Anthocyanin Extract from Flowers of *Lavender dentate* Beats Oxidative Stress *In vitro* and *In vivo*

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ABSTRACT: Free radicals, oxidative stress or antioxidants is more and more often used to explain different pathological disorders and their therapeutic approach. The aim of this study was to evaluate the protective effect of anthocyanin extract obtained by maceration of the flowers of *Lavandula dentata* in 0.1 % HCl/methanol (v/v) solution on oxidative stress. The antioxidant activity of anthocyanin extract *in vitro* was evaluated by reduction of iron (FRAP), DPPH and the ß-carotene tests. The *in vivo* oxidative stress was induced by intraperitoneal injection of 0.5 ml/kg of CCl_4 and treated orally by 500mg kg/day of the extract. Anthocyanins extract inhibited the free radical DPPH (IC_{50}:1.3 ± 0.23 mg/ml). Lavender extract prevented the oxidation of B carotene (28.34 ± 0.07%) and has an ability to reduce iron (0.736 ± 0.03). Intraperitoneal injection of CCl_4 has increased biochemical parameters, which was evidence of oxidative stress in *vivo*. In contrast, daily oral administration of anthocyanin extract has restored the biochemical parameters. Histopathological examinations of liver stained with haematoxylin and eosin showed loss of hepatic architecture. These injuries observed have been improved by treatment with anthocyanin extract. The findings revealed that anthocyanin extract from lavender possesses a significant antioxidant activity.

Key words: Anthocyanin, *Lavandula dentate*, DPPH, FRAP, β–carotene, Liver, Steatosis

INTRODUCTION

Anthocyanins, a category of flavonoids in broad sense, are a natural water soluble pigments of various flowers and fruits present in food. They are formed by the condensation of an aglycone (or anthocyanidin), non-carbohydrate portion of hetero-sides derived from flavylium ion which is linked to one or various sugar that can be further acylated by aromatic or aliphatic organic acids. Pelargonidin, cyanidin, delphinidin, pelargonidin, petunidin, peonidin, and malvidin are the main anthocyanins widely distributed in nature[1]. According to Khoo *et al.*[2], the protective effect of anthocyanidins and anthocyanins are due to their capacity to scavenging free-radical and by modulating cyclogenase pathway and inflammatory cytokines signaling.

Lavandula dentata is a species of Lamiaceae family native to the Mediterranean regions. It is known for its ornamental and decorative use, as well as for its economic and therapeutic importance.[3] Several studies have focused on the benefits of essential oils and polyphenol–extracts from flowers and leaves[4-8]. Lavander possesses various biological activities. Indeed, Blažeković *et al.*[9] have shown that the essential oil of lavender reduces bacterial and fungal contaminants which preserves the quality of food, cosmetics and pharmaceuticals products. Algeri et *al.*[5] have shown that extract of lavender displayed immunomodulatory properties in the TNBS model of rat colitis and anti-inflammatory effects in the carrageenan-induced paw edema in mice. *Angustifolia lavander* extract alleviated oxidative

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injury in the brain tissue and suppress epileptogenic effect produced by pentylenetetrazol in mice.[10]

However, no studies have been carried out on the anthocyanins of this species which make this study a unique initiative. Thus, we have evaluated the protective effects of anthocyanin extract of *L. dentata* flowers on oxidative stress produced *in vivo* and *in vitro.*

MATERIAL AND METHODS

Plant materials and phytochemical study. The species *Lavandula dentata* was harvested from Tenes located at Chlef (Algeria). The flowers were used in the fresh state. Before extraction of the anthocyanins, we carried out a phytochemical screening of the crude extract to identify of the classes of secondary metabolites present in flowers. Five (5) g of fresh flowers were infused into 100ml of boiling water for 15 minutes. The infused material was–filtered using Whatman fitter paper number 1. The filtrate thus obtained was used for the detection of saponosides, tannins, flavonoids, anthocyanins etc.

Extraction of anthocyanins. The extraction of anthocyanins was carried out according to the method described by Longo and Giuseppe.[11] After the detection of anthocyanins in the flowers, the extraction was made by maceration of 2.5 g of the flowers in 12.5 ml of 0.1 % HCl /methanol (v/v) solution for 20 hours at room temperature, in dark. After filtration, the residue thus obtained was washed with 12.5 ml of HCl / methanol (v/v) at 0.1 %, and dried using a rotary evaporator at 30 °C.

Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH). The DPPH test was–performed following the method described by Burits and Bucar.[12] Here, 50 µl of each of the methanolic solutions of the anthocyanin extract of lavender flowers tested at different concentrations (0.2, 0.4, 1, 2 mg/ml) was mixed with 5 ml of a methanolic solution of DPPH (0.004%). After an incubation period of 30 min at room temperature, the absorbance was read at 517 nm. The ascorbic acid was prepared under the same conditions for use as standard. According to Sharififar *et al.*[13] inhibition of the DPPH free radical as a percentage (I %) was calculated as follows:

$$I\,(\%) = (A_{Blanc} - A_{Sample} / A_{Blanc}) \times 100$$

Where, A_{Blanc} is the absorbance of the DPPH solution without extract

A_{Sample} is absorbance sample of the test compound.

The IC_{50} index defined as the concentration of antioxidant required to decrease the initial DPPH concentration by 50% was determined. All samples were prepared in three independent experiments.

β-carotene–linoleic acid method. This test was performed according to the method described by Kartal *et al.*[14] That consists in measuring at 490 nm the discoloration of β-carotene resulting from its oxidation by the decomposition products of linoleic acid. A mixture made of 0.5 mg of β-carotene, 1 ml of chloroform, 25 µl of linoleic acid and 200 mg of Tween 40 have been prepared. After shaking, the chloroform was removed by a rotary evaporator under vacuum. Subsequently, 100 ml of water saturated with oxygen was added to the preceding mixture with thorough shaking. Then 2.5 ml of the emulsion obtained are added to a series of tubes containing 350 µl of each sample (extract, BHT) prepared at a concentration of 2 mg / ml. After the incubation period, the absorbance of the mixtures were measured at 490 nm. Antioxidant capacity of the sample was compared with those of BHT and the blank (only methanol).

$$\% \text{ Inhibition} = A_{s(t=48h)}/A_{BHT(t=o).}$$

Where A_S represents the absorbance of test sample after incubation period (48h), A_{BHT} is the absorbance of control BHT at the moment of preparation (t = 0). All samples were prepared in three independent experiments.

Reducing power. The reducing power was determined according to the method advocated by Oyaizu[15]. In fact, 1 ml of different concentrations of each extract (0.1, 0.2, 0.3, 0.4, 0.5 mg) diluted in distilled water was mixed with 2.5 ml of the buffer

phosphate (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide [K3Fe (CN) 6]. The mixtures were incubated at 50 ° C for 30 minutes and 2.5 ml of trichloroacetic acid (10%) was added. The mixture was centrifuged at 3000 rpm for 10 min. Then 2.5 ml of the supernatant of each concentration was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%). The absorbance was–measured at 700 nm against a blank. Ascorbic acid was used as a positive control in this experiment under the same operating conditions.

Pharmacological study

Animal models. Twenty-one female albino mice obtained from Pasteur Institute of Algiers aged 3 weeks with an average weight of 27.5±13.75 g were used. The animals were maintained room temperature 22±2 °C and standard 12 h day/night cycle. The animals were fed on a standard diet and with free access to water. Experimental protocol prepared in accordance to the Guide for the Care and Use of Laboratory Animals was approved by the scientific committee of the University. Mice were acclimated for a few days before the experiment and were distributed in cages containing 7 mice per cage.

Group I: control group that received only water. Group II: used as positive control was injected with CCl_4 0.5 ml/kg in olive oil.[16] Group III: the test mice were treated intraperitoneally with the anthocyanin extract at the dose of 500 mg/kg/day. After the 7th day, an intraperitoneal injection of CCl_4 (0.5 ml/kg) was administered to the group III, 30 min after the last in-take of anthocyanin extract.[16]

After one week (7 days), the mice were sacrificed, the blood was collected in heparin tubes for biochemical analysis. The liver was obtained by a longitudinal abdominal opening and was fixed in formalin 10% for histological study by hematoxylin and eosin staining.

Statistical analysis. The data was subjected to analysis of variance (ANOVA) followed by Turkey's test. P values less than 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

Phytochemical screening. After shaking the infused flowers, a foam obtained greater than 1.8 cm which showed that the tested plant contain the saponosides. The appearance of the green color in the infused lavender flowers indicated the presence of catechin tannins after addition of iron chloride. On the other hand, the purplish pink color after the cyanide reaction indicated the presence of flavonones. The presence of anthocyanins was detected by the development of purple red color after addition of ammonia to the infused flowers acidified by HCl.

Antioxidant activity. The results obtained are expressed as percentage inhibition of the free radical (DPPH) as a function of the concentrations of the anthocyanin extract in the lavender flowers (Figure 1). The antioxidant activity exerted on the DPPH free radical by the extract was dose dependent. The anthocyanin extract effectively inhibited the DPPH free radical (61±0,36% at 2 mg/ml)**.** The reference antioxidant "ascorbic acid" showed a very potent capacity on the free radical DPPH (98±4.02%) at 2 mg/ml. Ascorbic acid appears to be the most active with an IC_{50} equal to 0.24±0.12 mg/l while the anthocyanin extract from *L. dentata* flowers have an IC_{50} of 1.3±0.23 mg/ml (Figure 1).

The ability of the anthocyanin extract to inhibit lipid peroxidation was evaluated by the β-carotene decolorization technique**.** Anthocyanins slightly protected the oxidation of β-carotene, with percentage inhibition of 28.34±0.07% for the 2mg/ml concentration. However, BHT displayed most potent activity with percentage inhibition of 98.59±0.12%. It protected the oxidation of β-carotene against the decomposition products of linoleic acid.

The antioxidant activity of the plant extract was investigated, by the iron reduction method, to test and determine the most active extract concentration. The results indicated an increase in absorbance which means an increase in the reducing power of the extracts tested (0.736±0.03). Ascorbic acid showed strong reducing power of iron (Fe^{3+}) in (Fe^{+2}) 2.99±0.18 . while lavender has shown a weak ability.

Biochemical study. The results obtained showed that the intraperitoneal (IP) injection of CCl_4 for 7 days caused metabolic disorder, which was confirmed by an increase in blood glucose, urea, cholesterol, triglycerides ($p > 0.05$) and a significant increase in creatinine compared to the control group (Table 1). The anthocyanin extract of *L. dentata* flowers caused a significant decrease in blood glucose level with intermediate values both markers of kidney function (urea, creatinine) and liver function (cholesterol, triglyceride) between the control and intoxicated mice ($p > 0.05$).

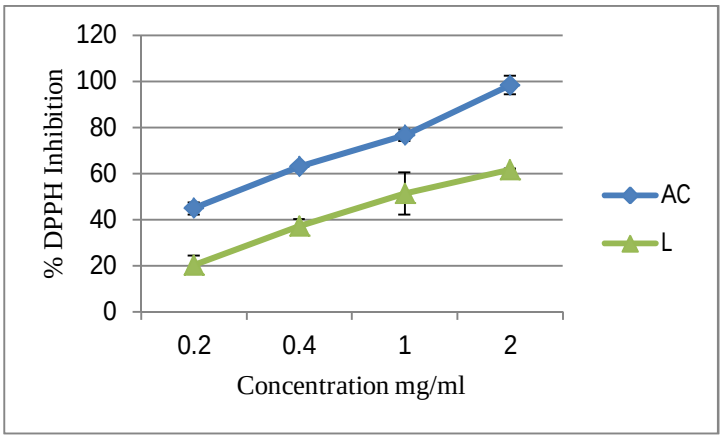

Figure 1. Antioxidant activities of anthocyanin extract of flowers of *L.dentata* as assessed by DPPH free radical scavenging (IC_{50}). AC: IC_{50}= 24±0.12 mg/l, L: IC_{50}= 1.3±0.23 mg/ml.

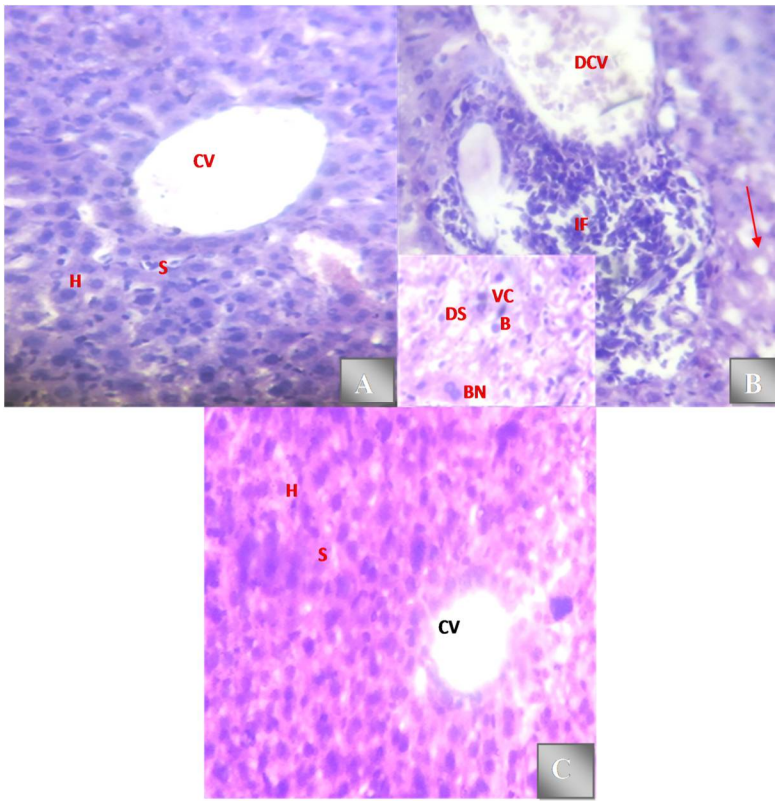

Figure 2. Photography of a section in the liver of control (A), Positive control (B), (C) treated group (H&E staining, Gr x 400). Hepatocyte (H), central vein (CV), sinusoids (S), infiltrate inflammatory (IF), dilated sinusoid (DS), vacuolated cells (V), Ballooning cell (B). red arrow: steatosis.

Histological study. The histopathological examinations of liver stained with hematoxylin and eosin showed that exposure to CCl_4 resulted in loss of hepatic architecture and necrosis such as, inflammatory infiltrate, an onset of steatosis compared to the control section. This section presents a normal hepatocyte cell and sinusoids. These observed lesions have been improved by treatment with the anthocyanin extract which has a cell architecture close to the control (Figure 2).

Anthocyanins are responsible for the color of fruits and vegetables. Many studies have shown that they have strong antioxidant properties suggesting that they have anti-inflammatory and anticarcinogenic activities. They are also able to prevent cardiovascular disease, control obesity, and alleviate diabetes.[17] According to Riaz *et al*.[18] anthocyanins have different pharmacological actions and health promoting effect such as improvement of visual performance, prevent liver toxicity, kidney diseases, colorectal cancer and enhance memory function and prevent age-related mental decline. All previous studies were devoted to lavender regarding essential oils as this medicinal plant is very aromatic. Our study with the anthocyanins extract of *L dentate* is the first. This extract has shown an important antioxidant activity against free radicals tested by *in vitro* and *vivo* methods.

Table 1. Results of biochemical parameters after 7 days. Control group I, positive control group II, treated group III. ($p < 0.05$).

Biochemical parameters	Group I	Group II	Group III
GLY(g/l)	0.97±0.09	1.03±0.22	0.69±0.09 *
UREE(g/l)	0.50±0.14	0.87±0.41	0.82±0.08
CREA(mg/l)	3.10±0.28	6.89±0.42*	5.40±1.55
TG(g/l)	0.88±0.20	1.07±0.46	0.95±0.07
CHOL(g/l)	0.61±0.49	1.51±0.36	0.82±0.26

In the liver, CCl_4 is metabolized by cytochrome (CYP) 2E1, CYP2B1 or CYP2B2, and possibly CYP3A inducing the formation of the free radical CCl_3^-, which attacks lipids causing fatty degeneration (steatosis) and with DNA, it forms adducts initiating liver cancer. Its reaction with oxygen forms a highly reactive species: CClOO* responsible for lipid peroxidation of various membranes of cell organelles such us mitochondrial, endoplasmic reticulum, and plasma membranes leading to alteration of calcium permeability and homeostasis which can substantially provide to subsequent cellular damage[19]. The IP injection of CCl_4 causes a biochemical imbalance marked mainly by increase in liver and renal markers accompanied by hyperglycemia and hyperlipidemia (a good indicator of stress). This means that the administration of carbon tetrachloride for 7 days causes a malfunction of liver confirmed the presence of steatosis.

Creatinine and urea are wastes produced by increased protein metabolism. They are eliminated by the kidneys, and generally used as an indicator of kidney function. When renal insufficiency sets in, the levels of these parameters increase.[20] CCl_4 also caused severe impairment of renal function with increased urea and serum creatinine compared to the control group[21]. The biochemical markers of renal function, creatinine, and urea were reduced in the treated group compared to the CCl_4-intoxicated group. These results suggest that *L. dentata* extract reduces CCl_4-induced hepatorenal intoxication.

Anthocyanin extract of flowers of lavender is considered effective in restoring the values of biochemical parameters, liver histology, demonstrating its antioxidant properties in vivo and in vitro. Several studies reported that anthocyanins decrease inflammation, oxidative stress, and liver damages (steatosis and fibrosis)[22-25] and improve mitochondrial functions.[25]

CONCLUSION

Lavandula dentata is among the plants that is widely used in traditional medicine in Algeria as a cure for anxiety and fertility. *L. dentata* is one of the plants commonly consumed for its culinary and medicinal properties. Our results highlight the beneficial effects of this medicinal plant. It may be considered as an ideal therapy against oxidative stress. The anthocyanin extract has antioxidant efficacy. An adequate intake of lavender in our diet would be beneficial to our health. However, further extensive studies should be conducted to ensure its safety and efficacy.

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