Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 11, 2015

Original Article

PHYTOCHEMICAL PROFILES, ANTIOXIDANT CAPACITY AND PROTECTIVE EFFECT AGAINST AAPH-INDUCED MOUSE ERYTHROCYTE DAMAGE BY *DAPHNE GNIDIUM* L. SHOOTS EXTRACTS

DJAMILA AMENI[1*], ABDERRAHMANE BAGHIANI[1*], SABAH BOUMERFEG[1], SALIHA DAHAMNA[2], SEDDIK KHENNOUF[2], MUSA H. ABU ZARGA[3], LEKHMICI ARRAR[1]

[1]Laboratory of Applied Biochemistry, Faculty of Nature and Life Sciences, Univ. Sétif, Setif 19000, Algeria, [2]Laboratory of Phytotherapy Applied to Chronic Diseases, Faculty of Nature and Life Sciences, Univ. Setif, Setif 19000,Algeria, [3]Department of Chemistry, Faculty of Sciences, The University of Jordan, Amman 11942, Jordan
Email: baghianiab@hotmail.co.uk

Received: 07 Jul 2015 Revised and Accepted: 22 Sep 2015

ABSTRACT

Objective: Various biological activities have been reported for *Daphne gnidium*, the aim of the present study was to determine polyphenols and some biological activities of extracts from the shoots of this plants.

Methods: Phenolic and flavonoids contents of *D. gnidium* extracts (DGE) were determined by Folin-Ciocalteau and identified by HPLC–DAD/MS. Free radical scavenging and antioxidant potential of the crude (CE), chloroform (CHE) and ethyl acetate (EAE) extracts of *D. gnidium* shoots were investigated using several *in vitro* and *ex vivo* assays, including 2, 2-diphenyl-picrylhydrazyl radical scavenging, superoxide anion scavenging (by both enzymatic and nonenzymatic methods) and hydroxyl radical scavenging capacity methods. The antioxidant activity of the extracts was measured using the xanthine oxidase (XO) inhibitory activity, reducing power and ß-carotene-linoleic bleaching assays. Inhibition of lipid peroxidation and oxidative hemolysis were also performed to confirm the protective effect of these extracts.

Results: It was found that values of phenolics varied between 130.84±5.99 and 137±7.66 mg gallic acid equivalent/g dry extract. HPLC analysis revealed the presence of cinnamic acid derivatives and other metabolites from the flavonoids family. All extracts exhibited a superoxide scavenging capacity. The EAE had the highest antioxidant activity as measured by DPPH radical and hydroxyl radical scavenging activity. The extracts showed an inhibitory effect on xanthine oxidase, the IC_{50} ranges from 0.021±0.001 to 0.061±0.001 mg/ml. The EAE showed also potent reducing power ability. CHE possess an inhibition ratio of (92.11%) in the linoleic acid oxidation assay close to that of BHT (96.77%). All extracts exhibited antioxidant activity in the linoleic acid emulsion system (3.87-61.11 %). Under the oxidative action of AAPH, EAE and CE showed higher protective effect against erythrocytes hemolysis than the CHE. The percentage of hemolysis (H%) determined for EAE and CE after 1 h of incubation were 0% and 1.9%, respectively.

Conclusion: This study indicates that DGE contains relevant antioxidant compounds responsible, at least in part, for its antioxidant and radicals scavenging activity. Flavone derivatives were determined as the main active component of the shoots part and the CHE was the most active extract.

Keywords: Xanthine oxidoreductase (XOR), Antioxidants, Radicals scavenger, *Daphne gnidium* L., DPPH, ß-carotene/linoleic acid, Hemolysis, Polyphenols.

INTRODUCTION

Reactive oxygen species (ROS) are various forms of activated oxygen, which includes free radicals such as superoxide ions ($O_2^{\bullet-}$) and hydroxyl radicals ($^{\bullet}OH$), as well as no free-radical species such as hydrogen peroxide (H_2O_2) [1]. In living organisms various ROS can be formed in different ways, including normal aerobic respiration, stimulated polymorph nuclear leukocytes, macrophages, enzymes and peroxisomes. One of the very important enzymes that has been reported to increase during oxidative stress is xanthine oxidase (XO), which is conventionally seen as a late enzyme of purine catabolism, catalyzing the oxidation of hypoxanthine to xanthine and of xanthine to uric acid which leads to increased superoxide production [2]. Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents, and pesticides [3]. *In vivo*, some of these ROS play a positive role such as energy production, phagocytosis, regulation of cell growth and a number of cellular signaling systems [4]. On the other hand ROS have been implicated in more than 100 diseases; it has been implicated in aging, as well as in a variety of diseases such as cancer [5] and neurological disorders, including Parkinson disease [6]. Nevertheless, all aerobic organisms, including human beings, have antioxidant defences that protect against oxidative damages [7]. However, this natural antioxidant mechanism can be inefficient, and hence dietary intake of antioxidant compounds is important [1]. Plants contain a wide variety of free radicals scavenging molecules such as phenols, flavonids, vitamins, terpenoids that are rich in antioxidants, tend to be safer and possess antiviral, antiinflammatory, anti-cancer, anti-tumour and hepato-protective properties [8]. During the last decade, the use of traditional medicine has expanded globally and is gaining popularity. In our study, we have investigated the antioxidant potential of a medicinal plant *Daphne gnidium* L. (Thymelaeaceae). The powdered roots of this species have been used in the traditional medicine as an abortifacient and the bark has been used as a diuretic agent and to treat toothache [9] and against hepatitis [10].

Recently, it has been demonstrated that different organic extracts of this plant have antiproliferative effects [11]. In addition, various biological activities have been reported for *D. gnidium* as insecticidal [12], anti-inflammatory [13], antibacterial [14] and antimycotic activity [15]. In this study, we examined the antioxidant, scavenging effect, hemolytic and lipid peroxidation inhibitory activity and phenolic compound identification of the extracts from the stems and leaves of *Daphne gnidium* L.

MATERIALS AND METHODS

Materials

Daphne gnidium L. was harvested at flowering stage from natural resources around Sétif, Algeria during spring (May–June). Areal parts were dried for 10-15 d in shadow at room temperature then powdered and stocked in darkness until use. The authenticity was confirmed by Pr Laouar Hocine (department of vegetal biology and

ecology, university Farhat Abbas Setif 1) and a voucher specimen was kept at the laboratory. Bovine milk was obtained from a local farm. All other reagents were purchased from Sigma chemicals (Germany) and Fluka.

Methods

Phenolic compounds extraction

The extraction was carried out using various polar and non polar solvents, according to the method of [16]. 100g of dried *Daphne gnidium L.* (DG) shoots (stems and leaves) were ground in warring blender. They were mixed with 1 litre of methanol (MeOH, 85 %). The mixture was placed on shaker for 24 h. The extracts were filtered through a Buchner funnel and the MeOH was removed on the rotary evaporator to give crude extract (CE). The aqueous solution was extracted with hexane several times to remove lipids, and then it was partitioned against chloroform to give chloroform extract (CHE). The remaining aqueous phase was exhaustively extracted with ethyl acetate (EtOAc) until the final EtOAc extract was colourless (EAE), the remaining aqueous extract was labelled (AE). All solvents were removed by evaporation under reduced pressure and the extracts were lyophilised and stored at-20 °C until use. The extracts used in this study were CE, CHE and EAE.

Determination of total polyphenols

Total polyphenols were measured using Prussian blue assay described by [17] modified by [18]. Phenolic compounds were expressed as gallic acid equivalent (GAE). Briefly 0.1 ml of *Daphne gnidium* extracts (DGE) are dissolved in methanol, 3 ml distilled water were added and mixed, then 1 ml of $K_3Fe(CN)_6$ (0.016 M) was added to each sample, followed by the addition of 1 ml of $FeCl_3$ (0.02 M dissolved in 0.1 M HCl). It was immediately mixed using a vortex, after adding the reagents to the sample, 5 ml stabiliser (30 ml Gum arabic (1 %), 30 ml H_3PO_4 (85 %) and 90 ml of distilled water) were added to the sample. The absorbance was measured at 700 nm. The amount of total polyphenols in different extracts was determined from a standard curve of gallic acid.

Determination of flavonoid

Flavonoids were quantified using aluminium chloride reagent $AlCl_3$ [19]. Flavonoids were expressed as quercetine equivalent (QE). 1 ml of DGE samples are dissolved in methanol, then 1 ml of $AlCl_3$ (2 % in MeOH) was added, after incubation for 10 min, the absorbance was measured at 430 nm.

Purification of milk xanthine oxidoreductase (XOR)

XOR was routinely purified in our laboratory from bovine milk, in the presence of 10 mM of dithiothreitol, by ammonium sulphate fractionation, followed by affinity chromatography on heparin-agarose [20]. XOR concentration was determined from the UV-visible spectrum by using an absorption coefficient of 36000 M^{-1} cm^{-1} at 450 nm. The purity of an enzyme was assessed on protein/flavin ratio (PFR = A_{280}/A_{450}) [20-22] and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10%) [23]. The XOR activity was specrophotometrically determined by measuring the production of uric acid from xanthine (100 µM, final concentration) at 295 nm using an absorption coefficient of 9600 M^{-1} cm^{-1} [20-22]. Assays were performed at room temperature in air-saturated 50 mM phosphate buffer, pH 7.4.

Effects of *Daphne gnidium L.* extracts on $O_2^{\bullet-}$radicals using enzymatic method

Anti-radical activity was determined according to the method of [24], by monitoring the effect of DGE on superoxide anion radicals ($O_2^{\bullet-}$) produced by xanthine/xanthine oxidase system. This radical is able to reduce cytochrome c. The reaction mixture contained xanthine (100 µM), horse heart cytochrome c (25 µM), in air-saturated sodium phosphate buffer (50 mM, pH 7.4), supplemented with 0.1 mM ethylene diamine tetra acetic acid (EDTA) and various concentrations of DGE. The reaction was started by the addition of XOR. The cytochrome c activity was calculated using an absorption coefficient of 21.100 M^{-1} cm^{-1}, and the sensitivity of the reaction was determined by using bovine erythrocytes superoxide dismutase.

Effects of *Daphne gnidium L.* extracts on Xanthine Oxidase (XO) activity

The effect of DGE on the xanthine oxidation was examined spectrophotometrically at 295 nm following the production of uric acid using an absorption coefficient of 9600 M^{-1} cm^{-1} [20]. Assays were performed at room temperature, in the presence of final concentration of 100 µM of xanthine, in air saturated sodium phosphate buffer (50 mM, pH 7.4) with various amounts of DGE dissolved in methanol. Control experiments revealed that solvent didn't influence the activity of XO at this concentration. The reaction was started by the addition of XOR (1176 nmol of urate/min/mg protein) for enzyme activity of the control sample was set to 100 % activity. The percentage inhibition was calculated using the following formula.

$$I\ \% = (A\ control - A\ sample) \times 100 / A\ control$$

Measurement of superoxide anion scavenging activity using non enzymatic system

The superoxide scavenging ability of DGE was assessed by a modified method [25]. Superoxide anions were generated in samples that contained 100 µl of NBT(1 mM), NADH (3 mM) and 0.3 mM PMS then the final volume was adjusted to 1 ml with 0.1M phosphate buffer (pH 7.8). The reaction mixture (NBT and NADH) was incubated with or without extracts at ambient temperature for 2 min and the reaction was started by adding PMS. The absorbance at 560 nm was measured. Decrease in absorbance in the presence of various plants extracts indicated $O_2^{\bullet-}$ scavenging activity.

ß-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 ß-carotene-linoleic acid mixture was prepared as follows: 0.5 mg ß-carotene was dissolved in 1 ml of chloroform (HPLC grade) and 25 µl linoleic acid and 200 mg Tween 40 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 µl of this reaction mixture were dispensed into test tubes and 350 µl of the DGE, 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.

Inhibition of lipid peroxidation by different extracts of *D. gnidium*, as measured by FTC method

The total antioxidant activity of DGE was carried out using linoleic acid system described by [27], with some modification. The linoleic acid emulsion was prepared by mixing 155 µl of linoleic acid, 175 µg of tween 20 as emulsifier and 50 ml of phosphate buffer (0.02 M, pH 7.0), and then the mixture was homogenized. A 0.5 ml of different extract of *D. gnidium* was mixed with 2.5 ml of linoleic acid emulsion and 2 ml of phosphate buffer. The reaction mixture was incubated at 40 °C in the dark to accelerate the peroxidation process. Aliquots of 0.1 ml were taken at different intervals during incubation.

The degree of oxidation was measured by sequentially adding ethanol (4.7 ml of 75%), an ammonium thiocyanate sample solution (0.1 ml, 30%) and ferrous chloride (0.1 ml, 0.02 M in 3.5 % HCl). After 3 min, the peroxide value was determined by recording the absorbance at 500 nm every 24 h until the absorbance of the control reached a maximum. Ascorbic acid and BHT were used as reference standards. A control was performed with linoleic acid but without tested extracts and compounds. All data reported are the average of triplicate analyses. Percentage inhibition of lipid peroxide generation was calculated using the following formula:

$$\%\ Inhibition = (1 - A_S/A_0) \times 100,$$

Where A_S is the absorbance value of the tested sample and A_0 is the absorbance value of the control sample.

Antihemolytic activity

Antihemolytic activity of the shoots extracts of *D. gnidium* was assessed by the method of [28] with slight modifications. The erythrocytes from mouse blood were separated by centrifugation and washed three times with 10 mM phosphate buffer (pH 7.4). It was then re suspended in PBS, so as to produce an RBC suspension at 2% (v/v) hematocrit. In order to induce free-radical chain oxidation in erythrocytes, aqueous peroxyl radicals were generated by thermal decomposition of AAPH (2, 2-azo-bis (2-amidinopropane) dihydrochloride) (dissolved in PBS; final concentration 300 mM). To study the protective effects of *D. gnidium* shoots extracts against AAPH-induced oxidative haemolysis, an erythrocyte suspension was pre incubated with different extracts or standards (0.1 mg/ml, dissolved in PBS) and AAPH solutions at 37 °C for 3 h 30 min. Samples were removed at regular intervals from the incubation mixture and the extent of hemolysis (%) was indicated via reductions in turbidity at 620 nm [29].

DPPH assay

The hydrogen atom or electron donation abilities of DGE and some pure compounds were measured from the bleaching of the purple-coloured methanol solution of 2,2'-Diphenyl-1-picrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent [30]. 50 µl of various concentrations of the extracts in methanol were added to 5 ml of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical DPPH in percent (I %) was calculated in the following way:

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

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.

Hydroxyl radical scavenging activity assay

The scavenging activity of extracts on hydroxyl radical was measured according to the method of [31] with some modifications. In this system, hydroxyl radicals were generated by the Fenton reaction. Hydroxyl radicals could oxidize Fe^{2+}into Fe^{3+}, and only Fe^{2+}could combined with 1, 10-phenanthroline to form a red compound (1, 10-phenanthroline-Fe^{2+}) with the maximum absorbance at 536 nm. The concentration of hydroxyl radical was reflected by the degree of decolourization of the reaction solution. A sample of 600 µl of 1,10-phenanthroline (5.0 mM), 600 µl of $FeSO_4$ (5.0 mM) and 600 µl of EDTA (15 mM) was mixed with 400 µl of sodium phosphate buffer (0.2 M, pH 7.4). Then 600 µl of *D. gnidium* extracts and 800 µl of H_2O_2 (0.01%) were added. The mixture was incubated at 37 °C for 60 min, and the absorbance was measured at 536 nm. Results were determined using the following equation:

Hydroxyl radical scavenging activity % = $(As-A_0)/(Ac-A_0) \times 100$,

Where A_S is the absorbance of the sample; A_0 is the absorbance of the blank solution using distilled water instead of sample; A_c is the absorbance of a control solution in the absence of H_2O_2. Ascorbic acid was used as positive control.

Reducing power assay

The reducing ability of the extracts from *D. gnidium*, BHT and gallic acid were measured according to the method described by [32] with some modifications. A 0.1 ml aliquot of each extract, BHT or 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 to stop the reaction, 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% $FeCl_3$ (0.5 ml) and then the absorbance was measured at 700 nm. The reducing power of the test samples was increased with the absorbance values.

LC/Uv-vis-DAD/MS analysis of *Daphne gnidium L.* extracts

Daphne gnidium extracts were evaluated by HPLC/UV-vis-DAD/ESI-MS using a Waters instrument (Waters Italia, Milano, Italy) consisting of a 1525 Binary HPLC Pump, a PDA 996 Photodiode Array Detector and a Micromass ZQ single quadrupole Mass Analyser equipped with a ESI Z-spray source; analyses were run in negative ion mode under the following conditions: capillary voltage 2.75 kV, extractor voltage, 2V; cone voltage 20V, source temperature 150 °C, desolvation temperature 250 °C; the gas (nitrogen) flow value used was 400 L/hr for the desolvation, 210 L/hr for the cone. Chromatographic runs were performed using a reverse-phase column (Luna C18, 250 x 4.6 mm, 5 µm particle size, Phenomenex Italia). Caper phenolics were eluted with the following gradient of B (acetonitrile: water 80:20) in A (formic acid, 0,1% solution in water)(ref.1): t = 0 min, 5% B; t =2 min, 5% B; t = 20 min, 20% B; t = 46 min, 38% B; t = 50, 100% B; then kept at 100% B for further 5 min. The solvent flow rate was 1 mL/min and the injector volume selected was 20 µL; the temperature was kept at 25 °C with a column oven (Hitachi L-2300, VWR International, Milano, Italy). Collected data were processed through a Mass Lynx processing system v. 4.0.

Statistical analysis

Statistical analysis was performed using Student's t-test and analysis of variance (ANOVA) followed by Dunnet's test for the multiple effects comparison of the different extracts. The *P*-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Extraction yields and total polyphenol contents

The estimated yields of extraction in relation to total weight (100g) of plant material showed that the CE has the highest yield 13.65±0.618% followed by AE with 7.06±1.04% while other extracts displayed lower yields (table 1).

Table 1: Total polyphenols and flavonoids content of *Daphne gnidium* L. extracts

Extract	Yields (%)	Total phenol content*	Flavonoids contents#
CE	13.65±0.618	137.08±7.66	22.22±1.48
CHE	02.03±0.325	129.65±4.82	36.55±1.721
EAE	2.71±s0.40	130.84±5.99	133.07±2.84
AE	07.06±1.04	nd	nd

Values are expressed as mean±SD (n=3-4). *: µg GAE/mg of extract, #: µg QE/mg of extract

Total phenolic and flavonoid contents were expressed as µg gallic acid equivalents per gram dry weight (µg GAE/g) and µg quercetine equivalents per gram dry weight (µg QE/g) respectively (table 1).

Xanthine oxidase purification

Bovine milk was found to yield around 23.21 mg XOR protein per litre, comparable to the amounts reported by [20], the obtained enzyme was largely (more than 90%) under the oxidase form. The XOR activity was specrophotometrically determined by measuring the production of uric acid from xanthine in the presence and absence of 500 µM NAD^+at 295 nm, using an absorption coefficient of 9600 M^{-1} cm^{-1}[20-22].

The freshly purified bovine milk XOR showed an ultraviolet/visible spectrum with three major peaks at 280, 325, 450 nm, with protein

to flavin ratio (A_{280}/A_{450}) (PFR) of 5.23 indicating a high degree of purity [20, 21, 22]. Run on SDS-PAGE purified enzymes showed one major band of approximately molecular weight of 150 KDa and traces of degradation bands appeared on storage. This is analogous to the well studied bovine, human and Camel XOR [20-22].

Effects of *D. gnidium extracts* on the $O_2^{\cdot-}$generated by the xanthine/xanthine oxidase system

The effect of DGE at different concentrations was studied for their ability to scavenge $O_2^{\cdot-}$generated by the xanthine/xanthine oxidase system. The amount of generated $O_2^{\cdot-}$was determined by measuring the reduction of cytochrome c. Under our experimental conditions, the activity of cytochrome c in the absence of extracts was (2135.91 nmols/min/mg proteins). The reduction of cytochrome $c^{3+}$was almost totally inhibited by SOD (330 U/ml) which used as negative control. The superoxide scavenging effect was found to increase with increasing concentration of DGE, and this is in agreement with our previous results [21, 33]. The EAE was the most potent scavenger of $O_2^{\cdot-}$with IC_{50} value of 0.051±0.0009 mg/ml ($P \leq 0.01$) followed by CE then CHE (0.064±0.001; 0.69±0.01 mg/ml, respectively). Xanthine oxidase-derived superoxide anion has been linked to post-ischaemic tissue injury and edema [34]. Hence, phytochemicals or extracts, which inhibit the superoxide anion regeneration by the enzymatic pathway, would be beneficial in preventing ischemia and edema. The scavenging action of plant constituents has been found to be related to polyphenolic compounds [35, 36] and to caffeic acid derivatives [37] and to flavonoids [48]. It is possible that the anti oxidative properties of DGE are caused, at least in part, by the presence of polyphenols and other yet to be discovered antioxidant compounds.

Effects of *D. gnidium extracts* on XO activity

There is a possibility that $O_2^{\cdot-}$ Scavenging ability of the DGE is due to suppressed effect of these extracts on the xanthine conversion to uric acid, thus the effects of DGE on the XO activity were checked. At identical concentrations, the DGE exhibited an inhibitory effect on xanthine oxidase activity in a concentration dependent manner (data not shown). These effects were compared to that of allopurinol, clinically used as XO inhibitor. The results demonstrated that EAE has the highest XO inhibitory effect with IC_{50} value of 0.021±0.001 mg/ml ($P \leq 0.01$) followed by CE (0.039±0.002 mg/ml) and CHE (0.061±0.001 mg/ml), however allopurinol gave an IC_{50} value of 0.0073±0.0001 mg/ml slighter than that of DGE ($P \leq 0.01$). Inhibiting XO is desirable for two principal reasons: first, it decreases the excess uric acid developed under hyperuricemic conditions that ultimately cause gout [39] second, it prevents the formation of superoxide radicals, thereby protecting against post-ischemic reperfusion injury [40]. The use of allopurinol is restricted by the formation of oxypurinol, which is known to cause side effects [43. Flavonoids might provide an interesting alternative for the treatment of radical-mediated diseases [42]. Several authors have demonstrated that these compounds have a high capacity to inhibit the xanthine oxidase [21, 33]. Our phytochemical study confirms the presence of many phenolic compound including flavonoids such as quercetine, apigenin, luteolin and genistein. Another study established that quercetine, myricetin, and genistein exhibited a competitive inhibition on XO. Apigenin had the comparable inhibitory effect as allopurinol. Genistein was a weaker inhibitor than quercetin and myricetin however naringenin did not show any xanthine oxidase inhibition activity [43].

***D. gnidium extracts* scavenging capacity on the $O_2^{\cdot-}$ radical**

To give a clear view, if DGE have really an $O_2^{\cdot-}$ Scavenging activity or not, the non-enzymatic method (PMS-NADH-NBT) was used. The $O_2^{\cdot-}$anions are generated in a reaction between PMS, NADH and the molecular oxygen. The generated free radical anions then reduce NBT to form a blue formazan colour with an absorbance band at 560 nm [25]. The decrease in the absorbance in the presence of antioxidants indicates the consumption of the generated superoxide anion in the reaction mixture. DGE showed a concentration-dependent scavenging activity by neutralizing superoxide radicals. The most potent scavenger of $O_2^{\cdot-}$ was CE with an IC_{50} of 0.54±0.01 mg/ml ($P \leq 0.01$) followed by EAE then CHE (IC_{50} of 0.872±0.009; 1.45±0.16, respectively). DGE scavenging effects were found to be higher than that of gallic acid, which showed an antioxidant capacity, determined by NBT [33] and higher than that of vitamin C and other phenolic compounds such as quercetin, epicatechin, catechin, rutin and chlorogenic acid), ABTS and DPPH assays [44].

ß-carotene/linoleic acid assay

The ß-carotene-linoleic bleaching inhibition assay is considered to be a good model for membrane based lipid peroxidation [45]. In this oil-water emulsion-based system, linoleic acid undergoes thermally induced oxidation, thereby producing free radicals that attack the ß-carotene's chromophore resulting in a bleaching effect [46]. An extract that inhibits ß-carotene bleaching can be described as a free-radical scavenger and a primary antioxidant [47]. The inhibition extent of lipid oxidation by DGE (2 mg/ml), when compared with BHT as positive control at the same concentration during 24h, showed a marked inhibition effect ($p \leq 0.01$) especially CHE (92.1 %) which was not far from that of BHT (96. 77 %) followed by that of EAE ($p \leq 0.01$) (fig. 1).

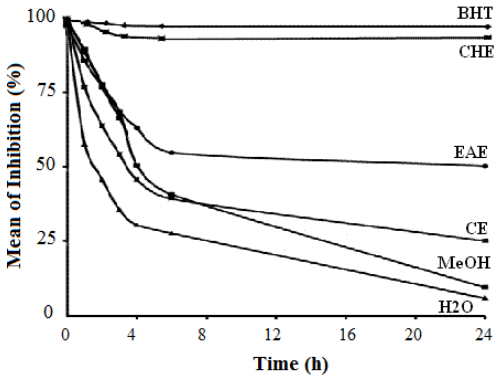


Fig. 1: Changes in the percentage of the linoleic acid oxidation inhibition ratios under the influence of DGE (2 mg/ml), compared to BHT as a positive control during 24 h. Each value represents the mean of numbers of triplicates±SD

We can notice that the activity of tested extracts in terms of polarity activity, the polar extract CHE is more active than EAE and CE. In multiphase systems antioxidants localization depends on their solubility and polarity. Frankel and Meyer (2000) [48] has suggested that antioxidants which exhibit a polar properties are most important because they are concentrated in the lipid-water interface, thereby preventing the formation of lipid radicals and β-carotene oxidation. While polar antioxidants are diluted in the aqueous phase and are thus less effective in protecting lipids.

Inhibition of lipid peroxidation by different extracts of *D. gnidium*, as measured by FTC method

The ferric thiocyanate method (FTC) measures the ability of antioxidants to scavenge peroxyl radicals produced during the initial stages of oxidation, which react with polyunsaturated fatty acids, through hydrogen donation [49]. During the linoleic acid peroxidation, peroxides are formed and these compounds oxidize $Fe^{2+}$to Fe^{3+}, the latter Fe^{3+}ion form a complex with SCN^-, which has a maximum absorbance at 500 nm. High absorbance (or low value of % of inhibition) is an indicator of high concentration of peroxide formed during the emulsion incubation. The inhibitory effect of DGE on lipid oxidation is in the same order as shown in ß-carotene/linoleic acid assay. Fig. 2 shows the time-course for the antioxidative activity of *D. gnidium* extracts, ascorbic acid, BHT, methanol and distilled water by the FTC method. Absorbance value of control samples (distilled water and methanol) increased up to (0.575 and 0.640, respectively) at 48 h, and then decreased. This is due to oxidation of linoleic acid, generating linoleic acid hydroperoxides, which leads to many secondary oxidation products or the intermediate products may be converted to stable end-products and the substrate was exhausted [50]. In the presence of

the *D. gnidium* extracts, ascorbic acid and BHT, oxidation of linoleic acid were very slow. The percentage inhibition of peroxidation in linoleic acid system by crud extract (CE), chloroform extract (CHE) and ethyl acetate extract (EAE) was found to be (3.87±0.03 %), (61.11±0.01)% and (37.11±0.02 %), respectively after 48h of testing. However, those values were lower than those of the positive controls such as BHT (64.549±0.007 %) and ascorbic acid (62.941±0.006) %. The extracts exhibited effective antioxidant activity at the concentration tested, revealing its ability to deter lipid peroxidation. 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.

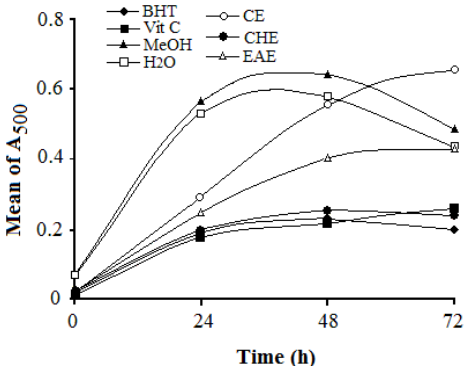


Fig. 2: Inhibition of lipid peroxidation by different extracts of *D. gnidium*, BHT, Vit C, MeOH and H_2O as measured by FTC method. Each absorbance value represents the mean of numbers of triplicates±SD

Antihemolytic activity *D. gnidium extracts*

AAPH generates peroxyl radicals ($ROO^{\cdot}$) that attack the erythrocytes to induce the chain oxidation of lipids and proteins, disturbing the membrane organization and eventually leading to hemolysis [51]. The hemolytic activity of AAPH-generated $ROO^{\cdot}$ was followed continuously at 620 nm to verify changes in the turbidity of RBCs. fig. 3 shows that RBCs underwent an initial increase followed by irreversible loss of cloudiness of suspended RBCs, following the addition of AAPH. This biphasic response was slightly observed in control erythrocytes (incubated only with PBS). The amount of time before the irreversible loss of cloudiness (lag phase) was significantly increased in RBCs incubated with EAE (60 min) versus RBCs incubated with AAPH and CHE (30 min). A difference of 15 min was observed between ascorbic acid, CE and AAPH. The hemolysis is lagged, indicating that endogenous antioxidants in the erythrocytes, namely glutathione, tocopherol, ascorbate and enzymes, such as catalase and superoxide dismutase, can efficiently quench radicals to prevent them from free radical-induced hemolysis, as described previously [52].

Both D. *gnidium* shoot extracts and standards protected the erythrocyte membrane from hemolysis induced by AAPH (fig. 4). A control erythrocytes were stable with little hemolysis within 3 h 30 min (data not shown). The EAE and CE showed higher protective effect against erythrocytes hemolysis than the CHE ($p \leq 0.001$). The percentage of hemolysis (H%) determined for EAE, CE and CHE after 1 h of incubation were 0%, 1.9% and 46.34%, respectively (fig. 4). Quercetine has a more protective effect than that of ascorbic acid and all extracts with the H% values of 2.42% and 7.69% after 1 h and 2 h of incubation, respectively. The H% values of ascorbic acid, EAE, CE and CHE after 2 h of incubation were 62.96%, 55.7%, 67.89% and 71.44%, respectively. Polyphenols are well-known effective scavengers of free radicals. Therefore, phenolic compounds of D. *gnidium* present in the incubation medium most likely quench the peroxyl radicals formed in the aqueous phase before these radicals attack the biomolecules of the erythrocyte membrane to cause oxidative hemolysis. Our results are in agreement with other studies showing that polyphenols are able to protect erythrocytes from oxidative stress or increase their resistance to the damage caused by oxidants [53, 54].

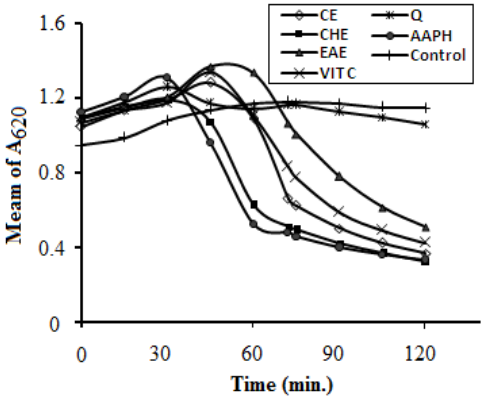


Fig. 3: Representative tracings showing changes in turbidity (OD at 620 nm) of RBC samples in response to AAPH following incubation with CE, CHE, EAE, Quercetine (Q) and Ascorbic acid (VIT C). Recording started immediately following the addition of AAPH. Each value is represented as mean of numbers of triplicates±SD (n = 3)

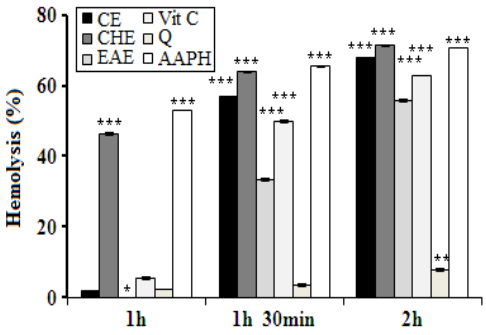


Fig. 4: Percentage of hemolysis of RBCs with 0.1 mg/ml of D. *gnidium* extracts (CE, CHE, EAE), Quercetine (Q) and Ascorbic acid (VIT C). Each value is represented as mean±S. D (n = 3). (*p ≤0.001;**p ≤0.01; *p ≤0.05)**

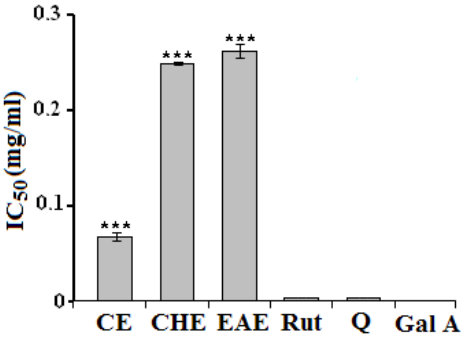


Fig. 5: DPPH radical scavenging activity of different DGE extracts; crud extract (CE) ethyl acetate (EAE), chloroform (CHE), rutine (Rut), quercetine (Q) and gallic acid (Gal A). Data are presented as IC_{50} values. Each value represents the mean±SD (n = 3). (*p ≤0.001; compared to Gal A)**

Scavenging activity of *D. gnidium extracts* on DPPH radicals

The results show that DGE have a concentration-dependent scavenging activity in quenching DPPH radicals. The CE was more

effective than other extracts but less effective than the standards used ($p \leq 0.01$) followed by CHE which gave an effect lower than CE by 4 folds (fig. 5). In most cases, the aqueous alcohol extracts showed better antioxidant activities than the chloroform extracts in DPPH-assay because it is considered the best solvent for extracting phenolic compounds from plant materials [55]. However, EAE showed the lowest effect than those of CE and CHE.

Although there is a great difference between EAE and CE extracts in flavonoid content, but the second one exhibited a higher antiradical effect, this may be explained by the interaction of different flavonoids and/or other compounds present in CE. It is reported that terpenoids and flavonoïds having glycosidic linkage are likely to be extracted into aqueous extracts [56]. It was demonstrated that DPPH• free radicals scavenger potential of polyphenolic compounds depends on the particular substitution pattern of free hydroxyl groups on the flavonoid skeleton, the highly active flavonoids possess a 3', 4'-dihydroxy occupied B ring and/or 3-OH group [57].

Hydroxyl radical scavenging activity of different DGE extracts

The hydroxyl radical is known to be detrimental and initiates auto-oxidation, polymerization and fragmentation of biological molecules [58]. Hydroxyl radicals are also known to be the most reactive species, causes damage to DNA, protein and other life essential biological molecules, leading to mutagenesis, carcinogenesis, and aging [59]. Therefore, the removal of the hydroxyl radical is probably one of the most effective defenses of a living body against various diseases [60]. The *D. gnidium* extracts and ascorbic acid showed significant inhibition of hydroxyl radicals generated by Fenton's reagent in a concentration dependent manner. The hydroxyl radical scavenging data (fig. 6) indicated that all extracts of *D. gnidium* possess the ability to scavenge this reactive oxygen species (ROS). Among the *D. gnidium* extracts, it was found that CHE was efficient in quenching the hydroxyl radical formation and expressed as an IC_{50} value of 0.425±0.011 mg/ml, followed by EAE (0.574±0.037 mg/ml) and CE (1.479±0.025 mg/ml) ($p \leq 0.001$). It is worth to mention that ascorbic acid was shown to be the strongest inhibitor than tested extracts (IC_{50}: 0.159±0.005 mg/ml) ($p \leq 0.001$). Many previous studies have investigated the relationship between the antioxidant activity of plant products and their poly phenolic content [61]. Poly phenolic compounds are very important antioxidants due to their hydroxyl groups that confer free radical scavenging ability to plant products. Flavonoids, probably the largest of the natural phenolics, possess antioxidant properties acting as an effective scavenger of deleterious free radicals and reactive oxygen species [62].

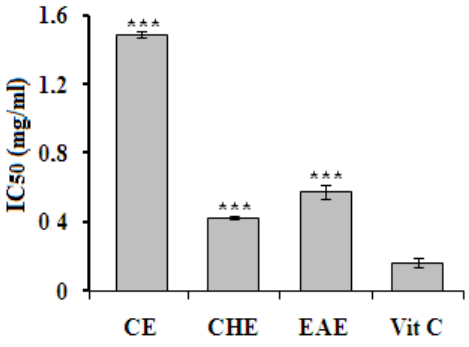


Fig. 6: Hydroxyl radical scavenging activity of *D. gnidium* extracts and ascorbic acid. Lower IC_{50} value indicates higher antioxidant activity. CE; crud extract, CHE; chloroform extract, EAE; ethyl acetate extract. Values are means±SD (n = 3) (* p ≤0.001; compared to Vit C)**

Reducing power of *D. gnidium* extracts

The antioxidant activities of natural components may have a reciprocal correlation with their reducing powers [63]. The reducing power assay is often used to evaluate the ability of the natural antioxidant to donate an electron or hydrogen [64]. 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 [65], so the reducing power of the sample could be monitored by measuring the formation of Perl's Prussian blue at 700 nm. It has been widely accepted that the higher absorbance at 700 nm, the greater is the reducing power. Fig. 7 shows the reducing power of the extracts of *D. gnidium* and standards such as gallic acid and BHT. The reducing ability of the extracts showed a dose-dependent trend increasing with increase in the concentrations of the extracts. Chloroform extract (CHE) exhibited the highest reducing capability (0.369±0.004) at the concentration of 9 µg/ml ($p \leq 0.001$), followed by ethyl acetate extract (EAE) (0.156±0.010) and crud extract (CE) (0.068±0.003). The reducing abilities recorded was in the following order, Gallic acid>BHT>CHE>EAE>CE. Gallic acid exhibited the highest reducing ability than both extracts and BHT with an absorbance value of (1.635±0.106) at 5µg/ml. The significant differences for the antioxidant activity among the different extracts may be attributed to the type of phenolics compounds present in this extract.

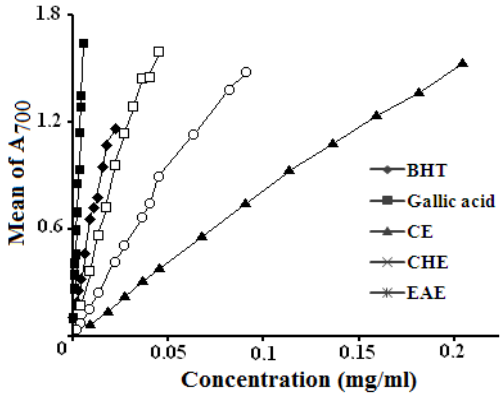
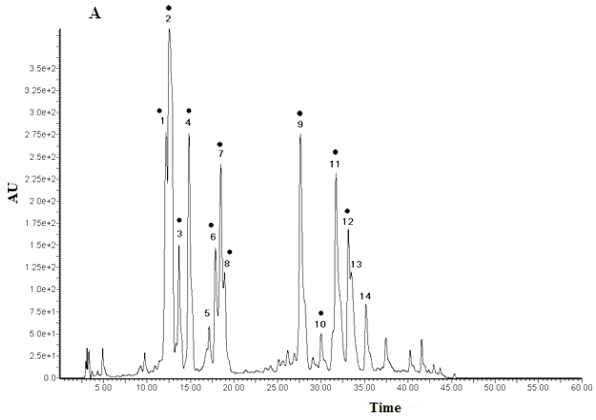


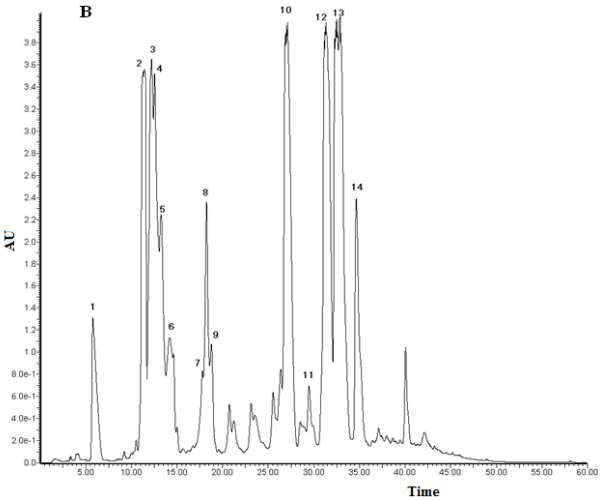
Fig. 7: Reducing power of *D. gnidium* extracts, gallic acid and BHT. CE; crud extract, CHE; chloroform extract, EAE; ethyl acetate extract, BHT; butylated hydroxyl toluene. Values are mean of numbers of triplicates±SD (n = 3)

Identification of phenolic compounds of DGE by HPLC-DAD

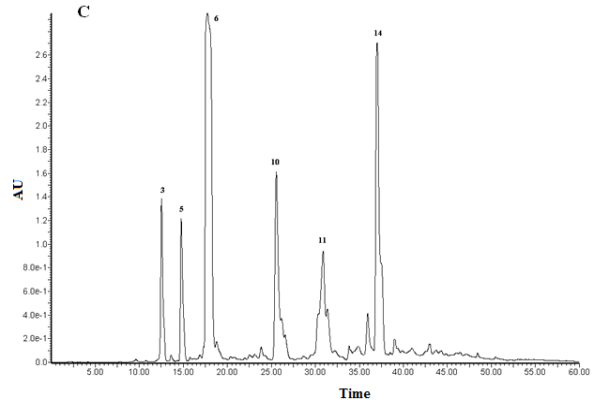
Phenolic cmpounds of DGE were determined using LC/Uv-vis-DAD. These compounds were identified as cinnamic acid derivatives and other metabolites from the flavonoid family particularly, quercetin, luteolin and apigenin (fig. 8. A. B. C)



HPLC chromatogram of methanol extract of *D. gnidium*. Peaks: 1, 2. ferulic acid derivatives; 3. genistein (isoflavone) derivative; 4. cinnamic acid derivative; 5. vicenin-2; 6. cinnamic acid derivative; 7,8. cinnamic acid derivatives; 9. quercetin 3-O-glucoside; 10. quercetin derivative; 11. luteolin glucoside; 12, 13 and 14. apigenin derivatives; 14. apigenin derivative



HPLC chromatogram of of ethyl acetate extract of *D. gnidium*. Peaks: 1. gallic acid; 2. ferulic acid derivative (likely rhamnoside); 3. genistein (isoflavone) derivative; 4,5. peak 2 isomers; 6. peak number 2 isomer; 7,8,9. *p*-coumaric acid derivatives; 10. quercetin 3-O-glucoside; 11. quercetin derivative; 12. luteolin glucoside; 13. methyl apigenin glucoside; 14. luteolin glucoside



HPLC chromatogram of the chloroform extract of *D. gnidium*. Peaks: 3. genistein (isoflavone) derivative; 5. vicenin-2; 6. cinnamic acid derivative; 10. quercetin derivative; 11. luteolin glucoside; 14. apigenin derivative

Fig. 8: LC/Uv-vis-DAD chromatograms of *D. gnidium* extracts visualised at 330 nm A: methanol extract; B: ethyl acetate extract C: chloroform extract

The observed antioxidant activity of DGE may be due mainly to one or more of the compounds found in *Daphne gnidium* such as phenolic compounds (mainly cinnamic acid) and flavonoids (flavones, flavonols and flavanones). It has been shown that quercetin and its glycosides exert inhibitory activity against lipid peroxidation [66]. Vicenin and luteolin-7-glucoside has shown the strongest antioxidant activity *in vitro* tests [67]. The positive effects of these antioxidant components come from their ability to inhibit lipid peroxidation [78]. Cos *et al.*, (2002) [69] measured the antioxidant activities of many phenolic acid derivatives. It has been found that gallic, protocatechuic, caffeic, and chlorogenic acids are good inhibitors of microsomal lipid peroxidation. Torel and Cillard (1986) [70] found that the inhibitory effects of flavonoids on autoxidation of linoleic acid increased in the order fustin<catechin<quercetin<rutin<luteolin<kaempferol<morin.

Previous studies have reported the antioxidant and free radical-scavenging activities of Ferulic acid. In addition genistein is a powerful antioxidant with cellular differentiation activity [71]. It has been proposed that the inhibition of DNA damage by genistein suggests its potential anticarcinogenic activity. (Noroozi *et al.*, 1998) [72] studied the inhibitory effects of flavonoids on the hydrogen peroxide-initiated oxidative DNA damage to human lymphocytes. The efficiency of flavonoids decreased in the range of luteolin>myricetin>quercetin>kaempferol>quercitrin>apigenin>quercetin-3-glucoside>rutin. Most of the flavonoids were more effective inhibitors than ascorbic acid.

CONCLUSION

To assess the antioxidant activities of single compounds or the antioxidant capacity of plant extracts, a variety of methods based on different mechanistic principles must be used in parallel, because different methods often give different results. We showed in the present study widely ranging results. All the extracts obtained from *D. gnidium* studied contained phenolics, flavonoids and antioxidant capacity, although with different efficiencies. The shoot extracts of the *D. gnidium* had high phenolic and flavonoid contents and antioxidant capacity. In addition, our results demonstrate for the first time that *D. gnidium* shoots extracts confers protection against free radical-induced oxidative damage on biological membranes. Flavones and flavonols were the major compounds in shoots of *D. gnidium*. The present study also indicates that the possible antioxidant mechanism of the extract may be due to its hydrogen or electron donating and direct free radical scavenging properties. The interpretation of data from the antioxidant assays is complicated; the antioxidant capacity of extracts was strongly dependent on the qualitative and quantitative phenolic profile. However, it cannot be ruled out that other non-phenolic compounds might be involved in the antioxidant activity. It is therefore suggested that *D. gnidium* shoots is a novel and promising natural antioxidant agent with high potential to prevent or slow the progress of human diseases mediated by oxidative stress. Considering its chemical composition and excellent antioxidant properties, further assays are being undertaken to assess other biological activities.

ACKNOWLEDGMENT

This work was supported by the Algerian Ministry of Higher Education and Scientific Research (Grant Nr F1220020) and by the Thematic Agency of Science Research in Health (DGRSDT, ATRSS). We express our gratitude to these organizations. The authors wish to also acknowledge Dr. Laura SIRACUSA and Dr. Giuseppe RUBERTO, and the support offered by the Istituto di Chimica Biomolecolare del CNR, which gave us an opportunity to conduct part of this type of work using its facilities.

CONFLIT OF INTERESTS

Declared None

REFERENCES

1. Halliwell B, Gutteridge J. Free radicals in biology and medicine. Oxford, UK: Oxford Univ. Press; 2007.
2. Harrison R. Physiological roles of xanthine oxidoreductase. Drug Metab Rev 2004;36:363-75.
3. Davies KJA. Oxidative stress the paradox of aerobic life. Biochem Symp 1994;61:1-34.
4. Fatehi-Hassanabad Z, Chan CB, Brian L, Furman BL. Reactive oxygen species and endothelial function in diabetes. Eur J Pharmacol Lharmacol 2010;636:8–17.
5. Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discovery 2009;8:579-91.
6. Shukla V, Mishra SK, Pant HC. Oxidative stress in neurodegeneration. Adv Pharmacol Sci 2011:572-634. doi.org/10.1155/2011/572634. [Article in Press]
7. Sun J, Chen Y, Li M, Ge Z. Role of antioxidant enzymes on ionizing radiation resistance. Free Radic Biol Med 1998;24:589-93.
8. Arya N, Prakash O, Verma AK, Vivekanand, Pant AK. Variation in antioxidant potential of *Curcuma Longa* L. collected from different ecological niches of western Himalayan region. Int J Pharm Pharm Sci 2015;7:85-90.
9. Borris RP, Blasko PG, Cordell GA. Ethnopharmacologic and phytochemical studies of the Thymelaeaceae. Ethnopharmacol J 1998;24:41-91.

10. Bellakhdar J, Claisse R, Fleurentin J, Younos C. Repertory of standard herbal drugs in the moroccan pharmacopoeia. Ethnopharmacol J 1991;35:123-43.
11. Chaouki W, Leger DY, Liagre B, Cherrah Y, Beneytou JL, Hmamouchi M. Roots of *Daphne gnidium* L. inhibit cell proliferation and induce apoptosis in the human breast cancer cell line MCF-7. Pharmazie 2009;64:542.
12. Maistrello L, López M, Soria F, Ocete R. Growth inhibitory activity of *Daphne gnidium* L. (Thymelaeaceae) extracts on the elm leaf beetle (Col., Chrysomelidae). Appl Entomol J 2005;129:418-24.
13. Harizi H, Chaabane F, Ghedira K, Chekir-Ghedira L. Inhibition of proinflammatory macrophage responses and lymphocyte proliferation *in vitro* by ethyl acetate leaf extract from *Daphne gnidium*. Cell Immunol 2011;267:94-101.
14. Cottiglia F, Loy G, Garau D, Floris C, Casu M, Pompei R, *et al*. Antimicrobial evaluation of coumarins and flavonoids from the stems of *Daphne gnidium* L. Phytomedicine 2001;8:302-5.
15. Iauk L, Aleo G, Caccamo F, Rapisarda A, Ragusa S, Speciale AM. Comparative evaluation of antibacterial and antimycotic activities of *Daphne gnidium* Leaf and bark extracts. Farmaci Terapia 1997;14:37-43.
16. Markham KR. Techniques of flavonoïds identification. London Academic Press; 1982;2:113.
17. Price MP, Butler LG. Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. J Agric Food Chem 1977;25:1268-73.
18. Graham HD. Modified prussian blue assay for total phenols. J Agric Food Chem 1992;40:801-5.
19. Quettier-Deleu C. Phenolic compounds and antioxidant activities of buckweat hulls and flour. J Ethnopharmacol 2000;72:35-42.
20. Baghiani A, Harrison R, Benboubetra M. Purification and partial characterisation of camel milk xanthine oxidoreductase. Arch Physiol Biochem 2003;111:407-14.
21. Baghiani A, Boumerfeg S, Adjadj M, Ameni D, Djermouni M, Khelifi-Touhami F, *et al*. Antioxidants, free radicals scavenging and xanthine oxidase inhibitory potentials of *Ajuga iva* L. Extracts. J Free Radical Antioxidant 2011a;1:21-30.
22. Baghiani A, Ameni D, Boumerfeg S, Djarmouni M, Charef N, Khennouf S, *et al*. Studies of antioxidants and xanthine oxidase inhibitory potentials of root and aerial parts of medicinal plant *Capparis spinosa* L. Am J Med Sci 2011b;1:1-9.
23. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
24. Robak J, Gryglewski RJ. Flavonoïds are scavengers of superoxide anions. Biochem Pharmacol 1988;37:837-41.
25. Ani V, Varadaraj MC, Akhilender, Naidu K. Antioxidant and antibacterial activities of polyphenolic compounds from bitter cumin (*Cuminum nigrum* L.). Eur Food Res Technol 2006;224:109-15.
26. Dapkevicius A, Venskutonis R, Van Beek TA, Linssen PH. Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania. J Sci Food Agric 1998;**77**:140-6.
27. Duh PD, Yen GC. Antioxidative activity of three herbal water extracts. Food Chem 1997;60:639-45.
28. Manna C, D'angelo S, Migliardi V, Loffredi E, Mazzoni O, Morrica P, *et al*. Protective effect of the phenolic fraction from virgin olive oils against oxidative stress in human cells. J Agric Food Chem 2002;50:6521-26.
29. Mukhina GL, Buckley T, Brodsky RA. A rapid spectrophotometric screening assay for paroxysmal nocturnal hemoglobinuria. Acta Haematol 2002;107:182-4.
30. Burits M, Bucar F. Antioxidant activity of *Nigella sativa* essential oil. Phytother Res 2000;14:323-8.
31. Li YH, Jiang B, Zhang T, Mu W M, Liu J. Antioxidant and free radical scavenging activities of chickpea protein hydrolysate (CPH). Food Chem 2008;106:444-50.
32. Chung YC, Chen SJ, Hsu CK, Chung CT, Chou ST. Studies on the antioxidative activity of graptopetalum paraguayense. Walther Food Chem 2005;91:419-24.
33. Boumerfeg S, Baghiani A, Djarmouni M, Ameni D, Adjadj M, Belkhiri F, *et al*. Inhibitory activity on xanthine oxidase and antioxidant properties of *Teucrium polium* L. Chin Med 2012;30:30-41.
34. Raedschelders K, Ansley DM, Chen DDY. The cellular and molecular origin of reactive oxygen species generation during myocardial ischemia and reperfusion. Pharmacol Ther 2012;133:230-55.
35. Jayaprakasha GK, Bhimanagouda S, Patil. *In vitro* evaluation of the antioxidant activities in fruit extracts from citron and blood orange. Food Chem 2007;101:410-8.
36. Youwei Z, Jinlian Z, Yonghong P. A comparative study on the free radical scavenging activities of some fresh flowers in southern China. LWT-Food Sci Tech 2008;41:1586-91.
37. Kimura Y, Okuda T, Hatono T, Agata I, Arichi S. Effects of extracts of leaves of Artemisia species and caffeic acid and chlorogenic acid on lipid metabolic injury in rats fed peroxidized oil. Chem Pharm Bull 1985;33:2028-34.
38. Tapiaa A, Rodriguezb J, Theodulozb C, Lopezd S, Egly Feresinc G, Schmeda Hirschmanna G. Free radical scavengers and antioxidants from *Baccharis grisebachii*. J Ethnopharmacol 2004;95:155-61.
39. Pea F. Pharmacology of drugs for hyperuricemia. Mechanisms, kinetics and interactions**.** Contrib Nephrol 2005;147:35-46.
40. Berry CE, Hare JM. Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. J Physiol 2004;555:589-606.
41. Richette F, Bardin T. Gout. Lancet 2010;375:318–28.
42. Koyama K, Kaya M, Ishigaki T, Tsujita J, Hori S, Seino T, *et al*. Role of xanthine oxidase in delayed lipid peroxidation in rat liver induced by acute exhausting exercise. Eur J Appl Physiol 1999;80:28.
43. Lin CM, Chen CS, Chen CT, Liang YC, Lina JK. Molecular modeling of flavonoids that inhibits xanthine oxidase. Biochem Biophys Res Commun 2002;294:167-72.
44. Kim DO, Lee KW, Lee HJ, Lee CY. Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. J Agric Food Chem 2002;50:3713-7.
45. Ferreria A, Proenc'a C, Serralheiro MLM, Arau´ jo MEM. The *in vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plant from portugal. J Ethnopharmacol 2006;108:31-7.
46. Koleva II, Van Beek TA, Linssen JPH, de Groot A, Evstatieva LN. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem Anal 2002;13:8-17.
47. Liyana-Pathirana CM, Shahidi F. Antioxidant properties of commercial soft and hard winter wheats (*Triticum aestivum L*) and their milling fractions. J Sci Food Agr 2006;86:477-85.
48. Frankel EN, Meyer AS. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. J Sci Food Agric 2000;80:1925-40.
49. Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J Agric Food Chem 2005;53:1841-56.
50. Vukovic N, Sukdolak S, Solujic S, Niciforovic N. Substituted imino and amino derivatives of 4-hydroxycoumarins as novel antioxidant, antibacterial and antifungal agents: Synthesis and *in vitro* assessments. Food Chem 2010;120:1011-8.
51. Mendes L, Victor de Freitas VD, Baptista P, Carvalho M. Comparative antihemolytic and radical scavenging activities of strawberry tree (*Arbutus unedo L.*) leaf and fruit. Food Chem Toxicol 2011;49:2285-91.
52. Zou CG, Agar NS, Jones GL. Oxidative insult to human red blood cells induced by free radical initiator AAPH and its inhibition by a commercial antioxidant mixture. Life Sci 2001;69:75-86.
53. Costa RM, Magalhães AS, Pereira JA, Andrade PB, Valentão P, Carvalho M, Silva BM. Evaluation of free radical scavenging and Antihemolytic activities of quince (*Cydonia oblonga*) leaf: a comparative study with green tea (*Camellia sinensis*). Food Chem Toxicol 2009;47:860-5.
54. Carvalho M, Ferreira PJ, Mendes VS, Silva R, Pereira JA, Jerónimo C, *et al*. Human cancer cell antiproliferative and antioxidant activities of *Juglans regia L*. Food Chem Toxicol 2010;48:441-7.
55. Negi PS, Jayaprakasha GK, Jena BS. Antioxidant and antimutagenic activities of pomegranate peel extracts. Food Chem 2003;80:393–7.

56. Satyanarayana S, Sushrutha K, Sarma GS, Srinivas N, Subba Raju GV. Antioxidant activity of the aqueous extracts of spicy food additives-Evaluation and comparison with ascorbic acid *in vitro* systems. J Herb Pharmacother 2004;4:1-10.
57. Amič D, Davidovic´-Amic´ D, Beslo D, Trinajstic´ N. Structure-radical scavenging activity relationships of flavonoids. Croat Chem Acta 2003;76:55-61.
58. Liu F, Ng TB. Antioxidative and free radical scavenging activities of selected medicinal herbs. Life Sci 2000;66:725-35.
59. Lee JC, Kim HR, Kim J, Jang YS. Antioxidant property of an ethanol extract of the stem of Opuntia flcus-indica var. sabote. J Agric Food Chem 2002;50:6490-6.
60. Qian ZJ, Jung WK, Byun HG, Kim SK. Protective effect of an antioxidative peptide purified from gastrointestinal digests of oyster, Crassostrea gigas against free radical induced DNA damage. Bioresour Technol 2008;99:3365-71.
61. Craciunescu O, Stefan LM, Balan M, Moldovan L. Evaluation of the radioprotective activity of new green tea-collagen materials. J Optoelectron Adv Mater 2007;9:2602-7.
62. Halliwell B, Aeschbach R, Lóliger J, Aruoma OI. The characterization of antioxidants. Food Chem Toxicol 1995;33:601-17.
63. Yen GC, Duh PD. Scavenging effect of methanolic extracts of peanut hulls on free-radical and active oxygen species. J Agric Food Chem 1995;42:629-32.
64. Shimada K, Fujikawa K, Yahara K, Nakamura T. Antioxidative properties of xanthum on the autoxidation of soybean oil in cyclodextrin emulsion. J Agric Food Chem 1992;40:945-8.
65. Gülçin I. Antioxidant and antiradical activities of L-carnitine. Life Sci 2006;78:803-11.
66. Cook NC, Samman S. Flavonoids-chemistry, metabolism, cardioprotective effects, and dietary sources. Nutr Biochem 1996;7:66-76.
67. Ulubelen A, Mabry TJ, Dellamonica G, Chopin J. Flavonoids from Passiflora palmeri. J Nat Prod 1984;47:384-5.
68. Le Marchand L. Cancer preventive effects of flavonoids-a review. Biomed Pharmacother 2002;56:296-301.
69. Cos P, Rajan P, Vedernikiva I, Calomme M, Pieters L, Vlietinck AJ, *et al*. *In vitro* antioxidant profile of phenolic acid derivatives. Free Radical Res 2002;36:711-6.
70. Torel J, Cillard J, Cillard P. Antioxidant activity of flavonoids and reactivity with peroxy radical. Phytochemistry 1986;25:383-5.
71. Kurzer MS, Xu X. Dietary phytoestrogens. Annu Rev Nutr 1997;17:353-81.
72. Noroozi M, Angerson WJ, Lean MEJ. Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. Am J Clin Nutr 1998;67:1210-8.