INTERNATIONAL JOURNAL OF
INTEGRATIVE AND
MODERN MEDICINE

Preparation of Extract of Atriplex Halimus Leaves Against Protoscolithic Activity as Well as Echinococcus Granulosus Protoscoleces

Abdiravidov Temurbek Abdimanonovich
Scientific researcher, 3rd year student of the direction of Clinical Pharmacy of the Faculty of Pharmacy of the Tashkent Pharmaceutical Institute

Sheraliyev Javakhir Ilhamzhonovich
Scientific researcher, 2nd year student of Pharmacy direction of the Faculty of Pharmacy of the Tashkent Pharmaceutical Institute

Abdusalomova Khumora Abduvaqqas qizi
Scientific researcher, 3rd year student, Clinical Pharmacy Department, Faculty of Pharmacy, Tashkent Pharmaceutical Institute

Khusainova Raykhona Ashrafovna
Scientific leader Professor of the Department of Pharmaceutical Chemistry of the Tashkent Pharmaceutical Institute

Abstract: Cystic echinococcosis, an endemic zoonosis in Algeria, is caused by the development of the helminth Echinococcus granulosus. Surgery remains the main treatment despite inducing relapse and several adverse reactions. In this context, natural scolicidal agents seem to be promising tools to overcome these reactions. In our study, we evaluated the phytochemical contents, antioxidant activity and scolicidal effect of Atriplex halimus. In this context, the aqueous extract from AH leaves (AHE) was subjected to preliminary phytochemical screening by HPLC. The in vitro antioxidant activity was determined by DPPH test. The cytotoxicity of AHE was evaluated in murine peritoneal macrophages and cell viability was examined by MTT assay. Moreover, different concentra tions of AHE (20, 40, 50, 60 and 100 mg/ml) were tested on E. granulosus protoscoleces (PSC) cultures, during different times of incubation (15, 30, 60, 90, 120 and 180 min). The viability was evaluated by eosin exclusion test. The morphological and ultrastructural damages were evaluated by SEM. Our results indicate that total phenolic and flavonoids contents were 37.93 µg of Gallic acid equivalent per mg of extract (GAE/mg E) and 18.86 µg of Quercetin equivalent per mg (QE/mg E) respectively. Furthermore, AHE has an antioxidant activity with an IC50 of 0.95 mg/ml. Interestingly, the extracts did not exhibit any cytotoxic effect against murine peritoneal macrophages. Moreover, our study indicated a significant scolicidal activity time- and dosedependent. At 60 and 100 mg/ml; and after 120 min of incubation; the mortality rate was 99.36 and 100%, respectively. The parasite's tegument is one of the plant's targets as demonstrated by SEM. Our findings show the benefits of Atriplex halimus extract as a new promising scolicidal tool in hydatid cyst treatment.

Keywords: Echinococcus granulosus, Atriplex halimus, Phytochemical composition, Antioxidant activity, Scolicidal activity.

1.Introduction.

Cystic echinococcosis (CE) or hydatidosis, a widely zoonotic disease caused by the larval stage of Echinococcus granulosus sensulato (s.l.), is characterized by long-term growth of hydatid cysts in humans and mammalian intermediate hosts. This parasitic infection is a chronic, complex, and still neglected disease. Human is accidently infected with eggs present in the definitive host feces. The larval forms of the parasite or metacestode can developed in almost any organ, but mainly in the liver

and lungs and less frequently in the kidneys, spleens, bones, brain and other organs. The metacestode produces protoscoleces (PSC) asexually. PSC are the fertile forms of the parasite released into the hydatid fluid. In some cases, a cyst rupture can occur and protoscoleces spillage leads to a secondary hy datid cyst formation Currently, there are three treatment options for CE: surgery, ultrasound-guided aspiration, and chemotherapy. The recommended chemotherapy drugs for the treatment of cystic echinococcosis are benzimidazole derivatives, such as mebendazole and albendazole. Despite advances in surgical techniques and chemotherapy, recurrence remains one of the major problems in the management of hydatid disease. Moreover, chemotherapy has different side effects. In this sense, the search of new scolicidal agents remains relevant. The aim of our current work was to study the in vitro protoscolicidal effect of AH leaves aqueous extract (AHE) against Echinococcus gran ulosus protoscoleces. In addition, the chemical composition (total phe nols and flavonoids) was analyzed by HPLC. Moreover, antioxidant activity and cytototoxicity against murine peritoneal macrophages were also evaluated by DPPH scavenging assay and MTT test respectively.

2. Materials and methods

2.1. Plant material Leaves of A. halimus were collected from Baniou (M'sila, North Algeria) (Coordinates: 35◦24′ 0″ N 4◦22′ 0″ E), in May 2016. The plant was identified by Mr Sarri Djamel (Department of Nature Sciences and Life, Faculty of Sciences, university of M'sila). A relative specimen voucher has been registered in the Herbarium of University of M'sila, Algeria. The leaves were washed and dried in the shade at room tem perature. Stems and thick veins were removed and leaves were then ground to a fine powder.

2.2. Preparation of A.halimus aqueous extract Fifty gram of the leaves powder was mixed with 500 ml of hot distilled water with constant stirring for 24 h. The solution was allowed to cool at room temperature and then filtered using muslin cloth before using Whatman filter paper number 1. Obtained filtrate was evaporated at 40 ◦C using a rotary evaporator. The extract was weighed and stored at 4 ◦C for future use.

2.3. Determination of total phenolic and flavonoids contents The total phenolic content of the extract was estimated using FolinCiocalteu's colorimetric method, with Gallic acid as standard. Briefly, 100 µl of each extract and standard (dissolved in methanol) are mixed with 200 µl of Folin-Ciocalteu reagent and 900 µl of water. After 5 min, 1000 µl of Sodium Carbonate (Na2 CO3) at 7% are added and the resulting solution was made up to 2.5 ml with distilled water. The mixture was vortexed for 15 s and allowed to stand for 90 min at room temperature in the dark. The absorbance was read using a spectropho tometer at 750 nm blanking with the control solution (prepared in the same way by replacing the extract by distilled water). Total phenolic content was determined as µg of Gallic acid equivalent per mg of extract (GAE/mg E). Values are expressed as mean ± SD of three independent experiments (Atere et al., 2018). Flavonoids content was determined by the Aluminum trichloride (AlCl3) method, based on the formation of a flavonoid-aluminum complex (Meda et al., 2005). Briefly, 5 ml of sample and the standard (prepared in methanol) with appropriate dilutions are added to the same volume of AlCl3 (2% in methanol). After 10 min of incubation in the dark, the absorbance is read at 415 nm. The experiment was done in triplicate. A calibration curve by Quercetin was established and Flavo noids contents were determined as µg of Quercetin equivalent per mg (QE/mg E). Values are expressed as mean ± SD of three independent experiments.

2.4. Identification of phenolic compounds by HPLC The identification of phenolic compounds in AHE was evaluated using the HPLC system (model YL9100) equipped with a reversed phase C18 analytical column (Zorbax Eclipse XDB C18). Clarity - Chroma tography SW Software (Data Apex, 2006; www.dataapex.com, Prague, Czech Republic) was used to collect and analyze data. The extract was weighted, dissolved in the mobile phase and then 20 µl were directly injected into HPLC. The injected sample volume was 20 µl and the flow rate of the mobile phase was 1 ml/min. Samples were eluted with a gradient of previously degassed 0.1% (v/v) acetic acid in water (eluent A, pH 2.2) and methanol (eluent B). Total run time was 60 min. The elution program was as follows: 95 A/5 B (0 min); 5 A/95 B (55 min); 95 A/5 B (60 min). Chromatographic profiles of the phenolic compounds were monitored

at 254 nm. The identification of compounds was ob tained by comparing their retention time values with those of pure standards.

2.5. Determination of antioxidant activity by DPPH radical scavenging assay The free radical scavenging capacity of A.halimus aqueous extract was determined by 1,1′ -diphenyl-2-picryl-hydrazil (DPPH) scavenging assay. Briefly, 50 µl of plant extract (0–2 mg/ml) and standard (BHT: Butylated hydrox ytoluene) were mixed with 1.95 ml of methanolic DPPH solution (0.025 g/l) and incubated in the dark at room temperature for 30 min. The absorbance was then read at 515 nm using a spectrophotometer. A control-containing DPPH solution and methanol only was also prepared. The DPPH scavenging activity was calculated using the following equation: % DPPH Scavenging Activity = [(Absorbance of the control – Absorbance of the test sample)/ Absorbance of the control] X 100. Data are presented as mean of triplicate and the concentration required for a 50% (IC50) reduction of DPPH radical was determined graphically.

2.6. Determination of AHE cytotoxicity by MTT test

2.6.1. Animals NMRI mice weighing 25–30 g were obtained from the Pasteur Institute (Algiers, Algeria). These mice were acclimated for 1 week before experiments starting and kept under normal conditions with a 12 h dark/light cycle and with ad libitum access to food and water.

2.6.2. Isolation of murine peritoneal macrophages Peritoneal macrophages were isolated from mice peritoneal cavity with cold phosphate buffered saline (PBS). Cells viability was deter mined in a hemacytometer by trypan-blue dye exclusion, and was found to be more than 95%. Macrophages (5 × 104 cells/ml) were cultured in 96-well plates with 200 µL of RPMI 1640 medium (10% FBS) for 2 h (37 ◦C, 5% CO2). Non-adherent cells were removed by washing the wells with PBS.

2.6.3. Cytotoxicity assay by MTT test Cultured peritoneal macrophages were incubated with increasing concentrations of AH extract (10, 20, 30, 40 and 50 mg/ml) in supple mented RPMI 1640 medium and cultured for 24 h under the same conditions. Macrophages without treatment were used as negative control and macrophages treated with 0.1% Triton X were used as positive control. After that, supernatants were removed and the MTT solution (5 mg/mL) was added and incubated for 3 h (37 ◦C, 5% CO2). Supernatants were discarded and 100 µL of DMSO (100%) was added to each well, to dissolve the formazan crystals. After 15 min of incubation, absorbance was measured spectrophotometrically at 550 nm. Experi ments in all the groups were performed in triplicate.

The percentage (%) of cell viability was calculated by the following formula:

(%) of cell viability= (Abs treated cells/Abs negative control) × 100

2.7. Determination of in vitro scolicidal activity

2.7.1. Protoscoleces collection E. granulosus hydatid cyst was obtained from sheep organs infected with Echinococcus granulosus metacestode (M'sila abattoir, Algeria). Protoscoleces (PSC) were collected aseptically and washed several times with sterile phosphate buffered saline (PBS). PSC viability was assessed microscopically using 0.1% eosin staining prior to incubation. At the time of the experiment, all samples have a viability superior then 98%; Amri et Touil-Boukoffa.

2.7.2. PSC culture and treatment with AH extract PSC were cultured in 48 well culture plates at a density of 2500 PSC/ ml in RPMI 1640 medium supplemented with 2 mM glutamine and 10% fetal calf serum, at 37 ◦C in humidified atmosphere of 5% CO2. PSC were cultured with different concentration of AHE (20, 40, 50, 60 and 100 mg/ml) for 15, 30, 60, 90, 120 and 180 min. All experiments were performed in triplicate. Data are expressed as the mean ± Standard deviation.

2.7.3. Determination of PSC morphological change by staining method The percentage of PSC viability was assessed prior to incubation and after culture by 0.1% eosin staining method under light microscopy. Assessment of viability was based on PSC vital staining, motility, and morphological criteria, as described by Amri et al. Eosin staining distinguishes between dead red protoscoleces and living refractive protoscoleces. All experiments were conducted in triplicate. Viability rate was calculated using the following equation: % Mortality= (died PSC/died + viable PSC) x 100

2.7.4. Determination of PSC ultrastructure by scanning electron microscopy (SEM) In order to analyze the ultrastructural changes of protoscoleces treated by different concentrations of AH extract, SEM studies were performed. Samples of protoscoleces cultured in vitro were fixed with 10% paraformaldehyde and processed as previously described by Eli ssondo.

2.8. Statistical analysis Statistical analysis was performed using GraphPad Prism 6. The re sults were expressed as mean ± SD. Data was analyzed statistically using one-way ANOVA and Tukey's multiple comparison test (for $p < 0.05$ differences were considered to be statistically significant).

3. Results

3.1. Phytochemicals content of AHE The phenolic and flavonoids content of the extract were 37.93 ± 0.002 µg Gallic acid equivalent (GAE)/mg E and 18.86 ± 0.01 µg Quercetin equivalent/mg E respectively (Table 2). By comparing the retention time values of the compounds with those of pure standards; HPLC analysis led to the identification of different phenolic compounds like: Gallic acid, Vanillic acid, Catechin, Ferulic acid, Rutin, Myrecetin and Quercetin; with predominance of Ferulic acid.

Table 1 HPLC analysis of AHE for phenolic compound contents.

Compounds	RT (min)	Area (mV. s)	Area [%]
Gallic acid	7.0	298.874	2.7
Chlorogenic acid	16.4	739.192	6.6
Caffeic acid	20.5 (20.4)	10.230	0.1
Catechin	21.39	224.119	2.0
Vanillic acid	22.84	215.275	1.9
Berberine	24.4	194.422	1.7
p-Coumaric	25.64	41.550	0.4
Trans-Cinnamic acid	27.093	661.531	5.9
Ferulic acid	27.560	868.773	7.7
3 Hydroxy 4-Methoxy Cinnamic acid	28.243	37.834	0.3
(Isoferulic acid)	28.677	898.816	8.0
Carboxylic acid			
Salicylic acid	30.9	220.655	2.0
Rutin	32.4	96.904	0.9
m-Anisic acid (3-Methoxy benzoic acid)	33.1	127.841	1.1
Myrecetin	34.3	744.882	6.6
Quercetin	38.527	31.995	0.3

RT: Retention time.

3.2. Antioxidant activity of AHE Our results show that AHE has an antioxidant activity expressed by an IC50 of 0.95 ± 0.02 mg/ml. The IC50 values of the extracts were compared to the IC50 of reference substance which is BHT with an IC50 of 0.21 ± 0.03 mg/ml.

3.3. Cytotoxicity assay Cytotoxic effect of AH extract was evaluated on murine peritoneal macrophages using the MTT assay. The percentages of cells viability were 98.66, 99.33, 98.64, 99.87 and 97.4% at at 10, 20, 30, 40 and 50 mg/ml of AHE for 24 h, respectively (Fig. 2).

3.4. In vitro scolicidal activity of AHE

3.4.1. Morphological damage of treated protoscoleces The scolicidal activity of Atriplex halimus was evaluated by studying the scolicidal effect of aqueous leaf extract on the viability of proto scoleces in vitro. With the concentration of 20, 40, 50, 60 and 100 mg/ ml, mortality rates were 30.25%, 59.66%, 63.05%, 96.86% and 99.95% after 90 min of incubation, respectively. Moreover, the scolicidal effect after 180 min of incubation with the aqueous extract was 50.81%, 70.05%, 91.08%, 99.33% and 100%, respectively for the same con centrations. Comparing to the control groups, the scolicidal effect of all concentrations of AHE was time and dose dependent ($p < 0.05$) (Fig. 3). As shown in Fig. 4, AHE induces strong morphological alterations.

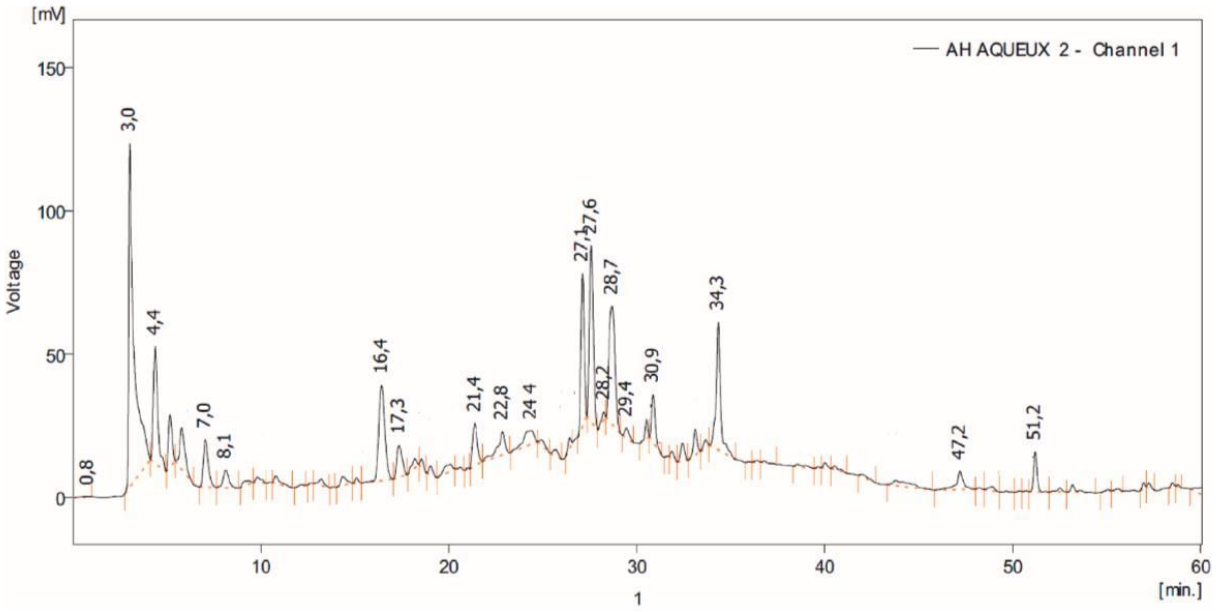

Fig. 1. HPLC chromatogram of Atriplex halimus leaves aqueous extract.

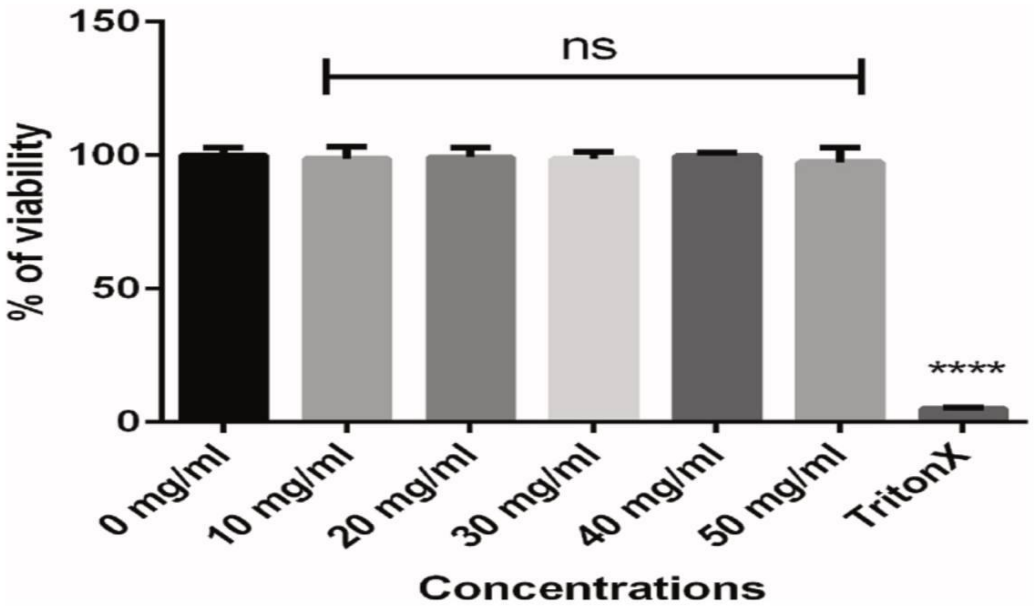

Fig. 2. Effect of AH extract on murine peritoneal macrophage viability. The cells viability was assessed by MTT test as described in materials and methods. The values represent mean ± S.E.M. of three independent experiments (n = 3) and results were analyzed using the ANOVA test followed by Dunnett's multiple comparison test (****$p < 0.0001$, ns: not significant compared to un treated group).

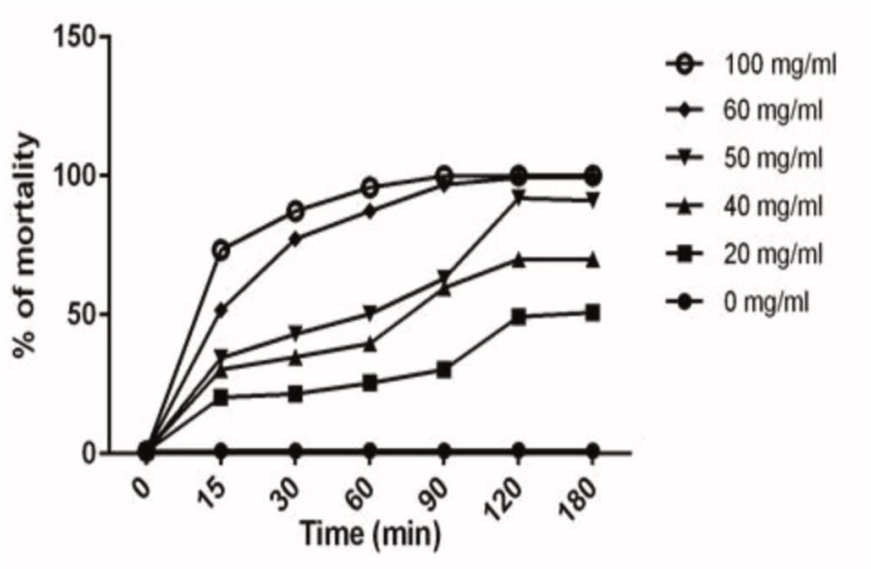

Fig. 3. Scolicidal effect of Atriplex halimus extract at different concentrations and after different exposure times. PSC viability was assessed by 0.1% eosin staining method. Each point represents the mean percentage of dead protoscoleces from three different experiments.

3.5. Ultrastructural damage of treated protoscoleces The alterations observed in protoscoleces under light microscopy were confirmed by SEM as shown in Fig. 5. Comparing with the control, SEM indicated that AH extract induced a morphological and ultra structural damage in treated protoscoleces (Fig. 5 c, d, e and f). Blebs formation, rostellum disorganization, hooks loss and membrane integ rity were also observed in treated PSC. Importantly, great damages in sucker region and microtrich were also observed (Fig. 5 c and d). At the concentration 40 and 60 mg/ml; SEM show a contracted and altered tegumental scolex (Fig. 5 e and f). Our data indicate that the primary site of drug's damage may be the parasite's tegument.

4. Conclusion

In the light of our results, we showed the beneficial properties of AH extract as new promising scolicidal agent as an adjunct to surgery or chemotherapy to prevent secondary echinococcosis. Our findings need to be more investigated in murine experimental models and further studies are needed to find the active components in AH extract.

CRediT authorship contribution statement

Samia Bouaziz: All experimentations, Methodology, Validation, Formal analysis, Resources, Writing – original draft, Writing – review & editing. Manel Amri: Methodology, Discussion, Review. Nadia Taibi: Methodology. Razika Zeghir-Bouteldja: Methodology. Abderrahim Benkhaled: Methodology. Dalila Mezioug: Methodology. Chafia Touil-Boukoffa: Conceptualization, Validation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project admin istration, Funding acquisition.

References

Albani, C.M., Pensel, P.E., Elissondo, N., Gambino, G., Elissondo, M.C., 2015. In vivo activity of Albendazole in combination with thymol against Echinococcus multilocularis. Vet. Parasitol. 212 (3–4), 193–199. https://doi.org/10.1016/j. vetpar.2015.06.030.

Ali, R., Khan, S., Khan, M., Adnan, M., Ali, I., Khan, T.A., et al., 2020. A systematic review of medicinal plants used against Echinococcus granulosus. PloS One 15 (10), e0240456. https://doi.org/10.1371/journal.pone.0240456. Amri, M., Aissa, S.A., Belguendouz, H., Mezioug, D., Touil-Boukoffa, C., 2007. In vitro antihydatic action of IFN-γ is dependent on the nitric oxide pathway. J. Interferon Cytokine Res. 27 (9), 781–788. https://doi.org/10.1089/jir.2007.0003. Amri, M., Mezioug, D., Touil-Boukoffa, C., 2009. Involvement of IL-10 and IL-4 in evasion strategies of

Echinococcus granulosus to host immune response. Eur. Cytokine Netw. 20 (2), 63–68. https://doi.org/10.1684/ecn.2009.0154. Amri, M., Touil-Boukoffa, C., 2015. A protective effect of the laminated layer on Echinococcus granulosus survival dependent on upregulation of host arginase. Acta Trop. 149, 186–194. https://doi.org/10.1016/j.actatropica.2015.05.027. Amri, M., Touil-Boukoffa, C., 2016. In vitro anti-hydatic and immunomodulatory effects of ginger and [6]-gingerol. Asian Pac J Trop Med 9 (8), 749–756. https://doi.org/ 10.1016/j.apjtm.2016.06.013. Assolini, J.P., da Silva, T.P., da Silva Bortoleti, B.T., Gonçalves, M.D., TomiottoPellissier, F., Sahd, C.S., Pavanelli, W.R., 2020. 4-nitrochalcone exerts leishmanicidal effect on L. amazonensis promastigotes and intracellular amastigotes, and the 4- nitrochalcone encapsulation in beeswax copaiba oil nanoparticles reduces macrophages cytotoxicity. Eur. J. Pharmacol. 884, 173392. https://doi.org/ 10.1016/j.ejphar.2020.173392. Atere, T.G., Akinloye, O.A., Ugbaja, R.N., Ojo, D.A., Dealtry, G., 2018. In vitro antioxidant capacity and free radical scavenging evaluation of standardized extract of Costus afer leaf. Food Science and Human Wellness 7 (4), 266–272. https://doi.org/10.1016/j.fshw.2018.09.004. Benarba, B., 2016. Medicinal plants used by traditional healers from South-West Algeria: an ethnobotanical study. J Intercult Ethnopharmacol 5 (4), 320–330. https://doi.org/10.5455/jice.20160814115725. Benhammou, N., Bekkara, F.A., et Panovska, T.K., 2009. Antioxidant activity of methanolic extracts and some bioactive compounds of Atriplex halimus. Compt. Rendus Chem. 12 (12), 1259–1266. https://doi.org/10.1016/j.crci.2009.02.004. Brunetti, E., Garcia, H.H., Junghanss, T., International Ce, P., Workshop in Lima, 2011. Cystic echinococcosis: chronic, complex, and still neglected. PLoS Negl Trop Dis 5 (7), e1146. https://doi.org/10.1371/journal.pntd.0001146. Clauser, M., Dall'Acqua, S., Loi, M.C., Innocenti, G., 2013. Phytochemical investigation on Atriplex halimus L. From sardinia. Nat. Prod. Res. 27 (20), 1940–1944. https:// doi.org/10.1080/14786419.2013.793684. Chan-Zapata, I., Canul-Canche, J., Fernandez-Martín, ´ K., Martín-Quintal, Z., TorresRomero, J.C., Lara-Riegos, J.C., Arana-Argaez, ´ V.E., 2018. Immunomodulatory effects of the methanolic extract from Pouteria campechiana leaves in macrophage functions. Food Agric. Immunol. 29 (1), 386–399. https://doi.org/10.1080/09540105.2017.1386163. Chikhi, I., Allali, H., Dib, M.E.A., Medjdoub, H., Tabti, B., 2014. Antidiabetic activity of aqueous leaf extract of Atriplex halimus L. (Chenopodiaceae) in streptozotocininduced diabetic rats. Asian Pac. J. Trop. Dis. 4, 181–184. https://doi.org/10.1016/S2222-1808(14)60501-6. Vuitton, D.A., McManus, D.P., Rogan, M.T., Romig, T., Gottstein, B., Naidich, A., et al., 2020. International consensus on terminology to be used in the field of echinococcoses. Parasite 27, 41. https://doi.org/10.1051/parasite/2020024. Wen, H., Vuitton, L., Tuxun, T., et al., 2019. Echinococcosis: advances in the 21st century. Clin. Microbiol. Rev. 32 (2) https://doi.org/10.1128/CMR.00075-18 e00075-18.