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***In vivo and in vitro* hepatoprotective effect of three endemic plants against carbon tetrachloride-induced liver damage in rats**

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Abstract: *The aim of the study was to investigate in vitro potential antioxidant and hepatoprotective effects of methanolic extracts from the aerial parts of Matricaria pubescens, Centaurea incana, and Santolina africana, against carbon tetrachloride induced liver damage in rats. The different aerial parts of three methanolic extracts, exhibited antioxidant activity in the DPPH free radical (IC₅₀, 0.032, 0.087 and 0.044 mg/ml) respectively. The reducing ability of the extracts showed a dose-dependent trend increasing with increase in the concentrations of the extracts. The antioxidant activity of samples at the concentration of 2 mg/ml was reflected in their ability to inhibit the bleaching of β -carotene. Treated groups with extracts of studied three plants showed decrease in plasma levels of AST, ALT, ALP, and an increase in plasma catalase levels activities, while hepatic MDA decreased and catalase increased as compared to carbon tetrachloride treated groups. In consistency with in vivo and in vitro results, extracts of all three plants ameliorated the carbon tetrachloride extensive damage to liver tissue of treated animals, but, Santolina africana extract was the most efficient.*

Keywords: *antioxidants, DPPH, β -carotene, carbon tetrachloride, rats, hepatoprotective effects*

1. Introduction

Reactive oxygen species (ROS) encompass a variety of diverse chemical species, including both free radicals, such as superoxide ($O_2^{\bullet-}$) and hydroxyl radicals ($OH^{\bullet}$), and other molecular species, such as hydrogen peroxide (H_2O_2) [1]. At high concentrations, ROS can be important mediators of damage to cell structures, nucleic acids, lipids and proteins [2]. ROS generation is increased in many pathological situations, including heart disease, diabetes, liver injury, cancer, aging etc. [3, 4]. Liver is well known to be the major organ responsible for the metabolism of drugs and toxic chemicals, and consequently is the primary target organ for nearly all the toxic chemicals [5]. Liver injury induced by drugs has become a primary reason for acute liver disease and therefore posed a potential regulatory and clinical challenge over the past few decades and has gained much attention [6]. CCL_4 is a classically known compound that causes hepatotoxicity by an acute exposure [7, 8]. It promptly reacts to the organs mainly the liver through its chemical reactivity properties to the surrounding molecules basically [9, 10], and through covalent binding of the compound to the microsomal lipids and membrane proteins and through lipids peroxidation [8].

Therefore, many folk remedies from plant origin are evaluated for their its possible antioxidant and hepatoprotective effects against different chemical-induced liver damage in experimental animals. *Matricaria pubescens* (Desf.) Sch.Bip. is a small plant rarely reaching 40 cm. Flowering takes place in spring in the northern Algerian Sahara, and any time after rain in the central Algerian Sahara [11, 12]. It is prepared as an infusion or powder and used internally. It has antiseptic properties. It is not reported as toxic by nomads. It is used for gastro-intestinal troubles and calculus, and is a much appreciated medicinal herb. It has an antibacterial activity [13] and it has an antiinflammatory activity [14]. *Centaurea incana* Desf. is a plant allgedly used in folk medicine [15]. *Santolina* L. is a smaller genus belonging to the Asteraceae family [16]. *Santolina africana* Jord. & Fourr. is endemic species of the North African region that grows naturally in the rocky slopes on the mountains [17]. It has been used since ancient times for medicinal, food and spice purposes, and known for its healing effects [18]. It is traditionally used for the treatment of the cardialgia ulcer and stomacal pain [19]. It has an antibacterial activity [20]. The aim of this study was to evaluate the antioxidative and hepatoprotective activity of methanolic extracts from the aerial parts of *Matricaria pubescens*, *Centaurea incana* and *Santolina africana*, an endemic species growing in Algeria, against CCL_4 induced liver damage in rats.

2. Materials and methods

2.1. Plant collection and identification

The aerial parts of *Matricaria pubescens*, were collected from Oued Souff; Algerian Sahara; *Centaurea incana*, and *Santolina africana* were collected from Oum el Bouaghi; Eastern of Algeria,

respectively. These three plants were identified by Pr. Khalfallah from the laboratory of Botanical Sciences, Faculty of Nature and Life sciences, University of Constantine 1, Algeria.

2.2. Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, Carbon tetrachloride (CCl₄), thiobarbituric acid, trichloroacetic acid (TCA), hydrogen peroxide, gallic acid, butylated hydroxytoluene (BHT), linoleic acid and β-carotene were obtained from Sigma Chemical Co. (St. Louis, MO). While, all other chemicals and solvents were available commercially.

2.3. Preparation of *Matricaria pubescens*, *Centarea incana* and *Santolina africana* aerial parts extracts

Samples of 100 g of air-dried powdered leaves and flowers of different plants were extracted, three times, with 96% methanol for 48h at room temperature. After filtration through whatman N°1 filter paper, the extract was evaporated in rotary evaporator at 40°C to obtain the methanolic extracts and stored at 4°C until further.

2.3.1. Determination of total phenolic content

Total phenolic compounds in methanolic extracts were determined by the method as described by Li *et al.* [21]. Briefly, extract reacted with Folin-Ciocalteu reagent in the presence of sodium carbonate to form a blue coloured complex which was read at 760 nm. Various concentrations of gallic acid were used to plot standard curve. Then, total phenolic content of extract was expressed as mg gallic acid equivalent/g dry weight of plants (AGE).

2.3.2. Determination of flavonoids content

Total flavonoids content in each extract was determined, using the aluminium chloride colorimetric method [22]. Briefly, 1 ml of diluted sample was mixed with 1 ml of 2% aluminium chloride in methanol. After incubation at room temperature for 10 min, the absorbance of the reaction mixture was measured at 430 nm. The flavonoids content was expressed in mg. Quercetin equivalent/g of dry weight of plants (QE).

2.4. In vitro study

2.4.1. DPPH radical scavenging assay

Free radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl radical was measured, using the method described by Burits and Bucar [23]. 50 µl of different dilutions of methanolic extracts were added to 5 ml of 4% solution DPPH dissolved in methanol. After 30 min at room temperature, the absorbance was measured at 517 nm. BHT and quercetin were used as standards. Inhibition of free radical DPPH in percent (I %) was calculated in the following way:

$$I \% = (A_{\text{blank}} - A_{\text{sample}}) \times 100 / A_{\text{blank}},$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}), was calculated from the plot of inhibition percentage against extract concentration. Tests were carried out in triplicate.

2.4.2. ABTS radical scavenging activity

ABTS radical scavenging activity of methanolic extracts was measured by the ABTS cation decolorization assay as described by Re et al. [24] with some modifications. The ABTS radical cation ($ABTS^{\bullet+}$) was produced by reaction of 7 mM stock solution of ABTS with 2.45 mM potassium persulfate and allowing the mixture to stand in dark at room temperature for 16 h before use. The absorbance of the $ABTS^{\bullet+}$ solution was equilibrated to 0.70 ± 0.02 by diluting with water at room temperature, methanolic extracts (0.1 ml) were allowed to react with 1.9 ml of the $ABTS^{\bullet+}$ solution and the absorbance was measured at 734 nm after 7 min. Trolox was used as a reference compound. The results were expressed as Trolox equivalent antioxidant capacity (TEAC) values and calculated as mean value $\pm$ standard deviation (SD) ($n = 3$).

2.4.3. Reducing power assay

The reducing ability of methanolic extracts and gallic acid were measured according to the method described by Oyaizu [25] with some modifications. A 0.1 ml aliquot of each extract and gallic acid was mixed with an equal volume of 0.2 M phosphate buffer (pH = 6.6) and 1% potassium ferricyanide, and then incubated at 50°C for 20 min. 0.25 ml of 10% trichloroacetic acid was added to the mixture, then the mixture was centrifuged at 3000g for 10 min. The supernatant (0.25 ml) was mixed with 0.25 ml distilled water and 0.1% $FeCl_3$ (500 μ l) and then the absorbance was measured at 700 nm. The reducing power of the test samples was increased with the absorbance values.

2.4.4. Beta Carotene /Linoleic Acid Assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation [26]. A stock solution of β -carotene-linoleic acid mixture was prepared as follows: 0.5 mg β -carotene was dissolved in 1 ml of chloroform (HPLC grade) and 25 μ l linoleic acid and 200 mg Tween₄₀ were added. Chloroform was completely evaporated. Then, 100 ml. distilled water saturated with oxygen (30 min; 100 ml/min) were added with vigorous shaking. 2500 μ l of this reaction mixture were dispensed into test tubes and 350 μ l of the different methanolic extracts, prepared at 2 mg/ml concentrations were added and the emulsion system was incubated for 48 h. at room temperature. The same procedure was repeated with synthetic antioxidant, BHT as positive control, and with a blank. After this incubation period, the absorbance of the mixtures was measured at 490 nm. The antioxidative capacity of the extracts was compared with that of BHT and of the blank.

2.4.5. *In vitro* lipid peroxidation determination

The extent of lipid peroxidation in liver rat homogenate was measured *in vitro* in terms of formation of thiobarbituric acid reactive substances (TBARS), according to the method of Ohkawa *et al.* [27]. Different concentrations of the different methanolic extracts were made up with methanol and expressed in terms of dry weight (mg/ml). The samples were individually added to the liver homogenate (1ml). This mixture was incubated with 1.15% KCL Lipid peroxidation was initiated by 5% of carboxymethyl cellulose (CMC). The reaction mixture was incubated at 37°C for 1 h. An equal volume of TBA: TCA (1:1, 1 ml). Tubes were then shaken and heated, in a water bath at 100°C for 20 min. After cooling with tap water; the tubes were centrifuged for 10 min at 4000 rpm. The absorbance of the supernatant was measured at 532 nm against a blank solution incubated without TBA. Peroxidation results were expressed as percentage of the inhibition in lipid peroxidation.

2.5. *In vivo* study

2.5.1. Animals

In this investigation, thirty male albino Wistar rats weighing 170-210 g (8 weeks old), purchased from the Pasteur institute in Algiers (Algeria) were used in these experiments. Rats were bred in our animal facility in stainless metallic cages (6 animals /cage). Before the experimentation, animals were allowed to adapt to the new housing environment for 1 week under standard laboratory conditions (12 h light : 12 h dark; 25±5°C). Rats were fed food and water *ad libitum*. All experimental procedures were carried out in conformity with international guidelines for the care and use of laboratory animals.

2.5.2. Hepatoprotective activity

The thirty experimental rats were divided into 5 groups, six rats in each group.

- Group I: served as controls and received an injection of vehicle (olive oil) alone;
- Group II: Acute liver injury in rats was induced by a single intraperitoneal injection (i.p) with CCl₄ dissolved in an equal volume of olive oil at a dose of 3 mL/kg body weight which is well documented to induce hepatotoxicity;
- Group III: was administered methanolic extract of *Matricaria pubescens* at a dose of 500 mg/kg and was injected by CCl₄ i.p, at a dose of 3 ml/kg body weight;
- Group IV: was administered methanolic extract of *Centaurea incana* at a dose of 500 mg/kg and was injected by CCl₄ i.p, at a dose of 3 ml/kg body weight;
- Group V: was administered methanolic extract of *Santolina africana* at a dose of 500 mg/kg and was injected by CCl₄ i.p, at a dose of 3 ml/kg body weight.

2.5.3. Plasma sample selection and hepatic function test

Twenty-four hours after the administration of CCl_4 , blood samples were collected by orbital sinus puncture into tubes containing anticoagulant. The samples were centrifuged at 6000 t/min for 15 min, and then the plasma was removed immediately and stored at -20°C until biochemical analyses, then. The rats were sacrificed by cervical dislocation and the liver of each rat was rapidly dissected and washed to remove excess blood and cut into two pieces; the first piece was fixed in formalin for histological analysis, and the second piece was homogenized in a teflon-glass homogenizer with a buffer containing 1.17% KCl, to obtain 1:10 (w/v) whole homogenate. The homogenates were centrifuged at 4000 t/min ($+4^\circ\text{C}$) for 10 min, to determine malondialdehyde (MDA) and catalase (CAT). Protein estimation was performed by Lowry *et al.* method [28].

2.5.4. Enzyme assays

2.5.4.1. Estimation of plasma AST, ALT and ALP levels

The Biochemical parameters like plasma enzymes: Aspartate transferase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were measured by using Auto analyzer (Architedt c system).

2.5.4.2. Catalase activity

Catalase (CAT) activity was measured by the method of Clairborne [29]. An aliquot of homogenate supernatants (50 μl) was added to a quartz cuvette and, the reaction was started by the addition of freshly prepared H_2O_2 (19 mM) in phosphate buffer (50 mM, pH = 7.0). The rate of H_2O_2 decomposition was measured spectrophotometrically at 240 nm during 120 s and the activity of CAT was expressed as UI/mg protein.

2.5.5. Estimation of thiobarbituric acid reactive substances (TBARS)

The TBARS level, an index of malonyldialdehyde (MDA), the last product of lipid breakdown caused by oxidative stress, was evaluated by the thiobarbituric acid reactive substances method (TBARS) and was expressed as nmol/g weight tissue production was determined by the method of Ohkawa *et al.* [27]. Briefly, an aliquot of 0.5 ml of tissue homogenates (10%, w/v) was mixed thoroughly with aqueous solution of thiobarbituric acid (0.67%, 1ml) and trichloroacetic acid (20%, 0.5 ml) and heated at 95°C for 15 min in a water bath. The suspension was then cooled to room temperature. After cooling, 4 ml n-butanol were added and mixed vigorously. The n-butanol layer was separated by centrifugation at 3000 r/min for 15 min the absorbance of the pink coloured supernatant was taken for spectrophotometry measurement at 530 nm for MDA assay. The results were compared to a standard curve of malondialdehyde (MDA) and results were expressed as nanomoles MDA per gram weight of tissue.

2.5.6. Histopathological study

For the histopathological study, liver tissues were fixed in 10% formalin, dehydrated in ethyl alcohol, cleared in xylol, and then embedded in paraffin. Sections of 5 μm thickness were stained with haematoxylin

and eosin (H&E) to study the effect of different methanolic extracts on the CCl₄ treated rats liver [30]. The sections were then examined under light microscope. Photomicrographs of the desired sections were obtained for further observations.

2.6. Statistical analysis

Results are expressed as Means $\pm$ S.E.M. and all statistical comparisons were carried out by means of one-way ANOVA test followed by Tukey-Kramer multiple comparisons test. The difference showing a P level of 0.05 or lower was considered to be statistically significant.

3. Results and discussion

3.1. *In vitro* assays

3.1.1. Extraction yields, total phenolics and flavonoids content

The estimated yields of extraction in relation to total weight (100 g) of plant material showed that the *Matricaria pubescens* and *Centaurea incana* have the highest yield (16% and 15% respectively), followed by *Santolina africana* with 10%.

The amount of total phenolics content (TPC), measured by Folin-Ciocalteu method, varied in the different methanolic extracts, varied from 15.056 to 27.304 mg Gallic Acid Equivalent (GAE)/g of dry weight. The results showed that *Santolina africana* and *Matricaria pubescens*, have the highest phenolic concentration (27.304 $\pm$ 0.008 and 25.698 $\pm$ 0.006 mg GAE/g of dry weight respectively), followed by methanolic extract of *Centaurea incana* (15.056 $\pm$ 0.016 mg GAE/g of dry weight) (Table 1).

The total flavonoids content (TFC), was determined using spectrophotometric method with Aluminium chloride. The TFC of each extract varied from 11.979 to 21.879 mg QE/g of dry weight. The results showed that *Santolina africana* contained the highest amounts of flavonoids (21.879 $\pm$ 0.048 mg QE/g of dry weight) compared to other extracts (Table 1).

3.1.2. DPPH radical scavenging activity

The results show that different methanolic extracts have a concentration-dependent scavenging activity in quenching DPPH radicals.

The IC₅₀ (DPPH[•]) values of different methanolic extracts and standards increased in the following order: *Centaurea incana* (0.087 mg/ml) > *Matricaria pubescens* (0.044 mg/ml) > *Santolina africana* (0.032 mg/ml) > rutin (0.0041 mg/ml) > quercetin (0.0034 mg/ml) > gallic acid (0.00056 mg/ml). The highest scavenging activity was observed for the extract from *Santolina africana*, whilst the lowest was observed for the extract from *Centaurea incana*. However, different methanolic extracts showed the lowest effect than standards (Fig. 1).

Fig. 1. DPPH radical scavenging activity of different methanolic extracts; *Matricaria pubescens* (MP) *Centaurea incana* (CI), *Santolina africana* (SA), rutine (Rut), quercetine (Q) and gallic acid (Gal A). Data are presented as IC₅₀ values. Each value represents the mean $\pm$ S.D (n = 3). (***)p $\leq$ 0.001; compared to Gal A)

3.1.3. ABTS radical scavenging activity

The antioxidant capacity of methanolic extracts of *Matricaria pubescens*, *Centaurea incana* and *Santolina africana* were valuated according to the ABTS decolorization method. The results of antioxidant activity of all samples or standard trolox were expressed as TEAC values, as shown in (Table 1). The methanolic extract of *Santolina africana* displayed the highest radical scavenging potential (0.11 $\pm$ 0.001 TEAC). The methanolic extract of *Centaurea incana* had the lowest antioxidant capacity of 0.07 $\pm$ 0.002 TEAC.

Table 1. Radical scavenging activity and total phenolic content of methanolic extracts of *Matricaria pubescens*, *Centaurea incana*, and *Santolina africana*

sample	Yiels (%)	DPPH assay IC50 (μ g/ml)	ABTS assay TEAC	Total phenol content*	Flavonoids contents#
<i>Matricariapubescens</i>	16	44.33 $\pm$ 0.29	0.10 $\pm$ 0.001	25.698 $\pm$ 0.006	11.979 $\pm$ 0.014
<i>Centaurea incana</i>	15	87.51 $\pm$ 0.42	0.07 $\pm$ 0.002	15.056 $\pm$ 0.016	12.782 $\pm$ 0.027
<i>Santolina africana</i>	10	35.64 $\pm$ 0.30	0.11 $\pm$ 0.001	27.304 $\pm$ 0.008	21.879 $\pm$ 0.048

Values are expressed as mean $\pm$ SD (n=3-4). *:mg. GAE/g. of dry weight, #:mg. QE/g. of dry weight.

3.1.4. Reducing power

As illustrated in Fig. 2, Fe^{3+} was transformed to Fe^{2+} in the presence of methanolic extracts of *Matricaria pubescens*, *Centaurea incana*, *Santolina africana* and the reference compound Gallic acid to measure the reductive capability. The reducing ability of the extracts showed a dose-dependent trend increasing with increase in the concentrations of the extracts. Methanol extract of *Santolina africana* exhibited the highest reducing capability (0.473 ± 0.009) at the concentration of 0.5 mg/ml ($p \leq 0.001$), followed by *Matricaria pubescens* extract (0.362 ± 0.147) and *Centaurea incana* (0.161 ± 0.027). The differences for the antioxidant activity among the different extracts may be attributed to the type of phenolics compounds present in this extract.

Fig. 2. Reducing power of methanolic extracts of *Matricaria pubescens* (MP), *Centaurea incana* (CI), *Santolina africana* (SA), and gallic acid (ac gal).

Values are mean of numbers of triplicates $\pm$ SD ($n = 3$)

3.1.5. Beta Carotene/Linoleic Acid Assay

The antioxidant activity of samples at the concentration of 2 mg/ml was reflected in their ability to inhibit the bleaching of β -carotene. In this assay, the *Santolina africana* and *Matricaria pubescens* also possessed better antioxidant activity than extract from *Centaurea incana* (Fig. 3).

Fig. 3. Changes in the percentage of the linoleic acid oxidation inhibition ratios under the influence of different methanolic extracts; *Matricaria pubescens* (MP), *Centaurea incana* (CI) and *Santolina africana* (SA) (2 mg/ml), compared to BHT as a positive control. Each value represents the mean of numbers of triplicates $\pm$ SD.

3.1.6. *In vitro* lipid peroxidation determination assay

In this study, *in vitro* lipid peroxidation was induced in rat liver by using CMC. The capacity of the different methanolic extracts to inhibit lipid peroxidation is shown in (Fig. 4). The present results show that *Santolina africana* possesses better dose-dependent generation of lipid peroxides than the other extracts.

Fig. 4. Scavenging effect on lipid peroxidation by different methanolic extracts. *Matricaria pubescens* (MP), *Centaurea incana* (CI), *Santolina africana* (SA)

3.2. In vivo assays

3.2.1. Effect of different methanolic extracts on CCl₄-induced hepatotoxicity and antioxidant enzyme

Fig. 5. Effect of different methanolic extracts on plasma ALT in CCl₄-induced hepatotoxicity in adult rats. Control, normal control; CCl₄, CCl₄ model control; MP+CCl₄, *Matricaria pubescens* (500 mg/kg b.w.) + CCl₄ (3 ml/kg b.w.); CI + CCl₄, *Centaurea incana* (500 mg/kg b.w.) + CCl₄ (3 ml/kg b.w.); SA + CCl₄, *Santolina africana* (500 mg/kg b.w.) + CCl₄ (3 ml/kg b.w.). Results are presented as the means $\pm$ SEM (n = 6). *, **, *** Significant difference at P < 0.05, P < 0.01 and P < 0.001 levels compared with the control group, respectively. ### Significant difference at P < 0.001 level compared with the CCl₄ group.

Fig. 6. Effect of different methanolic extracts on plasma AST in CCl₄-induced hepatotoxicity in adult rats. Control, normal control; CCl₄, CCl₄ model control; MP+CCl₄, *Matricaria pubescens* (500 mg/kg b.w.) + CCl₄ (3 ml/kg b.w.); CI + CCl₄, *Centaurea incana* (500 mg/kg b.w.) + CCl₄ (3 ml/kg b.w.); SA + CCl₄, *Santolina africana* (500 mg/kg b.w.) + CCl₄ (3 ml/kg b.w.). Results are presented as the means $\pm$ SEM (n = 6). *, **, *** Significant difference at P < 0.05, P < 0.01 and P < 0.001 levels compared with the control group, respectively. ### Significant difference at P < 0.001 level compared with the CCl₄ group

Fig. 7. Effect of different methanolic extracts on plasma ALP in CCl₄-induced hepatotoxicity in adult rats. Control, normal control; CCl₄, CCl₄ model control; MP+CCl₄, *Matricaria pubescens* (500 mg/kg b.w.) + CCl₄ (3 ml/kg b.w.); CI + CCl₄, *Centaurea incana* (500 mg/kg b.w.) + CCl₄ (3 ml/kg b.w.); SA + CCl₄, *Santolina africana* (500 mg/kg b.w.) + CCl₄ (3 ml/kg b.w.). Results are presented as the means ± SEM (n = 6). *, **, *** Significant difference at P < 0.05, P < 0.01 and P < 0.001 levels compared with the control group, respectively. ### Significant difference at P < 0.001 level compared with the CCl₄ group

The effects of different methanolic extracts pretreatment on the CCl₄-induced elevation of plasma ALT, AST and ALP activities are shown in Fig. 5, Fig. 6, and Fig. 7, respectively. A single dose of CCl₄ (3 ml/kg, i.p.) caused hepatotoxicity in rats, as indicated by the increases in ALT, AST and ALP plasma levels. However, pretreatment with methanolic extracts (500 mg/kg/day from *Matricaria pubescens*, 500 mg/kg/day from *Centaurea incana* and 500 mg/kg/day from *Santolina africana* respectively, *per os*, for 4 weeks) partially prevented the CCl₄-induced elevations in ALT, AST and ALP serum levels.

Fig. 8. Effects of different methanolic extracts on the hepatic MDA concentration in CCl₄-induced hepatotoxicity in adult rats. Contol, normal control; CCl₄, CCl₄ model control; MP+CCl₄, *Matricaria pubescens* (500 mg/kg b.w.) + CCl₄ (3 ml/kg b.w.); CI + CCl₄, *Centaurea incana* (500 mg/kg b.w.) + CCl₄ (3 ml/kg b.w.); SA + CCl₄, *Santolina africana* (500 mg/kg b.w.) + CCl₄ (3 ml/kg b.w.). Results are presented as the means ± SEM (n = 6). *, **, *** Significant difference at P < 0.05, P < 0.01 and P < 0.001 levels compared with the control group, respectively. ### Significant difference at P < 0.001 level compared with the CCl₄ group

3.2.2. Assessment of *in vivo* lipid peroxidation in liver cells

The concentration of MDA, an end product of lipid peroxidation, in the rats treated with CCl₄ was increased when compared with the vehicle control rats (Fig. 8). Consistent with the plasma AST, ALT and ALP activities, pre-treatment with methanolic extracts for 4 weeks to the rats resulted in a significant decrease in the concentration of hepatic MDA when compared with the CCl₄ group.

3.2.3. Catalase activity

CCl₄ administration alone significantly depleted antioxidant enzyme catalase, activity, whereas methanolic extracts pretreatment significantly protected against this depletion (Fig. 9).

Fig. 9. Effects of different methanolic extracts on the hepatic catalase activity in CCl₄-induced hepatotoxicity in adult rats. Contol, normal control; CCl₄, CCl₄ model control; MP+CCl₄, *Matricaria pubescens* (500 mg/kg b.w.) + CCl₄ (3 ml/kg b.w.); CI + CCl₄, *Centaurea incana* (500 mg/kg b.w.) + CCl₄ (3 ml/kg b.w.); SA + CCl₄, *Santolina africana* (500 mg/kg b.w.) + CCl₄ (3 ml/kg b.w.). Results are presented as the means $\pm$ SEM (n = 6). *, **, *** Significant difference at P < 0.05, P < 0.01 and P < 0.001 levels compared with the control group, respectively. ### Significant difference at P < 0.001 level compared with the CCl₄ group

3.2.4. Histopathology

Livers of rats in the control group showed a normal histological appearance (Fig. 10), whereas livers of rats treated with CCl₄ showed classic histology of cirrhosis, Coagulative necrosis, massive fibrosis, mononuclear cell infiltration, hemorrhage, fatty degeneration and formation of regenerative nodules were observed.

The protective effects exerted by methanolic extracts on CCl₄ as indicated by the biochemical data (ALT, AST and ALP activities and MDA levels approximated to the levels in normal rats) were confirmed by conventional histopathological examination.

Fig. 10. Photomicrographs of histopathological studies of liver sections of experimental groups. (A) control group I without any signs of liver damage. (B and C) CCl_4 group, liver sections with extensive macrovesicular and microvesicular steatosis, massive hepatocytes degeneration, apoptosis or necrosis, and inflammatory cells infiltration, sinusoids congestion (arrows). (D) *Centaurea incana* (500 mg/kg b.w. + CCl_4 3 ml/kg b.w) with microvesicular steatosis (arrows), (E) *Matricaria pubescens* (500 mg/kg b.w. + CCl_4 3 ml/kg b.w) and (F) *Santolina africana* (500 mg/kg b.w. + CCl_4 3 ml/kg b.w.) showed a reduction of the macrovesicular and microvesicular steatosis, and a decrease in area of degenerative hepatocytes. x 400, H & E).

4. Discussion

Many synthetic drugs protect against oxidative damage, but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplements and traditional medicines [31]. In almost all the traditional systems of medicine, medicinal plants play a major role and constitute their backbone. Medicinal plants are the most important source of life saving drugs for a majority of the world's population.

Plant phenolics and flavonoids represent major groups of plant constituents that work predominantly as powerful antioxidants or scavenger of free radicals. They play beneficial role in human health and

cure/prevent ailments such as inflammatory disorders, cancer, cardiovascular diseases and diabetes which occur due to the deregulation of free radicals generation in the cells [32].

The results of Ben Mansour et al. [19] show that ethanolic extracts contain 73.72 ± 1.98 QE/g of dry mass from *Santolina africana*. Other study of Béjaoui et al. [33] shows that methanolic extracts from *Santolina africana* contain 42.56 ± 0.980 QE/g of dry mass [33].

The difference between our results and their results can be attributed to the method of extraction and the polarity of solvents used in the extract preparation [34].

DPPH is very popular for the study of natural antioxidants [35]. The antiradical activity of tested compounds is expressed as a relative or absolute decrease of concentration of DPPH or as IC_{50} . DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [36]. The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. Quercetin, rutin and gallic acid are the reagents used as standards. The extracts are able to reduce the stable radical DPPH to the yellow-coloured diphenyl picrylhydrazine.

It was demonstrated that DPPH• free radicals scavenger potential of polyphenolic compounds depends on the particular substitution pattern of free hydroxyl groups on the flavonoids skeleton, the highly active flavonoids possess a 3', 4'-dihydroxy occupied B ring and/or 3-OH group [37].

Antioxidant activity depends on the number and position of the hydroxyl groups of the aromatic ring binding site and the type of substituent. ABTS.+ is a blue chromophore produced by the reaction between ABTS and potassium persulfate. Phenols with two hydroxyl groups linked to the aromatic ring at the ortho position more strongly quench ABTS•+ radical than compounds with hydroxyl groups substituted at the meta position [38]. The results were compared with those obtained using trolox and the TEAC value demonstrates that the methanolic extract of *Santolina africana* displayed the highest radical scavenging potential (0.11 ± 0.001 TEAC).

The antioxidant activities of natural components may have a reciprocal correlation with their reducing powers [39]. The reducing power assay is often used to evaluate the ability of the natural antioxidant to donate an electron or hydrogen [40]. In this assay, the presence of reductants in the antioxidant sample causes the reduction of the Fe^{3+} /ferricyanide complex to the Fe^{2+} /ferrous form [41], so the reducing power of the sample could be monitored by measuring the formation of Perl's Prussian blue at 700 nm.

The β -carotene-linoleic bleaching inhibition assay is considered to be a good model for membrane based lipid peroxidation [42]. The presence of antioxidants in the extract will minimize the oxidation of β -carotene by hydroperoxides. Hydroperoxides formed in this system will be neutralized by the antioxidants from the extracts. Thus, the degradation rate of β -carotene depends on the antioxidant activity of the extracts.

The systematic literature collection, pertaining to this investigation indicates that the plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators. Therefore, it is worthwhile to determine their total amount in the plants chosen for the study. Flavonoids as one of the most diverse and widespread group of natural compounds, are likely to be the most important natural phenolics [43]. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties.

Lipid peroxidation can be described generally as a process under which oxidants such as free radicals or non-radical species attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) [44]. Although the chemistry of initiation of peroxidation in membrane systems is not fully understood, iron ions are believed to play an important role in this mechanism. Thus, there have been various suggestions that iron-oxygen species are responsible for this initiation: perferryl radicals and specific Fe^{2+} - Fe^{3+} - O_2 complexes have been considered [45]. Phenolic compounds and other chemical components present in the extract may suppress lipid peroxidation through different chemical mechanisms, including free radical quenching, electron transfer, radical addition or radical recombination.

CCl_4 is a classical hepatotoxicant that causes rapid liver damage progressing from steatosis to centrilobular necrosis. Long-term administration of CCl_4 causes chronic liver injury, and is a widely accepted model to produce hepatic fibrosis [46, 47].

Serum levels of AST and ALT are the preferred indicators for evaluation of liver function, they usually reflect the altered permeability of hepatocellular membrane and the damaged structural integrity of hepatocytes [48]. In the present study, the single oral dose of CCl_4 at 3 ml/kg caused a dramatic elevation in serum AST, ALT and ALP activities, indicating an acute hepatotoxicity induced by administration of CCl_4 (Fig. 5, Fig. 6, and Fig. 7). These parameters have been attributed to hepatic structural damage because these enzymes are normally localized to the cytoplasm and are released into the circulation after cellular damage has occurred [47]. The acute hepatotoxic effects induced by CCl_4 administration were confirmed histopathologically, revealing extensive hepatocellular degeneration and necrosis, fatty changes, inflammatory cell infiltration, congestion, and sinusoidal dilatation (Fig. 10). The obtained results are in accordance with those of the previous reports [49, 50]. However, pretreatment with methanolic extracts (500 mg/kg/day from *Matricaria pubescens*, 500 mg/kg/day from *Centaurea incana* and 500 mg/kg/day from *Santolina africana* respectively, *per os*, for 4 weeks), partially prevented the CCl_4 -induced elevations in ALT, AST and ALP serum levels. This phenomenon was also confirmed by the results of histopathological examination, as evidenced by a dose-related decrease in the incidence and severity of histopathological hepatic lesions.

The liver injury induced by CCl_4 is resulted from free radicals and lipid peroxidation that cause hepatic cell damage. Lipid peroxidation is one of the principal causes of CCl_4 -induced liver injury [47, 51]

and is mediated by the free radical derivatives of CCl_4 . CCl_4 requires bioactivation by phase I cytochrome P_{450} system in liver to form reactive metabolic trichloromethyl radical ($\text{CCl}_3\cdot$) and peroxytrichloromethyl radical ($\cdot\text{OOCCl}_3$). These free radicals can bind with polyunsaturated fatty acid (PUFA) to produce alkoxy ($\text{R}\cdot$) and peroxy radicals ($\text{ROO}\cdot$), that, in turn, generate lipid peroxide, cause damage in cell membrane [51], change enzyme activity and finally induce hepatic injury or necrosis [50]. In addition, the antioxidant activity and/or the inhibition of free radical generation are important in terms of protecting the liver from CCl_4 induced damage [51, 53].

MDA is a major reactive aldehyde that appears during the peroxidation of biological membrane polyunsaturated fatty acid [54]. Therefore, the hepatic content of MDA is used as an indicator of liver tissue damage involving a series of chain reactions [27].

In the present study, a single intraperitoneal injection of CCl_4 at 3 ml/kg resulted in a significant increase in the hepatic MDA concentration, indicating increased lipid peroxidation caused by administration of CCl_4 (Fig. 8). The significant dose-dependent decrease in the hepatic MDA concentration confirms that pretreatment with different methanolic extracts (500 mg/kg/day from *Matricaria pubescens*, 500 mg/kg/day from *Centaurea incana* and 500 mg/kg/day from *Santolina africana* respectively, *per os*, for 4 weeks), could effectively protect against the hepatic lipid peroxidation induced by CCl_4 .

Generally, antioxidant enzyme such as catalase is easily inactivated by lipid peroxides or reactive oxygen species, which results in decreased activity of this enzyme in CCl_4 toxicity. Thus, antioxidant enzyme also plays an important role in the detoxification of xenobiotics, catalyzing their conjugation with reduced GSH. Free radicals in CCl_4 intoxication are a major pathway of non-enzymatically induced lipid peroxidation, which subsequently affect various enzyme activities in the body and therefore, may also be linked to enzymatically induced lipid peroxidation [55]. As a result, we suggest that crude extracts protect against significantly decreased catalase activities in rats treated with CCl_4 compared with control rats.

Histopathological studies were performed to provide direct evidence of the hepatotoxicity of CCl_4 . It has been reported that CCl_4 causes necrosis, steatosis, and foamy degeneration of hepatocytes and hence proved to be potent hepatotoxin [56-58]. Vacuolation of hepatocytes was described as microvesicular and macrovesicular steatosis [58]. Furthermore, cytoplasmic vacuolation was attributed to lipid peroxidation because of oxidative stress that damage cell membrane leading to increase in their permeability and disturbance of the ions concentrations in the cytoplasm [60]. Dilatation of central veins, blood sinusoids and portal veins was attributed to inflammatory changes or ischemia and hypoxia following high fat diet [61].

In the present study, marked disruption of the structure of hepatocytes was noted in liver tissue of rats exposed to CCl_4 alone after a single intraperitoneal injection with CCl_4 , at 3 ml/kg). Furthermore, it was determined that the histopathological changes observed after administration of CCl_4 had disappeared in rats

treated with CCl_4 and different methanolic extracts (500 mg/kg/day from *Matricaria pubescens*, 500 mg/kg/day from *Centaurea incana* and 500 mg/kg/day from *Santolina africana* respectively, *per os*, for 4 weeks), especially the methanolic extract from *Santolina africana*. This minimal disruption of the hepatocyte structure complemented the results of the liver enzyme studies (AST, ALT and ALP activities and MDA levels approximated to the levels in normal rats).

5. Conclusion

In conclusion, this study indicates that different methanolic extracts contains relevant antioxidant compounds responsible, for its antioxidant and radicals scavenging activity. In the other hand, our results demonstrate that methanolic extract from *Santolina africana* was able to reverse the pathological parameters, such as fibrotic histological feature and serum levels of ALT, AST and ALP against CCl_4 -induced hepatic damage. The mechanisms underlying hepatoprotection of the methanolic extract of *Santolina africana* may be related to both its radical scavenging properties and indicate effects as a regulator of antioxidative systems but further studies are needed in these directions.

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7. References

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