Acute and chronic toxicity of the methanolic extract of *Ajuga iva* in rodents

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**Summary.** *Ajuga iva*, known as “Chendgoura”, is widely used in North African countries in the traditional medicine. However, there is little toxicological informations available regarding its safety following repeated exposure. The present investigation describes the toxicity of a methanolic extract of *Ajuga iva*. The administration of the *Ajuga iva* methanolic extract (AIME) at 2 – 14 g/kg of bodyweight (bwt) did not produce mortality or significant changes in the general behavior of mice. However, single intraperitoneal injections of AIME (2 – 6 g/kg bwt) produced a dose-dependent increase in adverse effects in the general behavior and the mortality rate. The LD₅₀ by intraperitoneal route was 3.980 g/kg bwt. In chronic toxicological studies in rats, oral administration the AIME with daily doses of 100, 300 and 600 mg/kg bwt, did not cause any significant differences in the general conditions like growth, organ weights, hematological and biochemical parameters or in microscopic appearance of the organs (brain, liver and the kidney). In contrast, a transient rise in platelet counts and a decrease in serum glucose and cholesterol levels were noted. Therefore, the NOAEL for the AIME is 600 mg/kg/day administered orally for 13 weeks. So Al methanolic extract has low toxicity.

**Industrial relevance.** The extract of the plant *Ajuga iva* can be used as herbal teas or for the treatment of several disease such as diabetes, high cholesterol and increased serum triglycerides. However, information regarding its toxicological properties is not available. In this study, we have shown that the methanolic extract of *Ajuga iva* has low toxicity in rodent models and may be considered for human use after further studies.

**Keywords.** *Ajuga iva*; methanolic extract; acute toxicity; sub chronic toxicity

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**INTRODUCTION**

*Ajuga iva* Schreiber, locally named Chendgoura, is a plant of the Lamiaceae family¹ (Figure 1). *A. iva*, develops in deep soil of 2700 m height. It grows in period from spring to late summer. The flowering period is between May and June. It is widely distributed in the Mediterranean region: southern Europe and northern Africa, particularly in Algeria, Morocco, Tunisia, and Egypt. The aerial part of the plant is used as an infusion or decoction in Algerian traditional medication to treat different diseases like diabetes and it is known to have an anti inflammatory, antifongic and anti-microbial effects. Some species of *Ajuga* as *Ajuga shit* were used for the cicatrization of the wounds and like an anti-inflammatory drug. Some studies proved that this plant presented a range of biological and pharmacological activities, which can justify its therapeutic use in the Algerian traditional medicine. The extract of the plant *Ajuga iva* can be used as herbal teas or capsules in the Pharmacy and Phytotherapy industries, which use dried plants, extracts or isolated active ingredients for the treatment of several disease such as diabetes, high cholesterol and increased serum triglycerides.
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Studies on the aqueous extract of the *Ajuga iva* indicated its hypoglycemic, vasorelaxant and hypolipidemic effects. *Ajuga iva* has anti-lipid peroxidation, antioxidant, antiarthritic, antifungal, antibacterial, anti hypertensive, antiplasmodial properties. Furthermore, it has an effect on enzymatic activities in diabetes, an antifeedant (insecticide) action against the larvae and insects. The hematologic extract of *Ajuga iva* have shown an effective antifeedant activity against the *fragiperda of Spodoptera* and *the littoralis S.* The traditional use of the *Ajuga iva* was also supported by the isolation and the identification of several possible active chemical components. The pharmacological activity of this plant is due mainly to all the flavonoids and, the polyphenol present in the sheet and the fruits. The principal compound in *Ajuga iva* was determined by Camps et al. Four neoditerpenoids of clerodane (Ivain I - IV) were isolated from *A. iva*. Seven of the phytoecdysteroids were found in the whole plant, including the roots collected in Algeria. The studies of toxicity of *Ajuga iva* were carried out by El Hilaly et al., who have found that the aqueous extract does not have any toxic effects, the LD₅₀ by intraperitonial track was 3.6 g/kg. In this context and to confirm safety of the use of this plant, the present study was carried out on the extract obtained with the methanol, which is used for the extraction of the most polar molecules in order to provide conclusive scientific results for the sure use of this plant.

**MATERIALS AND METHODS**

**Plant material.** *Ajuga iva* (L.) Schreber was collected from Bordj Bou Arreridj, in the northeast of Algeria, and identified by Pr. H. Laouer (Department of Vegetable Biology and Ecology, University Ferhat Abbas, Setif 1). A voucher specimen was deposited at the Laboratory of Botany, Department of Vegetable Biology and Ecology (University Ferhat Abbas, Setif 1). All reagents were purchased from Sigma Chemicals (Germany), Fluka and Prolabo (France).

**Preparation of *Ajuga iva* extract.** The extraction of polyphenols was carried out according to the method described by Markham, with slight modifications. Dried plant material was ground in warring blender, mixed with 10-20 volumes of 85% aqueous methanol. The slurry was placed at room temperature for one week and the extract was filtered through a Buchner funnel and the methanol was removed by rotary evaporation using Buchi rotavapor. The dried extract was stored at -20°C temperature until use.

**Animals.** Albino mice of the weight ranging between 23 and 25 g and Wistar rats of 8 weeks of age were used (provided by Institut Pasteur d’Algérie). The animals were maintained for 7 days in the laboratory before the study. Mice or rats (5 males or 5 females) by group were placed in plastic cages with free access to food (ONAB, Bejaia) and water. They were maintained at 24°C under a cycle of 12 h of light/darkness. The animal studies were conducted after obtaining clearance from Institutional Animal Ethics Committee and the experiments were conducted in strict compliance according to ethical principles provided by Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA).

**Acute toxicity in the mice.** *Ajuga iva* methanolic extract (AIME) was dissolved in water and orally administered at a dose of 0, 2, 4, 6, 10 and 14 g/kg of bodyweight (bwt) and intraperitoneally at a doses of 0, 2, 3, 4, 5, and 6 g/kg bwt (n = 10; 5 males and 5 females). The mice were fasted during the night before the administration of the extract. The general behavior of the mice was continuously monitored for the first hour after the administration, and periodically during the first 24 hours and every day for 14 days. At the end of the experiment all the animals were killed by chloroform inhalation, and the organs (liver, heart and brain) were taken, weighed and macroscopically examined.

**Sub-acute toxicity in the rats.** AIME was dissolved in water and orally administered daily during 90 days (13 weeks) with amounts of 0, 100, 300, and 600 mg/kg bwt. All rats were anaesthetized with the chloroform at the end of each month and the blood samples were gathered by puncture of retro-orbital sinus in dry and EDTA containing tubes for biochemical and hematologic analyses, respectively. The hematologic analyses were carried out by a Coulter automate (laboratories of Abbott, IT, the United States), and biochemical tests were carried out using a COBAS Integra 800 (Rck,Germ). Histopathological examination. At the end of the experiment, rats were anesthetized with chloroform and sacrificed by decapitation, and then livers, brains and kidneys were carefully dissected out. Small slices of these freshly harvested tissues were fixed in buffered formaldehyde solution (10%), dehydrated by serial ethanol solution, diaphanized with ethanol–benzene and enclosed with paraffin. Micromiter sections, cut by a microtome, were stained with hematoxylin–cosin and examined under a light microscope then photomicrographs of the samples were recorded.

**Reproduction Test.** The extract of *Ajuga iva* was administered at a dose of 600 mg/kg bwt to a group which contains (5 males and 5 Females). The administration in the males was carried out over a period of four weeks. This period covers two weeks minimal period before and during the period of coupling and an approximate period two weeks after the coupling. At the end of this period the rats were killed and the testicles were weighed, fixed in Bouin solution and analyzed for histopathological changes.

In the females, the used dose was 600 mg/kg bwt throughout all the 40 days; two weeks before the coupling, the gestation period and at least four days after birth. During the period of the treatment, the animals were carefully observed at daily intervals to detect all signs of toxicity. The newborns were weighed and macroscopically observed on the signs of congenital malformation.

**Statistical analysis.** All parameters were expressed as mean±(S.E.M.). Statistical analysis was performed using Student’s t-tests. P-values ≤0.05 were considered to be statistically significant. Analysis was done with OriginPro 9.

**RESULTS**

**Acute toxicity.** Among all treated animals, there was no death and no observed signs of toxicity after the oral administration of *Ajuga iva* extract on any level of doses until the highest doses of 14 g/kg bwt, which was considred as the dose no-observed-
adverse-effect level (NOAEL). However, the death rate after the intraperitoneal administration of the methanolic extract is dose dependant. The death rate of 0% with doses ≤ 2 g/kg bwt, gradually reached 80% with 6 g/kg bwt, the highest dose in our study. The NOAEL for the intraperitoneal administration was 2 g/kg, whereas the low observed-adverse-effect level (LOAEL) was 3 g/kg. Some adverse effects, such as hypo-activity and diarrhea were observed just after the intraperitoneal injection, whereas others (anorexia and loss of the weight) were observed later, pronounced with the most raised dose and persisted until the death of the animals. The median lethal dose (LD₅₀) after intraperitoneal administration of the AIME in mice was 3980 mg/kg bwt (Figure 2).

**Figure 2.** Dose-mortality curve for *Ajuga iva* methanolic extract in mice (single intraperitoneal doses). Calculated LD₅₀ = 3.98 g/kg

**Chronic toxicity studies in rats. Effect of AIME on the body weight and mortality.** The body weight of the rats after the treatment by the AIME is presented in Figure 3. No significant difference in the profile of the body weight was noted between the treated groups and the control in any moment. Moreover, no mortality was recorded with any dose until the maximum of 600 mg/kg (NOAEL) during the 90 days of the treatment.

**Effect of AIME on the hematologic and biochemical parameters of rats.** The effect of the chronic oral administration of AIME on the hematologic parameters is presented in Table 1. An increase of the numbers of the platelet and hemoglobin were observed in the treated groups in the 60 and 90 day of the treatment compared to the control group (*p* < 0.05). This increase was observed until the end of the treatment (90 days). All the other parameters (hematocrit, WBC and RBC) remained within the normal limits along the period of the treatment.

The biochemical parameters of the treated and control rats are presented in Figure 4. The chronic oral administration of AIME (until an amount of 600 mg/kg bwt) did not cause crucial changes of creatinine and urea. However, the blood level of cholesterol, triglyceride and glucose were appreciably decreased in the treated animals (*p* < 0.05) compared to the control until the 90 days of treatment. The hypoglycemic effect was maintained at the end of the period of treatment (90 days). The activity of the enzymes (ALT, AST) were increased in the treated animals (*p* < 0.05) compared to the control.

Rats treated with 600 mg/kg bwt dose of AIME showed marked centrolobular sinusoidal congestion (F2), compared to the control groups (F1). There was no effect on the kidneys of rats treated with 100, 300 and 600 mg/kg bwt dose of AIME, as the glomeruli, distal and proximal tubules appeared normal (R2), compared to the control group (R1). There was no effect on the brain of rats treated with 100, 300 and 600 mg/kg bwt of AIME (C2), compared to the control group (C1), (Figure 5).

**Effect on reproduction.** After gestation, the new born were weighed and macroscopically observed. No difference was observed between treated and controls groups, concerning the weight of newborn, and the histological cuts of the testicles (Figure 6).

**Table 1:** Effect of sub-chronic oral administration of *Ajuga iva*-extract on hematological parameters of rats. The methanolic extract of the plant was given daily by the oral route to groups of Wistar rats (*n* = 10 per group) at the following doses: 0 g/kg (GR4, control), 600 mg/kg (GR1), 300 mg/kg (GR2), and 100 mg/kg (GR4) for 90 days. Hematological parameters were measured after 30, 60 and 90 days of treatment. Data are expressed as mean±S.E.M.

<table>
<thead>
<tr>
<th>Days</th>
<th>WBC*10⁹ L⁻¹</th>
<th>plaq*10⁹ L⁻¹</th>
<th>RBC*10¹² L⁻¹</th>
<th>HT%</th>
<th>HB g/L</th>
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<tr>
<td>GR1</td>
<td>30</td>
<td>7.86 ± 1.83</td>
<td>524 ± 113.4</td>
<td>8.57 ± 1.05</td>
<td>44.3 ± 5.59</td>
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<td></td>
<td>60</td>
<td>9.85 ± 3.26</td>
<td>906.7 ± 58.7***</td>
<td>9.93 ± 1.05</td>
<td>50.46 ± 3.09</td>
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Figure 3. Effect of sub-chronic oral administration of *Ajuga iva*-extract on the body weight of rats. The plant extract was given daily by the oral route for 90 days to groups of Wistar rats (*n* = 10 per group) at the following doses: 0 g/kg (GR4, control), 600 mg/kg (GR1), 300 mg/kg (GR2), and 100 mg/kg (GR3). The rats were weighed before the start of A1-extract treatment (D0) and then every 15 days (D15, D30, D45, D60, D75 and D90). Data are expressed as mean ±S.E.M.
Figure 4. Effect of sub-chronic oral administration of *Ajuga iva* extract on biochemical parameters of rats. The aqueous extract of the plant was given daily by the oral route to groups of Wistar rats (n = 10 per group) at the following doses: 0 g/kg (GR4, control), 600 mg/kg (GR1), 300 mg/kg (GR2), and 100 mg/kg (GR3) for 90 days. Biochemical parameters were measured after 30, 60 and 90 days of treatment. Panel A: glucose; Panel B: Urea, Panel C: creatinine; Panel D: Cholesterol, Panel E: TG, Panel F: alanine aminotransferase (ALT); Panel G: aspartate aminotransferase (AST). Data are expressed as mean ± S.E.M.; (**) *P* < 0.01; (***) *P* < 0.001 versus the control group.
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**Figure 5.** Photomicrographs of the liver (F1–F2), the brain (C1–C2), and the kidney (R1–R2); scale enlargement: ×40. Photomicrographs of the sections of the liver showing normal features in control rats (F1), and the liver of rats treated orally with 600 mg/kg of AI-extract for 90 days showing a marked centrolobular sinusoidal congestion (F2). (R1) Photomicrographs of the sections of the kidney showing normal features in control rats and the kidney of rats treated orally with 600 mg/kg of AI-extract for 90 days (R2). (C1) Photomicrographs of the sections of the brain showing normal features in control rats and the brain of rats treated orally with 600 mg/kg of AI-extract for 90 days (C2).

**Figure 6.** Photomicrographs of testicular (T1-T2), scale enlargement: ×40. (T1) Photomicrographs of the sections of the testicular showing normal features in control rats and the testicular of rats treated orally with 600 mg/kg of AI-extract for 30 days (T2).
DISCUSSION

El Hilaly et al.24. Showed that the aqueous extract of *Ajuga iva* does not have any toxic effects. To confirm the safety of this plant, we have carried out a study of toxicity of methanolic extract which is used for the extraction of most polar molecules. In this study, during the evaluation of acute toxicity, no mortality was observed in after oral administration. In the daily evaluation of the clinic signs, after intraperitoneal administration, we have observed some changes such as hypo-activity, anorexia and the diarrhoeas only in the animals of the groups which were treated with doses more than 3 g/kg bwt. These changes were irreversible because they persisted until the end of the experiment. The lethal dose was LD₅₀ = 3980 mg/kg. it is near to that found in the first study done by El Hilaly et al.24. LD₅₀ 3.6g / kg, and according to the classification of Hodge et Sterner26, the methanolic extracts of *Ajuga iva* was not toxic. In contrast, toxicity was observed in the intraperitoneal injection. the nonexistence of the harmful effects (toxic) after the oral administration of the methanolic extract of the *Ajuga iva* in the mice can be explained by the fall of the biodisponibility (low intestinal absorption or the effect of first hepatic passage)24.

In the chronic toxicity the daily clinical evaluation, no changes in the behavior were observed in the animals. In addition to these parameters, the changes of body weight are an indicator of the unfavorable side effects. In the present study no significant difference was found in the weight extended within the pre-established limits, justifying the selected doses p < 0.05. Also, no significant change was observed in the consumption of water and food. The determination of such parameters is important in the study of the safety of a product with therapeutic aim. For the hematologic parameters, in the first group which received a dose of 600 mg/kg, the observation of an increase in the platelets and hemoglobin agrees with the result obtained by El Hillaly et al.24. The other hematologic parameters did not show any significant changes. The analysis of blood parameters is appropriate to the toxicological evaluation of the risk because the hematologic system has a predictive value more raised for the human (91%) when the analyses imply rodents and not-rodent 27.

In the biochemical evaluation, the obtained results showed a significant reduction in the glycerina until the end of the experiment. The same result was obtained in the study of El Hilaly et al.24. For the other biochemical parameters, there were significant differences, of triglyceride and cholesterol in the treated groups compared to the control group. These results confirm the previous studies carried out by El Hilaly et al.8 and Boudrela et al.17, respectively on the role of *A. iva* in the reduction of cholesterol and triglycerides. But in the study on *A. iva* aqueous extract, El Hilaly et al.24 found normal rates of triglycerides and cholesterol.

The other evaluated parameters (AST and ALT) are considered markers of the function of liver28,24. The analysis of these parameters is important to evaluate the toxicity of the liver related to the use of the phytotherapeutic products29,30. In the present study, some changes of these parameters have been observed, but they did not correlate with the administered doses. This must be confirmed by the histopathological examinations 28,24,31. The toxicity of kidney was also reported after the use of the phytotherapeutic products 32,33, what makes essential its evaluation. In this case, the determination of creatinine and urea were used as markers of the kidney function. In the present study, no significant difference in these parameters was detected.

The studies carried out suggest that in the dose of 100 and 300 mg/kg (10 and 30 folds the indicated therapeutic dose), the product seems to be sure. However, the dose 600 mg/kg bwt, is much higher than that being usually used at human. Other studies in the non-rodents must be carried out to prove its safety. Moreover, the clinical use of *Ajuga iva* must be followed by a periodic hematologic tests.

REFERENCES

Toxicity study of the methanolic extract of *Ajuga iva*