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Hepatoprotective and antidiabetic activities of *Fraxinus angustifolia* Vahl extracts in animal models: characterization by high performance liquid chromatography analysis

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Background/aim: The present study was designed to explore antidiabetic and hepatoprotective potentials of *Fraxinus angustifolia* leaf (FAL) and bark (FAB) extracts in vivo.

Materials and methods: Streptozotocin (STZ)-induced diabetic rats, pretreated with the extracts (25 and 50 mg/kg), were monitored for fasting blood glucose (FBG) levels. Hepatoprotective potential was examined after injection of an excessive dose of paracetamol (10 g/60 kg) by analysis of biochemical parameters (transaminases, bilirubin), malondialdehyde (MDA) levels, and histological sections. high performance liquid chromatography analysis was also performed for partial characterization.

Results: A considerable hypoglycemic effect was noticed 2 h after the STZ-induction, with a higher efficiency ($P < 0.05$) for FAL (68%) as compared with FAB (57%). A significant ($P < 0.05$) reduction in MDA was observed for paracetamol-fed mice pretreated with FAL (50 mg/kg), FAB (50 mg/kg), or both (25 mg/kg each) extracts, and the MDA levels for the three conditions were 0.290 ± 0.034, 0.340 ± 0.038, and 0.25 ± 0.058 nmoles/mg of liver tissue, respectively). Hence, simultaneous treatment provided a better protection. Histological observations confirmed the higher hepatoprotective potential of FAL over FAB extracts

Conclusion: The obtained results indicate the possibility of pharmacological exploitation of *F. angustifolia* extracts in the treatment of diabetes and associated liver diseases.

Key words: *Fraxinus angustifolia*, antidiabetic, hepatoprotective, lipid peroxidation, α-amylase

1. Introduction

Diabetes mellitus, a complex chronic pathological condition, is considered a major health problem worldwide. This metabolic disorder is characterized by hyperglycemia and disturbances in carbohydrate, protein, and fat metabolisms secondary to an absolute or relative lack of insulin (1). Besides hyperglycemia, several other factors including dyslipidemia or hyperlipidemia are involved in the development of micro- and macrovascular complications of diabetes, leading to morbidity and mortality (2). For Algerian practitioners, diabetes is considered a serious public health problem since, according to the World Health Organization, 10% of the Maghreb inhabitants were diagnosed with type-2 (non-insulin-dependent) diabetes, with 24.1 million persons affected. Insulin therapy and other conventional drugs are not devoid of adverse side effects in addition to their unsatisfactory efficiency in certain cases, which urges the quest for the development of more effective antidiabetic natural agents. In fact, previous studies confirmed the efficiency of several medicinal plants in diabetes (3,4). Another therapeutic approach for treating diabetes is to inhibit the carbohydrate-hydrolyzing enzymes such as α-amylase, thus retarding the absorption of glucose in the digestive tract.

Liver disease constitutes another endemic problem in the world, causing the death of about 25,000 people annually due to liver cirrhosis caused by hepatitis (5) and lesions due to injury by free radical derivatives of paracetamol (6). Since the latter generates lipid peroxidation, the inhibition of the generation of free radicals is important in the protection against paracetamol-induced hepatopathy (7). In this perspective, natural antioxidants may play a key role in hepatoprotection. However, even though there is an increased assumption that natural products are safe, scientific studies have shown that several phytochemicals

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can be cytotoxic, genotoxic, and carcinogenic when consumed excessively and irrationally (8). The toxicity of medicinal plants depends on many factors such as the part of plant and the extraction solvent used, which will determine the composition and biological activity of the extracts as well as the bioavailability of their components. Moreover, poor handling of raw or processed materials may produce exogenous toxic contaminants, not inherent as plant phytochemicals (9).

In recent years, several authors investigated the antidiabetic potential of Algerian medicinal plants using experimental animals (10,11). Some species of *Fraxinus* have been examined for their hypoglycemic potential (12), but no data are available concerning *Fraxinus angustifolia*. The leaves and bark of this tree, endemic in Northwestern Africa, are traditionally used as diuretic, antirheumatoid, laxative, sudorific (13), hypotensive, hypoglycemic, and antiseptic (14–16). Therefore, the aim of the present study was to examine in vivo the subchronic toxicity and the antidiabetic and hepatoprotective potentials of ethanolic extracts of *F. angustifolia*. In order to understand the mechanism of antidiabetic action, the α-amylase activity of the extracts was also tested. Moreover, high performance liquid chromatography (HPLC) profiles of the extracts were established and compared with standards for partial characterization.

2. Materials and methods

2.1. Preparation of plant extracts

Fresh leaves and barks of *F. angustifolia* were harvested from Tizi Neftah, a remote place in the Province of Amizour, Bejaia, northeastern Algeria. The plant was identified in the Laboratory of botany (University of Bejaia) where a specimen was deposited. The leaves and barks were air-dried in the shade and ground to fine powders (63 μm in diameter); they were extracted separately with ethanol (1/4: w/v) at room temperature for 24 h. The extractive liquids were evaporated to obtain dry crude hydroalcoholic extracts of *Fraxinus angustifolia* leaves (FAL) and *Fraxinus angustifolia* bark (FAB).

2.2. HPLC fractionation and analysis

Extracts of FAB and FAL (3 g) were fractionated by HPLC with a photodiode array detector (HPLC-DAD) (pf 425–250 Interchim, C8 column Zorbax, 150 × 21.5 mm (250 bars)) at a concentration of 100 g/L. The mobile phase consisted of methanol (A) and water (B) with the following elution gradient: 0 min 0% A 100% B, 26.0 min 100% A 0% B; 26.1–29.4 0% A 100% B, 29.4–29.5 0% A 100% B. The flow rate was 20 mL/min and the column temperature was set at 25 °C.

Chemical characterization of FAL and FAB extracts was carried out by comparing the detected polyphenol peaks with respect to retention times with those of standard chemicals (such as chlorogenic acid, tyrosol, benzoic acid, caffeic acid, vanillic acid, syringic acid, sinapic acid, ferulic acid, oleuropein, quercetin, luteolin, cinnamic acid, and apigenin) that were monitored at 250 nm using the same HPLC system. All standards were purchased from Extrasynthese or Sigma-Aldrich. Identity and purity of the chemical standards were assessed by HPLC analysis.

2.3. Laboratory animals

Albino male mice (25 ± 3 g) and Wistar rats of either sex weighing 180–220 g obtained from Pasteur Institute (Algiers, Algeria) were provided with standard food and water ad libitum; the animals were kept in a constant controlled environment (temperature of 23 ± 1° C, relative humidity of 65 ± 5%, and 12/12 h light/dark cycle). After two weeks of acclimatization to laboratory conditions, experiments were conducted in strict compliance with internationally accepted principles for work with laboratory animals (directive N° 2010/63/EU of 22 September 2010, which updates and replaces directive N° 86/609/EEC of 24 November 1986).

2.4. Determination of total polyphenols and flavonoids

The quantification of total polyphenols was conducted according to the Folin–Ciocalteu procedure (17), using catechin as a standard. First, 0.1 mL of extract (3.1 mg/mL in methanol), 0.5 mL of Folin–Ciocalteu (1/10) reagent, and 1.5 mL of Na_2CO_3 (20%) were mixed. After incubation for 1 h, absorbance was measured at 765 nm. Results were expressed as mg of catechin equivalents per gram of extract obtained from a regression curve. Flavonoid content was determined using a colorimetric method (18) with some modifications. Then 1 mL of extract (0.15 mg/mL in methanol) was mixed with 0.5 mL of $AlCl_3$ and incubated for 10 min, after which the absorbance was measured at 430 nm. Results were calculated as mg rutin equivalents per gram of extracts obtained from a regression curve.

2.5. Subchronic toxicity

Twenty-five Wistar male rats were randomized into 5 groups (n = 5) and administered orally daily doses of the following solutions: group I received physiological saline (0.9%); groups II and III received FAL and FAB extracts at 20 mg/kg, respectively; and groups IV and V were given FAL and FAB extracts at 200 mg/kg, respectively. Rats were weighted on a daily basis and observations were made twice daily for mortality and changes in general behavior. At the end of the experiment (28 days), mice were anesthetized, sacrificed, and blood samples were collected for determination of hematological and biochemical parameters.

2.6. Antidiabetic activity in vivo

Diabetes was induced in Wistar rats fasted overnight by an intraperitoneal injection of 60 mg/kg of a solution of streptozotocin (STZ) (Sigma). Rats that developed glycemia with glucose levels higher than 200 mg/dL 72

h after STZ injection (19) were preselected and assigned to 6 groups (n = 5): group I (control) received vehicle (physiological saline 0.9%); group II received a reference molecule, Diabenil (50 mg/kg); groups III and IV received FAL and FAB extracts (25 mg/kg) respectively, and groups V and VI received FAL and FAB extracts (50 mg/kg), respectively. The volume of the suspension administered was based on body weight measured immediately prior to each dose (10 mL/kg). Blood withdrawn from the retro–orbital sinuses of the eyes was collected in heparinized tubes, after 1 and 2 h of treatment. Recovered blood was then centrifuged at 1500 × *g* for 10 min for glucose measurements using SPINREACT kit (cat. no-1001191).

2.7. α-Amylase inhibition assay

α-Amylase inhibition by FAL and FAB extracts was assessed using the method of Hasenah et al. (20). First, 400 µL of substrate solution prepared by using potato starch (0.5%) in phosphate buffer (20 mM, pH 6.9) and containing sodium chloride (6.7 mM) was mixed with 40 µL of plant extract (75–250 µg/mL) and 160 µL of distilled water. The enzymatic reaction was initiated by the addition of 200 µL of *Aspergillus oryzae* α-amylase (4 units/mL) (Sigma Aldrich) followed by incubation for 3 min at 25 °C. Subsequently, 200 µL of the reaction mixture was added to 100 µL (96 mM) 3,5-dinitrosalicylic acid sodium potassium tartrate (5.31 M) dissolved in NaOH (2 M) and incubated at 85 °C for 15 min. After addition of 900 µL of distilled water, the absorbance was measured at 540 nm. Enzyme inhibition was calculated using the following formula: % inhibition = $[1 - (A_t/A_{c)}] \times 100$, where A_t and A_c are absorbances of the test solutions with and without extract, respectively.

2.8. Hepatoprotective potential

The hepatoprotective effect of *F. angustifolia* ethanol extracts against paracetamol-induced toxicity was investigated using the method of Yeh and Hu (21). Forty male albino mice (25 ± 2 g) were randomly allocated to control and test groups (n = 8) in the following manner: group I (negative control) received saline solution (0.9%); group II (positive control) received an oral overdose of paracetamol (10 g/60 kg) on day 1 and serum saline (0.9%) on days 2 and 3; groups III, IV, and V were administered paracetamol (10 g/60 kg) on day 1 and FAL (50 mg/kg), FAB (50 mg/kg), and a mixture of both (25 mg/kg of each), respectively, on days 2 and 3.

2.8.1. Estimation of biochemical parameters

At the end of the experiment, mice were anesthetized and sacrificed. Blood samples were collected from their retro–orbital sinus and their livers were removed for further analysis. Blood samples were allowed to clot and sera were separated by centrifugation (2500 × *g*, 37 °C), stored at –20 °C until use for determination of the following hematological and biochemical parameters: transaminases (aspartate transaminase (AST) (cat.no-HBE06), alanine transaminase (ALT) (cat.no-HBE07), and total bilirubin) using commercial kits (Cypress Diagnostics).

2.8.2. Lipid peroxidation assay

Mice livers were excised promptly, weighed, and washed thoroughly in cold saline (0.9%). One gram of liver was chopped into 4–5 volumes of Tris buffer (0.15 M, pH 7.4) and homogenized. Lipid peroxidation was assayed according to standard procedures (22). Briefly, 1 mL of normal saline and 2 mL of 10% trichloroacetic acid (TCA) were added to 1 mL of tissue homogenate, and the mixture was centrifuged (3000 × *g*, 10 min). Then 2 mL of the obtained supernatant were removed, to which 0.5 mL of thiobarbituric acid (TBA) (1%) was added followed by heating at 100 °C for 30 min. The absorbance of the samples was measured at 532 nm and the MDA levels were expressed as nmoles MDA/mg of liver tissue using the extinction coefficient $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (23).

2.8.3. Histological analysis

Liver tissues isolated previously were fixed in 10% formalin solution. Paraffin blocks were prepared after completing the tissue processing in different grades of alcohol and xylene. Sections (4 µm) were prepared from paraffin blocks using a microtome and stained with hematoxylin and eosin. Microscopic examinations were conducted to detect signs of liver damage such as necrosis (neutrophil infiltration and dilatation of sinusoids), steatoses (fat deposits), and dilatation and congestion of the centrilobular vein.

3. Statistical analysis

Results were expressed as means ± standard deviation for in vitro tests or standard error of the means (SEM) for in vivo tests. Experimental data were analyzed using GraphPad Prism 5.3 software. Analysis of variance (ANOVA) was used, followed by Dunnett's test. The results were considered statistically significant at $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

4. Results

4.1. HPLC analysis

HPLC analysis of bark and leaf extracts (Figure 1) showed profiles with retention times that coincided exactly with some of these chemicals. Mainly, for bark, retention times of fractions 1, 2, 6, and 8 coincided with tyrosol, caffeic acid, pinoresinol, and luteolin, respectively. For leaves, retention times of fractions 1, 2, and 3 were found identical to those of oleuropein, quercetin, and cinnamic acid.

4.2. Determination of total polyphenols and flavonoids

Data shown in Table 1 indicate the presence of a substantial amount of polyphenols in the FAB extract compared with its FAL counterpart, while a greater amount of flavonoids was found in the FAL than in the FAB extract.

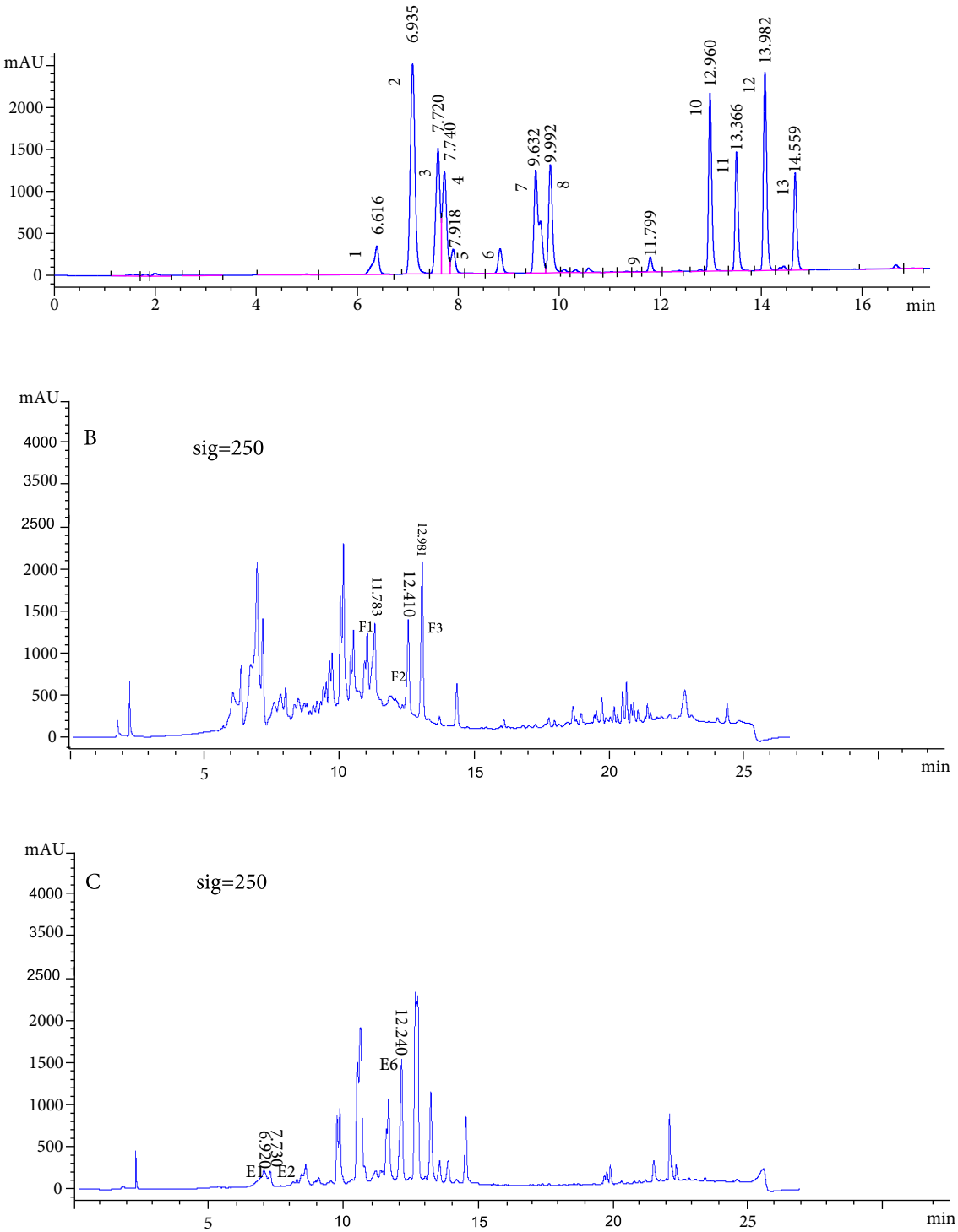

Figure 1. A) Chromatogram of standards: 1: chlorogenic acid; 2: tyrosol; 3: benzoic acid; 4: caffeic acid; 5: vanillic acid; 6: syringic acid; 7: sinapic acid; 8: ferulic acid; 9: oleuropein; 10: quercetin; 11: luteolin; 12: cinnamic acid; 13: apigenin. B) Chromatogram of *F. angustifolia* leaf extract: F_1: oleuropein; F_2: cinnamic acid; F_3: quercetin. C) HPLC chromatogram of *F. angustifolia* bark extract: E_1: tyrosol; E_2: caffeic acid; E_6: pinoresinol; E_8: luteolin.

Table 1. Determination of total polyphenols and flavonoids in *Fraxinus angustifolia* leaf and bark extracts.

	Total polyphenols mg Eq catechin/g extract		Total flavonoids mg Eq rutin/g extract	
Ethanol extract	FAL 194.05 ±14.08	FAB 715.96±59.62	FAL 100.29 ± 17.60	FAB 50.00 ± 17.64

FAL: *F. angustifolia* leaves; FAB: *F. angustifolia* bark.

4.3. Subchronic toxicity

Indications of toxicity are alterations in clinical signs such as diarrhea, weight loss, agitation, hispid hair, convulsions, tremors, dyspnea, and mortality (24). The close monitoring of mice during the whole treatment period indicated no such toxicity signs. Moreover, compared with the control group, no significant loss or gain of weight for any of the tested groups was observed during or at the end of the 28 days experiment (Figure 2).

No change in AST activity was observed in mice treated with either FAL (81.5 ± 3.8 and 79.0 ± 3.3 IU/L at 20 and 200 mg/kg, respectively) or FAB (77.0 ± 3.8 and 72.7 ± 2.1 IU/L at 20 and 200 mg/kg, respectively). Nevertheless, with respect to ALT, considered to be more specifically related to liver problems, a slight and dose-dependent toxicity was recorded in mice treated with FAL (45.5 ± 1.8 IU/L), while FAB (28.2 ± 1.9 IU/L) (200 mg/kg) reduced significantly the level of this enzyme (Table 2). At the same time, total bilirubin was unchanged with FAB (0.73 ± 0.07 mg/mL), but showed dose-dependent decreased levels with FAL (0.56 ± 0.04 mg/mL) (Table 3). In addition, blood sugar (1.03 ± 0.05 and 1.11 ± 0.04 g/L for FAL and FAB, respectively) or cholesterol levels (0.51 ± 0.03 g/L; 0.44 ± 0.04 g/L for FAL and FAB, respectively) were not affected (Table 3), while creatinine was either not altered for FAL at 20 mg/kg (9.80 ± 0.74 mg/mL) or significantly ($P < 0.05$) reduced for FAL at 200 mg/kg (4.16 ± 0.17 mg/mL) and for FAB at 200 mg/kg (4.06 ± 0. 35 mg/mL).

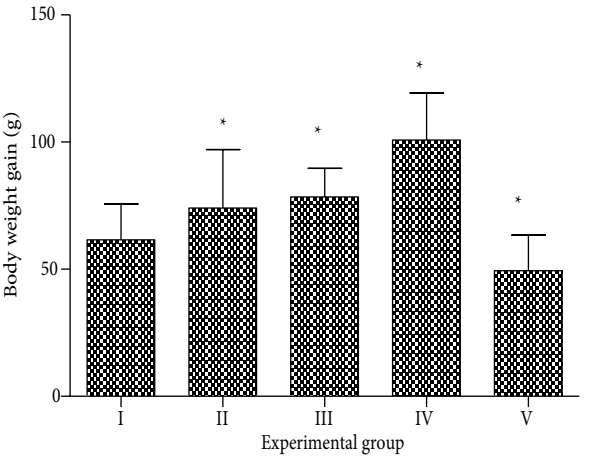

Figure 2. Bodyweight gain after 28 experimental days. FAL: *Fraxinus angustifolia* leaves, FAB: *Fraxinus angustifolia* bark. I: Control, II: FAL 20 mg/kg, III: FAB 20 mg/kg, IV: FAL 200 mg/kg, V: FAB 200 mg/kg. All values are expressed as means ± SEM of six animals. One-way ANOVA followed by Dunnett's test was used for statistical significance. *$P < 0.05$, compared with the normal control group.

4.4. Antidiabetic activity

4.4.1. Antidiabetic activity in vivo

Induction of diabetes in experimental rats was confirmed by a considerable rise in fasting blood glucose (FBG) levels (> 150 mg/dl) for the diabetic control rats (Figure 3). On the other hand, FAL reduced FBG by 45% and 68% at 25

Table 2. Effect of prolonged administration (28 days) of *F. angustifolia* leaf and bark ethanol extracts on mean (± SEM) serum enzyme parameters.

Groups	AST IU/L	ALT IU/L	ALP IU/L
I	73.0 ± 2.8^{a}	31.6 ± 1.8^{b}	236.0 ± 3.3^{b}
II	81.5 ± 3.8^{c}	39.0 ± 2.3^{c}	234.0 ± 7.1^{b}
III	77.0 ± 3.8^{ab}	33.6 ± 2.6^{b}	456.6 ± 6.9^{d}
IV	79.0 ± 3.3^{ab}	45.5 ± 1.8^{d}	296.6 ± 10.3^{c}
V	72.7 ± 2.1^{a}	28.2 ± 1.9^{a}	152.0 ± 3.8^{a}

IU/L: International unit/L. AST: aspartate amino transferase. ALT: alanine amino transferase. ALP: alkaline phosphatase G-I: Control, G-II: FAL 20 mg/kg, G-III: FAB 20 mg/kg, G-IV: FAL 200 mg/kg, G-V: FAB 200 mg/kg. Each value in the table is the mean ± (SEM) ($n = 6$). Values in the same column sharing different letters are significantly different ($P < 0.05$) (ANOVA followed by Newman–Keuls).

Tablea3. Effect of prolonged administration (28 days) of *F. angustifolia* ethanol leaf and bark extracts on mean (± SEM) biochemical parameters.

Groups	Creat mg/mL	Glc g/L	Chol g/L	TBil mg/mL
I	8.60 ± 0.48^{d}	1.01 ± 0.09^{ab}	0.47 ± 0.08^{ab}	1.10 ± 0.15^{c}
II	9.80 ± 0.74^{d}	1.03 ± 0.05^{ab}	0.59 ± 0.05^{c}	1.02 ± 0.23^{c}
III	4.94 ± 0.30^{b}	1.11 ± 0.04^{b}	0.42 ± 0.04^{a}	0.73 ± 0.07^{b}
IV	4.16 ± 0.17^{a}	1.026 ± 0.03^{ab}	0.51 ± 0.03^{bc}	0.56 ± 0.04^{ab}
V	4.06±0. 35^{a}	0.98±0.12^{a}	0.44±0.04^{ab}	0.61 ± 0.25^{a}

Creat: creatinine, **Glc:** glucose, **Chol:** cholesterol, **TBil**: Total bilirubin, G-I: Control, G-II: FAL 20 mg/kg, G-III: FAB 20 mg/kg, G-IV: FAL 200 mg/kg, G-V: FAB 200 mg/kg. Each value in the table is the mean ± (SEM) (n = 6). Values in the same column sharing different letters are significantly different (P < 0.05). (ANOVA followed by Newman–Keuls).

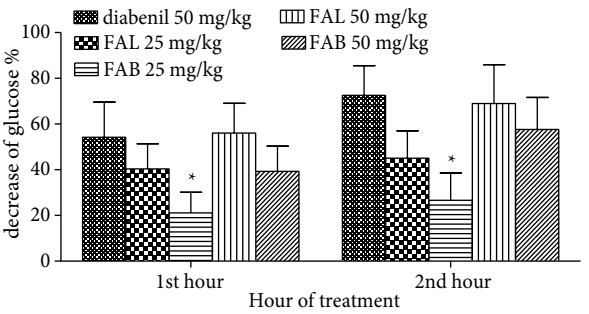

Figure 3. Percent decrease of glucose after the first and second hour of treatment. FAL: *F. angustifolia* leaves; FAB: *F. angustifolia* barks. All values are expressed as means ± SEM of six animals. One-way ANOVA followed by Dunnett's test was used for statistical significance. *P < 0.05, compared with the Diabenil group.

and 50 mg/kg, respectively, compared to 26% and 57% at the same respective doses for FAB. Diabenil (50 mg/kg), a well-known antidiabetic drug, caused a significant (P < 0.01) decrease of blood glucose (72%) after 2 h of treatment, comparable (P > 0.05) with that of FAL at 50 mg/kg.

4.4.2. Antidiabetic activity in vitro: α-amylase inhibition assay

In the α-amylase inhibition assay, the extracts exerted a moderate, dose-dependent inhibition on this enzyme (IC_{50} values of 187.42 and 360.75 µg/mL for FAL and FAB, respectively) (Figure 4), significantly (P < 0.05) lower than that of the standard acarbose (IC_{50} = 15.87 µg/mL).

4.5. Hepatoprotective potential

In order to estimate if liver necrosis was prevented by *F. angustifolia* extracts after paracetamol treatment, three parameters were evaluated: plasma transaminases, MDA levels, and liver histology.

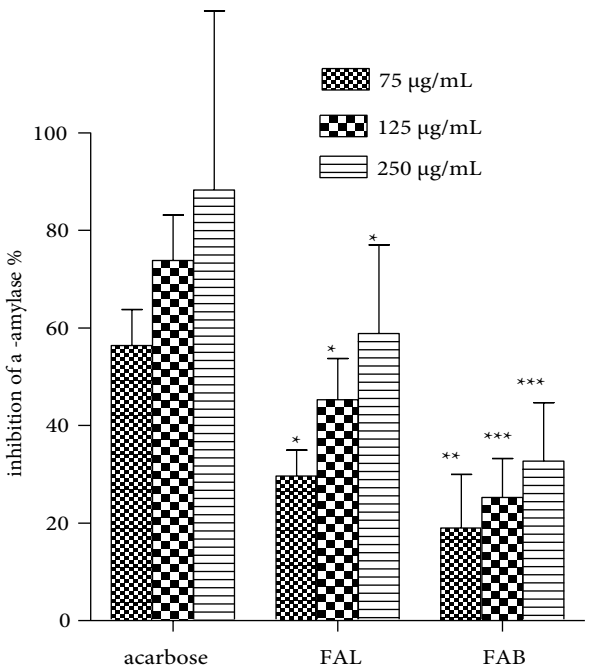

Figure 4. Percent inhibition of α-amylase by *Fraxinus angustifolia* ethanolic leaf and bark extracts. FAL: *Fraxinus angustifolia* leaves. FAB: *Fraxinus angustifolia* bark. All values are expressed as means ± SEM of six animals. One-way ANOVA followed by Dunnett's test was used for statistical significance. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the acarbose group.

4.5.1. Blood parameters

Paracetamol significantly (P < 0.05) raised hepatic enzyme levels (AST = 241.83 ± 67.33 IU/L, ALT = 66.66 ± 15.25 IU/L) and total bilirubin (1.41 ± 0.40 mg/mL) in the positive control group (Table 4, group II). While the FAB extract (50 mg/kg) failed to protect liver cells, the FAL extract led to a significant (P < 0.05) decrease in transaminase levels (AST = 200.20 ± 35.89 IU/L; ALT =

Table 4. Effect of *F. angustifolia* ethanol leaf and bark extracts on serum enzyme levels after treatment with paracetamol (10 g/60 kg).

Groups	AST IU/L	ALT IU/L	TBil mg/mL
I	78.90 ± 9.30[a]	59.5 ± 9.41[a]	0.95 ± 0.48[a]
II	241.83 ± 67.33[c]	66.66 ± 15.25[ab]	1.41 ± 0.40[b]
III	200.20 ± 35.89[cd]	63.98 ± 9.45[a]	1.42 ± 0.35[a]
IV	236.94 ± 27.45[d]	84.34 ± 12.53[b]	0.79 ± 0.08[a]
V	208.54 ± 48.63[b]	71.01 ± 17.22[ab]	0.85 ± 0.14[a]

IU/L: International unit/L. **AST:** aspartate amino transferase, **ALT:** alanine amino transferase, **TBil** : total bilirubin, G-I: Control-, G-II: paracetamol group 10 g/60 kg, G-III: FAL 50 mg/kg, G-IV: FAB 50 mg/kg, G-V: FAL/FAB 50 mg/kg. Each value in the table is the mean ± (SEM) (n = 8). Values in the same column sharing different letters are significantly different (P < 0.05) (ANOVA followed by Newman–Keuls).

63.98 ± 9.45 IU/L for group III and AST = 236.94 ± 27.45 IU/L; ALT = 84.34 ± 12.53 IU/L for group IV) (Table 4). Moreover, FAL extract prevented liver damage much better than the combined treatment of 25 mg/kg of each extract (FAB and FAL) (AST = 208.54 ± 48.63 IU/L; ALT = 71.01 ± 17.22 IU/L) (Table 4, group V). On the other hand, total bilirubin level was restored to its normal level completely and equally by all the treatments (1.42 ± 0.35 mg/mL for FAL; 0.79 ± 0.08 mg/mL for FAB and 0.85 ± 0.14 mg/mL for FAL/FAB).

4.5.2. Lipid peroxidation assay

MDA is a widely used marker of free radical-mediated lipid peroxidation, which explains why a significant increase (P < 0.05) of MDA (0.45 ± 0.040 nmol/mg liver tissue) in paracetamol-induced mice was observed compared with the negative control group (0.12 ± 0.07 nmol/mg liver tissue) (Figure 5). On the other hand, an ameliorative effect on the MDA levels (P < 0.05) in groups pretreated with FAL or FAB extracts (0.290 ± 0.034 and 0.340 ± 0.038 nmol/mg liver tissue) was noticed. Simultaneous treatment with both extracts provided even a better hepatoprotective effect (MDA = 0.250 ± 0.058 nmol/mg liver tissue) (Figure 5).

4.5.3. Histological analysis

Signs of extensive damage in livers of paracetamol-treated mice were obvious in the histological sections of the positive control group with massive centrilobular necrosis (leucocytes infiltration around the centrilobular veins) and some periportal degeneration (Figure 6). However, FAL and FAB provided significant protection against paracetamol-induced damage as the centrilobular area (i.e. the area in which covalent binding of paracetamol is preferentially localized) showed only little degeneration (Figures 6C and 6D). However, less protection was observed with the simultaneous treatment with both extracts (6E), which does not fully corroborate with MDA data.

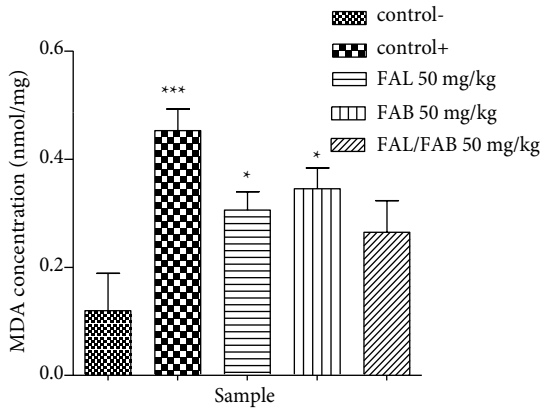

Figure 5. Malondialdehyde (MDA) levels in mice pretreated with *F. angustifolia* ethanol leaf and bark extracts. FAL: *Fraxinus angustifolia* leaves, FAB: *Fraxinus angustifolia* barks. All values are expressed as means ± SEM of six animals. One-way ANOVA followed by Dunnett's test was used for statistical significance. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the control group.

5. Discussion

5.1. HPLC analysis

The present HPLC system was able to detect all the standards as the respective simple peaks with good resolution (Figure 1A). The most remarkable observation from this data is that leaf and bark extracts do not share common chemicals and that all of the compounds with the exception of luteolin in bark were previously detected in this species (25,26). Moreover, the occurrence of secoridoids such as oleuropein and phenylethanoids such as tyrosol is a characteristic feature of *Fraxinus* species (26).

5.2. Determination of total polyphenols and flavonoids

Fraxinus angustifolia is a widely used shrub in folk medicine in Algeria. Its healing virtues are related to its high content of natural compounds, mainly polyphenols,

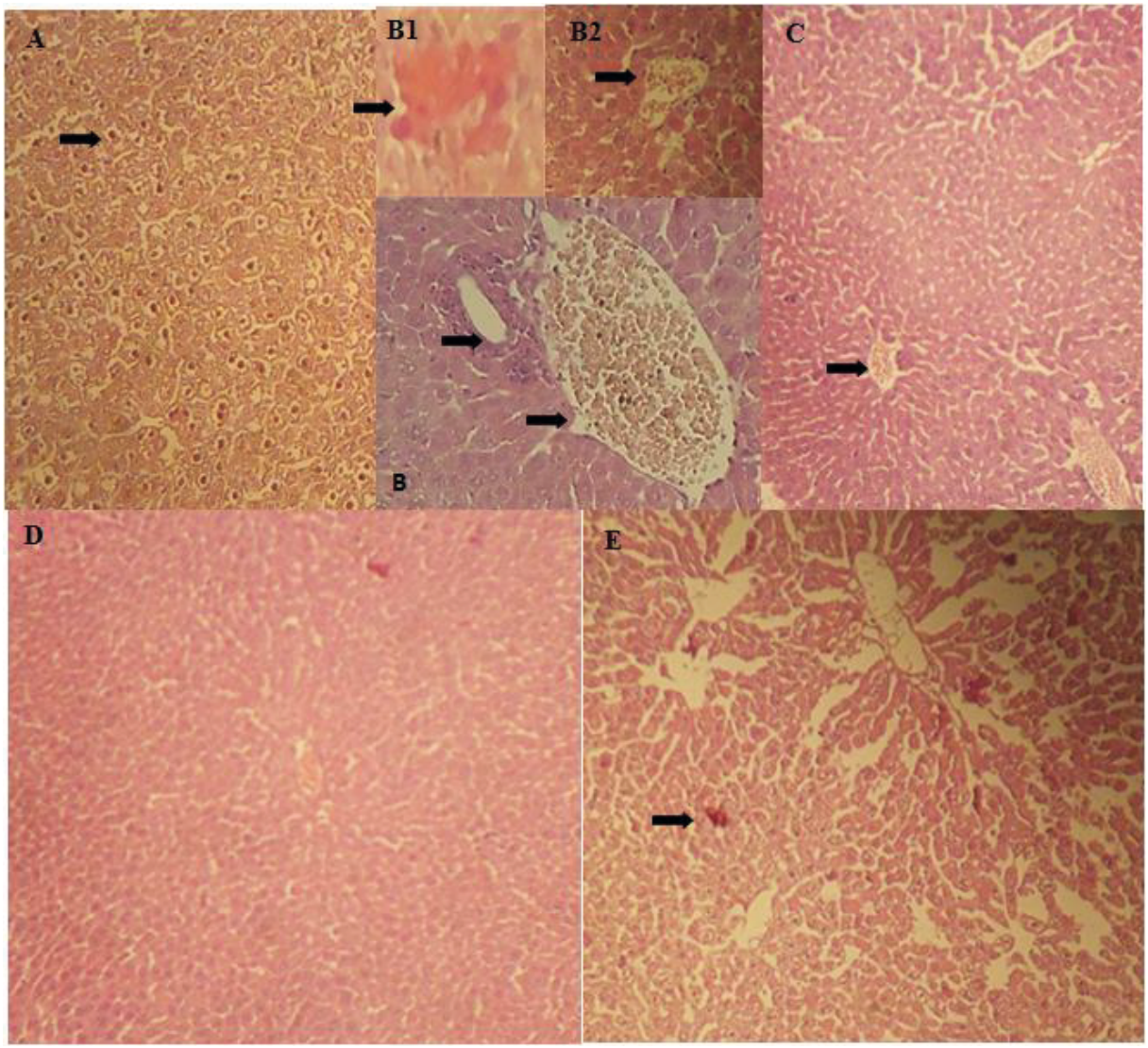

Figure 6. Histological sections of liver (400×). A) Untreated control group. B) Group supplemented with 10 g/60 kg of paracetamol: inflammatory mass, massive necrosis (B_1), centrilobular veins dilated and congested (B_2), dilated sinusoids. C) Group treated with 50 mg/kg of FAL, conserved architecture centrilobular vein dilated, no necrosis. D) Group treated with 50 mg/kg FAB conserved architecture with some necrosis. E) Group treated with leaves/barks extract, some necrosis.

which have been previously quantified (7,27). The results obtained in those studies are in full agreement with our findings. Additionally, the *Fraxinus* genus is described as a rich source of flavones and flavonols that are derivatives of quercetin and kaempferol (26). The above HPLC analysis confirmed the presence of a flavone, luteolin, and a flavonol, quercetin, in FAB and FAL extracts, respectively.

5.3. Subchronic toxicity

The extent of hepatic injury is assessed by the level of released cytoplasmic enzymes, AST and ALT, in circulation (28,29). The unaltered activity of AST, ALT, or bilirubin for both FAL- and FAB-fed mice (except for a slight rise in ALT in FAL-treated mice) (45.5 ± 1.8 IU/L) confirmed that the treatment did not cause hepatocellular injury. In addition, as revealed by glucose, cholesterol, and creatinine data, the extracts did not disrupt sugar or lipid metabolisms and did not cause impairment of kidney function. Aside from the interest in the efficacy of medicinal plant extracts against various diseases, safety (short and long term) is an important factor in traditional medicine. This experiment showed that the long-term administration of both parts of *F. angustifolia* did not present any toxicity.

5.4. Antidiabetic activity

5.4.1. Antidiabetic activity in vivo

The rise in glucose levels in the control positive group was counteracted by a maximal hypoglycemic effect in the test

groups, which was observed more specifically in 2 h, and more so in the case of FAL compared with FAB treatment. No previous data about the antidiabetic activity of *F. angustifolia* was reported, but findings on a related species, *Fraxinus excelsior* (12), were less promising because a single oral administration of an aqueous extract (20 mg/kg) of this plant reduced blood glucose levels from the 1st ($P < 0.01$) to the 6th h ($P < 0.001$) by only 13.6% in diabetic rats. The cytotoxic activity of STZ on pancreatic cells is based on the prooxidant action mechanism of STZ, acting as an $NO^{\cdot}$ donor and causing the fragmentation of DNA (30). Therefore, we assume that the high tolerance to glucose observed can be attributed to the antioxidant activity of the constituent flavonoids of *F. angustifolia* as higher amounts of these compounds in leaves led to higher protective activity against the toxic impact of STZ compared with that of the bark. The antioxidant potential of *F. angustifolia* bark extract was demonstrated in previous studies (27,31). Moreover, the current study revealed the presence of a number of phytochemicals that constitute a major group of compounds that act as primary antioxidants with high redox potentials and singlet oxygen quenchers (32). Some of these compounds, such as quercetin and luteolin, have shown strong antidiabetic activities (33), which corroborates earlier investigations that equally established a close relationship between the antioxidant properties of polyphenolic compounds and their antidiabetic potential (34,35).

5.4.2. Antidiabetic activity in vitro: α-amylase inhibition assay

Pancreatic α-amylase catalyzes the initial step in the hydrolysis of starch, a principal source of glucose in the diet (36). Therefore, the inhibition of this enzyme by FAL and FAB extracts would prevent more glucose release in the blood. The higher inhibition by FAL over FAB extract observed consolidates the above findings of the in vivo experiment in which the FAL extract was a more potent hypoglycemic agent than its bark counterpart. Moreover, this assay helped to gain some insight on the possible mechanism of action of *F. angustifolia* extracts by revealing that they contain α-amylase inhibitory metabolites, although other mechanisms cannot be excluded. In fact, past clinical studies have demonstrated that phenolic compounds, such as luteolin, that inhibit carbohydrate-hydrolyzing enzymes decrease postprandial hyperglycemia in noninsulin-dependent diabetes mellitus patients; however, it was less potent than acarbose (37).

5.5. Hepatoprotective potential

Paracetamol-induced hepatic injury is a commonly used experimental model for the screening of hepatoprotective drugs (38,39). The lesions produced are the result of the prooxidant effect of paracetamol, affecting blood parameters.

5.5.1. Blood parameters

The deleterious effect of paracetamol provoked an increase in transaminase levels, in agreement with previous findings (40), as a result of induced acute toxicity and destruction of liver cells. The administration of the FAL extract contributed to restoring the transaminase enzyme activities to their normal level, which was not the case for the FAB extract, which was insufficient to prevent the damage to liver cells by paracetamol.

5.5.2. Lipid peroxidation assay

Paracetamol is converted in the liver into its reactive metabolite N-acetyl [-p-benzo quinine imine] by hepatic cytochrome P-450 (40–42), leading to massive production of reactive species and depletion of protective physiological moieties such as glutathione and α-tocopherol. This ultimately leads to lipid peroxidation, which causes damage to the macromolecules in vital biomembranes (43,44), generating necrosis. Our experiment demonstrated that FAL was more efficient than FAB extracts in attenuating the MDA levels (produced as a result of lipid peroxidation). However, a more prominent effect was observed with simultaneous treatment, demonstrating a synergistic action between the compounds of both parts of the plant. HPLC analysis revealed the presence of some strong antioxidant compounds in both extracts such as caffeic acid and luteolin in bark and quercetin in leaves. However, limited information about the bioactive molecule responsible for the observed synergistic effect makes its identification imperative and even interesting. Indeed, knowledge of the molecular structures would help us gain insight into the mechanisms involved and widen the perspective of their exploitation in the pharmacological industry. Moreover, this route constitutes another research interest of our team in our continuous quest for identification of natural substances from local medicinal plants. For now, we can only assume that the counteracting effects of *F. angustifolia* against paracetamol toxicity may be due to the antioxidant potential of the extracts of this plant reported in an earlier in vitro study (25). The above data revealing higher quantity of flavonoids in FAL than in FAB may be an additional proof that the antioxidant character of the leaf constituents may lie behind their enhanced activity over FAB against reactive species generated by paracetamol. Patients with diabetes mellitus are characterized with elevated levels of oxidative damage and decreased levels of antioxidant defenses, and are prone to lipid abnormalities due to lipid peroxidation (45). Oxidative stress puts diabetes patients at high risk for the development of coronary disease and renal and liver failures. Treatment strategies that rely on insulin or Diabenil do not offer an antioxidant machinery to fight the prooxidant diseases that are associated with diabetes. Therefore, natural remedies of antioxidant character are good candidates to fight diabetes and associated problems, keeping in mind their lack of secondary effects that are common in modern drugs.

5.5.3. Histological analysis

The analysis of liver histology concluded that less necrosis and damage were generated in the extract-treated groups, and especially in those receiving FAL, compared with the control group. These results confirmed the above findings related to MDA and transaminase levels, with the exception of the group treated with both parts of the plant. The results of studies on the mechanism of hepatic necrosis suggest that lesion genesis proceeds in a sequence of events (46,47). The first can be metabolic activation of a toxic reactive intermediate as in the case of paracetamol. The latter might be irreversible destruction of cell components leading to disturbances in essential biochemical processes. This means that protection by *F. angustifolia* extracts against paracetamol-induced hepatotoxicity might involve inhibition of oxidative formation and/or detoxification of the paracetamol reactive metabolite. The high antioxidant capacities of the extracts (26) are probably implicated in those mechanisms.

In conclusion, the present work demonstrated that leaf and bark extracts of *F. angustifolia* are safe for use in folk medicine. It also shed light on their antidiabetic and hepatoprotective potentials, revealing higher activities for leave extracts than for their bark counterparts, which can be accounted for by their higher flavonoid content. The antioxidant power of flavonoids are surely implicated in the hepatoprotective potential of the plant and partially involved in its hypoglycemic action since other mechanisms, such as the inhibition of α-amylase, can play a considerable role. Therefore, *F. angustifolia* extracts might be considered for use in association with insulin therapy to alleviate the adverse side effects caused by that drug.

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References

1. Kuzuya T, Nakagawa S, Satoh J, Kanazawa Y, Iwamoto Y, Kobayashi M, Nanjo K, Sasaki A, SeinoY, Ito C et al. Report of the committee on the classification and diagnostic criteria of diabetes mellitus. Diabetes Res Clin Pr 2002; 55: 65-85.
2. Chaudhary P, Goel B, Ghosh AK. Anti-diabetic activity of *Adina cordifolia* (Roxb) leaves in alloxan induced diabetic rats. Asian Pac J Trop Biomed 2012; 2:1630-1632.
3. Puranik N, Kammar KF, Devi S. Anti-diabetic activity of *Tinospora cordifolia* (Willd.) in streptozotocin diabetic rats; does it act like sulfonylureas? Turk J Med Sci 2010; 40: 265-270.
4. Newman DJ, Cragg GM, Snader KM, The influence of natural products upon drug discovery. Nat Prod Rep 2000; 17: 215-234.
5. Shan S, Zhenyuan S, Cotler SJ, Cho M. Biomechanics and functionality of hepatocytes in liver cirrhosis. J Biomech 2014; 47: 2205-2210.
6. Barber DJ, Fairhurst S, Horton AA. Effect of old age on paracetamol-induced lipid peroxidation in rat liver (Glutathione; gerontological aspects of drug metabolism). Toxicol Lett 1983; 15: 283-287.
7. Castro JA, Acosta ND, Deferreyra EC, De Castro CR, Diaz Gomez MI, De Fenos OM. Studies on thioacetamide-induced liver necrosis. Toxicol Appl Pharm 1974; 79-86.
8. Hartung R, Cornish HH, Cosmet FD. Acute and short-term oral toxicity of 2-N-ethylaminoethanol in rats. Toxicol Lett 1969; 7: 595-602.
9. Ahmed AS, Elgorashi EE, Moodley N, McGaw LJ, Naidoo V, Eloff JN. The antimicrobial, antioxidative, anti-inflammatory activity and cytotoxicity of different fractions of four South African Bauhinia species used traditionally to treat diarrhea. J Ethnopharmacol 2012; 143: 826-839.
10. Allali H, Benmehdi H, Dib MA, Tabti B, Ghalem S, Benabadji N. Phytotherapy of diabetes in west Algeria. Asian J Chem 2008; 20: 2701-2710.
11. Boudjelal A, Henchiri C, Siracusa L, Sari M, Ruberto G. Compositional analysis and *in vivo* anti-diabetic activity of wild egyptian *Marrubium vulgare* L. infusion. Fitoterapia 2012; 83: 286-292.
12. Maghrani M, Zeggwagh NA, Lemhadri A, El Amraoui M, Michel JB, Eddouks M. Study of the hypoglycaemic activity of Fraxinus excelsior and *Silybum marianum* in an animal model of type 1 diabetes mellitus. J Ethnopharmacol 2004; 91: 309-316.
13. Goodman SM, Hobbs JJ. The ethnobotany of the Egyptian eastern desert: a comparison of common plant usage between two culturally distinct Bedouin groups. J Ethnopharmacol 1988; 23: 73-89.
14. Bellakhdar J, Claisse R, Fleurentin J, Younos C. Repertory of standard herbal drugs in the Moroccan pharmacopoeia. J Ethnopharmacol 1991; 35: 123-143.
15. Martin G, Iserin P. Les plantes par classe therapeutique. Phytotherapy 1993; 40: 29-31 (in French).
16. Legssyer A, Mekhfi H, Dassouli A, Serhrouchni M, Benjelloun W. Phytotherapy of hypertension and diabetes in oriental Morocco. J Ethnopharmacol 1997; 58: 45-54.
17. Montreau FR. Sur le dosage des composés phénoliques totaux dans les vins par la méthode Folin-Ciocalteau. Connaiss Vigne Vin 1972; 24: 397-404 (in French).
18. Mimica-Dukic IN, Ivanec-Tumbas R, Popovic IM, Gasic O. The content and composition of essential oil of *Hypericum perforatum* from Serbia. Pharm Pharmacol Lett 1997; 8: 26-28.

19. Singh R, Singh RK, Tripathi AK, Cornflissen G, Schwartzkopff O, Otsuka K, Halberg F. Chronomics of circulating plasma lipid peroxides and anti-oxidant enzymes and other related molecules in cirrhosis of liver. Biomed Pharmacother 2005; 59: 229-235.

20. Hasenah A, Houghton PJ, Soumyanath A. Amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to *Phyllanthus amarus*. J Ethnopharmacol 2006; 107: 449-455.

21. Yeh SI, Hu MI. Antioxidant and pro-oxidant effect of lycopene in comparison with β carotene on oxidant induced damage in Hs 68 cells. J Nutr Biochem 2000; 11: 548-554.

22. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thio-barbituric acid reaction. Anal Biochem 1979; 95: 351-358.

23. Azofeifa G, Quesada S, Perez AM, Vaillant F, Michel A. Pasteurization of blackberry juice preserves polyphenol-dependent inhibition for lipid peroxidation and intracellular radicals. J Food Compos Anal 2015; 42: 56-62.

24. Caparroz-Assef SM, Grespan R, Freire Batista RC, Bersani-Amado FA, Baroni S, Dantas JA, Nakamura Cuman RK, Bersani-Amado CA. Toxicity studies of *Cordia salicifolia* extract. Acta Scient Health Sci 2005; 27: 41-44.

25. Gaedcke F. Fraxinus. In: Hänsel R, Keller K, Rimpler H, Schneider G, editors. Hagers Handbuch der Pharmazeutischen Praxis. 5th ed. Berlin, Germany: Springer-Verlag; 1993. pp. 188-200 (in German).

26. Kostova I, Iossifova T. Chemical components of *Fraxinus* species. Fitoterapia 2007; 78: 85-106.

27. Atmani D, Chaher N, Berboucha M, Ayouni K, Lounis H, Boudaoud H, Debbache N, Atmani D. Antioxidant capacity and phenol content of selected Algerian medicinal plants. Food Chem 2009; 112: 303-309.

28. Slater TF. A note on the relative toxic activities of tetrachloromethane and trichloro-fluoro-methane on the rat. Biochem Pharmacol 1965; 14: 178-181.

29. Durmuş M, Yilmaz HR, Uz E, Özçelik N. The effect of caffeic acid phenethyl ester (CAPE) treatment on levels of MDA, NO and antioxidant enzyme activities in retinas of streptozotocin-induced diabetic rats. Turk J Med Sci 2008; 38: 525-530.

30. Kröncke KD, Fehsel K, Sommer A, Rodriguez ML, Kolb-bachofen V. Nitric oxide generation during cellular metabolization of the diabetogenic N-methyl-N-nitroso-urea streptozotozin contributes to islet cell DNA damage. Biol Chem 1995; 376: 179-185.

31. Berboucha M, Ayouni K, Atmani D, Atmani D, Benboubetra M. Kinetic study on the inhibition of xanthine oxidase by extracts from two selected Algerian plants traditionally used for the treatment of inflammatory diseases. J Med Food 2010; 13: 896-904.

32. Kähkönen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, Heinonen M. Antioxidant activity of plant extracts containing phenolic compounds. J Agr Food Chem 1999; 47: 3954-3962.

33. Brahmachari, G. Natural Products: Chemistry, Biochemistry and Pharmacology. 1st Ed. New Delhi, India: Narosa Publishing House Pvt. Ltd; 2009.

34. Palsamy P, Subramanian S. Resveratrol, a natural phytoalexin, normalizes hyperglycemia in streptozotocin nicotinamide induced experimental diabetic rats. Biomed Pharmacother 2008; 62: 598-605.

35. Kumar R, Pate DK, Prasad SK, Sairam K, Hemalatha S. Antidiabetic activity of alcoholic leaves extract of Alangium lamarckii Thwaites on streptozotocin-nicotinamide induced type 2 diabetic rats. Asian Pac J Trop Med 2011; 904-909.

36. Tarling CA, Woods K, Zhang R, Brastianos HC, Brayer GD, Andersen RJ, Withers SG. The search for novel human pancreatic α-amylase inhibitors: high-throughput screening of terrestrial and marine natural product extracts. Chem Bio Chem 2008; 9: 433-438.

37. Kim JS, Kwon CS, Son KH. Inhibition of alpha-glucosidase and amylase by luteolin, a flavonoid. Biosc Biotechnol Biochem 2000; 64: 2458-2461.

38. Sallie R, King R, Silva AE, Smith H, Tibbs C, Johnson P, Williams R. Prevalence of hepatitis "C" virus carriage in non-selected, otherwise healthy patients attending general practitioners in South London. J Hepatol 1991; 13: 167.

39. Packer JE, Slater TF, Willson RL. Reactions of the carbon tetrachloride-related peroxyl free radical ($CCl_3O_2^{\cdot}$) with amino acids: pulse radiolysis evidence. Life Sci 1978; 23: 2617-2620.

40. Chedella HK, Alluri R, Ghanta KM. hepatoprotective and antioxidant effect of *Ecbolium viride* (Forssk.) Alston roots against paracetamol-induced hepatotoxicity in albino wistar rats. J Pharm Res 2013; 7: 496-501.

41. Vande Straat R, Ries JV, Vermeulen NPE. Role of hepatic microsomal and purified cytochrome P-450 in one-electron reduction of two quinone imines and concomitant reduction of molecular oxygen. Biochem Pharmacol 1987; 36: 613-619.

42. Pesh-Imam M, Recknagel RO. Lipid peroxidation and the concept of anti oxygenic potential: Vitamin E changes in acute experimental CCl_4-, $BrCCl_3$-, and ethanol-induced liver injury. Toxicol Appl Pharm 1977; 42: 463-475.

43. Aldridge WN. Mechanisms of toxicity: new concepts are required in toxicology. Trends Pharmacol Sci 1981; 2: 228-231.

44. Asayama K, Uchida N, Nwakene T, Hayashibe H, Dobashi K, Amemia S, Kato K, Nakazawa S. Antioxidants in the serum of children with insulin-dependent diabetes mellitus. Free Radical Bio Med 1993; 15: 597-602.

45. Judah JD. Biochemical disturbances in liver injury. Brit Med Bull 1969; 25: 274-277.

46. McLean AEM, Nuttell L. An *in vitro* model of liver injury using paracetamol treatment of liver slices and prevention of injury by some antioxidants. Biochem Pharmacol 1978; 27: 425.

47. Yayla M, Halici Z, Unal B, Bayir Y, Akpinar E, Gocer F. Protective effect of Et-1 receptor antagonist bosentan on paracetamol-induced acute liver toxicity in rats. Eur J Pharmacol 2014; 726: 87-95.