or CP-induced genotoxicity.

TC concentration (mg/ml) and treatment	Statistics	Cells scored					(P+M)/(A+T)	MI	Genotoxicity	Modulatory effect on genotoxicity	
		Cells in interphase	Cells in mitosis			Total number of cells scored					
			NMC	AMC	Total cells in mitosis					HM on CP	HM on EMS
Water	Mean	1807.67	192.00	0.33	192.33	2000.00	2.69	9.62	0.17		
	SD	8.33	7.94	0.58	8.33	0.00	0.86	0.42	0.29		
CP (1.00)	Mean	1867.00	127.67	5.33	133.00	2000.00	3.24	6.65	4.06$^{\#}$		
	SD	51.68	50.08	2.08	51.68	0.00	1.63	2.58	0.92		
EMS (0.0375)	Mean	1824.00	161.67	14.33	176.00	2000.00	2.69	8.80	8.38$^{\#}$		
	SD	25.51	27.57	3.51	25.51	0.00	0.86	1.28	3.08		
HM (0.0156)	Mean	1821.67	167.67	10.67	178.33	2000.00	2.23	8.92	6.09$^{\#}$		
	SD	20.98	22.81	4.62	20.98	0.00	1.07	1.05	2.88		
HM (0.03125)	Mean	1857.67	132.33	10.00	142.33	2000.00	2.18	7.12*	7.02$^{\#}$		
	SD	27.59	26.31	5.20	27.59	0.00	0.19	1.38	3.14		
HM (0.0625)	Mean	1841.67	151.33	7.00	158.33	2000.00	3.08	7.92*	4.27$^{\#}$		
	SD	25.15	22.03	4.36	25.15	0.00	1.46	1.26	2.14		
CP + HM (0.0156)	Mean	1930.67	64.67	4.67	69.33	2000.00	3.43	3.47*	6.29$^{\#}$	0.57$^{+\dagger}$	
	SD	16.50	13.50	3.06	16.50	0.00	1.51	0.83	2.80		
CP + HM (0.03125)	Mean	1903.00	93.33	3.67	97.00	2000.00	3.36	4.85*	3.52$^{\#}$	-0.14$^{+\ddagger}$	
	SD	45.64	43.68	2.52	45.64	0.00	0.62	2.28	1.68		
CP + HM (0.0625)	Mean	1875.67	119.67	4.67	124.33	2000.00	2.71	6.22*	4.00$^{\#}$	-0.01$^{+\ddagger}$	
	SD	24.13	25.66	1.53	24.13	0.00	1.32	1.21	1.90		
EMS + HM (0.0156)	Mean	1986.00	4.00	10.00	14.00	2000.00	6.78	0.70*	70.77$^{\#}$		7.60$^{+\dagger}$
	SD	4.36	1.00	3.61	4.36	0.00	2.80	0.22	6.22		
EMS + HM (0.03125)	Mean	1966.00	24.67	9.33	34.00	2000.00	3.10	1.70*	28.42$^{\#}$		2.44$^{+\dagger}$
	SD	6.56	7.09	0.58	6.56	0.00	1.52	0.33	7.64		
EMS + HM (0.0625)	Mean	1990.67	3.00	6.33	9.33	2000.00	4.06	0.47*	71.43$^{\#}$		7.68$^{+\dagger}$
	SD	4.04	2.65	1.53	4.04	0.00	1.78	0.20	14.29		

TC = Test compound; NMC = Normal mitotic cells (comprising prophase, metaphase, anaphase and telophase); AMC = Aberrant mitotic cells; SD = Standard deviation; CP = Cyclophosphamide; EMS = Ethyl methanesulfonate; HM = Hyraceum; MI = Mitotic index; ↓ = P+M/A+T ratio (significant increase in ratio compared to negative control, P<0.05 in the t-test, n = 3); * = TC is Toxic (MI treatment significantly different from negative control, P<0.05 in the t-test, n = 3); $^{\#}$ = TC is genotoxic (significant difference from negative control, P<0.05 in the t-test, n = 3); $^{+\dagger}$ = HM + Mutagen mixture more genotoxic than mutagen or HM alone (Synergism); $^{+\ddagger}$ = HM + Mutagen mixture less genotoxic than mutagen or HM alone (antagonism); † = PC + Mutagen mixture more genotoxic than mutagen alone but less genotoxic than HM alone; ‡ = HM + Mutagen mixture less genotoxic than mutagen alone (antimutagenicity) but more than HM alone.

Modulatory effect of HM on EMS-induced genotoxicity

Examination of the ME in column 11 of Table 1 showed that the mixture of EMS (0.0375 mg/ml) separately with each of the three concentrations (0.016, 0.03125 and 0.0625 mg/ml) of HM induced a positive and significant (>2-fold) value of ME of 7.60, 2.44 and 7.68 respectively, and from the symbols against the ME values, each mixture was significantly more genotoxic than EMS or HM alone. The results indicated a synergistic interaction between HM extract at all three concentrations and EMS.

DISCUSSION

The results of the cytotoxicity and genotoxicity analysis in Table 1 showed that the concentrations of CP (1.00 mg/ml) and EMS (0.0375 mg/ml) used in the present study reduced the mitotic index (MI) of treated roots but the decreases were not significant ($p > 0.05$) and were not considered cytotoxic. They however induced GT in the root meristem cells of *A. cepa*. In one study, EMS at a concentration of 2×10^{-2} M (0.2484 mg/ml) was both toxic and mutagenic to root meristem cells of *A. cepa* (Çelik and Aslantürk, 2010). CP at a concentration of 1% (1 mg/ml) was also both toxic, that is, significantly reduced the mitotic index (MI), and clastogenic, significantly induced chromosome aberrations, in treated onion root meristem cells (Akeem et al., 2011). In the present study, the concentration (0.0375 mg/ml) of EMS induced more types of aberrations than CP (1.00 mg/ml).

Of the three concentrations of HM extracts tested (0.016, 0.03125 and 0.0625 mg/ml) (Table 1), only 0.0625 and 0.03125 mg/ml induced significant reduction of the MI compared to the water-treated negative control ($p < 0.05$) and were considered cytotoxic to the root meristem cells. The mixture of CP (1.00 mg/ml) or EMS (0.0375 mg/ml) with each of the three individual concentrations of extract of HM induced significant reduction of the MI compared to the water-treated negative control ($p > 0.05$) and were considered toxic to the root meristem cells.

A decrease in the proportion of dividing cells in A + T is an indication of metaphase arrest due to the poisoning of the spindle fibers, akin to the action of the well-documented spindle poison, colcemid (Parry et al., 1999). In the present study, none of the concentrations of the hyraceum extract tested significantly increased the (P + M)/(A + T) ratio. In a study by Ćenanović and Duraković (2015), treatment of *A. cepa* root tip cells with 825 µg/ml of ascorbic acid induced a decrease in the frequency of cells in metaphase, anaphase and telophase while the frequency of cells in prophase increased significantly ($p < 0.05$). Decrease in the number of cells in telophase had no statistical significance.

The three concentrations (0.016, 0.03125 and 0.0625 mg/ml) of HM used and their individual mixtures with CP (1.00 mg/ml) or EMS (0.0375 mg/ml) induced statistically significant levels of aberrant mitotic cells ($p < 0.05$) and were therefore adjudged to be genotoxic to the root meristem cells.

The change in the GT of the mixtures compared to the extract or mutagen alone was measured by the ME, which was defined as the number of times of the mutagen-induced GT by which the GT of the mutagen alone was reduced (ME is negative) or increased (ME is positive) in mixtures with HM extracts, was 0.57 for the mixture of HM (0.016 mg/ml) with CP (1.0 mg/ml) and ME of -0.14 and -0.01 for mixture of HM (0.03125 or 0.0625 mg/ml) separately with CP. The ME values were less than two-fold increase for mixture of the lowest concentration with CP or two-fold decrease for mixtures of CP with the middle or top dose. No interaction between HM extract and CP could therefore be inferred. However, the mixture of EMS (0.0375 mg/ml) separately with each of the three concentrations (0.016, 0.03125 and 0.0625 mg/ml) of HM induced a positive and significant (>2-fold) value of ME of 7.60, 2.44 and 7.68 respectively, and each mixture was significantly more genotoxic than EMS or HM alone which indicated a synergistic interaction between HM extract and EMS in all concentrations

There is little information on the toxicity of hyraceum. Hyraceum is reported to have been used in some areas as a poison ingredient (Watt and Breyer-Brandwijk, 1962) but it is not thought to be particularly poisonous and is used in perfumes and traditional medicine (Khoza and Hamer, 2013). As a tea, hyraceum is used to treat women's ailments but large doses are said to result in abortion (Laidler, 1928). Castoreum, the yellowish exudate from the castor sacs of the mature North American beaver (*Castor canadensis*) and the European beaver (*Castor fiber*) is another complex product of animal metabolic waste and plant materials, used also in traditional medicine but as an analgesic, analeptic, and nervine agent to treat conditions such as amenorrhea, dysmenorrhea, hysteria, and restless sleep (Müller-Schwarze, 2003). The activity of castoreum has been credited to the accumulation of salicin from willow trees (*Salix spp*.) in the beaver's diet, which is transformed to salicylic acid and has an action very similar to that of aspirin (Pincock, 2005). Camel urine, another animal metabolic waste showed cytotoxic and anti-tumor activity to Ehrlich ascite carcinoma (EAC) cells and a constituent of cow urine, creatinine, was highly cytotoxic to EAC cells (Al-Rejaie et al., 2007).

The organic matter of hyraceum contained traces of urea together with uric, hippuric and benzoic acids since hyraceum is partly derived from urine and faecal matter (Greene and Parker, 1879). In an *in vitro* study, sodium benzoate produced no significant chromosomal aberrations in human tissue culture cells at any of the

tested doses. The chromosome abnormalities as well as the mitotic indices were within normal values (Nair, 2001). In *in vivo* cytogenetic assays, the micronucleus test, or in other *in vivo* assays, sodium benzoate and benzyl alcohol were not genotoxic (OECD, 2001). Benzoate is known to be converted to uric and hippuric acids for excretion in urine (Bridges et al., 1970; Skinner et al., 2006).

In the present study, the three concentrations (0.016, 0.03125 and 0.0625 mg/ml) of HM were genotoxic and the two highest concentrations were, in addition, also cytotoxic to onion root tip meristem cells compared to the water-treated negative control ($p < 0.05$).

Many herbal extracts have been reported to inhibit mitosis and reduce the mitotic index (MI) (Çelik and Aslantürk, 2007; Akinboro and Bakare, 2007) which is attributed to the inhibition of DNA synthesis, blocking in the G2 phase of the cell cycle or inhibition of protein synthesis (Kim and Bendixen, 1987; Polit et al., 2003). The significance of the cytological and chromosomal aberrations such as chromosomal stickiness, chromosome fragments, lagging chromosomes, binucleated cells and micronuclei (MN) observed in these studies has been discussed by other authors (Patil and Bhat, 1992; Singh, 2003; Krishna and Hayashi, 2000; Çelik and Aslantürk, 2010). Inhibition of cytokinesis and occurrence of binucleated cells following treatment of root tip cells with plant extracts have been reported (Kaushik, 1996; Gömürgen et al., 2005). Levan (1938) described colchicine mitosis (c- metaphase or c-anaphase) as an inactivation of the spindle followed by a random scattering of the condensed chromosomes in the cell. The induction of c-metaphase as shown in the present studies agrees with the results of some other studies which examined the effects of different medicinal herbs (Soliman, 2001; Bidau et al., 2004).

Conclusion

Both HM alone and its mixture with CP or EMS were cytotoxic and genotoxic. No interaction between HM extract and CP could therefore be inferred however there was a synergistic interaction between HM extract and EMS in the induction of genotoxicity.

FUNDING

This work was supported by a Research grant provided by the Research and conference committee, National University of Lesotho, 2014.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Vol. 12(17), pp. 217-221, 10 June, 2018
DOI: 10.5897/JMPR2018.6582
Article Number: F45A0A457434
ISSN 1996-0875

http://www.academicjournals.org/JMPR

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Journal of Medicinal Plants Research

Full Length Research Paper

Acute toxicity evaluation of ethanol extract of red algae, Osmundaria obtusiloba, in BALB/c mice

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Received 22 March, 2018; Accepted 24 May, 2018

The genus *Osmundaria* (Rhodophyta, Ceramilaes, Rhodomelaceae) comprises tropical and temperate regions red marine seaweeds species. *Osmundaria obtusiloba* is distributed from the northeastern coast of Brazil to the state of Rio de Janeiro. Studies with ethanol extract of red seaweed *O. obtusiloba* showed the antiviral potential of this alga. Hence, this study examined in BALB/c mice the acute toxicity after oral administration of *O. obtusiloba* crude extract. Then, female BALB/c mice received a single dose of *O. obtusiloba* extract by gavage at 550 mg/kg and their behaviors were monitored for a 14 day period. The biochemical and histological changes in the liver, kidney, stomach and spleen were analyzed. *O. obtusiloba* extract did not significantly change behavior, body weight, hematological or biochemical profiles. The organs of the animals did not show significant alterations when submitted to treatment with *O. obtusiloba* extract. In conclusion, the *in vivo* results revealed that *O. obtusiloba* has low toxicity and it can be and may be the target of further studies of biological activity.

Key words: *Osmundaria obtusiloba*; acute toxicity; seaweeds; preclinical tests.

INTRODUCTION

A variety of natural substances have been isolated from extracts of the genus *Osmundaria* J.V. Lamouroux (Order Ceramiales, family Rhodomelaceae), however, there is a predominance of bromophenols (Popplewell and

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Northcote, 2009), which are molecules that have one or more rings benzene, with varying degrees of halogenation and hydroxyl groups (Liu et al., 2011). These molecules present interesting biological activities described in the literature, for example, antimicrobial (Barreto and Meyer, 2006), antiviral (de Souza et al., 2012) and cytotoxic (Popplewell and Northcote, 2009).

The *Osmundaria obtusiloba* (C. Agardh) R.E. Norris species is characterized as a robust plant (Rhodophyta, Ceramiales, Rhodomelaceae), which can measure from 10 to 15 cm in height, with flat apices of 3 to 4 mm wide, showing dark red coloration (Carvalho et al., 2006). Several biological activities have been described from *O. obtusiloba*, mainly for their extracts, such as the fraction of *O. obtusiloba* rich in lectin that inhibited the trypsin and α-amylase enzymes, indicating its potential use in the production of drugs against diabetes (de Oliveira et al., 2009) or the ethanol extract that was able to inhibit the replication of Zika virus while maintaining low cytotoxicity (CC_{50}=525 µg/ml) (Cirne-Santos et al., 2017). Antiviral activity against HSV-1 and HSV-2 has also been described for glycolipids extracted from *O. obtusiloba* (de Souza et al., 2012). In the work of de Alencar et al. (2016) the 70% EtOH was most effective solvent for extracting phenolic compounds from red seaweeds when compared to hexane, also *O. obtusiloba* EtOH extract presented high antioxidant activity. Already, the *O. obtusiloba* methanolic extract showed to present bromophenols (Carvalho et al., 2006).

Due to the discovery of several biological activities previously described, in particular the activity against the Zika virus, it is necessary to make the first preclinical tests to investigate the degree of toxicity of the ethanol extract of this seaweed, aiming the development of new drugs. Toxicity studies provides information on toxic doses and therapeutic indices of drugs and this type of studies in animals is vitally needed to determine the safety of medicinal plants for a future clinical study (Al-Afifi et al., 2018). There are still few toxicity studies of seaweed extracts in animals and the present work aims to evaluate the acute toxicity of the *O. obtusiloba* ethanol extract.

MATERIALS AND METHODS

Algae and extraction

Specimens of *O. obtusiloba* were collected at Rasa Beach, Armação de Búzios, Rio de Janeiro State, Brazil (lat. 22° 45'40", long. 41° 54' 32"). The seaweeds were washed with local water and separated from sediments, epiphytes, and other associated organisms. The material was dried at room temperature for about seven days, triturated using an industrial blender and weighed (140 g) on a semi-analytical scale. The crushed seaweed was exhaustively extracted with ethanol at room temperature. The extract was evaporated under reduced pressure, yielding a brownish residue (5 g). For oral administration the extract was diluted in 1% dimethyl sulphoxide (DMSO).

Biological studies

Animal model

Three groups of six female BALB/c mice, three months old, weighing 19 to 25 g were used for acute toxicity tests (Garrido, 2016). The animals were observed into our bioterium in Virology laboratory kept in polypropylene cages at 25 ± 2°C, under a 12/12 h light/dark cycle, with food and water *ad libitum*. All the tests were performed according to the protocols already approved by the Ethics Committee on Animal Use of the Fluminense Federal University (CEUA-UFF) with certificate number 798.

Experimental protocol: Acute toxicity (14 days)

To evaluate the acute toxicity over 14 days, the animals were divided into three groups: I) *O. obtusiloba* extract – 550 mg/kg (n = 6); II) 1% DMSO - vehicle (n = 6); III) Saline – negative control (n=6). These were administered in single oral dose of 200 µl. The animal behavior was observed throughout both experiments. At the end of the experimental period (14 days), the animals were euthanized by anesthetic overdose (ketamine + xylazine). Body weights were measured on D0 (first day), before extract administration, D7 (7th day) and D14 (14th day) of the experimental period. The protocol and concentrations used in this study were based on the OECD 423 guidelines (OECD, 2008). The concentration of 550 mg/kg of the extract was chosen based in low *in vitro* cytotoxicity of this extract and the dose-response curve in the OECD 425 guidelines (OECD, 2001). Blood samples were collected at the end of the experiment (14th day). Biochemical parameters tests used samples collected in BD-Microtainer® (Clot Activator/SSTTM Gel–Amber) vials to analyze: alanine aminotransferase (ALT), aspartate aminotransferase (AST) and blood urea nitrogen (BUN) (Garrido, 2016). The results were obtained with an automatic biochemistry meter (BS-210-Bioclin).

Histological analysis

At necropsy, the liver, kidney, heart, spleen and stomach were removed for histological processing. Thus, the organs were fixed in 10% Carson formalin and after tissue slices of all organs were routinely processed for paraffin embedding. After the processing of the organs, the pieces were cut into 5 mM microtome according to the literature (Musumeci, 2014) and were prepared and stained by Mayer hematoxylin/eosin (Sigma–Aldrich). Then, the slides were analyzed by conventional microscopy.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test using GraphPad Prism version 5 program. A P value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

To evaluate the acute toxicity of *O. obtusiloba* extract (550 mg/kg) three groups were formed, a group receiving the ethanol extract, another group receiving only the vehicle and a group receiving saline by gavage. All animals treated with *O. obtusiloba* extract, 1% DMSO and saline survived for 14 days. There were no significant adverse clinical signs or changes in body weights (p >

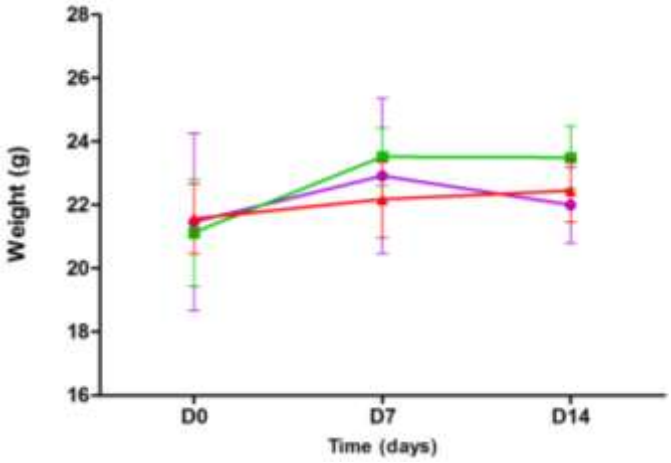

Figure 1. Body weight of BALB/c mice treated with *O. obtusiloba* extract and controls (1% DMSO and saline) on days 0 (D0), 7 (D7) and 14 (D14). Saline – negative control, 1% DMSO – vehicle and *O. obtusiloba* extract. p>0.05 in Tukey Test.

0.05) (Figure 1). From the serum were determined the transaminases ALT and AST, parameters used for the evaluation of liver function and BUN, which can be used to estimate the renal function (Roy et al., 2015). The results showed that the group that received the oral administration of *O. obtusiloba* extract had no significant difference compared to the control group (saline) and vehicle (p>0.05) in all the biochemical parameters analyzed (Table 1) and remained within normal range (Kifayatullah et al., 2015).

Histological findings corroborate with those clinical observed during the experiment and biochemical analyses. We analyzed organs extracted from the experiment mice and they did not show significant changes in morphology in the 550 mg/kg *O. obtusiloba* extract group when compared with control groups (vehicle and saline) with spleen exception. Therefore, the liver was histologically normal, and structures of the hepatic wire and centrilobular veins may be evident corroborating with the AST and ALT analyzes (Figure 2A, B and C). Sasidharan et al. (2010) by testing another red seaweed observed no significant signs of toxicity, nor did a single administration of 2000 mg/kg methanol extract of *Gracilaria changii* cause death during the 14-day observation period. In another acute toxicity work of our group (period of 10 days) with dolabelladienotriol, a natural product derived from brown algae, *Dictyota friabilis (*as *Dictyota pfaffii*), all of the animals that received dolabelladienotriol presented a moderate increase in mitosis of hepatocytes and focal areas of hydropic cells in the medulla of the kidneys, even though this study indicates that dolabelladienotriol has low toxicity in administered dose range (Garrido et al., 2011).

The architecture of the kidneys presented normality in the tubules and glomeruli (Figure 2D, E and F) and urea parameters (Table 1). The low toxicity was later confirmed by the subchronic toxicity study of this product (Garrido et al., 2017). Already for the extract of *O. obtusiloba* has been reported the presence of bromophenols (Osako and Teixeira, 2013). The heart showed no changes in the architecture with cardiac fibers very visible to the cut (Figure 2G, H and I). Finally, we analyze the histological structure of stomach and observed no changes (Figure 2J, K and L), just as Garrido et al. (2011) found in the acute toxicity study of dolabelladienotriol. However in the spleen, as noted in the 1% DMSO group and *O. obtusiloba* group, was presented an increase of white pulp with a suggestive activation of the germinal center, possibly due to the DMSO solvent used for solubilization of the extract (Figure 2M, N and O). These results, together with the biochemical parameters analyzed, demonstrate the low acute toxicity of the extract of this alga in tested concentration.

Conclusions

The result indicates that the single oral dose of administration of *O. obtusiloba* ethanol extract (550mg/Kg) in our acute toxicity study after 14 days did not produce any significant toxic effect in BALB/c mice. Hence, further studies should be carried out to confirm the low toxicity of this extract with its continuous and prolonged use.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

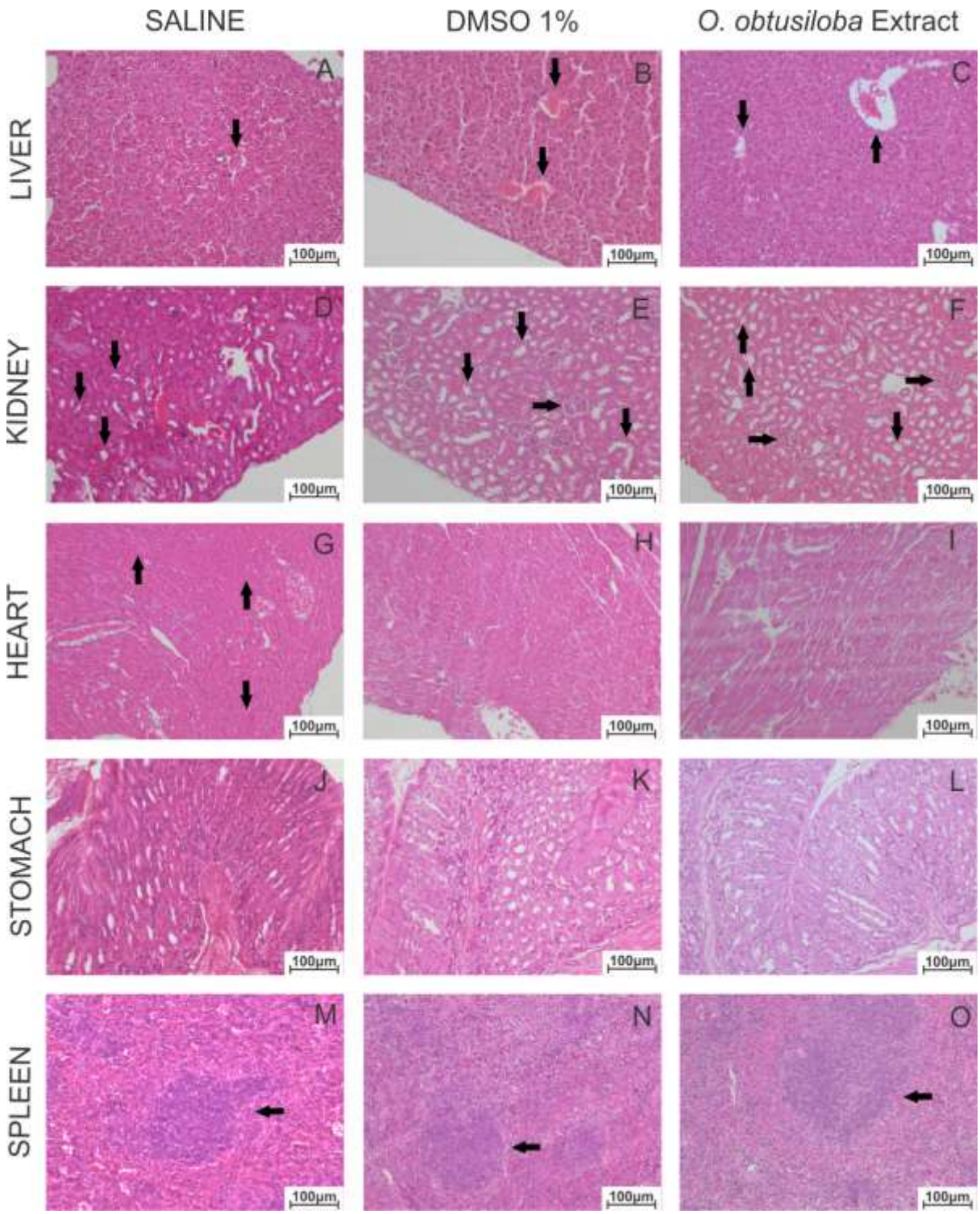

Figure. 2 Histological analysis of liver, kidney, heart, stomach and spleen in female BALB/c mice administered with a single oral dose of saline (A,D, G, J and M), 1% DMSO (B, E, H, K and N) or *O. obtusiloba* ethanol extract (550 mg/kg) (C, F, I, L and O), at 14 days after the administration. (A, B and C) liver: Histologically normal, and structures of the hepatic wire and centrilobular veins (vertical arrow) may be evident; (D, E and F) kidney: Presented normality architecture in the tubules (vertical arrow) and glomeruli (horizontal arrow); (G, H and I) heart: No changes in the architecture with cardiac fibers (vertical arrow) very visible to the cut; (J, K and L) stomach: Architecture preserved; (M,N and O) spleen: Saline group (M) presented spleen with preserved architecture, the horizontal arrow indicates the germinal center, 1% DMSO group (N) and *O. obtusiloba* group (O), presented an increase of white pulp with a suggestive activation of the germinal center (horizontal arrow) (200×). Mayer hematoxylin and eosin staining.

Table 1. Effect of *O. obtusiloba* extract on biochemical analyses in acute toxicity study in BALB/c mice.

Parameter	Saline	1% DMSO	*O. obtusiloba* extract
ALT (U L^{-1})	24.50 ± 3.91	22.50 ± 2.51	20.50 ± 2.12
AST (U L^{-1})	133.00 ± 51.36	79.50 ± 9.14	96.00 ± 42.14
BUN(mg dl^{-1})	42.83 ± 5.24	42.83 ± 6.05	40.33 ± 7.66

Values are expressed as mean ± SD. No significant changes in the parameters ($p>0.05$). ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen.

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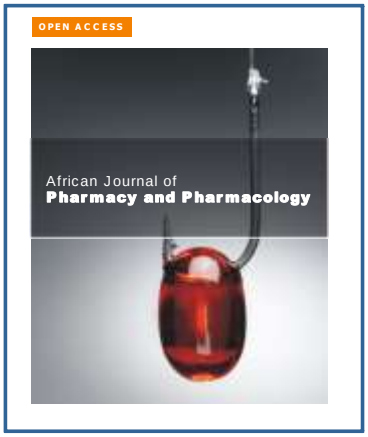

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