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THEME

BIOCHEMICAL AND HISTOLOGICAL INDICATORS OF *ATRACTYLIS GUMMIFERA* L. TOXICITY

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SUMMARY

Herbal drugs are widely used and often contain high active pharmacological compounds. Hepatotoxicity of herbal remedies ranges from mild liver enzyme alterations to chronic liver disease and liver failure. Atractylis gummefera is one of the toxic plants in Algeria, it's toxicity is due to its active substances mainly atractyloside and carboxyatractyloside. Hepatotoxic reactions have been observed often by ingestion of Atractylis gummefera.The intoxications, usually lead to collective death in rural places. Especially the children between 4-13 years. In this study animals were treated with different doses of rhizome extract according to their body weight. At one experiment, acute toxicity, one dose was used on rats which corresponds to 1/5 LD50 Atractylis gummifera and sacrified after the 1st day, 3rd 5th, 7th, 10th, and 14th. Biochemical, haematological and histological studies were carried on. The results showed an important alteration in liver tissue; necrosis in the hepatic parenchyma. Fluctuations in plasma glucose levels were observed in all treated groups. These generally followed by hypoglycaemia. Enzymes activities of plasma GOT and GPT were increased. These results indicate that Atractylis gummifera rhizome extract causes structural and functional changes of liver. The study of the toxicity of Atractylis gummifera rhizome extract on rabbits (Oryctolagus cunilicus & New Zealand White rabbits), treated with 1/5LD50 and 1/20 LD50 compared with K atractylate (6.5, 13, 32 umole/kg), The histopathological results showed lesions and morphological changes of the hepato-cellular and confirmed disturbances of the biochemical parameters , these changes were much underlines during the animal toxicity. The acute toxicity of this plant extract on rat male reproductive system showed a clear modification of sperm morphology, especially the flagella. Testicular and epididymal morphology was also impaired. It is concluded that Atractylis gummifera may cause morphological and functional alteration of the male reproductive tract. Another study, was focused on oxygen consumption and glucose absorption by rat intestine loops to get some knowledge about the effect of AG extract. Warburg technique was used and oxygen consumption was reduced by 30% . The inhibitory effect of rhizome extract on oxygen consumption, was clear in the presence of glucose, and reached approximately 56%.

Key words: *Atractylis gummefera,* Biochemical parameters, Toxicity, Histological alterations

Résumé

Les drogues végétales sont largement utilisées suite à leur contenu élevé en composés pharmacologiques actifs. La toxicité hépatique des médicaments d'origine végétale varie des altérations modérées de l'enzyme du foie à des maladies chroniques voire l'arrêt fonctionnel de cet organe. *Atractylis gummifera* est une des plantes toxiques algériennes, elle tire sa toxicité de ses substances actives notamment l'actractyloside et les carboxyatractyloside. Les réactions de toxicité hépatique ont été souvent observées suite à l'ingestion de l'*Atractylis gummifera*. Ces intoxications conduisent les plus souvent à la mort collective, en milieu rural, des enfants de 4 à 13 ans. Dans la présente étude les animaux ont été traités avec différentes doses l'extrait rhizomateux, en fonction du poids corporel. Dans une expérience, la dose utilisée sur des rats correspond au 1/5 de la DL_{50} d'*Atractylis gummifera* et ces sujets ont été sacrifiés après 1[ier] , le 3[ieme] , 5 [ieme], 7 [ieme], 10 [ieme] et 14 [ieme] jours. Des examens biochimiques, hématologiques et histologiques ont été opérés. Les résultats indiquent une altération importante des tissus du foie et des nécroses du parenchyme hépatique. Des fluctuations du niveau du plasma glucosique (vs glucose plasmique) ont été notées chez tous les groupes traités, et sont généralement suivies par une hypoglycémie. Les activités enzymatiques du plasma GOT et GPT augmentent. Ces résultats indiquent que l'extrait rhizomateux d'*Atractylis gummefera* induit des changements structurels et fonctionnels du foie.

L'étude de la toxicité de l'extrait rhizomateux d'*Atractylis gummifera* sur les lapins *(Oryctogalus cunilicus* et les lapins blancs de la Nouvelle Zélande), traités avec 1/5 DL_{50} et 1/20 DL_{50}, est comparée au K atractylate (6.5, 13 et 32 µmole/kg) Les résultats histo- pathologiques montrent des lésions et des changements morphologiques des cellules hépatiques et confirment les changements des paramètres biochimiques. Ces changements ont été mis en relief au cours de l'intoxication de l'animal. La toxicité de l'extrait de cette plante, sur le système reproducteur des rats mâles, montre une nette modification de la morphologie du sperme, notamment le flagelle. La morphologie des testicules et de l'épididyme a été aussi altérée. . Il a été conclu que l'*Atractylis gummifera* cause des altérations morphologiques et fonctionnelles de l'appareil reproducteur du rat mâle. L'autre expérience s'est focalisée sur la consommation de l'oxygène et l'absorption du glucose par l'intestin des rats pour avoir des informations sur l'effet de l'extrait AG. La technique Warburg a été utilisée et la consommation de l'oxygène a été réduite de 30%. L'effet inhibiteur de l'extrait rhizomateux sur la consommation de l'oxygène est net, en présence du glucose et atteint approximativement 56%.

Mots clés: *Atractylis gummifera,* Paramètres biochimiques, Toxicité, Altérations histologiques

ملخص

العقاقير العشبية واسعة الاستعمال و غالبا ما تحتوي على مركبات صيدلانية ذات فعالية عالية. سمية الكبد بواسطة التداوي بالأعشاب تعمل على إحداث خلل في إنزيمات الكبد مؤدية إلى مرض حاد بالكبد و فشل كبدي ، نبات *Atractylis gummefera* و المعروف في الجزائر بالأداد هو أحد النباتات السامة و ترجع سميته إلى احتوائه على مواد فعالة خاصة (atractyloside (ATR و carboxyatractyloside (CATR). كما أن حوادث التسمم بنبات الأداد غالبا ما تتكرر و تكون مميتة خاصة في الأوساط الريفية وعند الأطفال الذين تتراوح أعمارهم ما بين 4-13 سنة، ويرجع السبب إلى استعمال النبات كمصدر علاجي لبعض الأمراض ومذاق جذوره الحلو. عوملت الحيوانات (فئران وأرانب) بمستخلص نبات الأداد و هذا اعتمادا على الجرعة و على حسب وزن الحيوان. تم خلال أحد التجارب استعمال الجرعة الحادة ، أين عوملت الفئران بجرعة واحدة مقدرة ب 5/1 LD_{50} من مستخلص نبات الأداد على حسب وزن الحيوان. قتلت الحيوانات بعد يوم واحد و3 و5 و7 و10 و14 يوما من المعاملة. تمت الدراسة البيوكيميائية والدموية والنسيجية. أظهرت النتائج تغيرات هامة على مستوى النسيج الكبدي ونكرزة ببرانشيمية الكبد. وكذلك تذبذب في مستويات الغلوكوز عند كل الحيوانات المعاملة مقارنة بالشواهد. وقد كانت متبوعة بهبوط في الجلوكوز Hypoglycemie. وكذلك ارتفاع في نشاطية انزيمات TGOو TGP . تبين النتائج بأن المستخلص جذر نبات الأداد يسبب تغيرات تركيبية ونسيجية بالكبد. في دراسة لسمية جذر نبات الأداد على الأرانب من نوع Oryctolagus cunilicus و New Zealand White المعاملة بجرعةLD_{50}5/1 و20LD_{50}/1 مقارنة بمادة Atractylate البوتاسيوم وبمعدل (6.5 و13 و32 ميكرومول/كلغ). اظهرت النتائج النسيجية حدوث تمزقات وتغيرات مورفولوجية للخلايا الكبدية حيث تثبت المؤشرات البيوكميائية حدوث اختلالات بالكبد. عموما تلاحظ هذه التغيرات خلال تسمم الحيوان.أظهرت دراسة السمية الحادة بمستخلص جذر نبات الاداد على الجهاز التناسلي لذكور الفئران تغيرات واضحة بعد المعاملة وتشوهات في الشكل الخارجي للحيامن وعلى مستوى السوط .وكذلك كان تأثير مستخلص جذر نبات الاداد ، و بشكل واضح على الخصية وطلائية البربخ. ونستخلص من هذه الدراسة بأن نبات الأداد يسبب تغيرات مورفولوجية (شكلية) ووظيفية على الجهاز التكاثري الذكري. في دراسة مغايرة، تم تقدير استهلاك الأوكسجين اعتمادا على تقنية WARBURG وامتصاص الغلوكوز *من طرف حلقات معي الفأر لمعرفة تأثير مستخلص نبات* *Atractylis gummifera* L. *أظهرت النتائج انخفاض نسبة الأوكسجين المستهلكة بحوالي 30% كما أن التأثير التثبيطي لمستخلص جذر النبات على استهلاك الأوكسجين كان واضحا في وجود الغلوكوز حيث بلغت نسبته تقريبا 56%* .

الكلمات الدالة: ***Atractylis gummifera*** *،* ***المؤشرات البيوكيميائية، السمية ، التغيرات النسيجية***

ABREVIATIONS

ADP :	Adenosine Di Phosphate .
AG:	Atractylis Gummifera
ATP:	Adenosine Tri phosphate
ATR:	Atractyloside
GPT :	Glutamate pyruvate Transaminase .
GOT :	Glutamate Oxaloacetate Transaminase .
GSH:	Reduced Glutathione
PAH:	Phosphatase Alkaline .
CSA :	Cyclosporin .
LD 50:	Lethal dose.
ANT :	Adenine nucleotide Translocase.
GSSG:	Oxidized Glutathione .
DTT:	Dithiothreitol Transaminase .
VRP:	Verapamil
NAC:	N-Acetylcysteine
SDH:	Sorbitol dehydogenase
STEV :	Steviosid
GLDH :	Glutamate Lactate Dehydrogenase .
MDH:	Malate Dehydrogenase
8-GT :	Gamma Glutamyle Transferase.
RBC :	Red Blood Cells
WBC :	White Blood Cells
HB :	Hemoglobine
Qo2 :	Oxygene Consumption.

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GENERAL INTRODUCTION

In some regions of the world, plant poisoning continues to be an infrequent though important problem causing clinical morbidity and mortality. Among plants occasionally involved in human poisonings is *Atractylis gummifera* L., especially in countries where the plant grows spontaneously (Hamouda *et al*., 2000). Because *Atractylis gummifera* L. is easily confused with a wild artichoke most poisonings are unintentional. Many of the victims are children because *Atractylis gummifera* has sweet tasting juice and children enjoy chewing the root-like chewing gum (Stickel *et al*., 2000).

Atractylis gummifera L., is a toxic plant widely distributed through the worldwide, but especially in North Africa and the Mediterranean countries. This plant has been used in folk medicine or taken mistake (Caravaca *et al*., 1985). Atractyloside (ATR) is one of a group of diterpenoide glycosides. The primary mechanism of atractyloside poisoning is known to be inhibition of the mitochondrial ADP transporter and thus blocks oxidative phosphorylation, which prevents the synthesis of ATP and leads failure of gluconeogenesis and ultimately cell death (Obatomi *and Bach*., 1998).

The consumption of this plant, containing ATR causes acute fatal renal and hepatic necrosis in animals and humans (Obatomi *and Bach*, 1998).Reversible hypoglycaemia accompanied by depletion of hepatic glycogen was observed during *in vivo* investigations on the carbohydrate metabolism of rats and rabbits treated with the glycosidic fraction of the extract obtained from unroasted coffee-beans of coffee robusta which contains three glycosides closely related to ATR, which is a powerful competitive inhibitor of the ADP transport (Casio *et al*., 1994).

Clinical manifestations are related to the induced hypoglycemia and neurovegetative disorders or subsequent renal failure (Larrey, 1994). Epigastric pain, vomiting, respiratory depression and general anxiety have been reported. Patients poisoned demonstrated also an elevated serum alanine and aspartate aminotransferases (ALT and AST), indicating liver damage, which was associated with a 50% reduction of the prothrombin index, sharply demarcated and severe centrilobular hepatocellular necrosis (Stuart *et al.*, 1981;Bhoola, 1983; Caravaca *et a*l., 1985; Georgiou *et al.*, 1988 and Hedili *et al.*, 1989).

It has been previously demonstrated that ATR causes inhibition of energy processes in both liver and kidney tissues and exhibits cell-specific cytotoxicity *in vitro* (Obatomi *et al.*, 1998a). It has been shown that tissue slices offer concrete scientific advantages for studying organ specific activity (Obatomi *et al.*, 1998a, b).

Despite significant human and animal exposure and many reported deaths (Bhoola, 1983; Hutchings and Terblanche, 1989) there is no established mechanism of injury and thus there has been no rational approach to limit or prevent ATR toxicity. It is also not clear whether ATR metabolism is the same in man and in the laboratory animals although both species have been reported to exhibit common target toxicity *in vivo* and pig has been shown to be specifically sensitive to this compound (Georgiou *et al.*, 1988; Stewart *et al.*, 2000).

Although its toxicity, the antidote is not known so far. There are many studies associated with the toxicology and the effects of this plant, and its active substances (Lemaigre *et al.*, 1975; Daniel *et al.*, 1993). However the decrease of the toxicity or the anti-toxic response at the cellular level or body level has yet to be identified, but a better understanding of the mechanisms of toxicity may lead to the application of a number of compounds (natural or chemical compounds) that are effective *in vitro*

(Stewart and Steenkamp, 2000). These important developments emphasize the need to bring the current knowledge on ATR to the attention of the food, clinical and research toxicologists. More importantly, many aspects of ATR toxicity have not been investigated. There are only limited or no data available on pharmacokinetics, subchronic and chronic toxicity, reproductive toxicity, carcinogenicity and metabolism.

Although, the studies of the effects of some protective agents on renal and hepatic toxicity, in general, indicated that these protective agents exert cytoprotective effects in atractyloside-induced biochemical perturbation, which acted differently in liver and kidney (Obatomi *et al.*, 2001).

The depletion of the tissue level of GSH was found to contribute significantly to the ultimate cytotoxicity of ATR. In the kidney tissue, PAH accumulation was also markedly reduced, while lipid peroxidation was more pronounced in the liver.

Flavonoides including flavones, isoflavones, and flavonones acted as antioxidant against peroxyl and hydroxyl radicals and several proxidants in the presence of cu^{+2} (Guohua *et al.*, 1996).

It has been shown that cyclosporin A (CsA) and glutathione (GSH) can reduce ATR toxicity *in vivo* (Haouzi *et al.*, 2002).

CHAPTER I: LITERATURE REVIEW

1. Literature review

1.1. Botany and ethnopharmacology of *Atractylis gummifera* L.

Atractylis gummifera is a thistle distributed worldwide and it is especially abundant in the Mediterranean regions: North Africa (Algeria, Morocco and Tunisia) and South Europe (Italy, Greece, Spain and Portugal). The plant has a long rhizome that can reach 30–40 cm length with a 7–8 cm diameter. The leaves are deeply divided into prickly lobes and grouped into rosettes. The flowers are pink and grouped into capitulum surrounded by bracts covered with spikes (Fig. 1). Yellowish white latex exudes from the base of the bracts, once the fruit is ripe (Bruneton, 1999). The rhizome contains the plant's lactiferous system. The parenchymal cells of the rhizome abound in crystalline masses and the cortical parenchyma has numerous secreting lacunae.

Linnaeus named the species and classified it among the Asteraceae, in the *Atractylis* genus and as *gummifera* species. He also proposed that *Atractylis gummifera* could be the white chamaleon described by Theophrastus (1949). The species is also known as *Acarna gummifera* W. and *Carlina gummifera* Less. Two varieties of *Carlina gummifera* were proposed: α-typical and β-Fontanesii (Fiori and Paoletti, 1973), which differed in their plant morphology and geographical distribution: α-typical grows in the south of Italy and Sicily whereas β-Fontanesii is found especially in Sardinia and Corsica. *Atractylis gummifera* was known as 'masticogna' (Sicilian), 'musciurida' (Sardinian), 'chardon `a glu' (French) and 'el-addad' (Arabian). Other common names are 'birdlime', 'blue thistle' and 'chamaelon', because its flowers continually change colour. The court-physician and botanist-pharmacologist of the khalif el-Hakim of Cahira, Mesuè the Younger (1000 a.d.), called it 'mezereon';

the same author, in his ‘De Simplicibus’ commented that the Persians named this deadly plant ‘rapiens vitam’ (Santi and Luciani, 1978).

Fig. 1: *Atractilis gummifera L.* (Daniele *et al*., 2005)

The therapeutic as well as the toxic properties of *Atractylis gummifera* have long been recognized. In the first century a.d., the Greek pharmacologist and physician Pedanius Dioscorides of Anazarbos gave a thorough description of the plant and its properties. Theophrastus (300 BC) also noted the toxicological effects of the plant in animals; he described two kinds of ‘chamalaeon’, white and dark, differing in the colour of their root and properties. The root of the ‘chamalaeon’ type is white, thick and sweet and has a strong smell. Theophrastus wrote also that the white root had been used against worms, and as a poison for dogs and pigs when mixed with oil and wild cabbage. The dark ‘chamalaeon’ type resembles white chamelaeon, but has smaller and smoother leaves; the main root is thick and black and was known for its properties against leprosy (Theophrastus, 1949). Galen recommended the plant only for external applications (Bruneton, 1999). In the renaissance the plant was also well known, especially under the name of chameleone. Matthioli described, in his book, the 'chameleone bianco’ as a sweet, aromatic plant with a strong odour. He suggested using its root for various purposes, as a vermifuge boiled in wine, vinegar and wild marjoram; as a drink, mixed with wine against snake poison and mixed with ‘polenta’, sugar, water and oil as poison against dogs, pigs and mice (Matthioli, 1957).

In another renaissance book (Durante, 1585), the author accurately described both ‘chameleone bianco’ and ‘chameleone nero’. He recommended a root decoction of ‘chameleone bianco’ against urinary retention, and somnolence; and the root of ‘chameleone nero’ to decrease toothache, to refresh the breath and to remove skin stains such as freckles. In folk medicine, *Atractylis gummifera* has been used to treat several conditions including intestinal parasites, ulcers, snake-bite poisoning, hydropsy and drowsiness.

In traditional Arabic medicine it was used to cauterize abscesses. The plant was also known for its antipyretic, diuretic, purgative and emetic properties (Larrey and Pageaux, 1995). In the popular medicine of Northern African, it is still used to treat syphilitic ulcers, induce abortion and bleach the teeth (Capdevielle and Darraq, 1980; Georgiou *et al*., 1988). It is also used against parasites in folk veterinary medicine (Viegi *et al*., 2003). The dry rhizome is also usually burned in Arabic countries as incense to ward off bad fate (Hamouda *et al*., 2004).

1.2. Phytochemistry

All the underground parts of this plant contain two toxic diterpenoid glucosides: atractyloside (ATR) and carboxyatractyloside (CATR) (Fig. 2 and Fig. 3). ATR was isolated from the roots of *Atractylis gummifera* for the first time by Lefranc (1868). The corresponding aglycone (atractyligenine) is a non-volatile diterpene of the (–) kaurene family, with a perhydrophenanthrenic structure. The carbohydrate portion consists of a single D(+) glucose molecule, with only one free hydroxyl group (C-6); it is linked in C-2' to a residue of isovaleric acid, in C-3' and C-4' to two residues of sulphuric acid and in C-1', through a β-linkage, to the C-hydroxyl of atractyligenin (Piozzi, 1978). CATR was isolated for the first time in 1964 and called gummiferin (Stanisls and Vignais, 1964) and subsequently identified as 4-carboxyatractyloside (Danieli *et al*., 1971). CATR differs from ATR owing to the presence of a second carboxylic group in position C-4' of the diterpene ring. CATR is present in fresh but

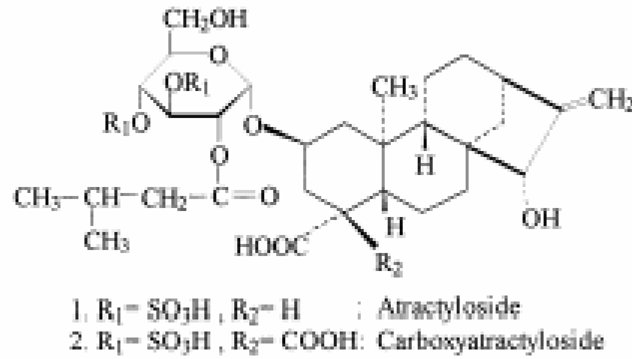


Fig. 2: Chemical structure of toxic compounds from *Atractylis gummifera* (Danieli *et al.*, 1971)

R1 = SO_3H; R2 = H; Atractyloside

R1 = SO_3H; R2 = COOH ;Carboxyatractyloside

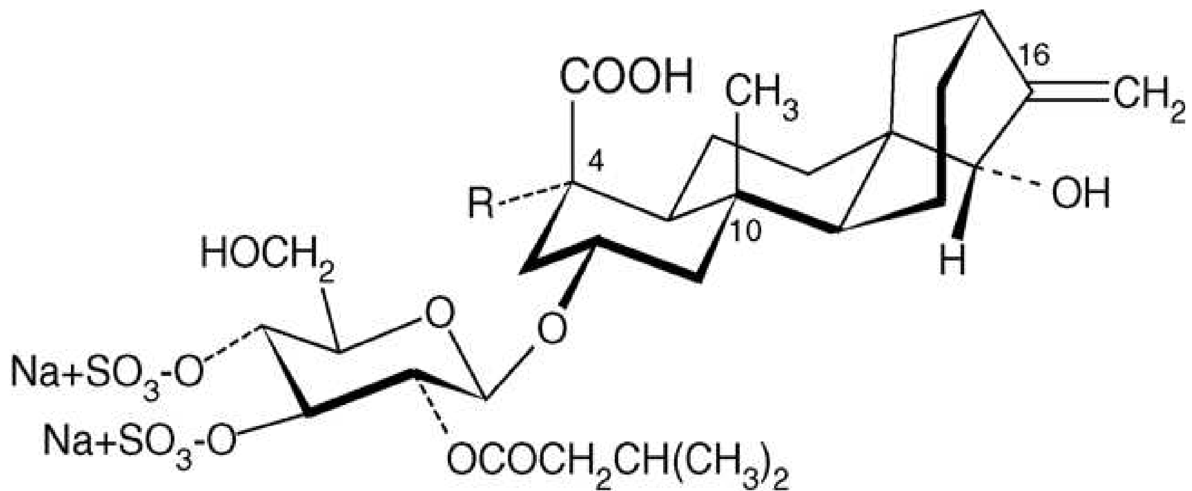


Fig. 3: Toxic compounds from *Atractylis gummifera* (Danieli *et al.*, 1971)

not in dried plants because it is decarboxylated to ATR during ageing or desiccation. CATR is also more toxic than ATR (Luciani and Carpenedo, 1978). Several factors, including the climate, the composition of the soil, the time of harvest and genetic factors, influence the content of diterpenoid glucosides in the rhizome of *Atractylis gummifera*. For example, differences reportedly exist between the rhizome content of ATR from *Atractylis gummifera* grown in Sardinia or in Sicily (Table. 1) (Fassina *et al.*, 1962; Contessa and Fassina, 1978). A higher amount of ATR was found in rhizomes collected in autumn or winter, than in spring or summer (Table. 1), in agreement with the higher content of reserve substances and active compounds in the underground part of a plant during the time of quiescence. And last, a quantitative study showed that *Atractylis gummifera* of Sicilian origin had a higher ATR content than that of Sardinian origin, even though the two plants had been acclimatized in the same territory different from the original habitat (Toth, 1964).

ATR and CATR have also been also isolated from other plants from different genera including *Callilepsis laureola*, *Xanthium strumarium*, *Iphiona aucheri*, and *Wedeila glauca* (Obatomi and Bach, 1998). ATR analogues were also found in aqueous extracts of green and roasted beans of *Coffea arabica*. In particular, three glycosides were identified: 2-O-(2-O-isovaleryl-beta-d-glucopyranosyl)-atractyligenin,2-O-beta-D-glucopyranosyl-atractyligenin and 2-O-(3-O-betad-glucopyranosyl-2-O-isovaleriyl-beta-d- glucopyranosy)-atractyligenin (Fig. 4) (Obermann and Spiteller, 1976; Richer and Spiteller, 1978). Green beans of *Coffea arabica* contain 34.5– 62.4 mg/kg of ATR analogues whereas the roasted beans contain only 17.5–32.5 mg/kg (Obatomi and Bach, 1998).

Table 1: Contents of atractyloside in rhizomes of *Atratylis gummifera* L. from Sardinia , Sicily and Algeria in different time of the years (Fassina *et al.*, 1962 ; Contessa and Fassina, 1978)

	Time of the harvest	ATR content gram% dry weight
Sardinia	June	0.12±0.013
	October	0.19±0.036
	December	0.33±0.045
Sicily	May	1.21±0.038
	December	1.57±0.101
Algeria	May	0.97
	December	1.34

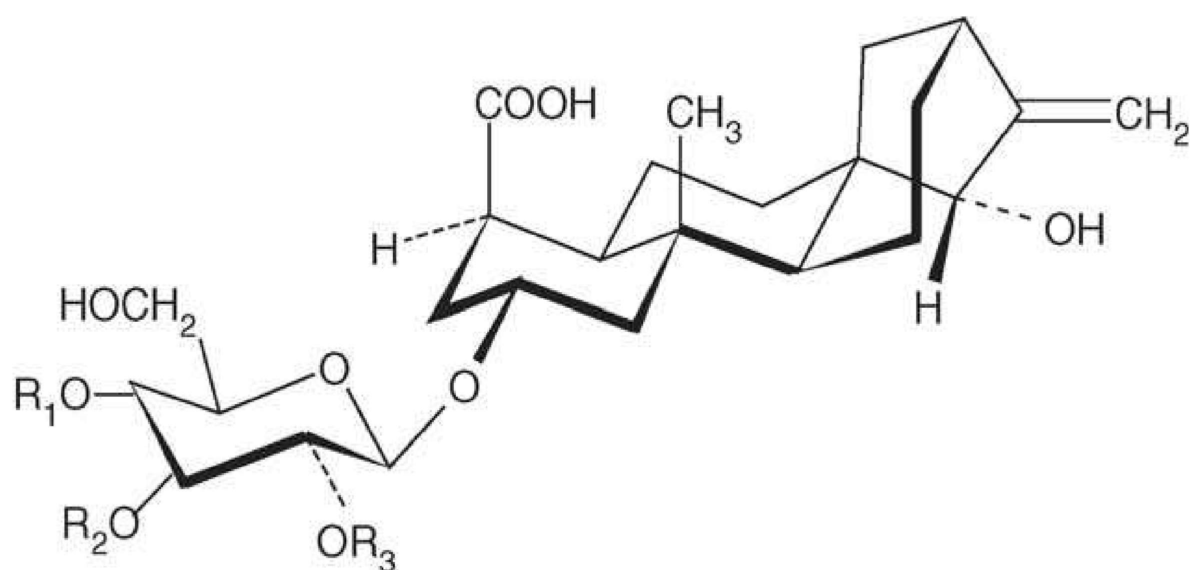


Fig. 4: Chemical structure of atratylosides of *Coffea arabica* (Richer and Spiteller, 1978)

No published information is available on the ATR plant levels required to produce toxicity. Considering the quantity of ATR analogues present in coffee beans and the fact that some of them are non-toxic (Fontana *et al.*, 1994), the risk related to coffee consumption is presumably negligible. Indeed, no published reports have described cases of coffee-induced ATR toxicity even in heavy coffee drinkers. ATR inhibits the oxidative phosphorylation of ADP. Because of its genin, atractyligenin, which is about 150-fold less toxic, has a similar but weaker activity, the glucose moiety, disulphoric and isovaleric acids probably increase the inhibitory effect of the atracyligenin moiety of ATR (Vignais *et al.*, 1978).

Various atractyloside analogues were prepared and tested, to evaluate structure– activity relationships and to better understand the mechanism underlying the action of ATR. One of the most crucial groups for ATR toxicity is the carboxyl group in C-4' of the diterpene ring: its reduction to alcohol produces a non-toxic compound, atractylitriol. ATR toxicity depends also on the C-16 methylene group: its reduction leaves a compound two- or three-fold less potent than ATR. Acetylation of the two free hydroxyl groups of ATR, C-15 of atractyligenin and the C-6 free hydroxyl group on the glucose molecule, decreases by 100-fold the inhibitory potency of ATR. The derivatives obtained by the removal of isovaleric acid or the sulphate groups are also less effective than ATR (Vignais *et al.*, 1978). For example, Fontana *et al.* (1994) studied the effects of two atractylosides of *Coffea arabica* 2-O-(2-O-isovaleryl-beta-d-glucopyranosyl)-atractyligenin and 2-O-beta-d-glucopyranosyl-atractyligenin on carbohydrate metabolism in rats by measuring blood glucose levels, lactate and pyruvate, as well as hepatic, muscular and cardiac glycogen. In this study, the ATR analogues were used at a dose of about 150 mg/kg, corresponding to the ATR DL50 in rats, when administered by the S.C. route (Luciani and Carpenedo, 1978); 2-O-(2-

O-isovaleryl-beta-dglucopyranosyl)-atractyligenin showed the same activity as atractyloside, conversely the other analogue glycoside without the isovaleric group, was nearly inactive.

1.3. Clinical aspects of *Atractylis gummifera* poisoning

About 100 cases of *Atractylis gummifera* poisoning have been described in the literature (Table 2) since the XIXth century (Hamouda *et al*., 2004). Larribaud (1954) reported the intoxication of two children aged 4 and 6 years, after ingestion of *Atractylis gummifera* rhizome in a region of Dellys (Algeria). The children arrived at the hospital with violent abdominal colic and abundant vomiting, followed by generalized contractions, cyanosis, agony and coma. The next morning both children died. The histopathologic findings showed congested intestine, peritoneal exudates and haemorrhages in stomach and kidney. In 1955, a report, describing the accidental poisoning of several Italian school children including three fatal cases, raised the interest of the scientific community in *Atractylis gummifera*(Santi and Cascio, 1955). Catanzano (1969) described the intoxication of two children who drank a decoction of *Atractylis gummifera*. The observed symptoms were vomiting, convulsions, muscular hypertonia and mydriasis; the arterial pressure values were 100/50mmHg in one child and 100/75mmHg in the other and the pulse rate was 100 bpm for both children. The laboratory findings showed increased leucocyte numbers (20,000/mm3) while the histopathologic findings showed hepatic necrosis. Georgiou *et al.,* (1988) described the case of a 7-year-old boy, admitted to hospital 2 days after drinking an extract of *Atractylis gummifera*, taken to treat oxyuriasis. The symptoms were epigastric pain, vomiting and general anxiety. The patient's condition progressively worsened and, despite treatment, the boy died 8 days after admission.

In Spain, five people were poisoned after taking an infusion of *Atractylis gummifera*, owing to a misidentification.

The patients suffered extensive liver damage and kidney failure. One of them died after gastrointestinal haemorrhage but the other four survived after dialysis (Caravaca-Magarinos *et al.*, 1985). A report by Hamouda *et al.* (2000) stated that from 1983 to 1998 the Tunisian Poisoning Center collected 56 medical records of patients admitted to the toxicological intensive care unit for poisoning with 11 species of plants. The principal plants involved were *Atractylis gummifera* (18 cases; 32%) *Datura stramonium* L. (14 cases; 25%) and *Ricinus communis* L. (5 cases; 9%). Of these 56 cases 16 were lethal and all of them involved *Atractylis gummifera*.

These case reports provide useful information on the symptoms and laboratory findings that help to identify victims of *Atractylis gummifera* poisoning. The symptoms begin 6–36 h after the ingestion of the extract of *Atractylis gummifera* rhizome (Capdevielle and Darraq, 1980). The typical symptoms are gastrointestinal problems including nausea, vomiting, epigastric and abdominal pain, and diarrhoea (Capdevielle and Darraq, 1980; Georgiou *et al.*, 1988). Some reports also describe general anxiety, headache, drowsiness, arrhythmia and convulsions (Hamouda *et al.*, 2000). In several cases, these symptoms are followed by coma (Capdevielle and Darraq, 1980).

The laboratory findings (marked increased in SGOT, SGPT and bilirubin) may indicate severe hepatocellular damage and acute renal failure (Georgiou *et al.*, 1988; Nogue *et al.*, 1992). Post-mortem histopathologic examination discloses massive gastrointestinal haemorrhage, diffuse necrosis of the hepatic parenchyma with collapse of the interstitial connective tissue and accumulation of macrophages (Caravaca *et al.*, 1985).

Table. 2: Case reports of *Atratylis gummifera* L. poisoning

Author		Case reports
Madani	2006	*Atractylis gummifera* poisoning in a pregnant woman
Skalli	2002	*Atractylis gummifera* L. poisoning: a case report
Nogue	1992	Acute kidney failure caused by *Atractylis gummifera* L. poisoning
Georgiou	1988	Hepatotoxicity due to *Atractylis gummifera* L.
Caravaca	1985	Renal and hepatic injuries in human intoxication with *Atractylis gummifera* L.
Capdevielle	1980	Poisoning by bird-line thistle (*Atractylis gummifera* L.)
Lemaigre	1975	Fulminating hepatitis caused by glue thistle (*Atractylis gummifera* L.), poisoning. Anatomo-pathological study of 4 cases
Catanzano	1969	2 cases of poisoning due to "gum thistle" (*Atractylis gummifera* L.). Clinical development and anatomo-pathologic lesions
Thiodet	1961	Poisoning by the glue thistle (*Atractylis gummifera* L.). Clinical study apropos of 11 cases
Thiodet	1960	A fatal poisoning frequent in Algeria and little known: poisoning by a gummiferous thistle (*Atractylis gummifera* L.)
Santi	1955	Ricerche farmacologiche sul principio attivo dell' *Atractylis gummifera* L.
Larribaud	1954	Two cases of poisoning by gummy thistle, *Atractylis gummifera* L.

1.4. Toxicology and pharmacology

The toxic effect of *Atractylis gummifera* arises from ATR, a powerful inhibitor of oxidative phosphorylation in mitochondria. This action is exerted especially in cells rich in mitochondria such as hepatocytes and in proximal tubular epithelial cells, which contain carriers that allow ATR to cross the cell membrane. ATR interacts with the adenine nucleotide translocator (ANT) (Roux *et al*., 1996), a mitochondrial protein contained in the inner membrane. ANT has two major functions: it is responsible for the antiport of ATP and ADP, an important system for oxidative phosphorylation and it is part of the permeability transition pore complex, a non-specific pore involved in mitochondrial membrane permeabilization, an important event during apoptosis (Haouzi *et al*., 2002). ATR and ADP both interact in a similar way with ANT because they resemble one another in geometric and charge distribution (Stewart and Steenkamp, 2000): the polar character of the sulphate groups in ATR corresponds to the phosphate group in ADP; the glucose in the glycoside group of ATR corresponds to the ribose of ADP, and the condensed rings of ATR resemble the purine moiety of adenine.

The selective binding of ATR to ANT has two important consequences. First, ATR inhibits ADP transport and inhibits the access of extra mitochondrial ADP to a phosphorylation site located in the mitochondrial compartment, thus blocking oxidative phosphorylation and Krebs cycle oxidative reactions (Quintanilla *et al.,* 1979; Kholodenko *et al*., 1988). Second, ATR induces opening of the mitochondrial permeability transition ATN-containing pores leading to membrane permeabilization and release of soluble intermembrane proteins, including cytochrome C. The translocation of cytochrome C from mitochondria to the cytosol is a crucial step in

Fasinduced apoptosis (Vancompernolle *et al.*, 1998). The toxic effects due to the inhibition of mitochondrial phosphorylation are hepatic necrosis and renal failure in animals and humans.

The acute toxicity of ATR nevertheless differs according to the animal species and route of administration. Toxicity is higher in dogs than in mice and rats, suggesting an even higher toxicity in humans (Luciani and Carpenedo, 1978). Renal toxicity also differs among the various species: for example, rabbits and guinea pigs show no renal necrosis at ATR doses that are nephrotoxic for rats (Carpenedo *et al.*, 1974).

Toxicity differs also in male rats between *albino* and *Wistar* strains: in albino rats ATR is not toxic at doses up to 200 mg/kg, whereas in *Wistar* strains 60 mg/kg can lead to death. Luciani and Carpenedo (1978) described the action of ATR in different species.

The major effects are on glycidic metabolism: ATR depletes glycogen *in vivo* by inhibiting glycogen synthesis, it increases blood lactic acid concentrations and decreases oxygen consumption. Animals poisoned with ATR usually die in hypoglycaemic convulsions.

An interesting feature of ATR poisoning is the long latency between the administration of ATR and the appearance of the first toxic symptoms, also when the compound is given intravenously. After an initial hyperglycaemic phase caused by depletion of skeletal muscle and hepatic glycogen a hypoglycaemic phase ensues that has important consequences on the whole organism, such as respiratory depression, hypoxaemia, acidosis due to increased plasma lactic acid, and finally convulsions. Because ATR toxicity remains after vagotomy and pancreatectomy

(Santi, 1958) it cannot depend either on an interaction with the autonomic nervous system or on glands implicated in glycaemic control.

In a study in rats, Hopps *et al.* (1997) described the effects of the ATR and some of its derivatives on renal function. They determined the urinary excretion of enzymes (beta-Nacetyl-d-glucosaminidase, alanine amino peptidase, gamma glutamyltransferase) and electrolytes (Na^+, K^+ and Cl^-), and the blood and urinary concentrations of creatinine in rats treated with ATR, atractyligenin and two ATR derivatives (one lacking both the sulphate groups and the isovalerate group, the other lacking the sulphate groups alone). These experiments showed that ATR is highly toxic to the kidney, as shown by enzymuria and reduction of creatinine clearance, whereas atractyligenin leaves renal function almost unchanged. ATR toxicity is related to its chemical structure and increases when the hydroxyl groups in C-4' is esterified with isovaleric acid or when the hydroxyl groups in C-3' and C-4' are esterified with sulphuric acid. Other important information on the toxic effects of ATR comes from *in vitro* studies using tissue slices (Obatomi *et al*., 1998; Obatomi and Bach, 1998).

This method allowed comparison of enzyme leakage, mitochondrial viability, changes in ATP levels, lipid peroxidation, oxidized glutathione (GSSG) and reduced glutathione (GSH) levels in kidney and liver tissues from different animals. ATR nephrotoxicity involved only the proximal tubule cells, whereas the glomerular cells appeared unaffected (Carpenedo *et al*., 1974). In the proximal tubule cells, ATR caused a significant concentration-dependent decrease in ATP content and a depletion of cellular GSH (Obatomi and Bach, 1998). The investigators proposed that ATR could act by interfering with cell transport; the presence of a sulphate moiety, suggested that ATR may undergo anion transport and then accumulate in the renal

cells. ATR also inhibited organic anion uptake in a dose- and time-dependent manner (Koechel and Krejci, 1992; Obatomi *et al.*, 1998).

In the liver, the major effects are lipid peroxidation, GSH depletion and GSSG elevation. These changes suggest that ATR may induce its toxic effects through an oxidative process involving its methylene moiety thus producing a free radical. The reactive intermediate produced has not been identified, but reactive oxygen species such as superoxide anion, hydrogen peroxide or hydroxyl radicals may be involved (Obatomi *et al.*, 1998).

1.5. Herbal hepatotoxicity

The liver is the central drug-metabolizing organ and is, therefore, a prime target of drug-related pathologies. Foreign compounds are predominantly bio transformed in the liver by the action of drug-metabolizing enzymes including microsomal cytochrome P450 enzymes, mixed-function mono-oxygenases, glutathione-S-transferases, sulfotransferases and UDP-glucuronosyltrans-ferases. Some of these can be induced through variable mechanisms which may lead to large inter individual variability in susceptibility for drug-related liver damage. Hepatic damage from conventional drugs is widely acknowledged and most physicians are well aware of them. Herbals as a cause of adverse hepatic reactions, however, have only recently been recognized as their use has become more widespread. Certain herbals have been identified as a cause of acute and chronic hepatitis, cholestasis, drug-induced autoimmunity, vascular lesions and even hepatic failure and cirrhosis. Risk factors for herbal toxicity have not been well identified, largely since hepatotoxic incidents have mostly been published as isolated case reports or small series

(Table. 3) However, a certain risk pattern has become evident, such as the observation that most affected individuals were females. This gender difference does not reflect a higher likelihood of women to use these preparations, but their higher susceptibility towards herb-induced liver damage (Flora *et al.,* 1997), as is observed for the majority of adverse hepatic reactions induced by conventional drugs. As with chemically defined drugs, adverse hepatic reactions towards herbals cannot be predicted through diagnostic means, which makes the early recognition of liver damage important. Most individuals who take herbals do not admit their intake, even on repeated questioning, either because they do not consider herbals as 'drugs', or because they fear not to be taken seriously by their doctors for using herbals. Furthermore, doctors who recommend herbals or patients who take them advocate the long-standing use of herbals in traditional medicine as proof of safety, in particular, since most herbals are available without prescriptions and at low costs. Therefore, self-medication is frequent and, sometimes, patients even increase the dosage as liver disease worsens. Another problem is that herbals are usually mixtures of several ingredients or plants harvested during different seasons and extracted through variable procedures, which makes the identification of both the pharmacologically active and toxic compounds difficult. Also, contamination of herbals with microorganisms, fungal toxins such as aflatoxin, with pesticides, heavy metals, and synthetic drugs has been described (Yamamura *et al.*, 1997).

Table 3: Selection of Herbal Preparations With Proven Hepatotoxicity (Larrey and Pageaux, 1995; Kaplowitz, 1997)

Causative Plants	Toxic Agents	Symptoms	Mechanism/Pathology
Crotalaria *Senecio* *Heliotropium* *Symphytum officinale* (Comfrey)	Pyrrolizidine alkaloids	Veno-occlusive disease	Endothelial cell glutathione depletion, central vein necrosis, thrombosis, and fibrosis
Atractylis gummifera	Atractylate, gummiferin	Hepatitis	Inhibition of oxidative phosphorylation, hepatic necrosis
Callilepsis laureola	Atractylate	Hepatitis	Hepatocyte necrosis
Chelidonum majus (greater celandine)	Chelidonine, sanguinarine, berberine, coptisine?	Hepatitis (cholestatic)	Lymphocyte infiltration
Larrea tridentata (chaparral)	Guaiaretic acid derivatives	Hepatitis	Not known
Teucrium chamaedrys (germander)	Furano-diterpenoids	Hepatitis	Hepatocyte glutathione depletion and apoptosis
Chinese herbal mixtures (artemisia, hare's ear, chrysanthemum, plantago seed, gardinia, red peony root, etc.)	Largely undefined	Hepatitis	Not known

1.6. *Atractylis gummifera* and toxic hepatitis

Atractylis gummifera is a known cause of toxic hepatitis in the Mediterranean. It is used as an antipyretic, emetic and diuretic, and a bright fluid secreted from the plant is enjoyed by children as chewing-gum (Chauhan *et al.*, 1991). The onset of hepatitis is usually acute and commences few hours after ingestion and following unspecific symptoms such as nausea, abdominal pain and headache. It is associated with a syndrome of neuro vegetative symptoms, hepato renal failure and pronounced hypo glycemia; the latter being caused by the inhibition of gluconeogenesis. Death due to fulminant hepatic failure is frequent. Consumption of *Atractylis gummifera* is particularly dangerous during spring time when toxins are concentrated in roots or when the plant is confused with wild artichoc (Abatroun. 1986). Toxicity has been ascribed to atractylosides and gummiferin which are inhibitors of the Krebs cycle and other mitochondrial functions and exert selective toxicity to hepatocytes and kidney epithelia *in vitro* via induction of oxidative stress, as mirrored by glutathione depletion and increased lipid peroxidation (Obatomi *et al.*, 2000) .

1.7. Treatment of *Atractylis gummifera* poisoning and studies on possible therapeutic approaches

No specific pharmacological treatment is currently available to treat *Atractylis gummifera* intoxications. All therapeutic approaches, including fluid and electrolyte replacement, cardiovascular and respiratory support, seizure control and conventional therapeutic methods for severe hepatic and renal failure, are symptomatic (Stewart and Steenkamp, 2000). Some recommend that standard therapeutic practice should include induction of vomiting, bowel evacuation, gastric lavage and administration of activated charcoal (Ben Salah *et al.*, 2001).

In an attempt to develop better pharmacological treatments for *Atractylis gummifera* intoxication, various compounds were evaluated. Catanzano *et al.* (1969)

showed the inefficacy of hydrocortisone (500 and 25 mg i.v.) administered to two poisoned children. *In vivo* experiments performed in dogs (Chardon *et al.*, 1964) evaluated the efficacy of an intravenous administration of 100 ml of a solution containing 6–7% glucose, 0.1% dinitrophenol, 5mg of ATP and 11.25 mg of cytochrome C against poisoning induced by intravenous administration of an extract or dry root of *Atractylis gummifera* in saline solution. This treatment corrected the hypoglycaemia, hypotension and anuria and delayed death only when administered immediately after the extract. In an other study, Ishii and Bracht (1986) investigated the protective activity against ATR-induced liver toxicity of stevioside, a sweet glycoside isolated from the plant Stevia rebaudiana (*Asteraceae*) that can interfere with the transport of ATR across the cell membrane. In isolated perfused rat liver, stevioside decreased the effects of ATR on glycolysis, gluconeogenesis and oxygen uptake.

The cytoprotective effects of verapamil (VRP) and dithiothreitol (DTT) against ATR toxicity were investigated using precision-cut renal and liver slices assays (Obatomi *et al.*, 2001a). The beneficial effect of VRP reflects its ability to reduce the ATR-induced increase in cytosolic Ca^{+2}. VRP, a well-known calcium-channel blocker, completely blocked ATR-induced cell death, depletion of ATP, inhibition of gluconeogenesis in both kidney and liver slices, and provided protection against ATR-induced depletion of GSH in liver slices but not in kidney slices. Dithiothreitol (DTT), a sulphydryl-reducing agent and a metal chelator, reduced the reactive methylene double bond of ATR. DTT protected against ATR-induced enzyme linkage, inhibition of gluconeogenesis and depletion of GSH in kidney but exerted no protective effects against GSH and ATP depletion in the liver slices. Both verapamil and dithiothreitol nevertheless had protective effects only if used 30 or 60 min before

ATR exposure, but were ineffective after the exposure to ATR. Hence they cannot be used for therapeutic purposes.

In another study, Obatomi et al., (2001b) compared the role of ADP, calpain inhibitor I (CPI), stevioside and probenecid in protecting against ATR-induced toxicity in rat renal cortical slices. ADP, CPI and stevioside prevented the depletion of ATP and the reduction in gluconeogenesis, whereas probenecid gave no protection at all. Both *in vivo* and in vitro Haouzi *et al*., (2002) showed that ATR-permeability transition is counteracted by cyclosporin A (CsA) and GSH. CsA inhibited necrosis and apoptosis induced by ATR in human hepatic cells and pre-incubation with the GSH precursor N-acetylcysteine (NAC) reduced cytotoxic effect of ATR. *In vivo* ATR lethality was reduced by a regime, which enhances mitochondrial GSH levels; a diet enriched in sulphur amino acids or CsA administration significantly reduced both the lethal effect of ATR and cytoplasmic vacuolisation in hepatocytes and in proximal renal tubular cells.

1.8. Plasma Marker Enzymes

The history of the use of enzymes in diagnostic studies began with the introduction by Wolegemuth (1908) of the determination of amylase in plasma. Since that time further investigations have been carried out. It was reported that carbon tetrachloride can cause a decrease in cholinesterase (Brawer and Root, 1946). In recent decades many investigators have correlated clinical and serum tests which became routinely used and applied diagnostically in both medicine and toxicology. It was Ladenson *et al.,* (1974); and Haymond and knight, (1975) who emphasised the superiority of plasma samples to serum samples for diagnostic purposes.

The classification of plasma enzymes is based on their function and tissue origin rather than upon their enzyme action (Bücher, 1958; Bücher *et al* , 1959) there are two major groups :

1. Plasma specific enzymes which are characterised by their secretion into the plasma, where they will express activity. These enzymes are found to be at higher activity level in plasma than in the tissue of origin. Examples of these are prothrombin, plasminogen, pseudocholinesterase. The level of activity of these enzymes decreases when there is no damage to the tissue from where they originate.

2. Non_specific plasma enzymes which can be divided into those of exocrine secretions and those concerned only with cellular metabolism; these enzymes are not normal functional constituents of blood and are only present because the blood was circulated through tissues of high enzyme activity.

The enzymes of cellular metabolites can be clearly associated with their source in certain pathological condition and include pancreatic α- amylase, prostatic phosphatase and pepsinogen. The cellular enzymes include those of tissue metabolism that are not active in blood because their substrates and co-factors are mainly absent. Enzymes of key metabolic pathways that may occur include glutamate ,lactate,malate and α - glycophosphate-dehydrogenase , 1.6-diphosphofructoaldolase glutamate oxaloacetate and glutamate pyruvate dehydrogenase , some of these are bound within the same two organelles such as mitochondria. In addition enzymes involved in the ~~in~~ cellular metabolism that are mainly associated with specific tissues and organ functions include the urea cycle enzymes such as arginase and ornithine carbamyl transferase, also alcohol and sorbitol dehydrogenase, glucose-6-

phosphatase and 1-phosphofructoaldolase all of those from liver; also glycerokinase from liver and kidney and alkaline phosphatase from bone.

1.9. Mechanism of Enzyme Release from Cells

Plasma enzymes are good indicators of cell injury; they are commonly used in tissue function tests along with other tests designed to elucidate subtle response injury (Burke, 1978). Enzymes can be released from the tissue or the place of origin by different ways such as diffusion, active transport, permeability change or the shedding of structures such as the glycocalyx of plasma membranes (Fishman and Doellgast, 1975).

It has been shown that the appearance of cell necrosis (Schmidt *et al.,* 1963; Schmidt and Schmidt, 1974; Wilkinson, 1978). However cell death may lead to the discharge of cell contents into the extra cellular fluid. The enzyme release may be due to an imbalance between energy production and cellular expenditure of energy. This can be shown in certain cases of histopathology such as hypoxia, when little hepatic necrosis occur with large increases in cell enzyme activities (Schmidt and Schmidt, 1974).

A schematic mechanism of enzyme leakage under pathological condition was made by Hess (1963) (Fig. 5), damage to the cellular respiration leading to pathological disturbance in energy metabolism. The consequent alteration in the water content of the cell, cellular swelling and permeability, sometimes possibly ends in cellular death.

This theory is supported by evidence provided by many investigators. It was found that in the incubated cells, the direct measurement of the intracellular ATP (Wilkinson & Robinson, 1974) showed little leakage occurred until it had fallen to at least

20% of the original level after which efflux was greatly increased. There are three types of enzymes that can be distinguished

Firstly, cytoplasmic enzymes, e.g. lactate dehydrogenase (SDH), secondly, mitochondrial enzymes such as glutamate dehydrogenase (GLDH), and finally from both cell compartments, for instance, glutamate oxaloacetate (GPT) and malate dehydrogenase (MDH). The first analysis of serum enzymes was made by (Schmidt and Schmidt, 1962) who suggested that the ratio of GOT: GPT may be useful in the diagnosis of chronic inflammatory lesions of the liver from necrotic processes.

Many studies have been carried out upon the distribution of the enzymes. It was reported (Schmidt and Schmidt, 1962) that the value of the ratio GOT + GPT/GLDH was useful for the differential diagnosis of obstructive jaundice. In addition patho-physiological conditions such as haemolytic syndromes (Gerlach *et al*., 1961) can be distinguished by enzyme ratios that include LDH: GOT, and (δ-GT): GOT ratios for hepatitis, toxic liver damage and alcoholic cirrhosis (Schmidt and Schmidt, 1976). Because (δ-GT) is located in the epithelium of intra hepatic biliary tracts, it serves as sensitive indicator of biliary obstruction, rising earlier and faster than either leucine or amino peptidase or alkaline phosphates. Ratios of GPT: (δ-GT), GOT: δ-GT and GPT. GLDH are also used as aids in differential diagnosis of the condition. The release of plasma enzymes from tissues was considered by Rees and Sinha (1960) and Zimmerman *et al.,* 1965a) to reflect the sequence of cellular injury since a delayed plasma elevation from mitochondrial enzymes was obtained compared to cytoplasmic enzymes.

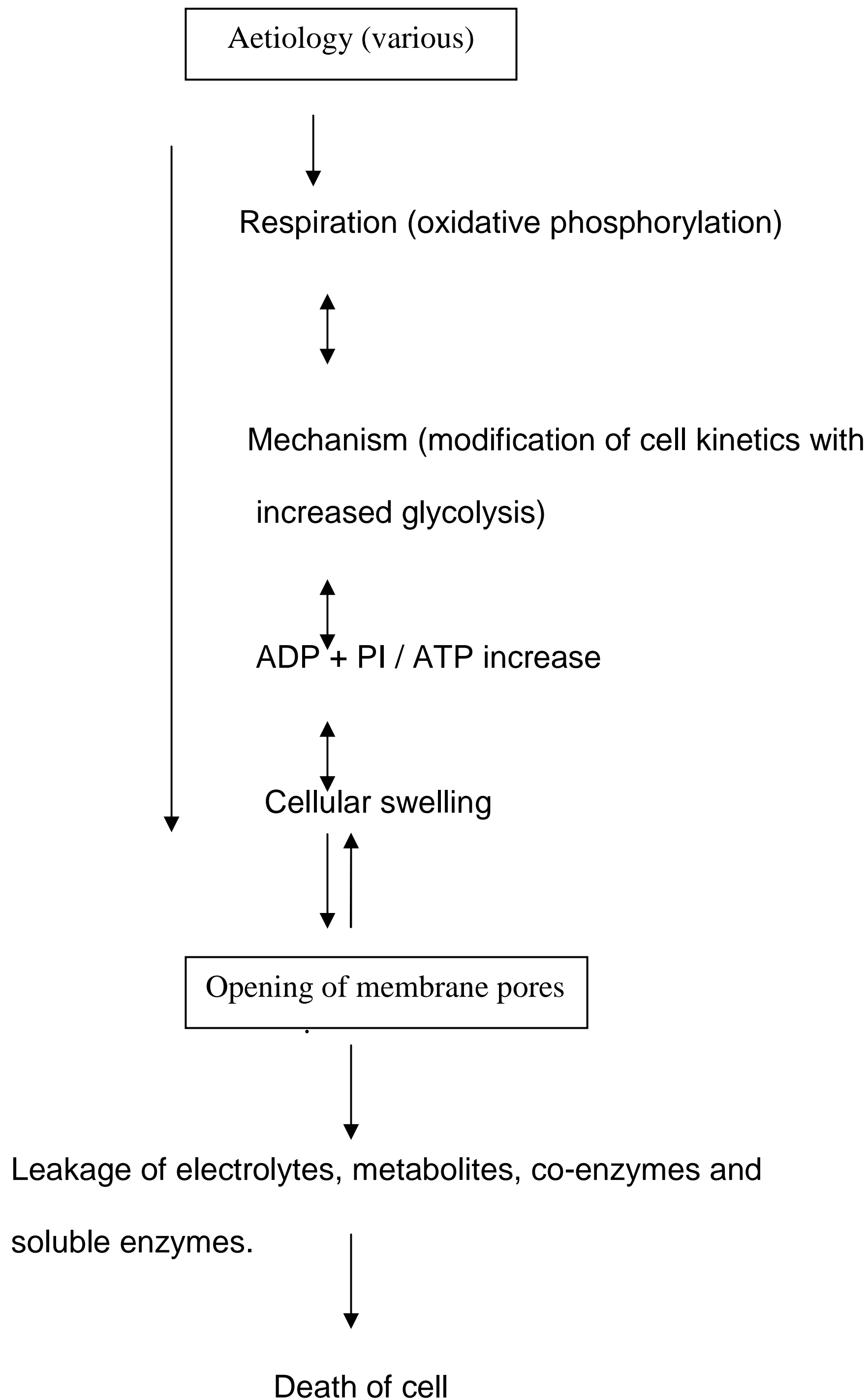


Fig. 5: A schematic mechanism of enzyme leakage under pathological conditions (Hess, 1963).

CHAPTER II: GENERAL MATERIALS AND METHODS

2. General materials and methods

2.1. Plant material

Atractylis gummifera plant samples (rhizome and aerial part) used in these studies were collected from different sites at Setif and Bordj- Bou- Arreridj regions. The plant samples were washed, cleaned and cut into small peaces then left to dry at room temperature in the shadow and then finely grinded to powder in a rotating knife grinder. The powder was sieved through a 1 mm mesh to remove large fragments. Each plant part powder was then used for the extraction procedure.

2.2. Extraction of plant materials

The extraction procedure was conducted according to the method used by Bey (1990). This method was used either to extract toxic compounds from rhizomes or aerial parts (Fig. 6 a and b; Fig. 7)

a

b

Fig. 6: *Atractylis gummifera* L., Rhizome (a) aerial part (b)

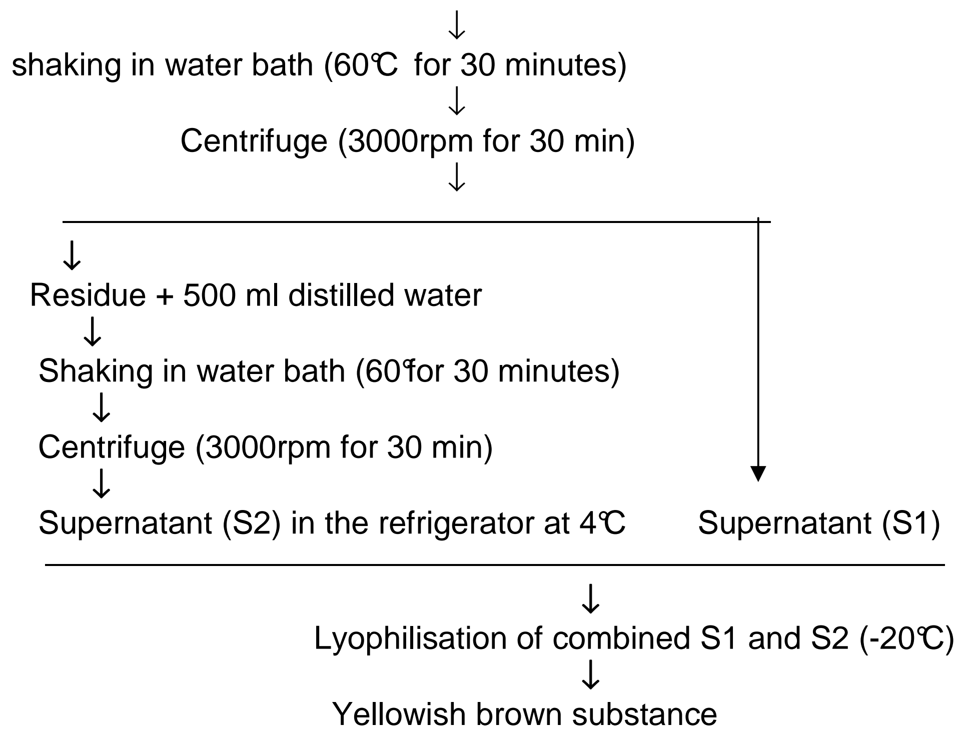


Fig. 7: Steps of the plant material extraction of *Atractylis gummifera* L. (Bey, 1990) after sleight modification.

2.3. Animal Tests

In the experiments performed two adult animal species were used. *Albino Wistar* rats and New Zealand rabbits. Rats were purchased from Pasteur Institute in Algiers and rabbits were provided by a local breeder in Setif region (Société d'exploitation agricole, Mezloug, Setif). All animals were kept in the animal house with a natural lighting schedule for 1 to 3 weeks before experiment. They were fed with a standard pellet food and tap water.

2.4. Toxicological study experiments

The LD_{50} was determined using rats according to the method described by Abatroun (1986). The symptoms of acute toxicity and post mortem finding were recorded.

2.5. Study design and dosage

The animals were divided into two groups: check and treated.

2.6. Assays

Plasma glucose, creatinine, urea , alkaline phosphatase , transaminases "AST and ALT", CPK and total proteins were measured by Bio Merieux kits (Anonymous, 1972). Hb, hematocrit , RBC and WBC were measured according to Dimianova, (1986) , Sultan *et al.*, (1978), Dacie , (1982) respectivelly.

2.7. Histological techniques

2.7.1. Tissue preparation

Following the treatment with the *Atractylis gummifera* extract, animals were stunned and killed. This was done to coincide with the time when the level of certain Glutamate Oxaloacetate Transaminase (GOT) enzymes had increased in the plasma samples. The liver was then removed, washed with saline (0.9% NaCl) and cut into small cubes (approximately 5mm), and fixed in formalin (pH=7.0) or in Bouin's solution, (Pantin.1959), for at least 24 hrs.

- Bouin 's solution

Picric acid (saturated aqueous solution)	75ml
Formalin (40% formaldehyde)	25ml
Glacial acetic acid	05ml

- Neutral 10% formal saline, pH 7.0

Formalin (40% formaldehyde	250ml
Sodium chloride	02ml
Distilled water	250ml

2.7.2. Light microscopy

1. **Fixation and dehydration of specimens**

Specimens which had been fixed in Bouin's solution or formalin washed in 70% alcohol for 24 hours (3-5 changes), dehydrated in series of alcohols 90%,100% and cleared in chloroform (3 changes) according to Mahoney (1973). After this, specimens were embedded in fresh wax and sectioned at thickness of 5-8 µm with microtome.

2. Staining method

Many staining techniques were tried. The useful chosen stain found was Hould method (1984) .

3. Hould's staining method

Staining procedure

Treatment	Time of treatment
1. Xylene 1	02 minutes
2. Xylene 2	02 minutes
3. Absolute alcohol	02 minutes
4. 90% alcohol	01 minutes
5. 70% alcohol	01 minutes
6. Distilled water	01 minutes
7. Haematoxylin	20 minutes
8. Tap water	03 minutes
9. Differentiation acid alcohol (1% hydrochloric acid in 70% alcohol)	10 seconds
10. Wash in tap water	02 minutes
11. 70% alcohol	02 minutes
12. 1% eosin in 70% alcohol	01 minutes
13. 90% alcohol	01 minutes
14. absolute alcohol 1	02 minutes
15. absolute alcohol 2	02 minutes
16. xylene 1	03 minutes

2.8. Chemicals

All drugs were reagent grade, and were purchased from Sigma chemical Corp., St Louis (MO, USA) or from Merck (Darmstadt, Germany) unless otherwise stated.

2.9. Statistical analysis

Data are shown as means ± standard error (SEM). The statistical analyses were performed with the analysis of variance. The results obtained at the end of each time phase were compared with those obtained from zero time for the same group using Student’s t-test for paired observations. At the same time, the results obtained at the end of each time phase were compared with those obtained for the control group at the same time phase. Biochemical data were analysed by Fischer and Dunnet tests.

CHAPTER III: EFFECT OF RHIZOME EXTRACT ON SOME BIOCHEMICAL AND HEMATOLOGICAL PARAMETERS RELATED TO THE LIVER FUNCTION AND STRUCTURE.

3. Effect of rhizome extract on some biochemical and hematological parameters related to the liver function and structure

3.1. Introduction

Atractylis gummifera L. is a plant growing in North Africa and in most of the Mediterranean countries. It is called Added in the Maghreb, and it is used in folk medicine as a remedy for many nutritional disorders aliments or taken by mistake as an infusion of *Atractylis gummifera's* roots (birdlime thistle), thinking it was another common root often used as at home herbal remedy (Caravaca *et a*l., 1985), because of its antihemorragic, emetic and purgative properties.

The toxicity of the "glue thistle" is well known in Algeria. Ten (10) children were involved in a case of collective poisoning (Lemaigre, *et al*., 1975). One of its active products is atractyloside, which is known to be a competitive and specfic inhibitor of the adenine nucleotide translocation, across the inner mitochondrial membrane (Vignais *et al*., 1971).

The aim of the present study is to examine the toxic effect of *Atractylis gummifera* L. rhizome on some biochemical and hematological parameters related to the liver function and structure.

3.2. Materials and methods

3.2.1. Rhizome extract effect on rabbit liver

The experiment was carried out on male rabbits *Oryctolagus cuniculs*, weighing about 1Kg. The plant was collected form Megress (Setif), in April, 1999. The rhizome was extracted (Bey, 1991). Animals were, divided in 3 groups of five rabbits each-group. They were treated orally with an extract, dissolved in 0.9% NaCl and with a dose of 1/5 LD_{50} (76 mg/kg, b/w), and 1/20 LD_{50} (19 mg/kg, b/w). Control animals were treated with saline only (0.5ml/100g, b/w). Blood was collected, centrifuged at 3000 rpm for 15 minutes. GOT, GPT, Alkaline phosphatase, CPK enzyme activities were measured in the plasma, and also the total proteins, Hb, Ht and glucose. Morphological study of blood cells was carried out by the method of

May-Grunwald-Giemsa (Sultan *et al.*, 1978). After that the histopathological studies were followed. The organs were macroscopically observed, removed, and rinsed in 0.9% NaCl, sections of 5 μm thickness were then fixed in the formol, followed by alcohol and eosin (Hould, 1984). Slides were coated with a cover slip. The results were statistically analyzed using Statistica software.

3.2.2. Rhizome extract effect on rat liver

The experiment was carried out on female rats "albino wister" weighting about 150-170g. The plant was collected from "AIN EL KEBIRA" (SETIF) in February, 1996. The rhizome was extracted. Animals were divided into 7 groups of 5 individuals each. They were treated orally with an extract of 76 mg/kg (b/w) in one dose, dissolved in 0.9% NaCl and then killed by cervical dislocation after 1, 3, 5, 7, 10 and 14 days from the treatment. Control animals were treated with saline solution only (0.5 ml/100g) Blood was collected, centrifuged at 3000 r/pm for 15 minutes. GOT and GPT enzyme activities were measured in the plasma and glucose as well. Histopathological studies were followed. The organs were macroscopically observed, removed and rinsed in 0.9 % NaCl , then fixed in the formol, sectioned (5 μm thickness), followed by alcohol and embedded in the paraffin, then coloured by Hematoxylin and eosin (Hould, 1984). Slides were coated with a cover slip. The results were statistically treated by using Statistica (v) software.

3.3. Results and Discussion

Studies on aerial parts showed slight effect on blood parameters in rats and rabbits compared to rhizome extract. Therafter and for this reason only rhizome extract was used in the whole study.

Biochemical studies of plasma of rabbits treated with the rhizome extract of *Atractylis gummifera* L. (76 and 19 mg/kg b/w), showed a clear alteration especially glucose metabolism (Capdevielle and Darracq, 1980). It has been noticed a slight decrease of plasma glucose after the treatment with the dose of 1/5 LD_{50}, and a significant increase ($p \leq 0.007$) with the dose of 1/20 LD_{50} hyperglycemia (Fig. 8).

The hyperglycemia that happened is probably due to the ATR and CATR glycolsides which have rapidly deplete hepatic glycogen, resulting in an initial hyperglycemia phase and a subsequent severe hypoglycemia phase. As it has been shown also weakness, ataxia, convulsion, saliva, respiratory depression, coma and death occurred in the two groups, after the treatment. The most probable mechanism by which these glycosides induce severe toxicity is by binding to the ADP/ATP translocase of these by nucleotides, inhibition of these enzymes provokes a partial blockage of aerobic metabolism (blockage of Krebs's cycle and respiratory chain). Translocated by decrease in the consumption of glucose by cells, which explains probably by hyperglycemic action (Daniel *et al.,*1993).

Biochemical studies of plasma parameters of animals treated with the rhizome extract of *Atractylis gummifera* showed a significant increase of plasma enzyme activity in GOT in both doses ($p \leq 0.007$; $p \leq 0.004$ respectively) (Fig. 9), also a slight increase in GPT (Fig 10) and CPK (Fig. 11) were noticed. At the same time a significant decrease in alkaline phosphatase was observed after the dose of 1/5 and 1/20 LD_{50} respectively (p=0.02; 0.004 respectively) (Fig. 12).The total proteins were increased (Fig. 13).

The toxic effects of *Atractylis gummifera* could be connected to the function of new toxic metabolites. The release of blood enzymes was as a result of tissues damage.

Hematological results showed a decrease in RBC after the treatment with the dose of 1/5 LD_{50}, (87.8%) and also for the dose of 1/20 LD_{50} (10.61%) (Fig. 14). However a significant decrease found in WBC after the dose of 1/5 and 1/20 LD_{50}, (45.30% ; 60.22%) ($p \leq 0.0004$; $p \leq 0.0009$ respectively) (Fig. 15). For the hemoglobin and hematocrite a significant decrease noticed after the treatment with the of 1/5 and 1/20 LD_{50} doses ($p \leq 0.001$; $p \leq 0.013$; $p \leq 0.0004$; $p \leq 0.036$ respectively) (Fig. 16 & 17).

The previous results probably indicated the disturbance of erythropoiesis and also the hemolysis (Chardon *et al*., 1964; Owen and Halestrap, 1993). In fact the decrease of RBC after the dose of 1/20 LD_{50}, resulted from the glutation reductase (Hedili *et al.* 1989).

The red blood cells showed echinocytes transformation (Fig. 18). This is probably due to the penetration of the bilayer lipid in the RBC, which has the

selective characteristics of the substance uptake in the extra-cellular layer, according to its physical and chemical properties (Wacjman *et al.,* 1992).

Histopathological studies of the liver were made. The histological pictures -of the liver- are being that of centro-lobular hemorragy (Fig. 19a), necrosis (Fig. 19b), and an important congestion of sinusoids (Fig. 19c), (Tebbi, 1974; Georgiou *et al*., 1988). The increase of GOT is probably by the parenchyme necrosis.

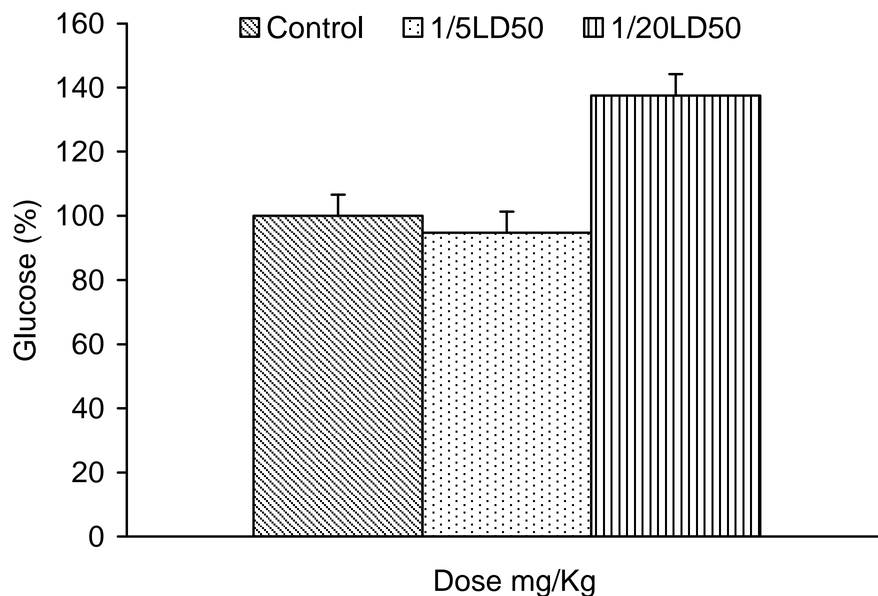


Fig. 8: Effects of *Atractylis gummifera* L. extract on plasma glucose (g/L) in rabbits after the treatment. Each point is the mean+SEM (n=5).

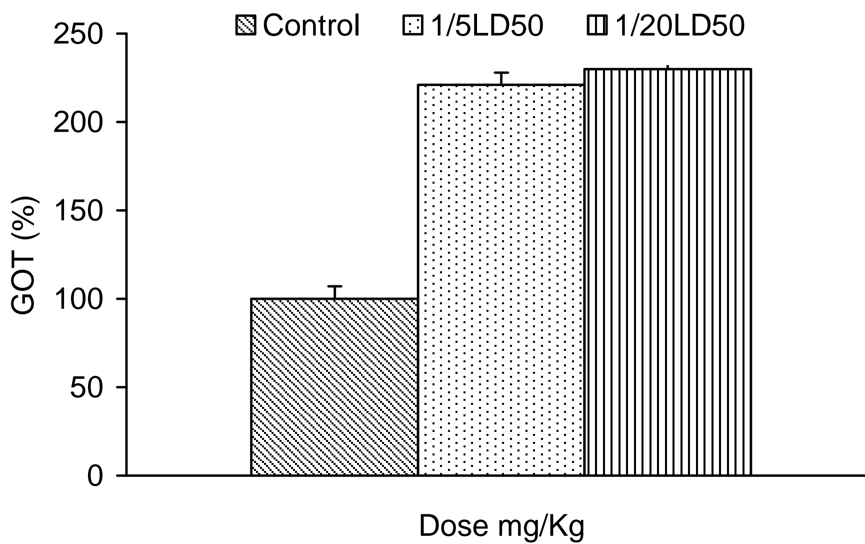


Fig. 9: Effects of *Atractylis gummifera* L. extract on plasma GOT(IU/L) in rabbits after the treatment. Each point is the mean+SEM (n=5).

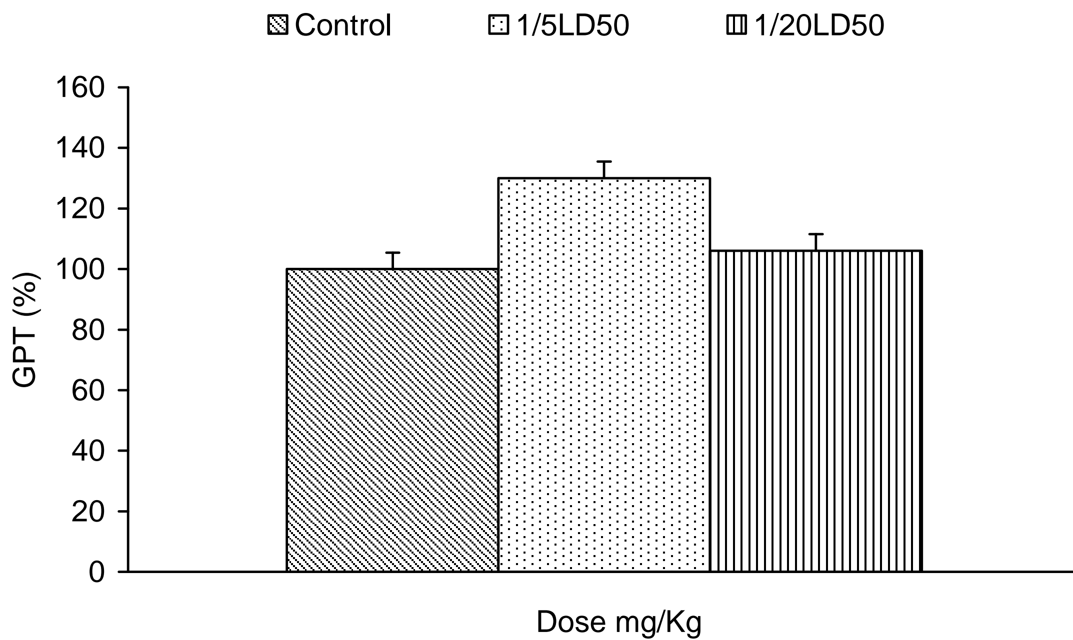


Fig. 10: Effects of *Atractylis gummifera* L. extract on plasma GPT (IU/L) in rabbits after the treatment. Each point is the mean+SEM (n=5).

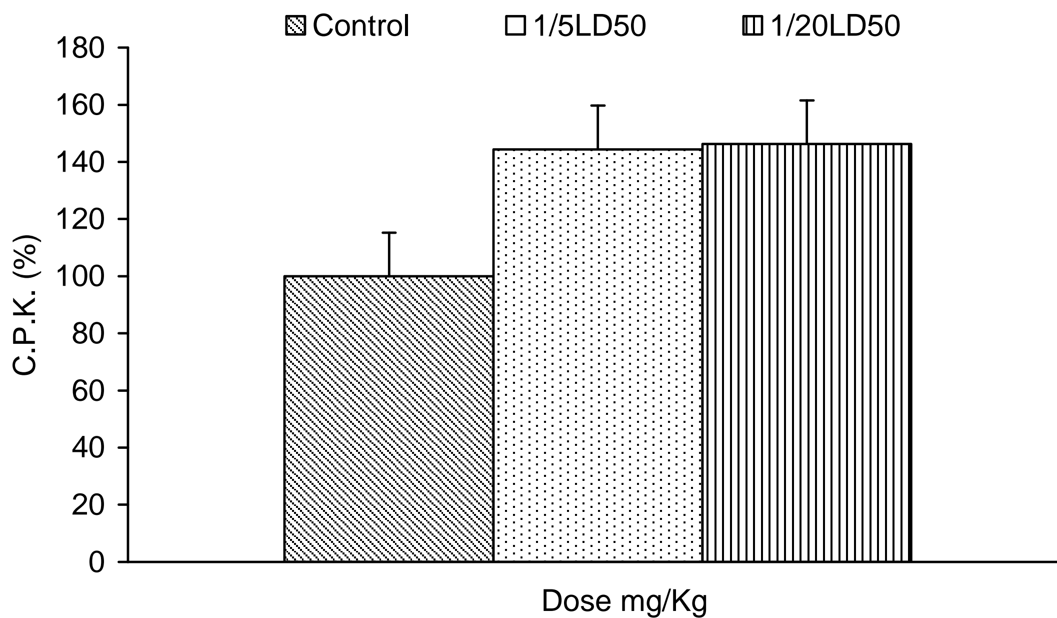


Fig. 11: Effects of *Atractylis gummifera* L. extract on plasma CPK (IU/L) in rabbits after the treatment. Each point is the mean+SEM (n=5).

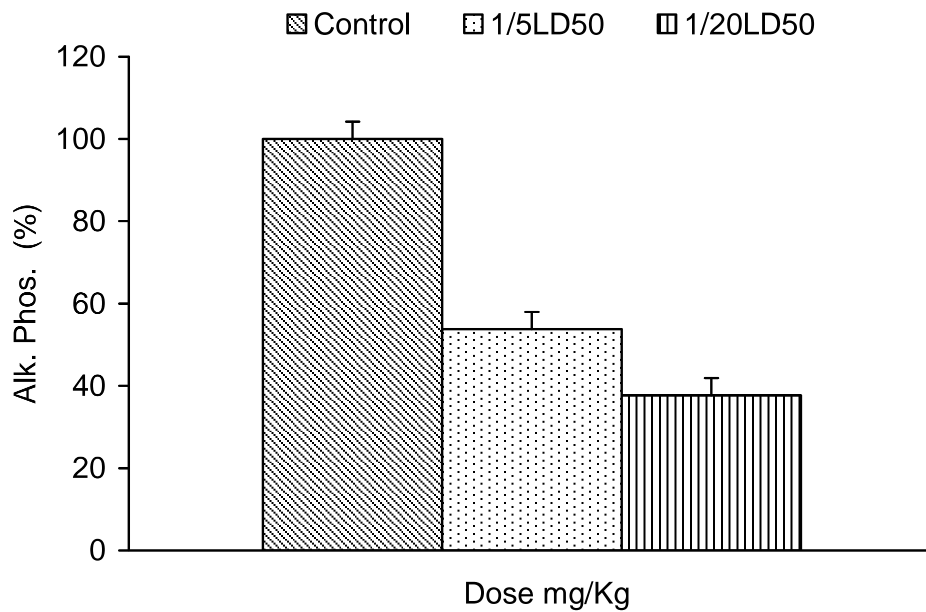


Fig. 12: Effects of *Atractylis gummifera* L. extract on plasma Alkaline phosphatase (IU/L) in rabbits after the treatment. Each point is the mean+SEM (n=5).

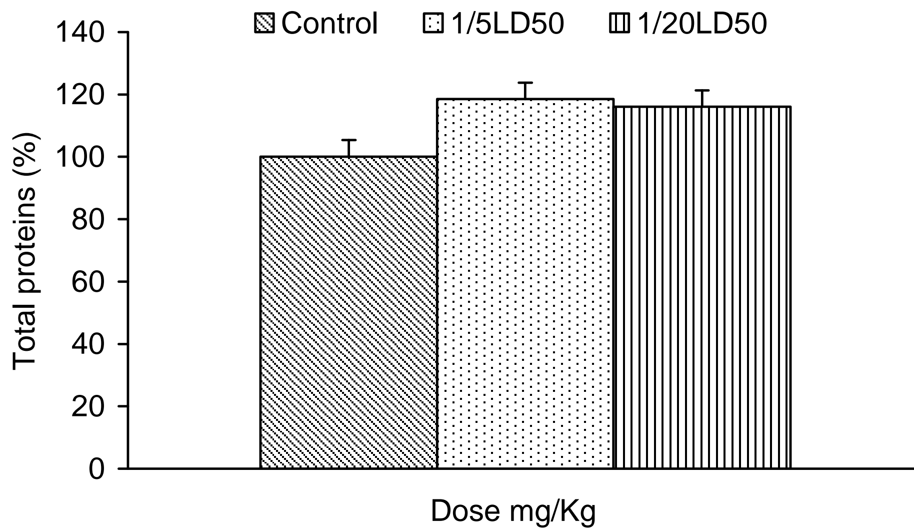


Fig. 13: Effects of *Atractylis gummifera* L. extract on plasma proteins (g/L) in rabbits after the treatment. Each point is the mean+SEM (n=5).

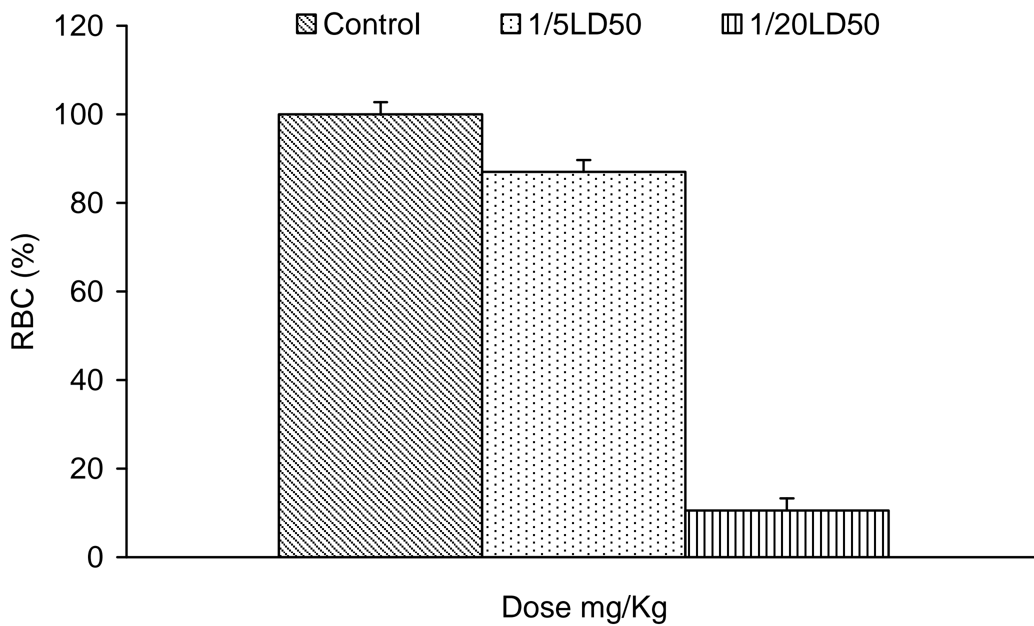


Fig. 14: Effects of *Atractylis gummifera* L. extract on red blood cells(RBC) in rabbits after the treatment. Each point is the mean+SEM (n=5).

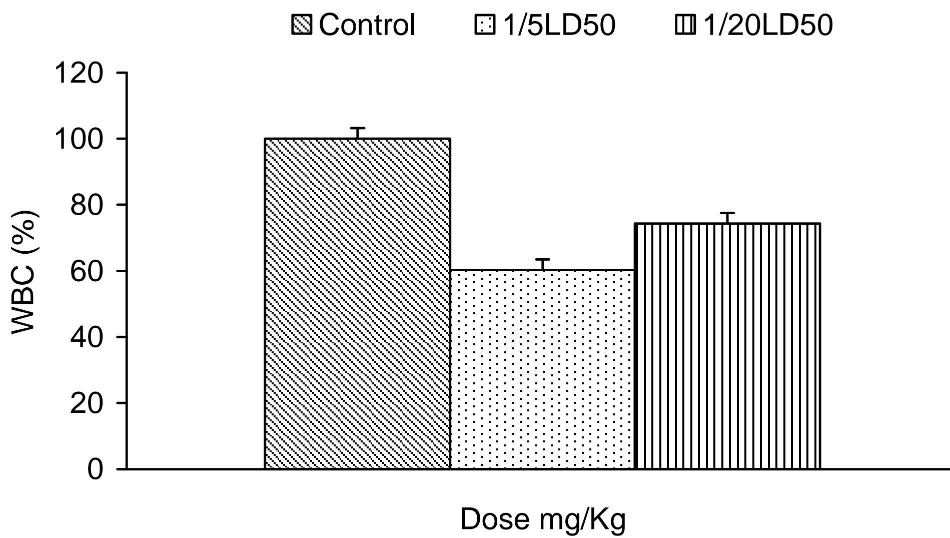


Fig. 15: Effects of *Atractylis gummifera* L. extract on white blood cells (WBC) in rabbits after the treatment. Each point is the mean+SEM (n=5).

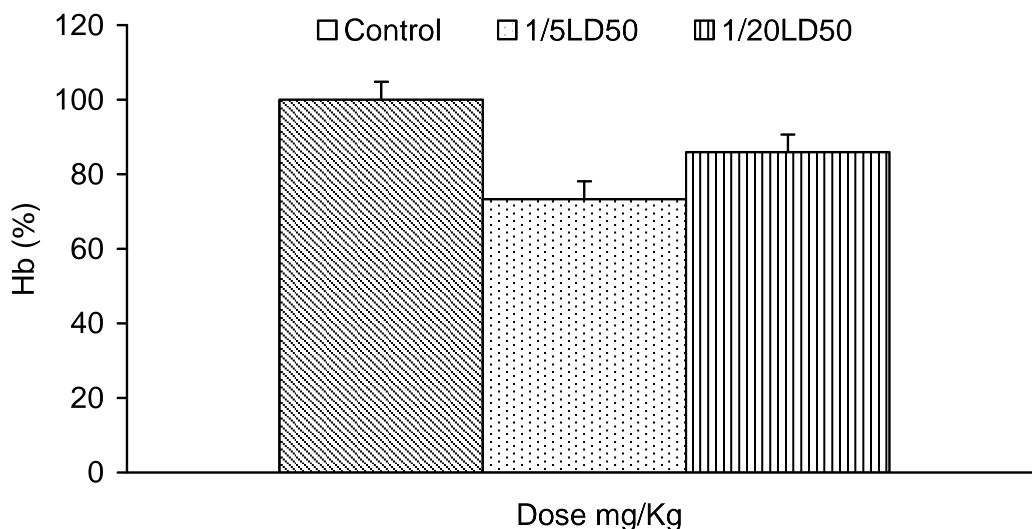


Fig. 16: Effects of *Atractylis gummifera* L. extract on plasma Hb (g/dl) in rabbits after the treatment. Each point is the mean+SEM (n=5).

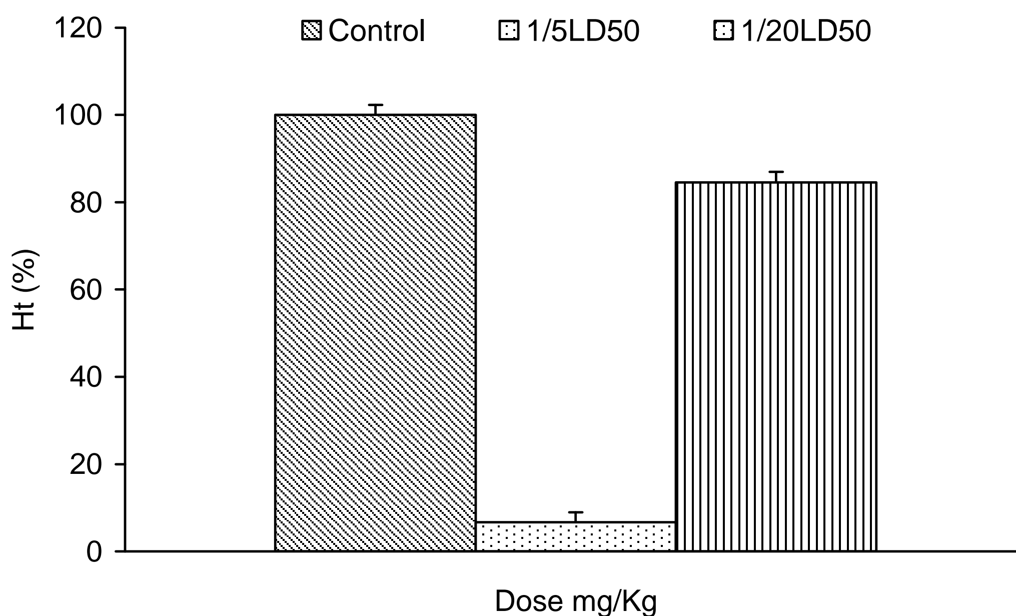


Fig. 17: Effects of *Atractylis gummifera* L. extract on plasma hematocrite (%) in rabbits after the treatment. Each point is the mean+SEM (n=5).

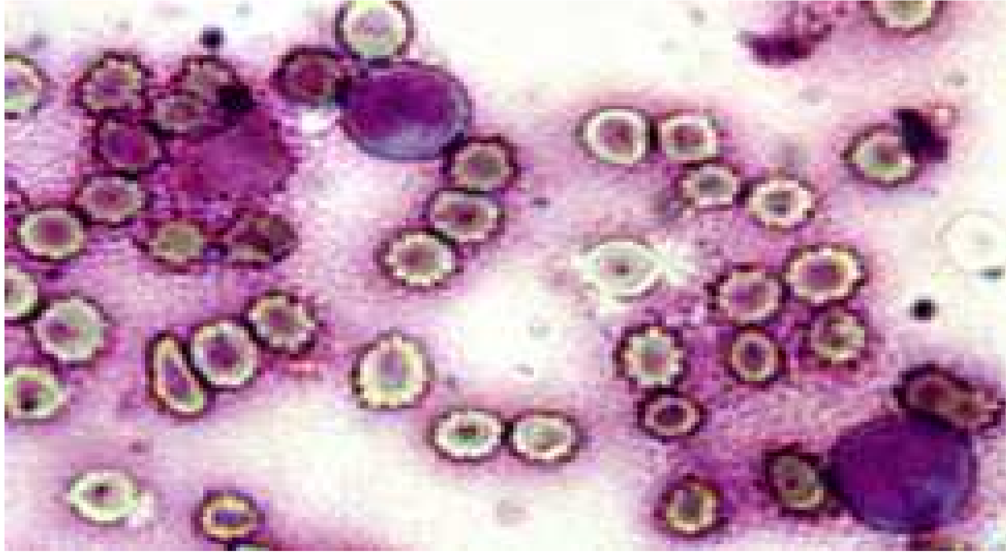


Fig.18: Micrograph of rabbit blood stream treated with 76mg/kg of *Atractylis gummifera* extract, shows echinocytes transformation (arrows).

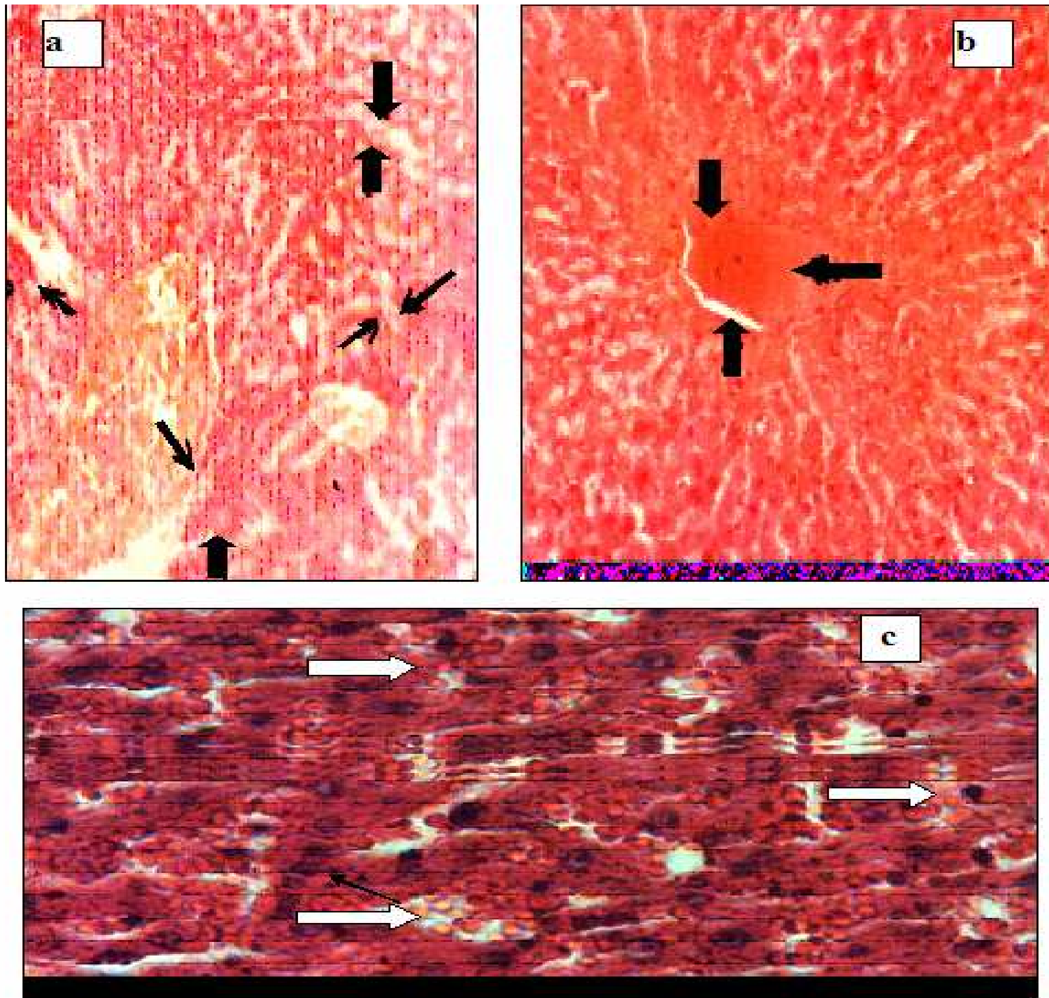


Fig. 19: Micograph of a liver section from rabbit treated with 76 mg/kg *Atractylis gummifera* L. rhizome extract, showing centro-lobular hemorragy (arrows, a), necrosis (arrows, b) and congestion of sinusoids (arrows, c).

Plasma parameters studies of rats treated with the rhizome extract of *Atrctylis gummifera* L. (76 mg/Kg, b/w) showed a clear alteration especially glucose metabolism. It has been recorded an increase of plasma glucose (225 %) after 24 h, followed by a decrease after ~~the~~ 3 days (94.89 %) then hyperglycaemia occurred after 5, 7, 10 days and after 14 days a high decrease by 42.8 % was noticed, (fig. 20).

The hyperglycaemia that happened after 24 hours probably is due to the ATR and CATR glycosides which have rapidly deplete hepatic glycogen resulting in an initial hyperglycaemic phase and a subsequent severe hypoglycaemic phase. As it has been showed also symptoms of intoxication similar to those sated before.

Plasma parameters of rats treated with the rhizome extract of *Atractylis gummifera* showed an increase of plasma GOT and GPT in all groups after 24 hours and until the end of the experiment (fig 21a and b).

Histopathological studies on rat liver showed a hepato-cellular necrosis (fig. 22A) and also peri- centroglobulare (fig. 22B) in the group of 7, 10 and 14 days, an important congestion of sinusoids and a microvascular steatose.

The Liver is the first target organ of active substances such as ATR & CATR which cause severe liver failure (Stickel *et al.*, 2000; Krejci and Koechel, 1992) and hepato-lobular necrosis (Snyder,*et al.*, 1992).

The biochemical and histological studies showed that this toxic plant is a high hepatotoxicity.

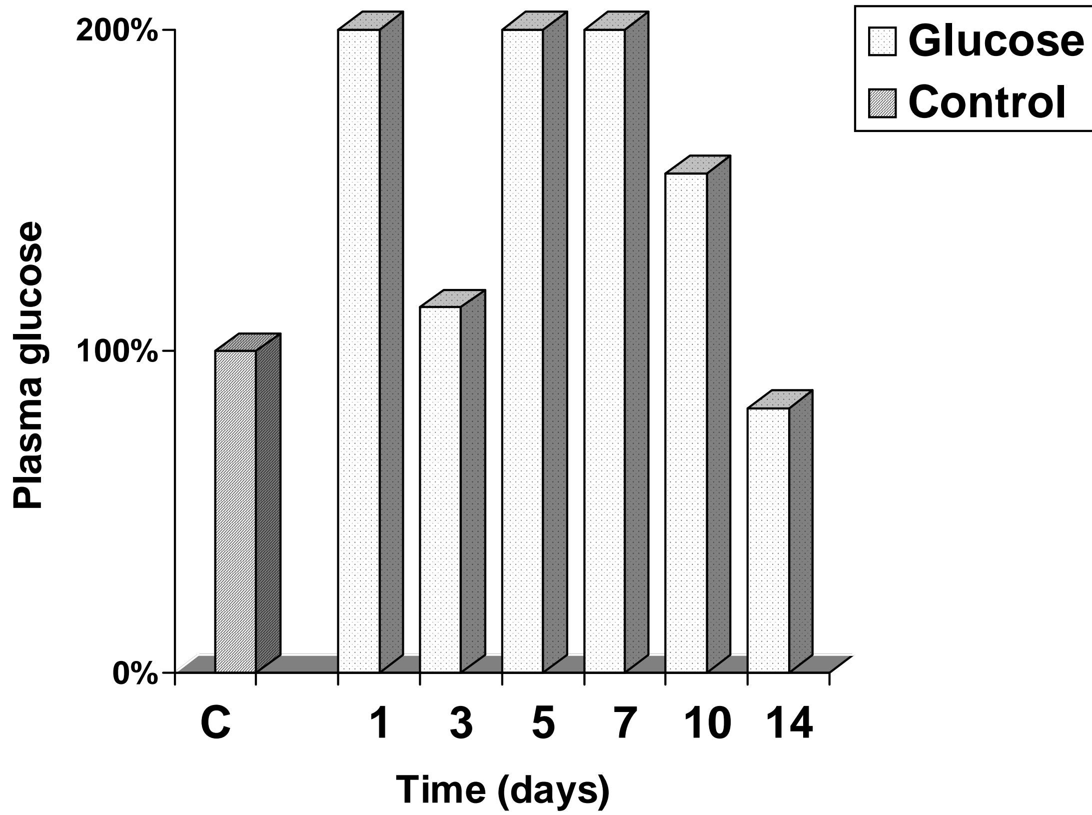
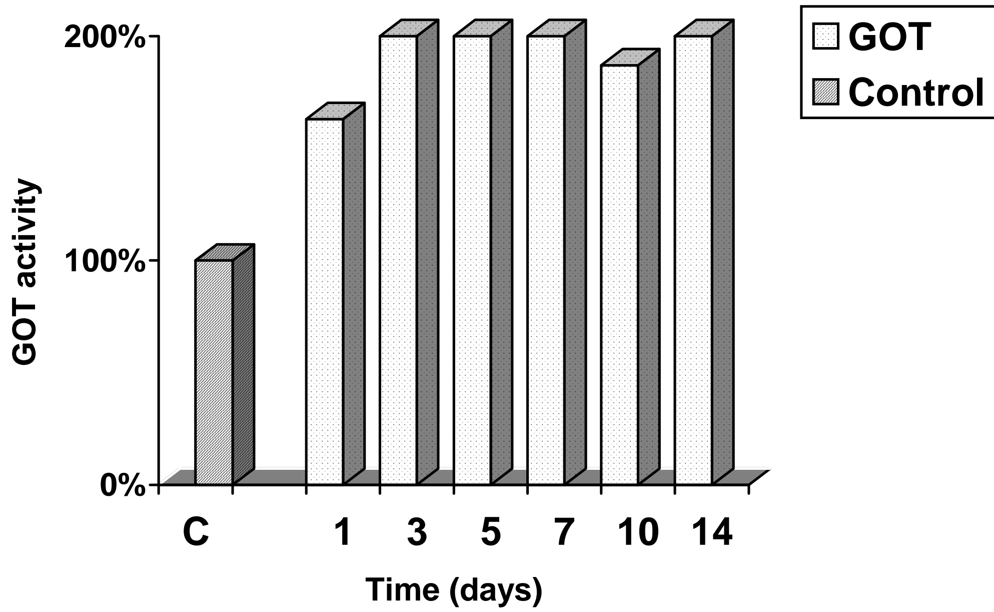
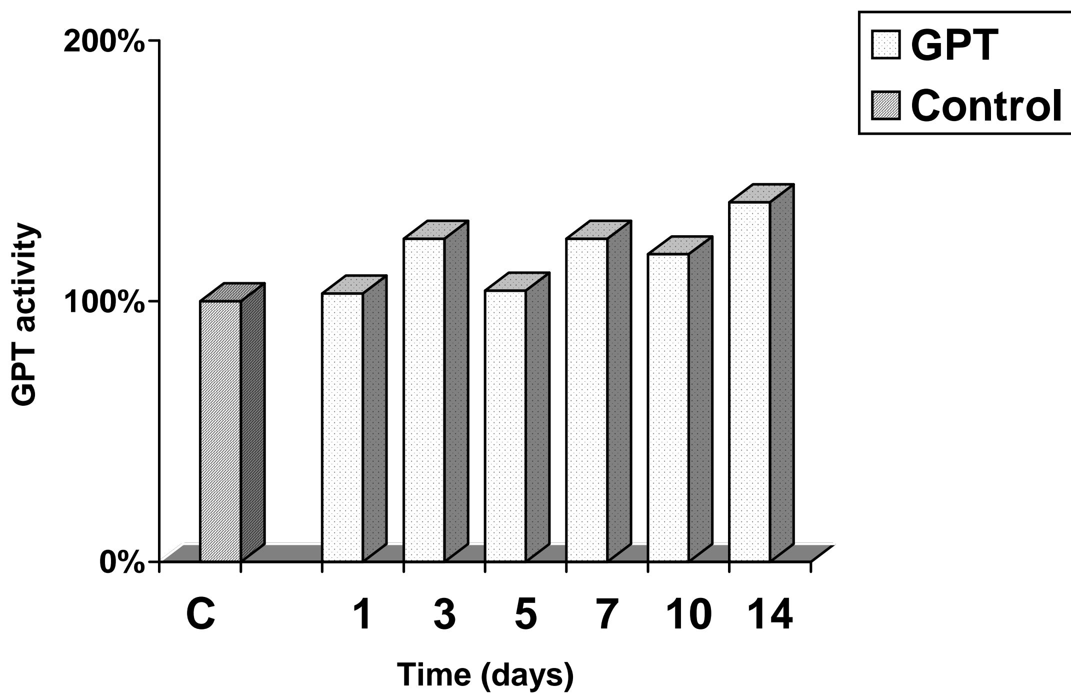


Fig. 20:: Effects of Atractylis gummifera L. rhizome extract (76mg/Kg) on glucose in rats (n=5), after 1,3,5,7,10 and 14 days expressed as a percentage. Each point represents the mean ± SEM.



a



b

Fig.21: Enzymatic activities of plasma GOT (**a**) and GPT (**b**) in female rats (n=5) treated with *Atractylis gummifera* rhizome extract (76mg/Kg) L. The results were expresed as percentage. Each point represents the mean ± SEM.

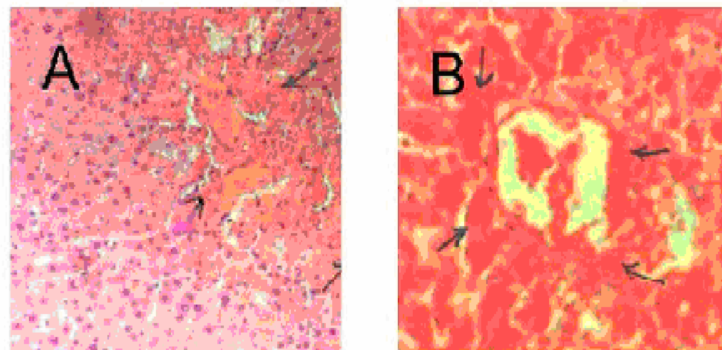


Fig.22: Micrograph of a section of liver from female rat treated with 76 mg/Kg *Atractylis gummifera* L. rhizome extract showing hepatocellular necrosis (A), Peri-centro-lobular necrosis (arrows) (B),

CHAPTER IV: COMPARISION BETWEEN ATRACTYLIS GUMMIFERA EXTRACT AND POTASSIUM ATRACTYLATE ON BLOOD PARAMERTERS, LIVER AND KIDNEY STRUCTURE

4. Comparison between *Atractylis gummifera* extract and potassium atractylate on blood parameters, liver and kidney structure

4.1. Introduction

Atractylis gummifera L. is a plant growing in north Africa and in most of the Mediterranean countries. It is used as ethnomedicine throughout Africa, the Mediterranean areas and the far Eastern countries Debetto,(1978), Obatomi and Bach (1998) The plants are known to cause acute fatal renal and liver damage in humans and domestic animals foraging on atractyloside- containing plants (Stuart *et al.*, 1981; Martin *et al.*,1986). *Iphiona aucheri* is responsible for poisoning racing camels (*Camelus dromedrius*) in the United Arab Emirates. The toxicity of these plants are deterpenoids, atractyloside (ATR) and carboxyatractyloside (CATR). (Roeder *et al.*, 1994). These two components are both specific inhibitors of mitochondria oxidative phosphorylation (Vignais, 1971).

The aim of the present study is to examine the toxic effects of *Atractylis gummifera* extract and different doses of potassium atractylate on some biochemical, hematological paramaters related to the liver and kidney function and structure.

4.2. Materials and methods

The experiment was carried out on male New Zealand White rabbits, weighing about 1.2 Kg. The plant material was collected from Bordj Bou-Arreridj area (70 km from Setif) in April. The rhizome was extracted using the method of Bey (1990) after modification. Animals were divided in 5 groups, 5 rabbits in each group. The animals

gavaged with one dose each. Control animals were treated with saline solution only (0.5 ml/ 100g). The second group with an extract, dissolved in 0.9% NaCl and with a dose of 1/5 LD_{50} (76 mg/kg, b/w), the 3rd, 4th and 5th group with 6.5, 13.0 and 32.0 µmole/kg respectively. After 48 hours the Blood was removed, centrifuged at 3000 rpm for 15 minutes.

GOT and GPT enzyme activities were measured in the plasma and also the glucose and total proteins. The RBC, WBC, Hb and hematocrite were measured as well. Morphological study of blood cells was carried out by the method of May-Grunwald-Giemsa (Sultan *et al.,* 1978). After that the histopathological studies were followed. The organs were macroscopically observed, removed, and rinsed in 0.9% NaCl, then fixed in the formol, sectioned 5 µm thickness, followed by alcohol and eosin (Hould, 1984). Slides were coated with a cover slip. The results were statistically treated by using the student t test.

4.3. Results and discussion

Biochemical studies of plasma treated with the rhizome extract of *Atractylis gummifera* L. (76 mg/kg b/w), showed a clear alteration especially glucose metabolism Capevielle and Darraq (1980). It has been noticed a slight decrease of plasma glucose after the treatment 6.5 and 13 µmole/kg body weight (96%, 78% respectively), and an increase after the treatment with 32 µmole/kg b/w, Hyperglycemia (Fig. 23). The , Hyperglycemia that happened probably is due to the ATR glycoside which has rapidly deplete hepatic glycogen resulting in an initial hyperglycemia phase and subsequent severe hypoglycemia phase.

In general all treated animals showed symptoms of intoxication (weakness, ataxia, convulsion, saliva, respiratory depression), and death occurred in the 1st group and the 5th group (80% and 88% respectively). The most probable mechanism by which these glycosides induce severe toxicity is by binding to the ADP/ATP translocase of these key nucleotide (Daniel *et al.*, 1993; Mark and Daniel , 1992). Inhibition of this enzyme provoke a partial blockage of aerobic metabolism (blockage of Krebs cycle and respiratory chain (Larry, 1994), translocated by a decrease of the use of glucose by cells explain probably hyperglycemia action.

Biochemical results of plasma parameters of animals treated with both *Atractylis gummifera* extract and K atractylate showed an increase of plasma GOT and GPT in most all the groups (Fig. 24 and 25 respectively). Total proteins was registered in all animals (Fig. 26). The toxic effect of these compounds could be connected to the formation of new metabolites. However the release of transaminases (GOT and GPT) may be related to cells damage. ATR was also found to alter catabolic and anabolic functions *in vivo* (Georgiou *et al.*,1988), and its consumption in dried roasted beans (*coffea arabica*) has been linked to pancreatic cancer (Pegel,1981).

Hematological results showed a slight decrease in RBC and WBC in most of the animals and a decrease was registered in the hemoglobin and the hematocrit (results not shown). The previous results probably indicate the disturbance of erythropiesis and the hymolysis (Chardon *et al.*, 1964; Owen and Halestrp,1993).

The red blood cells showed also echenocytes transformation. This is probably due to the penetration of the bilayer lipid in the RBC, which has the selective characteristics of the substance uptake in the extracellular layer, according to its physical and chemical properties (Wacjman *et al.*,1992).

Histopathological studies were made in the liver and kidney. The histological pictures of the liver show necrosis (Fig. 27), in the animals treated with 76 mg/kg b/w and with 32 μmole/kg and also centro-lobular hemorragy (Fig. 28) and an important congestion of sinusoids in the kidney (Fig. 29). The increase of GOT and GPT are probably by the parenchyma necrosis. The liver is the first target organ of active substances such as ATR and CATR which cause severe liver failure and hepato-lobular necrosis.

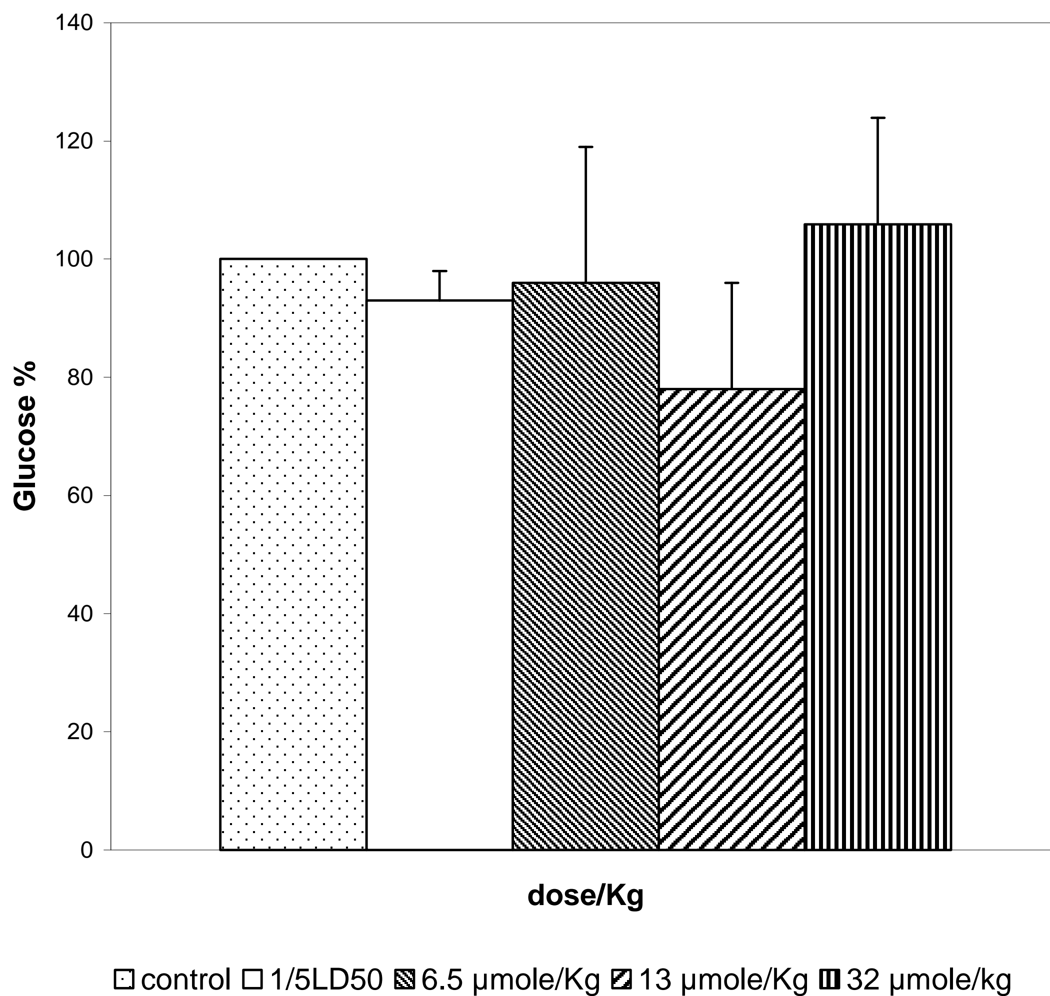


Fig. 23 Effect of Atractylis gummifera L. extract and K atractylate on plasma glucose . Each histogram is the mean+SEM (n=5).

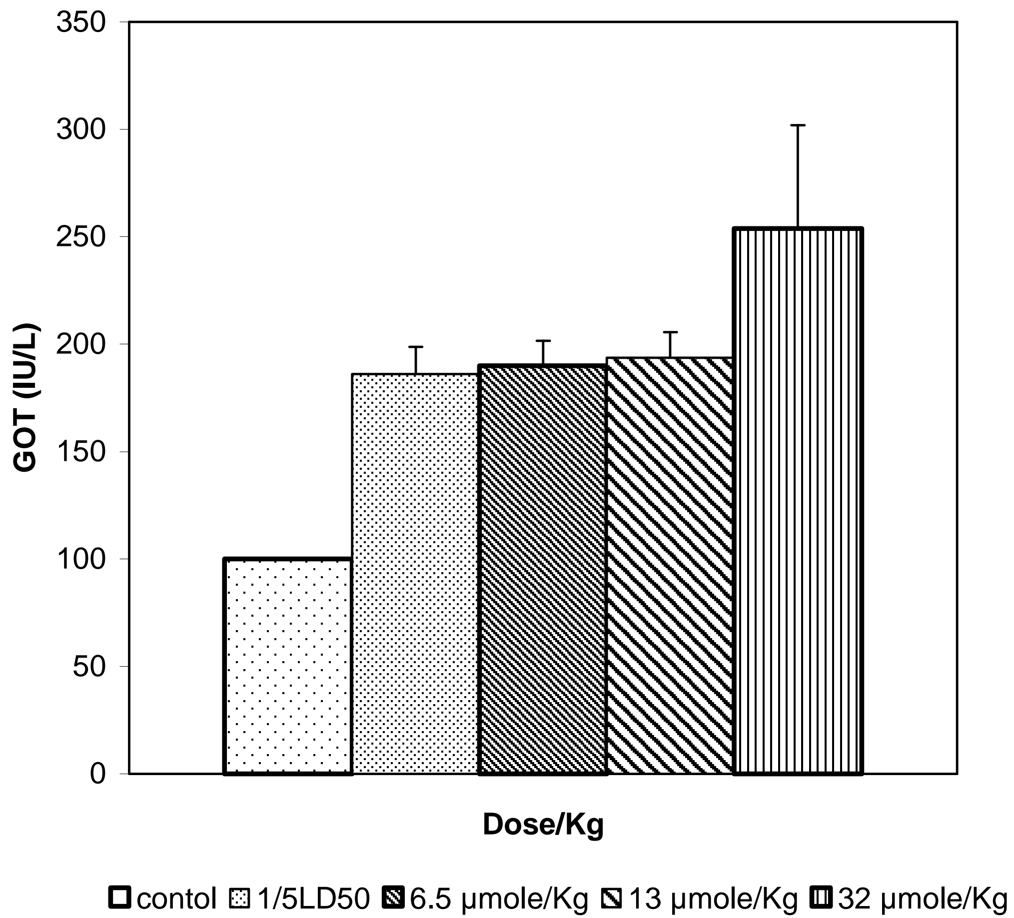


Fig. 24: Effect of Atractylis gummifera L. extract and K atractylate on plasma GOT . Each histogram is the mean+SEM (n=5).

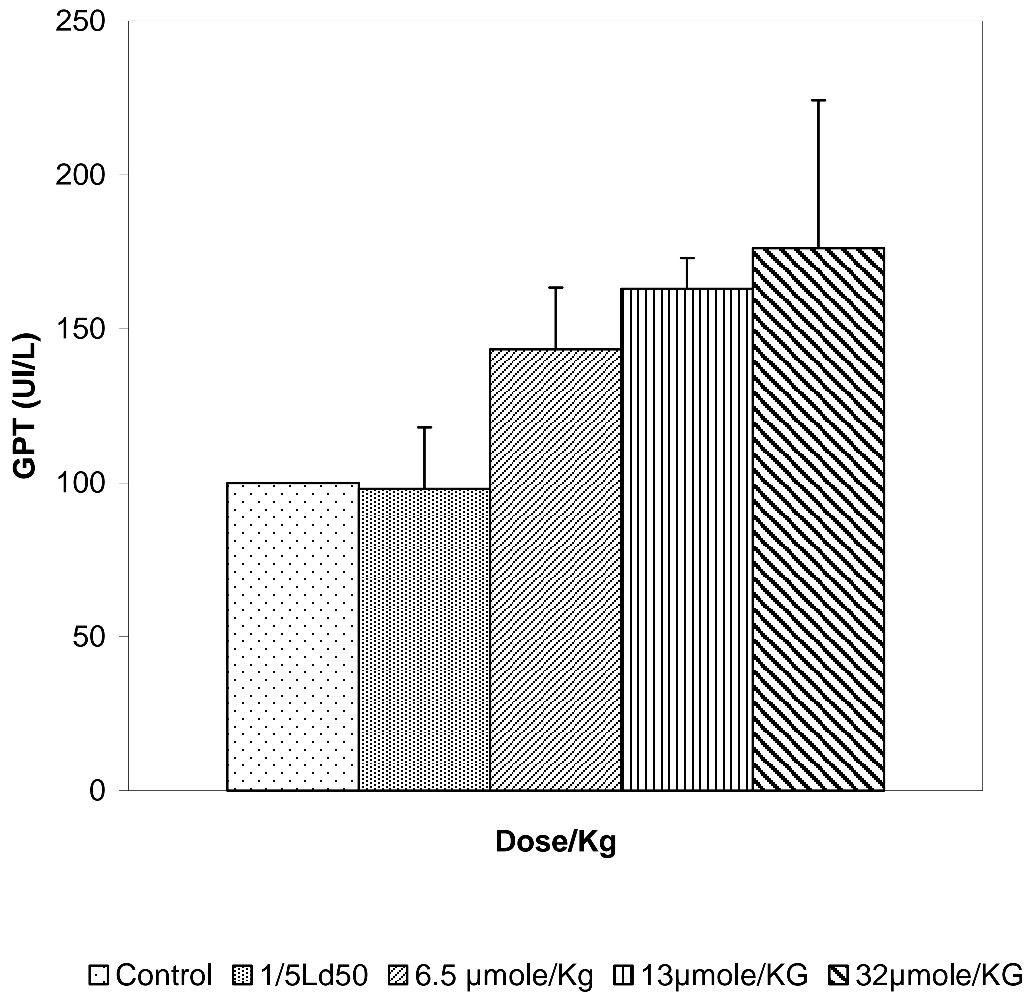


Fig. 25: Effect of Atractylis gummifera L. extract and K atractylate on plasma GPT . Each histogram is the mean+SEM (n=5).

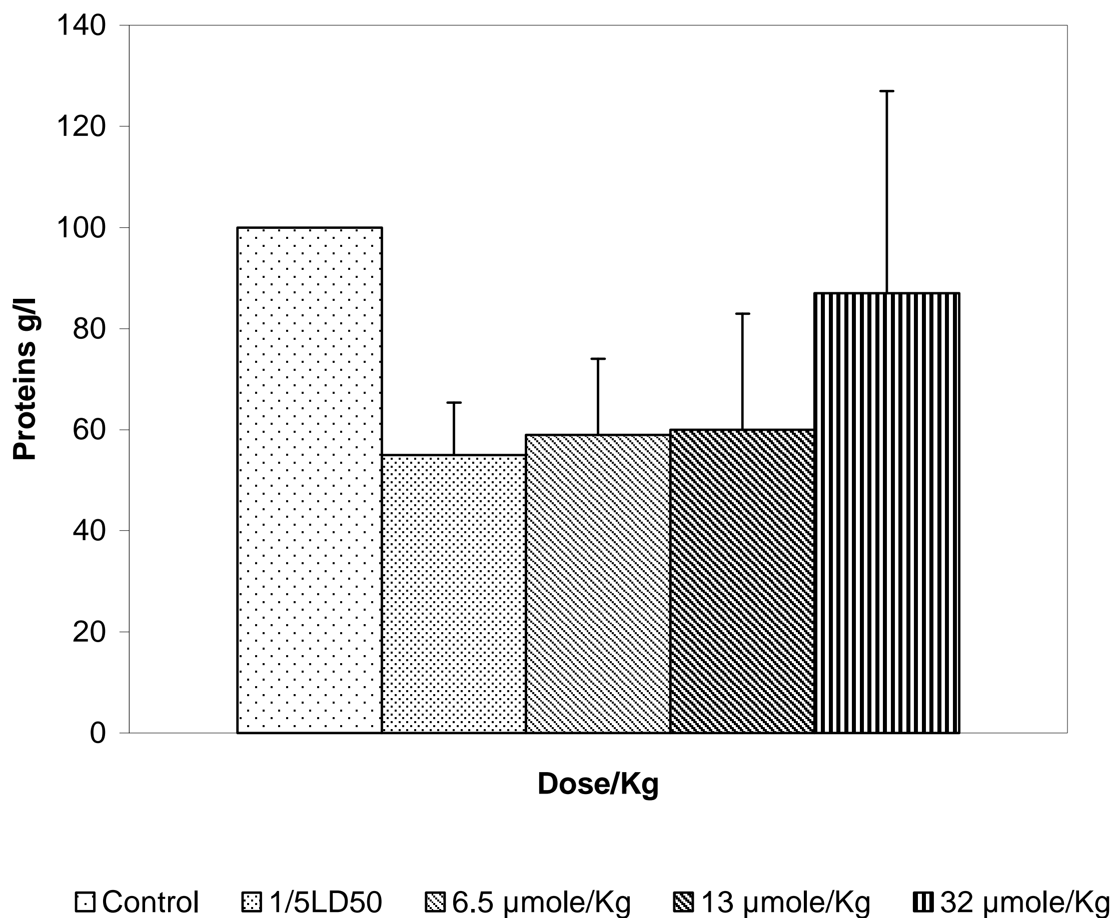
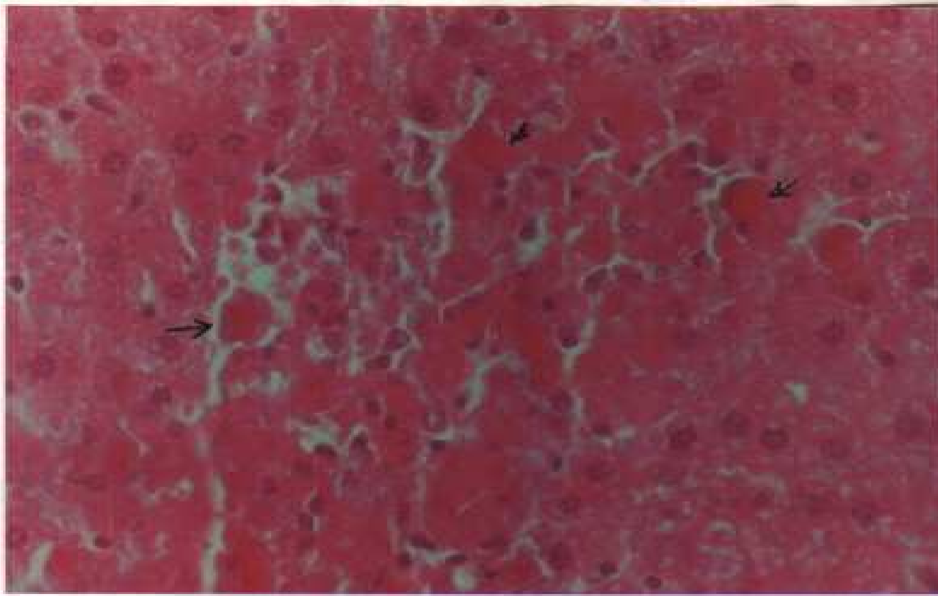
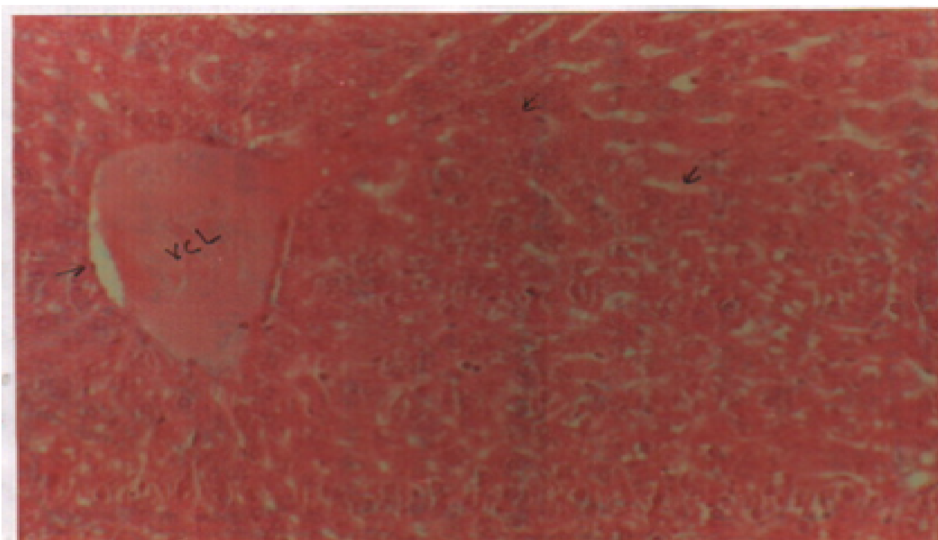


Fig. 26: Effect of Atractylis gummifera L. extract and K atractylate on plasma protein . Each histogram is the mean+SEM (n=5).



A



B

Fig 27: Micrograph of liver section from female rabbit treated with 76 mg/kg *Atractylis gummifera* rhizom extract showing (A) hepato-cellular necrosis (arrows) and (B) control.

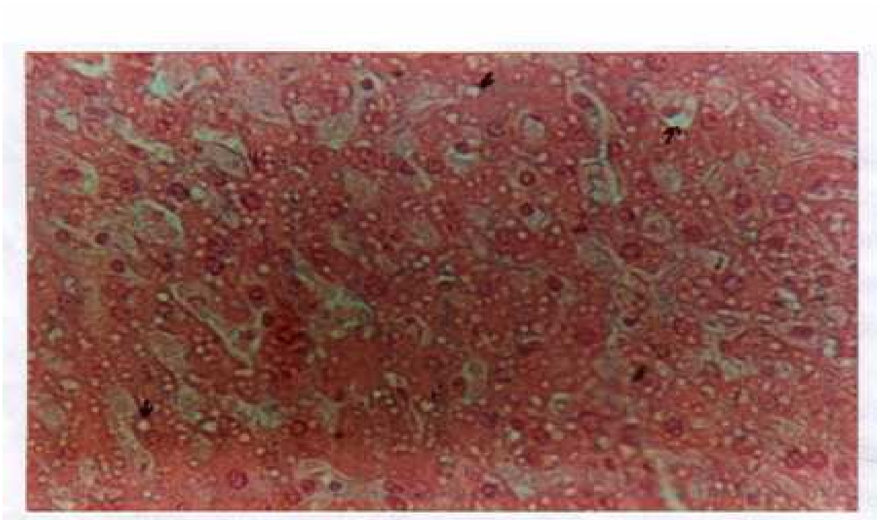


Fig. 28 : Micrograph of liver section from female rabbit treated with 32 µmole/kg K atractylate showing hepato-cellular necrosis and hemorragy (arrows)

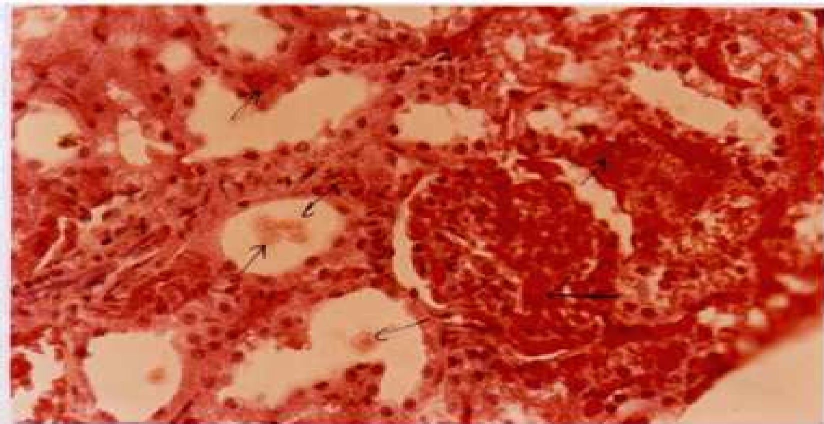


Fig 29: Micrograph of kidney section from female rabbit treated with 76 mg/kg Atractylis gummifera rhizom extract showing congestion (arrows) and (B) control.

CHAPTER V: TOXIC EFFECT OF *ATRACTYLIS GUMMIFERA* L. ON SPERMATOGENESIS AND RAT MALE REPRODUCTION ORGANS

5. Toxic effect of *Atractylis gummifera* L. on spermatogenesis and rat male reproduction organs

5.1. Introduction

Atractylis gummifera L grows in the mediterranean and its surrounds. Accidental poisoning occurs to children when they eat the fresh roots of plant which are very sweet . The subsequent clinical features are severe and often lethal. This toxicity is related mainly to two heterosides: atractyloside (ATR) and carboxyatractyloside (CATR). ATR and CATR are both specific inhibitors of mitochondrial oxidative phosphorylation, this inhibition is more important from CATR (Calmes et al., 1994). These two compounds are of high hepatoxicity and nephrotoxicity in animals and man but there is no data on their effects on male fertility. The present study was conducted to evaluate the action of acute *Atractylis gummifera* L. rhizome extract ingestion on some parameters related to male fertility.

5.2. Materials and methods

Male albino wistar rats were administred oraly with only one dose of 120 mg/Kg of the rhizome extract and killed by cervical dislocation 48 hours and 21 days after treatment. Control rats received only saline. The sperm count and percentage motility from epididymis were done using haemocytometer. Epididymal sperm morphology of treated and control animals was evaluated using light microscopy. Histology of testes was also studied using H.E. staining (Nezolof *et al*., 1972).

5.3. Results

5.3.1. Sperm content and motility

Content of spermatozoa decreased by 20.54 % after 48 h of treatment when compared with data obtained from the control group animal. Whereas, after 21 days

of treatment, the sperm count appeared to increase as much as in the control group. Sperm velocity in the epididymal cauda suspension decreased significantly by 32.41 % after 48h of treatment, whereas after 21 days of treatment there was a slight increase in sperm velocity compared to control animals (Fig. 30 and 31).

5.3.2. Sperm morphology

Light microscopic study of sperm morphology showed that normal rat sperm from epididymis suspension has characteristically sickle shaped head and the tail end piece was straight (Fig. 32a). Anomalies of sperm of treated animals were grouped into 2 major categories:

1- Head anomalies include microcephaly (Fig. 32b), amorphous (Fig. 32c), elongated and lacking in hook (Fig. 32d), and rotated (Broken neck) (Fig. 32e).

2- Tail anomalies: Cut up (Fig. 32f), angular (Fig. 32f) and irregular with node (Fig. 32 g). Figure 32 shows significantly greater number of sperm with tail and head anomalies in the treated animals.

5.3.3. Organ histology

The seminiferous tubules of control rat testes possess different stages of spermatogenesis having sperm in their lumen. Leydig cells are situated in between those tubules (Fig. 33A). The histological features of testes in rats after 48 h of treatment brought about severe damage and lysis of spermatogenic elements in seminiferous tubules. Degeneration of basement membrane and absence of spermatozoa in most tubules were also evident.Leydig cells revealed signs of atrophy (Fig. 33B): After 21 days of treatment, it has been shown a depopulation of spermatogenesis stages in seminiferous tubules and distortion between tubules (Fig. 33C).

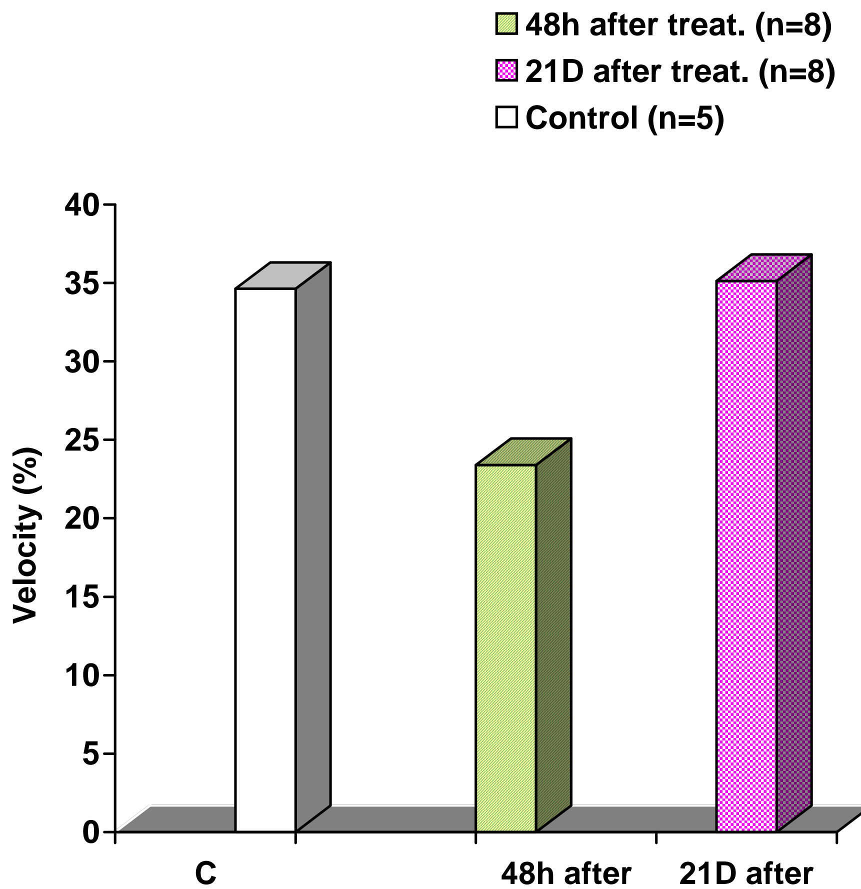


Fig.30: Epididymal sperm velocity after acute *Atractylis gummifera* L. rhizome extract ingestion (120 mg/Kg). Forward progression velocity is the average ± SD of the mean velocities obtained for each male rat.

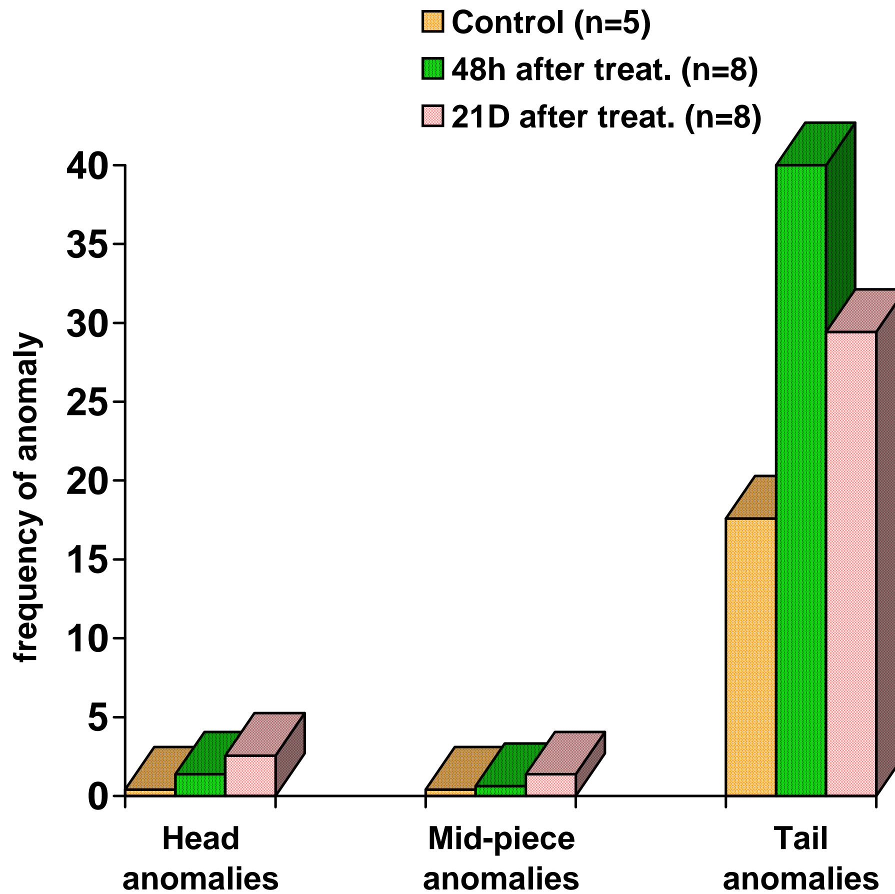


Fig. 31: Increased spermatozoal dysmorphology associated with acute *Atractylis gummifera* L. rhizome extract ingestion (120 mg/Kg). Details are given in the legend of Fig. 30. Values are reported as average ± SD of 30 spermatozoa from each male rat.

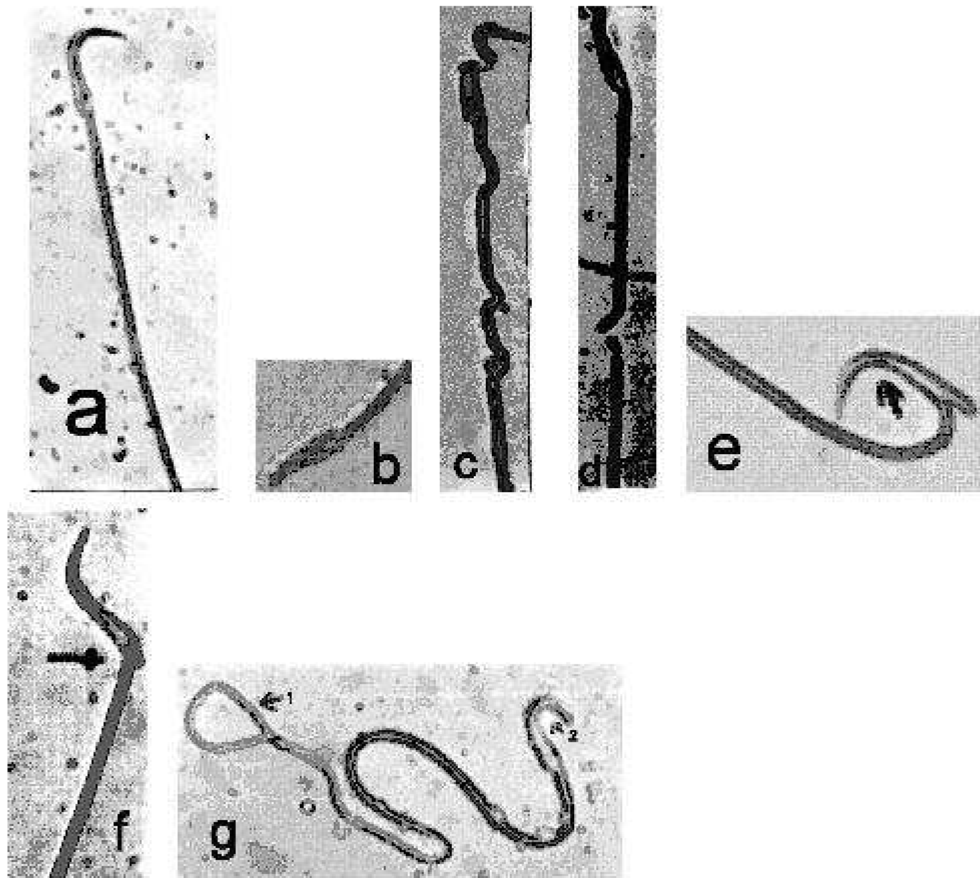
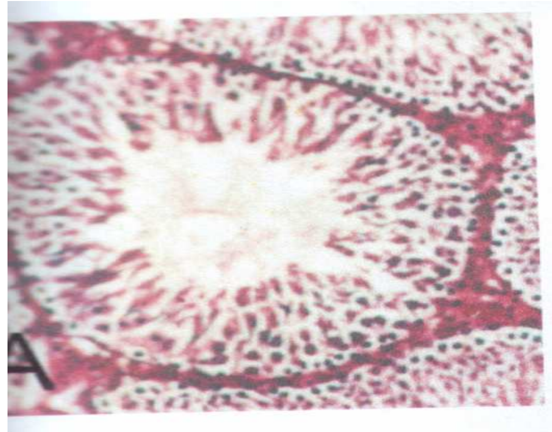


Fig. 32: *Atractylis gummifera* L.-induced sperm dismorphology: epididymal spermatozoa were fixed, stained and examined for morphological anomalies as described in the text. The light micrographs showed:

- Normal spermatozoa (a). Note the typical sickle shaped head, the orientation
  of the head with the sperm body and the elongated tail
- Head anomalies: microcephaly (b), amorphous (c), elongated lacking in hook (d) and rotated (e).
- Tail anomalies: cut up (f), angular (f) and irregular with node (g).



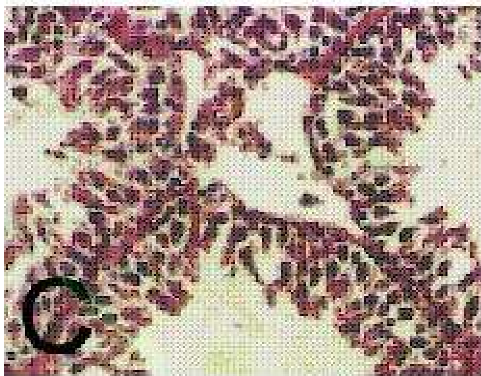


Fig. 33: Transverse sections of testes of normal and treated male rats with 120mg/Kg. of Atractylis gummifera L.rhizome extract.

A: Normal testis. Note the different stages of spermatogenesis having sperms in the lumen of the control rats.

B: A severe damage and lysis of spermatogenic elements and absence of sperms in most seminiferous tubules after 48 hours of treatment.

C: Depopulation of spermatogenesis stages after 21 days of treatment and distortion in situ (arrow) in interstitial tissue.

5.4. Discussion

After treatment with *atractylis gummifera* rhizome extract both a decrease in sperm count and an increase in abnormal sperm morphology were produced, and these findings may be of relevance for the decrease in reproductive potential of male rats, although some other mechanisms might also probably reflects diminished spermatogenesis. This conclusion is supported by the finding of poor quality spermatogenesis within the somniferous tubules and an increased frequency of inactive tubules in testes and sperm dysmorphology.

Atractyloside is known to be a competitive and specific inhibitor of the adenine nucleotide translocation across the inner mitochondrion membrane (Vignais *et al*., 1973). Moreover, one of the contractile protein, dynein is rich in ATPase activity, necessary for the conversion of chemical energy to mechanical energy for sperm flagellar movement (Zanevald and Polakoski, 1977; Vaissaire, 1977) . Therefore, changes in metabolism, morphology and count in sperm epididyms may be contributory to failure of fertility (Thibault and Levasseur, 1991).

CHAPTER VI: EFFECT OF ATRACTYLIS GUMMIFERA L. RHIZOME EXTRACT ON CELLULAR RESPIRATION AND GLUCOSE ABSORPTION BY RAT INTESTINE.

6. Effect of Atractylis gummifera L. rhizome extract on cellular respiration and glucose absorption by rat intestine.

6.1. Introduction

Metabolic process depends on gas exchange, which could be measured manomatecally by Warburg method. Cellular respiration depends also on certain number of factors: intense metabolism of substrate, functional respiratory chain. To test the influence of *Atractylis gummifera* rhizome extract , oxygen consumption of intestinal loops will be measured in the presence and absence of glucose .

6.2. Materials and methods

6.2.1. Plant material

The plant was collected from Beni Fouda (Setif) in the autumn. The rhizome weighted 38.70 g and measured 51 cm length X 7.5 width. The external layer was removed, then the rhizome was cut into small peaces and left to dry at room temperature, in the shadow. The procedure of extraction described above was followed.

6.2.2. Preparation and incubation of intestinal loops

The modified technique of Laval and Mazliak*.,* (1979) was used, seven female Wistar rats weighing 206 g (average weight) were used in respiratory experiments. Animals fasted for 48 hours with free access to water, were anesthetized by intraperitoneal injection of uretane (0.12 mg/100g, b/w).

The abdominal cavity was opened by a mild line incision. The small intestine (10-12 cm long) from the junction of stomach with duodenum after being saved from remaining visceral membranes was excised. The intestine was washed with physiological solution (with agitation) then hydrated on absorbable paper moisten, cut

into equal loops (circles) (0.5 cm long) and collected in Petri dish containing physiological solution.

The intestinal loops were incubated in 2 ml of physiological solution (Kreb's) in the presence and absence of glucose with 12 mM concentration and treated with AG rhizome extract (15 mg/ml dissolved in DMSO, test experiment) and with only DMSO solution (control experiment).

6.2.3. Calculation of O_2 consumption

Since the introduction by Warburg (Warburg, 1928) of a procedure for determination of tissue respiration in which the oxygen consumption is measured manometrically by the reduction in volume of a gas phase in contact with the tissue suspension, most investigations on tissue respiration have been performed based on this technique.

All absorption or gas escaped in the internal flask as a reaction lead to gas pressure change, which is proportion to the gas quantity to be changed in between the gas changed (x) and pressure variation measured in the manometer (h) the reaction is :

$$X = h * k$$

- X: Gas volume in mm^3 escaped in normal conditions of temperature and pressure, (0℃,760 mm/Hg).
- H: Height in mm of level liquid in the opened branch.
- K: Constant flask: is the factor lead in mm pressure change in µl gas change.

$$K = \frac{Vg \times 273/T + Vf \times \alpha}{Po}$$

Vg: Volume of gas retained in the flask and the in tubes linked as a reference, 150, in closed manometer branch.

Vf: Total volume of liquid found in the flask + alive material.

Po: Normal pressure (760 mm/Hg) equivalent of 1000Mmof Bordie liquid

T: Absolute temperature in which measures are effective.

α : Gas solubility coefficient, considered in the reactional liquid when the gas is the normal pressure and the temperaute T. (α 30°C = 0.026).

6.2.4. Effect of A.G rhizome extract on glucose absorption by that intestine *in vivo*

Eight female Albino Wistar rats (weighing 180g) were used in this study. Rats were anaesthetized as described previously. The abdominal cavity was opened by a mild line incision.

The small intestine (10 -12 cm long) from the junction of stomach with duodenum after being saved from remaining visceral membranes was excised. The two extremities were tied after placing a syringe needle in one extremity,
2 ml of glucose solution (5.4% in krebs solution) was injected into the control intestine. The test intestine was injected with 2 ml of A.G rhizome extract (15 mg /ml DMSO diluted 10 fold by glucose solution (5.4%).The same experiment was repeated with DMSO (control). After 30 mn the intestinal loop was excised, the external part was rinsed by NaCl (9%).The contents were collected into flasks (250 ml) containing 50 ml of TCA (5%).

The internal content of the intestine was collected, then the intestine rinsed with 20 ml of physiological solution. The collected solution of both was added to the previous flask and completed with distilled water (250 ml). Flasks were shacked for 15 min. The weight was recorded after drying the intestine using paper cloth.

6.2.4. Effect of AG rhizome extract on glucose absorption by intestinal loops *in vitro*

After respiration measurement in Warburg apparatus (Warburg tool (B-Braun Melsungen type V166), each flasks contnent was centrifuged (3000 rpm for 30 min) . 0.5 ml of each supernatant was added to 2.5 ml Orthotoluidine (revealing solution). The mixture was boiled (8 minutes) and optical density was measured spectophotometrically at 630 nm. Glucose concentrations were calculated according to calibration curve (Fig.34)

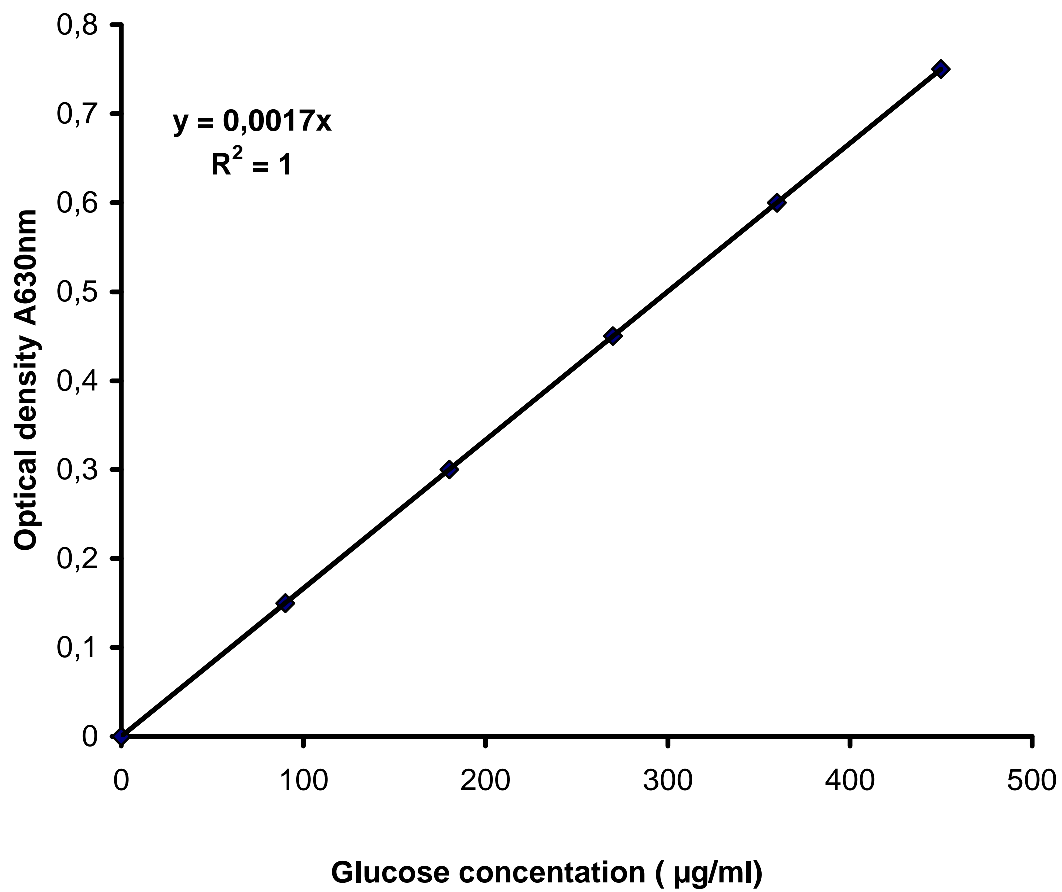


Fig 34 : Glucose calibration curve

6.3. Results and discussion:

Measurements of oxygen consumption (QO_2) from intestinal loops every 10 mn for 60 mn incubated in physiological solution (krebs) was done. Where a concentration of 15 mg /ml dissolved in DMSO of AG rhizome extract in the absence and presence of glucose was tested.

According to the results, it was observed that, in the absence of glucose a considerable increase in oxygen consumption through all measurement time; QO_2 is noticed (figure 35), but in the presence of glucose a considerable increase in QO_2 through every measuring time is observed and DMSO presence is observed as well (figure 36).

It seems that there is no effect of DMSO solution on QO_2. Whereas, QO_2 in the presence and absence of glucose increased considerably ,especially in the presence of glucose and this is appeared clearly in the 50 minutes to the 60 minutes where the concentration increased by 27% . This might indicate the glucose influence effect on QO_2 (figure 37,38)

A same increase was observed in QO_2 in the presence of AG rhizome extract in the first 10 minutes followed by considerable decrease in the 40 minutes (figure 39). This situation indicate clearly the effect of AG rhizome extract on QO_2 , where the amount of inhibition was 30%. In the presence of glucose with or without AG extract , it is observed a considerable increase in QO2 at the first 20 minutes followed by a decrease all remaining measurement time (figure 40). The inhibition action of AG rhizome extract was noted starting from 20 minutes and the percentage of inhibition at the end of the experiment was about 56%.

The second part of this study treated the effect of rhizome extract on glucose absorption. This was carried on rat intestinal loops *in vivo* and *in vitro*. From the results of the *in vivo* experiment, it is observed that AG rhizome extract has an inhibition effect on glucose absorption in small intestine, where the percentage of inhibition reached 19%. However, in the *in vitro* experiment, a slight effect of AG rhizome extract was noted on glucose absorption by rat intestinal loops incubated in physiological solution where the percentage of inhibition was 7%.

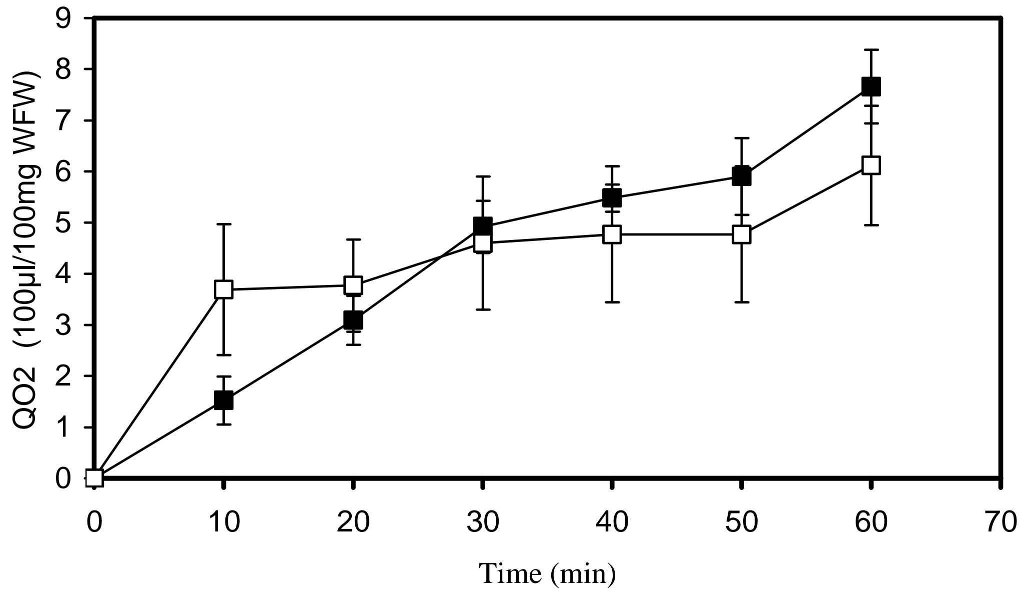


Fig.35: QO2 consumption (100µl/100mg wet fresh weight) from intestinal rat loop in presence ( ) and in absence ( ) of DMSO

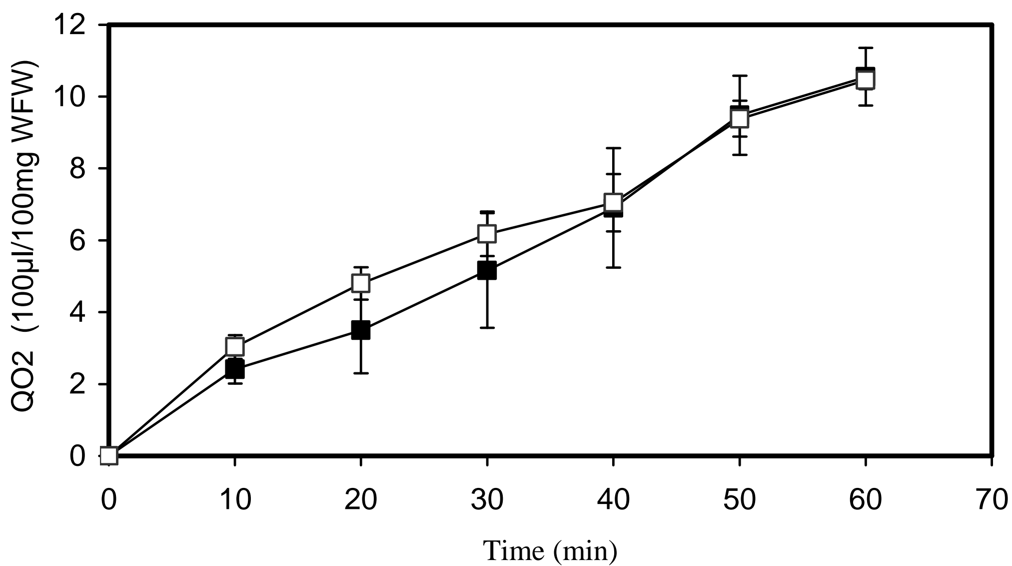


Fig.36: QO2 consumption (100µl/100mg wet fresh weight) from intestinal rat loop in presence( ) of glucose (12mM) and in presence of glucose + DMSO ( )

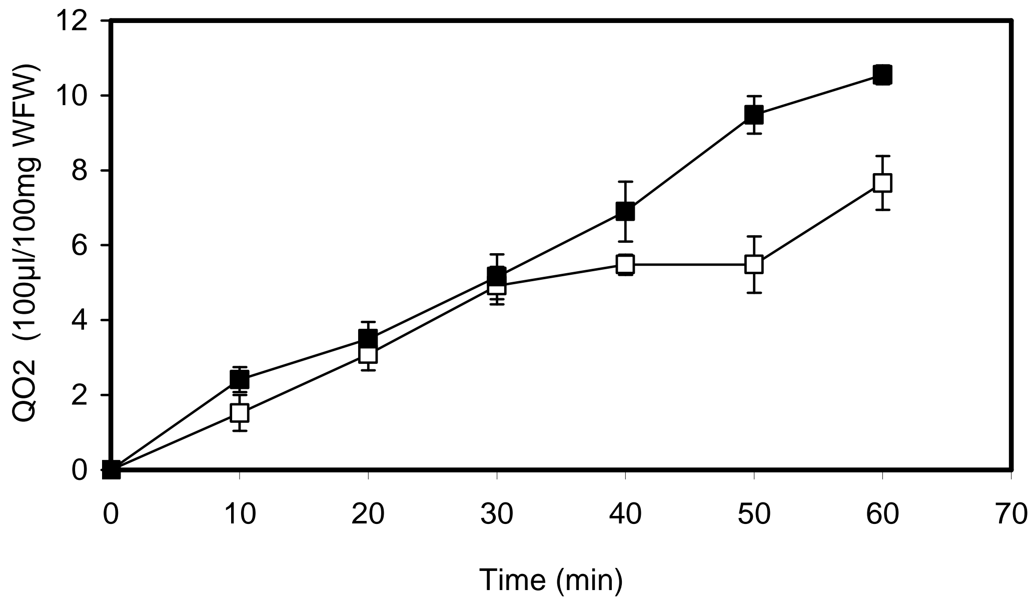


Fig.37: QO2 consumption (100µl/100mg wet fresh weight) from intestinal rat loop in presence ( ) and absence of glucose (12mM)

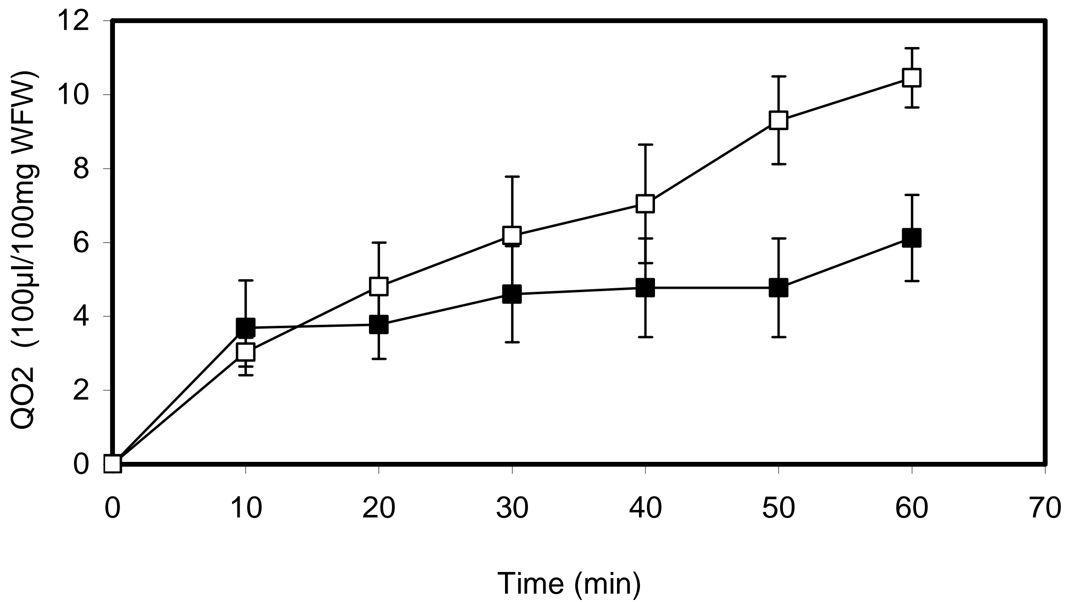


Fig.38: QO2 consumption (100µl/100mg wet fresh weight) from intestinal rat loop in presence DMSO ( ) and in presence of glucose dissolved in DMSO ( ) .

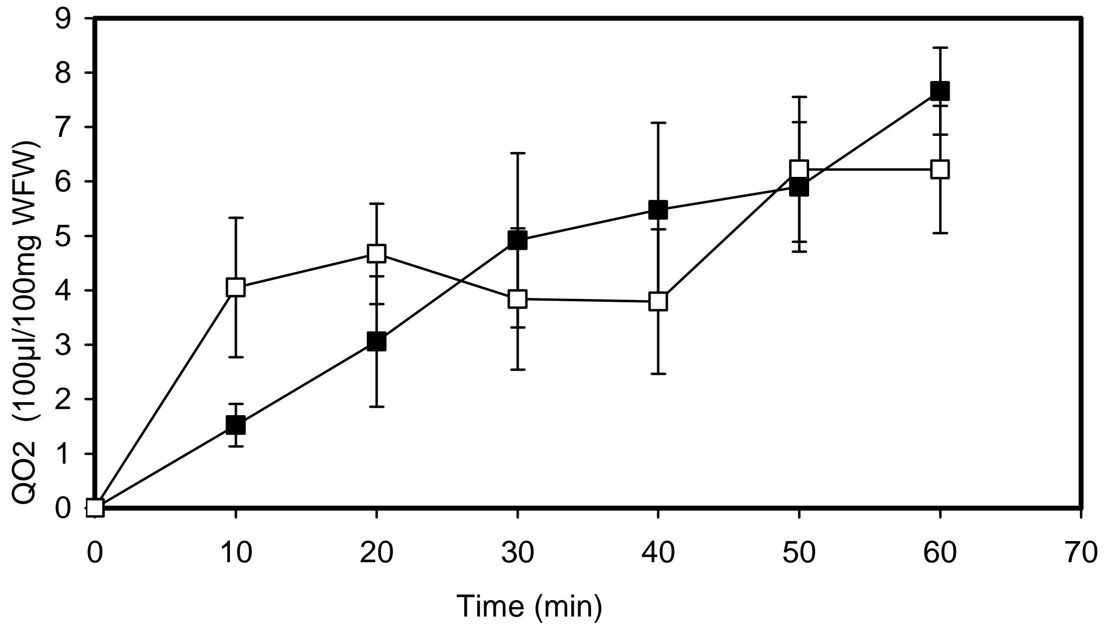


Fig.39: QO2 consumption (100µl/100mg wet fresh weight) from intestinal rat loop in presence ( ) and absence ( ) of AG (15 mg /ml) dissolved in DMSO

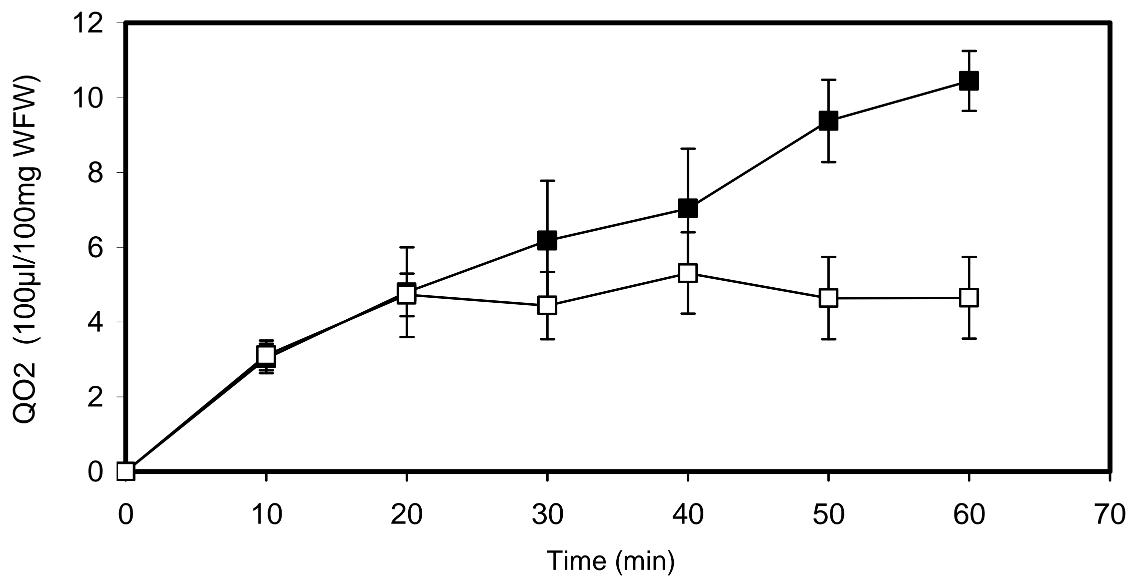


Fig.40: QO2 consumption (100µl/100mg wet fresh weight) from intestinal rat loop in presence of glucose (12mM) ,in presence( ) and absence( ) of AG (extract,15mg/ml) dissolved in DMSO.

The effect of oxygen consumption by rat intestinal loops in the presence of considerable quantity of glucose agreeing with what found in literature, so QO_2 increase by average 34% in 10 millimolar of glucose concentration. Both Bronk and Parson (1965) related this to active transports of Na ions, which stimulate glucose transport. Frizzel *et al*..,(1974) related this stimulation to the partial inhibition of lipids metabolism which stimulate glucose metabolism. Brank and Parson (1965) found that the increasing of consumable oxygen percentage from rat intestinal mucus alone by about 34% compared to kidney tissue.

Bruni *et al*.,(1962) demonstrated that the consumption of O_2 increased strongly in the presence of ATR (0.05 mM), also he found that 0.05 mM ATR stopped completely catching phosphate from rat liver and mitochondria as a result of inhibition of phosphorylation.

There was QO_2 prevention in rat liver mitochondria (Bucheler *et al*., 1991) besides this it was observed that 5 µmolar of CATR concentration prevent ANP exchange through ATP/ADP carrier then increased ATP concentration (Darnell et al., 1989). This is due to CATR and ATR which are fixed on external filling –places of ADP and ATP carriers (Hagen *et al*.,, 1993)

Our results did not show a considerable effect of AG plant extract on glucose absorption, nevertheless, mechanism of ATR and CATR prevention remain obscure even though Aprille (1993) suggested the possibility of ATR penetration, through plasma membrane. Ishii and Bracht (1986) Studies noticed that STEV does not penetrate into plasma membrane of human red blood cells after two hours of incubation at 37℃ (Aprille,1993).

CHAPTER VII: GENERAL DISCUSSION

7. General discussion

The World Health Organization estimates that 65-80% of the world's population rely on traditional medicines as their primary mode of healthcare and their use in developed countries ranges from 9-65%. Rates of poisoning with these agents, whether intentional or unintentional, are less easily determined (Flora *et al.*, 1996).

The most accessible way to study poisoning is via hospital admissions or calls to national poison centers. Such studies have shown traditional medicines account for 1.7-15.8% of all poisoning admissions. Reported mortality rates range from 8.5-15% for emergency department hospital admissions (Malek *et al.*, 1992).

Atractylis gummifera L., contains a toxic glucoside, the atractyloside, which inhibits oxydating phosphorylation process. Atractyloside poisoning is an infrequent but often fatal form of herbal poisoning, which occurs worldwide but especially in Africa and the Mediterranean regions. The primary mechanism of atractyloside poisoning is known to be inhibition of the mitochondrial ADP transporter. Poisoning in humans may present with either acute hepatic or renal pathology and it is possible that there is a second mechanism of toxicity to the hepatocyte. Atractyloside in large amounts gives rise to massive necrosis, but in vitro studies have shown that at lower doses cells progress to apoptosis.

After treatment of rats and rabbits with different doses of Atractylis gummifera extract 1/5, 1/12 and 1/20 LD_{50} and with respect to different periods of time, the activity of transaminases increased,This was evident with the decrease of glucose concentration and red blood cells (Dahamna *et a*l., 1998 ; Dahamna *et a*l., 2000 ;Dahamna *et a*l., 2002; Nogue *et al.*, 1992). The consumption of this plant as an extract containing ATR causes acute fatal renal and hepatic necrosis in animals and human (Daniele *et al.*, 2005; Dahamna *et al.*, 1998 ; Tebbi, 1974; Dahamna *et al.*, 1999; Dahamna *et al.*, 2000; Obatomi and Bach, 1998; Lemaigre *et al.*, 1975; Georgiou *et al.*, 1988, Nogue *et al.*, 1992; Temamna, 1985).

Our results showed changes of biochemical values ,these are generally observed in organ failure or damage. Increase in AST, ALT, and GGT enzymes activities suggested an hepatic damage (Boyd, 1980; Hardaway, 2000), and changes in creatinine and urea levels are suggestive of renal damage (Turgut, 2000). A

decrease in RBC after the treatment with the dose of 1/5 LD_{50}, (87.8%) and also for the dose of 1/20 LD_{50} (10.61%).These results probably indicate the disturbance of erythopoiesis and also the haemolysis (Chardon *et al.*, 1964; Owen and Halestrap, 1993). The decrease of RBC is due probably to the glutation reductase (Hedili *et al.* 1989). The cell turnover of GSH is associated with its transport through the cell enzyme of key metabolism of GSH. The GGT is a glycosylic enzyme of plasma membrane catalysis the synthesis of gamma glutamyl amino acids and consequently it reacts as a mechanism of transport. In addition this enzyme is important in detoxification pathway of GSH and other metabolic processes, for example the conversion of leucotriene C4 to leucotriene D4. It has a flux of GSH starting from the liver to the plasma; however, the cells with elevated rates of plasma GGT were unregistered in chronic hepatitis, liver neoplasm, cardiac deficiency and pancreatic diseases (Clealand, 1963; Schirmer et al., 1987; Li and Li, 1996).

The primary mechanism of atractyloside poisoning is known to be inhibition of the mitochondrial ADP transporters and thus blocks oxidative phosphorylation, which prevents the synthesis of ATP and leads to failure of gluconeogenesis and ultimately cell serious problem in Africa (Mark and Daniel, 1992).

The inappropriate use of medicinal herbs has resulted in numerous fatalities, especially in young children (Lemaigre *et al.*, 1975; Stewart et al.,2000). The obtained results clearly showed that, *Atractylis gummifera* extract (including its active substances), led to a failure of the important organs, such as liver and kidney.

The study on reproductive system showed a decrease in sperm count and an increase in abnormal sperm morphology. This phenomenal alteration, confirmed frequency of inactive tubules in testes and sperm dysmorphology (Zanevald and Polakoski, 1977; Thibault and Levasseur, 1991).

Another study of the effect of *Atractylis gummifera* extract is on cellular respiration and glucose absorption, this finding showed an inhibition in the presence of Atractylis gummifera extract by about 56% (Dahamna *et al.,* 2001), This, coincide with that found in the literature review. Both Ishii and Bracht (1980), unregistered the inhibition of QO_2 of liver cells by about 24% and by using ATR (50 μM). To screen the relevant ATR-derivatives as possible inhibitors of the adenine nucleotide translocation, their effect on the ADP-stimulated respiration of rat liver mitochondria was taken as indicative of their interference with the ADP translocation. The removal of the sulphate groups or of the isovaleric acid from the ATR and gummiferin

molecules results in a significant decrease of inhibitory power. Reduction of the methylene group or acetyltion of the alcohol groups also decreases the activity of ATR. Using 5 mg of mitochondrial protein, 50% inhibition was found for a concentration of ATR or gummiferin of 0.4 – 0.5 ug/ml of medium (Vignais *et al.*, 1972). Gummiferin is as a potent an inhibitor of the ADP translocation as ATR, is also specific in its action. As for ATR, concentration of gummiferin which totally inhibit the oxidative phosphorylation of extra-mitochondrial ADP, do not alter the oxidative phosphorylation of intra-mitochondrial ADP (Vignais *et al.*, 1971). However, high concentrations of ADP which reverse the inhibition by ATR of the ADP translocation have no effect on the gummiferin-dependent inhibition of the ADP-stimulated respiration (Vignais *et al.*, 1971).

The focus of future research should be directed on surveys of human foodstuff for ATR, avoidance of exposure to ATR-containing ethnomedicines through educational initiatives, comparative toxicity studies of ATR and ATR analogues occurring naturally in plants (especially human foods) and systemic studies to establish whether the toxicity of ATR -containing plants of known ATR content is attributable solely to the components that can materially influence ATR toxicity. In addition, there is also the need to provide appropriate therapeutic intervention to limit or eliminate its toxicity in human poisoning.

Simple methods for the detection of atractyloside poisoning are at present restricted to thin-layer chromatography in urine and are useful only in the case of severe poisoning. Immunoassays, high-performance liquid chromatography, nuclear magnetic resonance, and a recently developed high-performance liquid chromatography -mass spectrometry method have yet to be applied to clinical diagnoses. There is at present no treatment, but a fuller understanding of the mechanisms of toxicity may lead to the application of a number of compounds that are effective *in vitro*.

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