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Anti-inflammatory and hemostatic Activities of Methanolic Extract from *ATRIPLEX HALIMUS* Leaves

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Abstract

Introduction: Chenopodiaceaefamily species are known for their important biological activity, in which Atriplexhalimus belongs.However, the inflammatory effect of this plant leaves has not been studied. This work aims to assess the anti- inflammatory and hemostatic activities of the methanolic extract AHMeOH of *Atriplexhalimus*'s leaves.

Methods: The extract was obtained using sonication of powder's leaves in 80 % methanol. The analysis of phenolic compounds was carried out using thin-layer chromatography (TLC). The anti-inflammatory activity was determined by studying the plasmic membrane stabilization and albumin denaturation inhibition, the hemostatic activity was evaluated by measuring the plasma level in blood.

Results: Quantitative determination of total flavonoids reveals that AHMeOH is rich in flavonoids (16 ± 0.88 µg Q / mg extract) and polyphenols (20 ± 0.20 µg AG / mg extract).

About the anti-inflammatory activity, the tests show that AHMeOH has a significant effect (P≤0.05) of inhibiting hypotonic-induced hemolysis with concentrations (100 and 200 µg / ml) with 77.55 and 90% respectively, and heat-induced hemolysis with percentages 81.75% and 87.44% respectively with a significant difference (P ≤0.05).

The obtained results reveal that the inhibition of albumin denaturation has dependent dose. The concentration of 400 µg / ml gives denaturation inhibition effect of 81.00 ± 17.70% and the concentration of 600 µg / ml gives an effect of 82.95 ± 17.40%.

Regarding the hemostatic activity our extract with the doses 10 mg / ml, 20 mg / ml and 30 mg / ml confer a decrease of the plasma recalcification time in the tube, these concentrations could prolong the time of coagulation significantly compared to the control (P≤0.001). This result is an interesting indication in favor of hemostatic activity of AHMeOH.

Conclusion: *Atriplexhalimus* has a strong anti-inflammatory activity and constitutes a potential source to the development of new treatments.

Keywords: Albumin,*Atriplexhalimus*, hemostatic activity, methanolic extract.

Introduction

Reports have shown that inflammation is usually triggered by the damage of the living tissues resulting from bacterial, viral, fungal infections; physical agents; and defective immune response. The fundamental aim of inflammatory response is to localize and eliminate the harmful agents; secondly, to remove the damaged tissue components to culminate in healing of the affected tissues, organs, or system [Chen et al., 2018]

Phytotherapy refers to medicine based on plant extraction and natural active ingredients. The use of plants as a therapeutic goes back to antiquity, and concerns a large number of civilizations across time [Yahia et al., 2018 a, Yahia et al., 2018 b).

It is in the context of the enhancement of our natural heritage that our study fits. The approach pursued in this research consists of an ethnobotanical study, followed by an extraction and a qualitative analysis of different chemical compounds likely to have a pharmacological activity. Among these compounds, we are more particularly interested in those endowed with anti-inflammatory and hemostatic activity[Benhouda et al., 2015].

Various nonsteroidal anti-inflammatory drugs can reduce pain and inflammation by blocking the metabolism of arachidonic acid through iso form of cyclooxygenase enzyme (COX-1 and/or COX-2), thereby reducing the production of prostaglandin[Bruneand Patrignani, 2015]. Unfortunately, there are many side effects associated with the administration of nonsteroidal anti-inflammatory drugs. However, there are medicinal plants with anti-inflammatory therapeutic effects with low or no side effects. The African continent is richly endowed with diverse medicinal plants with anti-inflammatory activities that have been shown to be effective in the treatment of inflammatory conditions in traditional medicine[Oguntibeju ,2018].

The species studied the ***Atriplexhalimus*** which belong to the Chenopodiaceae family. A plant recognized in traditional therapy with their remarkable property in the treatment of pathologies.

This plant is used as a medicinal plant in the traditional pharmacopoeia [Dutuitet al. , 1999]. It was recommended for diabetic patients because of the chromium, manganese and magnesium salts present in the *Atriplexhalimus* [Marles and Farnsworth, 1995]. As a result, scientists have explored the possibility that *Atriplexhalimus* has an anti-diabetic effect and is hypolipidemic [Mirsky and Nitsa, 2001].

Non-steroidal anti-inflammatory drugs (NSAIDs) represent one of the most common classes of medications used worldwide with an estimated usage of >30 million per day for inflammation and related disorders[Horl,2010]. Most of the NSAIDs are carboxylic acid containing drugs including salicylate derivatives (aspirin), carboxylic and heterocyclic acid derivatives (indomethacin), propionic acid derivatives (ibuprofen, ketoprofen, flurbiprofen) and phenyl acetic acid derivatives (diclofenac). This organic acid containing drugs act at the active site of the enzyme preventing the access of arachidonic acid (AA) to the enzyme and stop the cyclooxygenase pathway[Zarghi et Arfaei ,2011].Unfortunately, besides the excellent anti-inflammatory potential of the NSAIDs, the severe side effects such as gastrointestinal (GI) ulceration, perforation, obstruction, and bleeding has limited the therapeutic usage of NSAIDs[Sostres et al.,2013].

The inflammatory effect of *Ariplexhalimus* has not been studied. The present work aims to assess the anti- inflammatory and hemostatic activities of the methanolic extract (AHMeOH) of *Atriplexhalimus's* leaves in vitro.

Methods

Plant material

The studied plant was collected on February 2018 from the region of Arris,Batna, Algeria. The harvested plant leaves were washed and then dried in the shade for 40 days in a dry and ventilated place for later use such extracting the active ingredients.

The plant leaves were subjected to grinding and extraction of bioactive substances.

Plant Extraction

The extraction is carried out according to the method of Diallo et al., (2004) in which, 500 g of powder of the leaves of the plant is macerated with 2.5L of methanol, the extraction is carried out with continuous stirring at room temperature, for 24 hours and we used the filter paper and the cotton for the filtration, after filtration, the filtrate is concentrated by rotary evaporation in a Rotavapor at a temperature of 40 ° C and we obtained methanolic extract (AHMeOH).

Total phenolic content measurement

1 ml of the extract (0.2 mg / ml) is diluted in 5 ml of distilled water and 1 ml of 20% Na2CO3. After mixing, the whole was incubated at room temperature. Subsequently, 1 ml of Folin-Ciocateu reagent was added, then incubated for 30 minutes in an oven at 40 ° C.

The absorbance was read at 760 nm against a blank. The level of polyphenols is expressed in µg equivalents of gallic acid per mg of extract (µg GAE / mg of extract) via a calibration curve of gallic acid (0-200 µg / ml) [Singeltonet al., 1999].

Total flavonoid content

Total flavonoid content is estimated according to the process described through [Park et al., 2008]. A 0.3 ml of extracts dissolved with 3.4 ml of 30% methanol, 0.15 ml of $NaNO_2$ (0.5 M) and 0.15 ml of $AlCl_3.6H_2O$ (0.3M) in a 10 ml test tube. After 5 min, 1 ml of NaOH (1 M) was delivered to that combination. The blend was liberated well and the absorbance taken at 506 nm. The standard curve for whole flavonoids was made utilizing rutin solution (0 to 100 mg/l) under the similar process as earlier described. Total flavonoids have been expressed as milligrams of rutin equivalents per g of dried crude extract.

In vitro biological activities assay

Hemostatic activity test

We followed the method described by Aouissa, (2002); this test is performed in vitro on blood plasma from a healthy adult person of 25 years. The principle of this test is to measure the coagulation time of decalcified plasma after recalcification. That's why, the blood is collected on a tube of sodium citrate in a healthy subject. The plasma is obtained after centrifugation at 3600 rpm for 10 minutes.

Then, concentrations of 10, 20, 30, mg / ml of extract were dispensed into test tubes for each dose. Another empty test tube was used as a control did not receive any dose of the extract. The tubes are kept in a water bath at 37 ° C. Next, a volume of 200 µl of plasma and 200 µl of calcium chloride (CaCl 2) at 0.025 M are added to each tube. The chronometer is triggered as soon as the plasma is added to each tube. The observations were recorded every 30 seconds, until a clot was formed, noting the coagulation time for the 8 tubes of each dose.

Coagulation assessment is done by tilting the tube at a 45 ° angle to see whether or not coagulation occurs every 30 seconds. The test is positive if the coagulation time of plasma containing an extract is less than that of the control plasma.

Stabilization effects of the plasma membrane

According to the method described by Shinde et al., (1989), 5 ml of human blood was collected and transferred to the EDTA tube. The tube was centrifuged at 2000 rpm for 5 min, and washed three times with an equal volume of normal saline. Blood volume is measured and reconstituted as a 40% suspension with isotonic buffer solution (pH = 7.4). The composition of the buffer solution (g / l) was [NaCl (4.4 g), NAH 2 PO 4 (1.6 g) and Na 2 HPO 4 (7.6 g)].

Inhibition of albumin denaturation

The protective effect of the AHMeOH extract against heat-induced membrane albumin denaturation was evaluated according to the method described by Sakat et al., (2009). The turbidity measurement was made at λ = 660nm with spectrophotometry and the results were compared with those of diclofenac. The percentage of inhibition of the denaturation of albumin is calculated according to the following formula:

Denaturation inhibition percentage = 100 - [A1 / A0 × 100].

A0: Absorbance of the sample or standard.

A1: Absorbance of the control solution.

This test consists of adding to 1 ml of the extract prepared with different concentrations (400 and 600 µg / ml) or standard reference sodium diclofenac (400 and 600 µg / ml) 1 ml of fetal bovine albumin solution (1 mM) and then incubated at 27 ° C for 15 min.

Denaturation was induced by incubating the albumin solution at 60 ° C in a water bath for 10 minutes. After cooling, the turbidity was measured spectrophotometrically at 660 nm.

Statistical analysis

The statistical study was carried out by the statistical software Graph Pad prime 5. The results are expressed in mean ± SD. The results are analyzed by the univariate ANOVA test followed by the Dunnet / tukey test for multiple comparisons and determination of significance rates.

The values of P≤0.05 are considered statisticallysignificant.

Results and Discussion

Phytochemical study

The phytochemical screening carried out in this work from the plant extract reveals the presence of several secondary metabolites.

Preliminary tests have indicated the presence of polyphenols, gallic tannins, terpenoids and flavonoids and with an increased presence of alkaloids in *Atriplexhalimus* extract. Concerning catechin tannins and saponins are revealed absent in the extract which is showed on **table 1**

Effect of membrane stabilization

a-Hypotonia

The effect of AHMeOH on the stability of the membrane of human erythrocytes in vitro is shown in Figure (1).

The examination of these results shows that our extract was able to inhibit the hemolysis induced by hypotonia with the concentrations (200 and 400 µg / ml) with percentages 77, 55% and 90% respectively with significant difference (P <0.05).

Indomethacin, which is used as a standard reference , gave an effect of 61% which was lower compared to the effects of the extract (100 µg / ml and 200 µg / ml) with a significant difference (P <0.05).**Figure 1**

b-Heat

Examination of these results showed that the extract AHMeOH was able to inhibit heat-induced hemolysis with the two concentrations (200 and 400 µg / ml) with percentages 81.75% and 87.44% respectively without significant difference (P> 0.05) (Fig2).

Indomethacin, which is used as the standard reference , gave a 51.28% effect which was less than the one of the extract (200 mg / ml and 400 µg / ml) with a significant difference (P <0.05).**Figure 2**

In vitro hemostatic activity test

The choice to study the hemostatic activity of the extract of *Atriplexhalimus* was made because of its use in traditional medicine.

This plant is used to treat blood bleeding after an injury in the form of a poultice, the latter is mostly prepared by maceration or decoction of the chopped leaves in water.

The results of this activity are shown in the table in **Figure 3**.

The extract AHMeOH with the doses 10 mg / ml, 20 mg / ml and 30 mg / ml confers a reduction in the recalcification time of plasma in vitro in the tube.

We found that the extract with the concentrations could significantly increase the clotting time compared to the control (P≤0.001).

(It's a dose dependent decrease of this extract), so our extract decreases plasma coagulation. This result is an interesting indication in favor of hemostatic activity of AHMeOH.

This plant is considered as a natural source of high-value anticoagulant compounds.

Our extract contains high phenolic compounds and flavonoids.

The high content of phenolic compounds and the significant linear correlation between the values of the concentration of phenolic compounds and hemostatic activity indicated that these compounds contribute to anticoagulant activity. This may be due to phenolic hydroxyl groups, tannins capable of reacting with strong hydrogen bonds with the atoms of the peptide binding protein by inhibiting thrombin for example, a proteolytic enzyme which transforms fibrinogen from a soluble molecule into an insoluble molecule, fibrin [Pushpamali et al., 2008].

Experimental studies are interested in investigating the anticoagulant activities of a various natural extracts, they are primarily focused on the study of the anticoagulant properties of brown and red marine algae [Pereira et al., 2005; Athukorala et al., 2007].

The compounds most responsible of the anticoagulant effect are the polysaccharides [Yoon et al., 2002; Pawlaczyk et al., 2009], these compounds are the most studied because of their anticoagulant activity thanks to the presence in their chemical structures of the hydroxyl group (-OH), thus undergoing modifications of the carboxylation or sulfation type in synergy can cause inhibition of the endogenous coagulation pathway [Yang et al., 2005].

Among the compounds endowed with this activity, peptides [Mieszczanek et al., 2004], glycoproteins, and polyphenols [Pawlaczyk et al., 2011] in particular coumarins and some tannins [Bae,1993]. Although there are several research projects focused on the anticoagulant activity of various plant extracts, this activity has not been studied for polyphenolic extracts of *A. halimus*, therefore the subject of this dissertation is considered to be the first of its kind which is part of the studies interested in the prevention and therapy of thrombotic diseases, and this by the search for a possible anticoagulant activity of the polyphenols of *A. halimus.*

Inhibition of albumin denaturation

Protein denaturation is a phenomenon which the protein loses its three-dimensional structure, following its exposure to heat, to an infectious or chemical agent (acid or strong base) [Jacquier-sarlin and Polla, 1994].

The results obtained with the extract AHMeOH reveal that the inhibition of denaturation is dose dependent dose. The

concentration of 400 µg / ml gives an inhibition of denaturation of 81.00 ± 17.70% and the concentration 600 µg / ml gives an effect of 82.95 ± 17.40%.

The standard used (Diclofenac) gave an inhibition effect of approximately 58.77 ± 12.82% with the concentration 400 µg / ml and a percentage of approximately 56.42 ± 6.92% with the concentration 600 µg / ml, but without any difference significant with the extract **(fig 4).**

Albumin is a small globular protein of 66 k Da and the most abundant in plasma. It represents approximately 60% of plasma proteins and has 585 amino acids with a thiol group at the level of its cysteine 34 in reduced form [Mira, 2008] which plays a role in the aggregation of albumin under the effect of temperature increase [Barone, 1992]. It is responsible for maintaining oncotic pressure It also has an anti-thrombotic and anticoagulant role which may be due to its ability to bind NO (nitric oxide), which prolongs the anti-aggregating effect of NO on platelets [Evans, 2002].

The anti-denaturing activity of the extract could be due to the interaction of certain components with two sites (present at the level of certain exp proteins: albumin) of bonds rich in Tyrosine, Threonine and Lysine [Williams et al., 2002].

Albumin undergoes structural changes with loss of its three-dimensional shape and exposure of certain hydrophobic sites (such as the cysteine residue 34) which are inaccessible in the normal physiological case (native functional protein). These hydrophobic zones can interact and form aggregates that are harmful to the cell [Arrigo, 2005].

According to the study by Dufour and Dangles, (2004) on the interaction of flavonoids with albumin, the latter has a strong affinity for quercetin, which could explain the protective activity of polyphenols against thermal denaturation of albumin.

Various authors have shown that flavonoids exhibit a moderate affinity for albumin with association constants varying from 1 to 15 × 104 M-1 [D'Archivio et al., 2007, Skerget, 2005]. Using site markers (warfarin and DNSA for the IIA subdomain, ibuprofen and diazepam for the IIIA subdomain), it has been shown that flavonoid albumin complexation takes place mainly in the IIA subdomain, in agreement with the fluorescence quenching of the Trp-214 residue (itself located in this sub-domain) that it produces [D'Archivio et al., 2007].

The stoichiometry of complexes is 1: 1 in most studies [D'Archivio et al., 2007]. The main interactions responsible for the protein-flavonoid association are distributed between hydrogen bonds, ionic interactions and van der Waals interactions [Hertog et al., 1992]. Polyphenols show a moderate affinity for β-CD [Bouaziz et al., 2008; Erbay and Icier, 2009]. This macrocycle can interact with polyphenols thanks to Van der Waals bonds, hydrogen bonds and by hydrophobic effect [Bourvellec et Renard., 2012].

Conclusion

The purpose of this work was to carry out a phytochemical and biological study of the *Atriplexhalimus* plant of the Chenopodiaceae family, chosen on the basis of their traditional uses.

To do this, we previously performed a preliminary screening of the various secondary metabolites contained in the species and we found that this plant contains mainly flavonoids; tannins, and alkaloids.

The results of the anti-inflammatory activity have shown that the methanolic extract of *Atriplexhalimus* has a role in inhibiting hemolysis which induces hypotonia and heat and a role in inhibiting the denaturation of albumin.

The evaluation of the hemostatic activity shows that AHMeOH has an anti-coagulant effect, with a significant difference between the

coagulation time of the control and that of plasma with AHMeOH.

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Conflict of interest :

There is no conflict of interest.

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Table 1: Phytochemical screening of the extract (AHMeOH) of the leaves of Atriplexhalimus.

Plant	Extract	Polyphenol	Flavonoids	Tannins catéchic	Tannins gallic	Terpenoids	Alkaloids	Saponins
Atriplex Halimus	EMe	++	++	-	+++	+++	++++	-

++++: Very abundant +++: abundant; ++: medium; +: - suspicious reaction.

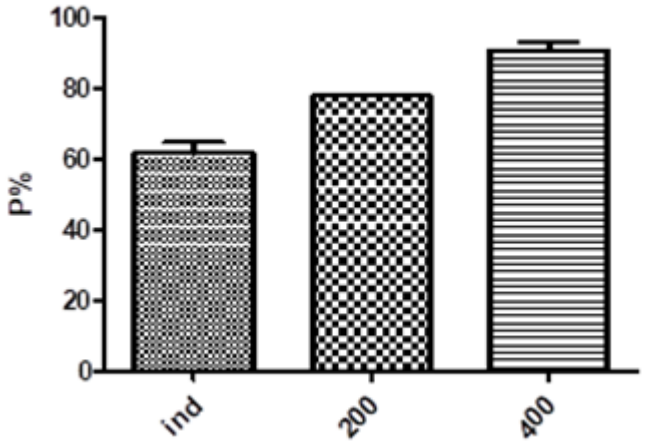

Figure 1. Hemolysis induced by hypotonia. Each bar represents the mean ± SD of 3 experiments.

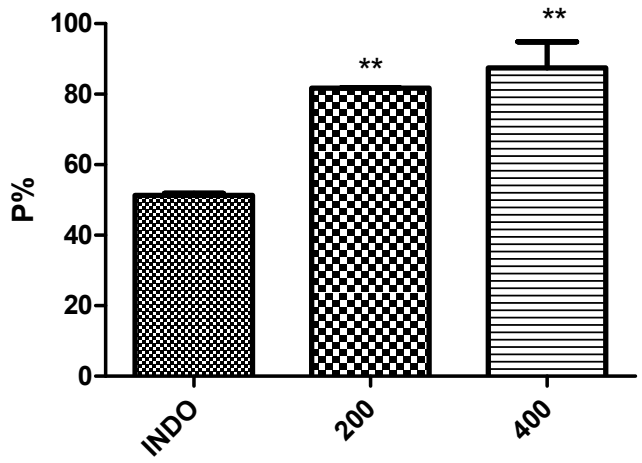

Figure 2. Heat induced hemolysis. Each bar represents the mean ± SD of 3 experiments; ** P <0.001: significant difference compared to indomethacin

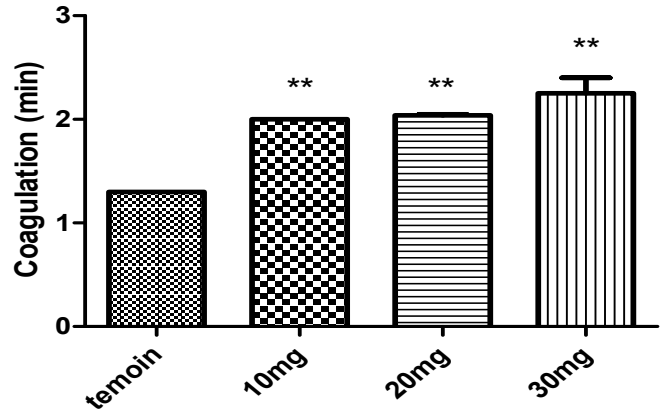

Figure 3. Histogram representing the statistical differences obtained for hemostatic activity. Each bar represents the mean ± SD of 3 experiments; ** P <0.001: significant difference compared to the control.

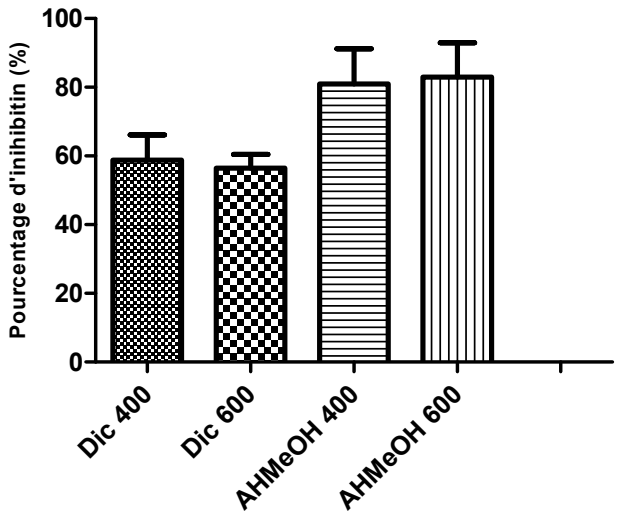

Figure 4. Inhibition of albumin denaturation. Each bar represents the mean ± SD of 3 experiments; P> 0.05: no significant difference.