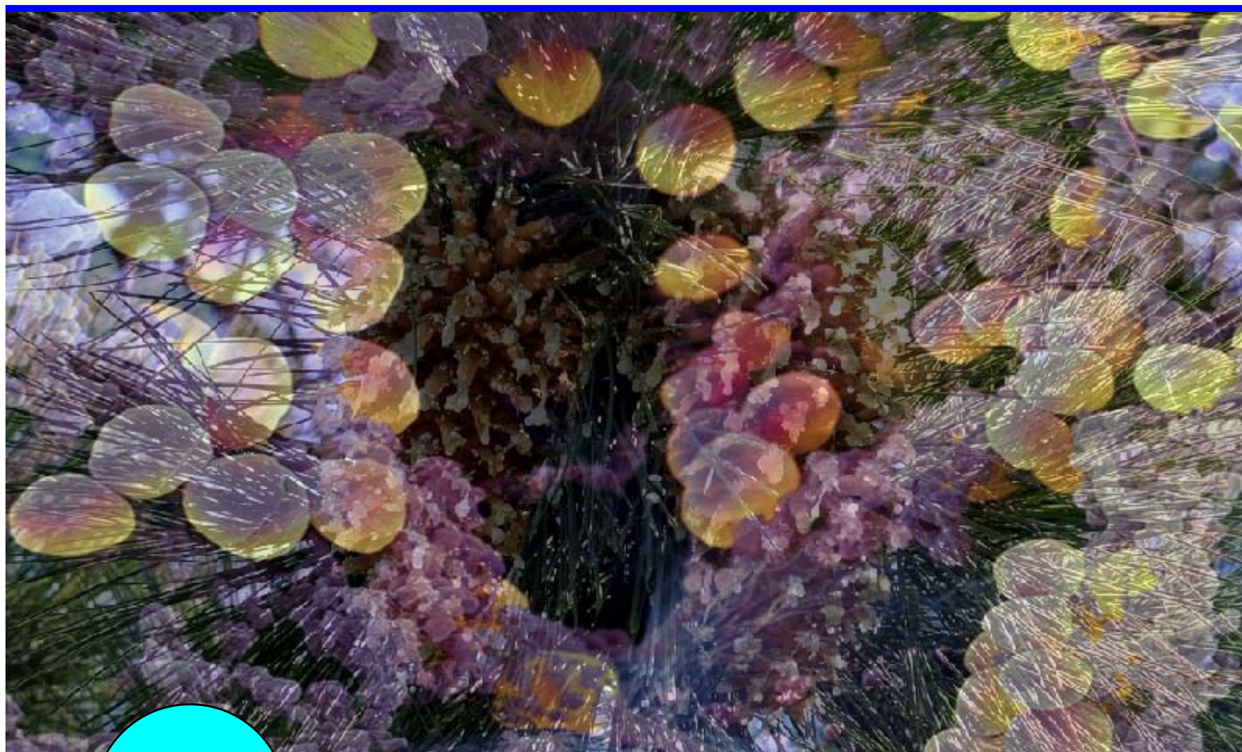

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Chemical analysis of phenolic compounds and determination of antioxidant, antimicrobial and cytotoxic activities of organic extracts of *Pinus coulteri*

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Abstract

New bioactive natural products, the phenolic composition and the biological activities of organic extracts from the needles of the Algerian *Pinus coulteri* were investigated. The analysis by HPLC-DAD of crude extract revealed the presence of 10 phenolic acids and nine flavonoids. *In vitro* antioxidant activities were performed using four different tests. The greatest antiradical activity was found in the ethyl acetate fraction (IC_{50} = 3.2 ± 0.3 µg/mL), whereas the diethyl ether fraction had the higher contents of total phenolics and flavonoids and exhibited a highest activity in reducing power and β-carotene–linoleic acid tests with EC_{50}= 67.1 ± 0.4 µg/mL and 71.5 ± 0.2% of inhibition, respectively. Furthermore, a low to moderate antimicrobial activity according to all extracts was revealed against eight bacteria tested. The MIC value of chloroform fraction showed a strong degree of antibacterial activity (<0.09 mg/mL). The crude extract was found toxic with LC_{50} value of 15.2 µg/mL by brine shrimp toxicity assay. The needle extract of *P. coulteri* is rich in valuable biologically active compounds and could represent a new resource of antioxidant agents for the treatment of diseases.

Introduction

Plant products are the rich sources of a variety of biologically active compounds, mainly flavonoid and phenolic, those phytochemicals have been found to possess various biological properties (Kaneria et al., 2012). Moreover, it has been reported that the use of natural antioxidants may protect the harmful effects of the reactive oxygen and nitrogen species, which are produced in biological systems and foods. They play an essential role in the prevention of human diseases such as diabetes, cancer and neurodegenerative diseases (Sun et al., 2002; Liu, 2003).

On the other hand, the recent situation of antimicrobial resistance of human pathogens to current antimicrobial agents becomes serious medical and economical problems which are the necessity for novel antibiotic prototypes. This need oblige scientists to investigate another source for antimicrobial such as plants. Where some research proves that higher plants may serve as promising sources of novel antibiotics prototypes as well (Chew et al., 2012).

Exploration of natural products as antioxidant, antimicrobial or cytotoxic agents is a research field with great potential. Numerous groups are currently performing further activity-guided studies to find of new molecules of interest, including extraction prepared

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from divers parts of pine (Ustun et al., 2012; Cui et al., 2005; Kwak et al., 2006; Amalinei et al., 2014; Yesil-Celiktas et al ., 2009).

Pinus is the largest genus of conifers occurring naturally in the northern hemisphere. A literature survey shows that this genus is a good source of phenolic compounds, which are known for their diverse biological and pharmacological proprieties. A long tradition explains that pines retain a solid reputation for beneficial trees. Disscoride advised the vinous decoction of pine cones for the cough inveterate to phthisics and ulcerated lungs, while the leaves are used for liver diseases (Lucienne, 2010).

Some pine such as *P. pinaster, P. halepensis, P. brutia, P. radiata, P. pinea*, *P. nigra* and other have been extensively researched (chemical composition and biological activities, morphological and geographical variation, gene ecology and genetic side) (Ait Mimoune et al., 2013; Dob et al., 2007; Ustun et al., 2012; Yesil-Celiktas et al., 2009; Calamassi, 1986), there are comparably few studies that have been conducted on the biological activities and the phenolic composition of the extracts from other pine species.

Among this genus, *Pinus coulteri* D. Don is an evergreen tree that naturally occurs in the coastal mountains of Southern California and Northern Baja California (Mexico) and also grows in the Maghreb countries after it was introduced in arboretums for more than a century. The leaves in clusters containing three needles measure 15–30 cm long, the outstanding characteristic of this pine is the large spiny cones which are 20-40 cm long, and weigh 2–5 kg when fresh (Gernandt et al., 2005). This species is considered among the coniferous of choice for reforestation in Algeria (Meddour, 1992).

The chemical composition and biological activity of the essential oil of *P. coulteri* obtained from needles, has been reported (Hanana et al., 2014; Ioannou et al., 2014). To the best of our knowledge there is no work has been published on its phenolic composition and its biological activities. The objective of the present work was to investigate and evaluate the antioxidant, antimicrobial and cytotoxic activities using *in vitro* assays of crude extracts and its fractions obtained from the needles of Algerian *P. coulteri*. In addition, we determined the phytochemical constituents by characterization of different classes of secondary metabolites, HPLC-DAD analysis of crude extract and quantification of total phenolic and total flavonoid contents.

Materials and Methods

Plant material

Fresh aerial parts of *P. coulteri* were collected in May 2017 from the Medea of Algeria (latitude 36°27′ N, longitude 3°17′E and altitude 874 m). The plant material was taxonomically identified by the botanical survey and the voucher specimen was deposited in the herbarium of the Department of Botany of Ecole National Superieure d'Agronomie. The needles were cleaned and air-dried at room temperature in the shade, and then crushed into fine particles.

Preparation of plant extracts

Ten grams of dried plant materials were extracted for 48 hours with 100 mL of 70% (v/v) aqueous methanol. This process was repeated thrice. The hydromethanolic solutions were pooled and evaporated under reduced pressure at 40°C using vacuum rotary evaporator to obtain dry extract. The hydromethanolic crude extract (HME) was subjected to fractionation using different solvents. The crude extract was resuspended in warm water and then partitioned with equal volumes of chloroform, diethyl ether, ethyl acetate and *n*-butanol. Chloroform fraction (CF), diethyl ether fraction (DEF), ethyl acetate fraction (EAF) and *n*-butanol fraction (BF) were collected separately and concentrated using a vacuum evaporator to remove the solvent. All extracts obtained were then redissolved in methanol and were conserved at +4°C until use.

Phytochemical screening

The powder of hydromethanolic extract or aqueous extract of *P. coulteri* was qualitatively tested for the presence of the various bioactive compounds constituents. Phytochemical screening was performed using the following reagents and chemicals: Alkaloids with Dragen Dorff's reagent, Wagner's reagent and Bouchardat's solutions, anthocyanines with concentrated sulfuric acid and ammonium hydroxide, flavonoids with the use of magnesium and hydrochloric acid, tannins with concentrated hydrochloric acid and ferric chloride solution, anthraquinones free and glycosides with ammonium hydroxide solution, steroids and terpenes with sulfuric acid, coumarins with ammonium hydroxide solution and saponins with ability to produce suds. These were identified by characteristic color changes using standard procedures (Paris and Moyse, 1976).

Determination of total phenolics

For the assessment of total phenolic contents (TPC), the colorimetric method of Singleton and Rossi (1965) was used. Briefly, an aqueous aliquot (0.2 mL) of the extract was added to 3.8 mL of distilled water in a test tube, 0.3 mL of Folin-Ciocalteu's reagent was mixed and the solution was incubated at 40°C for 40 min before adding 0.8 mL of 20% sodium carbonate. The blue coloration was read at 760 nm. Thus, the calibration curve was drawn using gallic acid as standard for total phenolics which was measured as mg gallic acid equivalents (GAE) per g of dry weight.

Determination of total flavonoids

To analyze the total flavonoid contents (TFC), the method introduced by Lamaison and Carnet (1990) was adapted. 1.5 mL of the diluted extract was added to equal volumes of 2% $AlCl_3$. After 10 min, the absorbance was measured at 440 nm. Quercetin was used as standard and flavonoid contents were expressed as mg quercetin equivalents (QE) per g of dry weight.

HPLC analysis

High performance liquid chromatography (HPLC) was performed using a Waters Alliance 2695 chromatography system and UV detector with bars of diode surveyor (DAD). Chromatographic runs were performed using Ascentis C18 column (250 x 4.6 mm, 5 µm). The chromatographic data system was controlled by the Empower Software from Waters. The extract was analyzed using solvent system A (0.2% formic acid dissolved in water) in solvent system B (0.2% formic acid dissolved in acetonitrile) with the following gradient: 0-15 min (95 to 90% A); 15-50 min (90 to 70% A); 50-60 min (70 to 10% A). The solvent flow rate was 1 mL/min and the temperature was fixed to 40°C.

Antioxidant activity

Total antioxidant capacity

The total antioxidant capacity (TAC) was evaluated according to the method described by Prieto et al. (1999). This assay is based on the reduction of molybdenum (VI) to molybdenum (V) by the plant extracts. An aliquot (0.2 mL) of plant extracts (1 mg/mL) was combined to 2 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95°C for 90 min. Then, the samples were cooled to room temperature and the green coloration was read at 695 nm against a blank. The antioxidant capacity was expressed as mg gallic acid equivalent per gram of dry weight (mg GAE/g dry weight).

DPPH radical scavenging activity assay

The capability of the plant extracts to donate hydrogen atoms or electrons was measured from the bleaching of purple colored methanol solution of 1,1- diphenyl-2-picrylhydrazyl (DPPH) (Braca et al., 2002). Different methanolic dilutions of extracts (1 to 750 µg/mL) were mixed with equal volumes of freshly prepared DPPH methanol solution (0.004% w/v). The reaction mixture was vortexed and incubated in the dark for 30 min. The absorbance was read at 517 nm using a blank containing the same concentration of extracts without DPPH. Ascorbic acid, α-tocopherol and BHT were taken as standards. Inhibition of the DPPH free radical (I %) was calculated using the following equation:

$$I\ \% = [(AC - AS)/AC] \times 100$$

Where, AC and AS are the absorbance of control and sample, respectively

The extract dose providing 50% inhibition (IC_{50}) was extrapolated from the graph of scavenging effect percentage against extract concentration.

Reducing power assay

The ability of *P. coulteri* extract to reduce Fe^{3+} was revealed by the method of Oyaizu (1986). Different concentrations of plant extracts in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. Then, 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 3,000 rpm. An aliquot of the supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 0.1%). The absorbance was measured at 700 nm against a blank. Ascorbic acid, α-tocopherol and BHT were used as authentic standards. EC_{50} value is the effective concentration of the extract which corresponds to 0.5 of absorbance for reducing power was obtained from the linear regression analysis.

β-Carotene/linoleic acid bleaching assay

After using a slightly modified Shon et al. (2003) method was based on the aptitude of various extracts to decrease the oxidative discoloration of β-carotene in an emulsion. 2 mg of β-carotene was dissolved in 10 mL of chloroform. 1 mL of this solution was pipetted into a round-bottom flask containing 20 mg of linoleic acid and 200 mg of Tween 40. Then, 50 mL of distilled water was added slowly to the residue and the solution was vigorously agitated to form a stable emulsion. 4.8 mL of the obtained emulsion were transferred into different test tubes containing 0.2 mL of extract (2 mg/mL). The mixture was then placed in a water bath at 50°C for 120 min. Absorbance at 470 nm was measured every 30 min for 120 min using UV-Vis spectrophotometer. Blank solution was prepared in a similar way except that addition of β-carotene was omitted. BHT was used as standards. The bleaching rate (R) of β-carotene was calculated according to first-order kinetics:

$$R = (\text{In}\ (A_{t=0} / A_{t=t}))/t$$ (Al- Saikhan et al., 1995)

Where, ln is natural log, t is the time in minutes, $A_{t=0}$ and $A_{t=t}$ are the absorbance at time t (0, 30, 60, 90, and 120 min)

The percent of antioxidant activity (AA) was calculated using the equation:

$$AA = (R_{control} - R_{sample}) / R_{control} \times 100$$

Where, $R_{control}$ and R_{sample} are average bleaching rates of the negative control and the antioxidant (plant extract or BHT), respectively

Antimicrobial activity

Microbial strains

The hydro-methanolic crude extract and its fractions

were individually tested against a panel of nine pathogenic microorganisms including eight bacteria species, three Gram positive *Staphylococcus aureus* (methicillin-resistant MRSA 639c), *Bacillus subtilis* (ATCC 6633) and *Listeria monocytogenes* (ATCC 13932), five Gram negative *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (CIP A22), *Enterobacter cloacae* (E13), *Salmonella typhimurium* (ATCC 14028) and *Klebsiella pneumonia* (E40) and one yeast *Candida albicans* (M3). All microorganisms were obtained from the Microbiological laboratory, Department of Biology, ENS, Algiers, Algeria.

Disc diffusion assay

Antimicrobial activities of extracts were first screened for their inhibitory zone by the agar disc diffusion method. The microbial suspension was prepared by diluting the cell mass in saline water (0.9% NaCl) and adjusted to 0.5 McFarland scale. 20 mL of sterile agar [Mueller Hinton for bacterial strains and Sabouraud dextrose agar for yeast (Institut Pasteur, Algeria)] were poured in the petri plates and allowed to dry. The inoculums were swabbed on the top of the solidified media. A sterile paper discs (5.5 mm of paper) impregnated with 10 µL of the plant extracts (100 mg/mL) were applied in the petri dish. Before incubation, all petri dishes were stored in the dark at +4°C for 2 hours, to allow the diffusion of the extracts from disc to medium without microbial growth. Positive control was prepared using the levofloxacin (10 µg/disc) for bacteria and nystatin (10 µg/disc) for yeast. The activity was determined by measuring the inhibitory zone diameter ID in mm, after incubation at 37°C/24 and 30°C/48 hours for bacteria and yeast, respectively. The data used was the mean of three replicates.

Agar dilution method

The minimum inhibitory concentration (MIC) was determined by the agar dilution method as described by Ebrahimabadi et al. (2010). The appropriate amount of the plant extract was added aseptically to the sterile medium to produce the concentration range of 25–0.097 mg extract/mL medium. The resulting agar solutions were immediately mixed and poured into the petri plates. The plates were spot inoculated with 1 µL of microorganism. The MIC was defined as the lowest concentration of the extract which prevents visible growth of microorganisms.

Brine shrimp lethality test

Brine shrimp larvae were used as indicator animals for preliminary cytotoxicity assay of the extracts as reported by Turker and Camper (2002). 1 g of *Artemia salina* (Linnaeus) cysts (obtained from CNRDPA, Algeria) was aerated in 1 L capacity of rectangular plastic (jar) containing seawater (36 g of sea salt dissolved in 1 L of distilled water) and positioned near a lamp (60 W) to provide direct light and heat (27 ± 01°C). After 10-12 hours incubation newly hatched free-swimming pink-colored nauplii were harvested from the bottom outlet. Two days was allowed for the shrimp to mature as nauplii. The assay system was prepared with 2.5 mL of seawater prepared containing respective concentration of hydro-methanolic crude extract (10000, 1000, 100, 10, 1 µg/mL). In each, 10 nauplii were transferred and the setup was allowed to remain for 24 hours, under constant illumination. Therefore, the dead nauplii were counted. Based on the percent mortality, the median lethal concentration, LC_{50} value (the concentration at which 50% mortality of brine shrimp nauplii occurred) of the plant extract was determined. Three replicates were prepared for each concentration. A negative control was used in the same saline solution without extract.

Statistical analysis

All experiments were carried out in triplicate. Data were expressed as mean± standard deviation (SD). Differences were evaluated by one-way analysis of variance (ANOVA) test completed by a student's t-test. The concentration needed for 50% inhibition (IC_{50}) was estimated graphically by linear regression analysis. LC_{50} value was obtained by a plot of percentage of dead shrimps against the logarithm of the sample concentration. The correlations between antioxidant activities and total phenolic and/or flavonoid contents were calculated using the Pearson coefficient (r) and linear regression analysis by Microsoft Excel program. Differences were considered significant at $p<0.05$.

Results

Preliminary phytochemical screening

The important phytochemical anthocyane, terpenes, steroids, tannins, *O*-heterosids, flavonoids and saponins were screened for their presence (Table I).

Yield and total phenolic and flavonoid contents

From Table II the extraction yield varies from 1.3 to 24.3% (w/w). Among all the fractions, BF obtains the highest extraction yield (6.5 ± 0.9%) while DEF yield the lowest (1.3 ± 1.1%).

Whereas the total phenolic and flavonoid contents (TPC, TFC) show that *P. coulteri* extracts exhibited an important amount of polyphenol content followed by flavonoid. The amount of total phenolics varied from 4.2 ± 0.3 to 20.6 ± 0.1 mg GAE/g of dry weight. HME had the higher content (20.6 ± 0.1 mg GAE/g of dry weight). The fraction with the highest total phenolic content was diethyl ether (8.5 ± 0.1 mg GAE/g of dry weight). The same tendency is observed for flavonoid contents (Table II).

Table I	
Phytochemicals detected in *P. coulteri*	
Phytochemicals	Results[a]
Anthraquinones	-
Alkaloids	-
Anthocyane	+++
Saponins	+
Coumarins	-
Flavonoids	+
Tannins	++
O-heterosids	++
C- heterosids	+
Steroids and terpenes	+++

[a](+) present, (-) absent

HPLC analysis

The HPLC analysis obtained from the hydromethanolic extract (Figure 1), showed that *P. coulteri* is a plant rich in secondary metabolites reported by a chromatogram comprising 23 peaks, and comparing the retention times and UV spectra of these peaks with those standards injected in the same conditions as the extract allowed us to identify 19 compounds: Ten phenolic acids, nine flavonoids and four were unidentified (Table III).

Table II			
Extraction yields (%) and polyphenol contents of *P. coulteri* crude extract and its fractions			
Extracts/ Fractions	Extraction yield[a] (%)	Phenolics[b]	Flavonoids[c]
HME	24.3 ± 2.3	20.6 ± 0.1	0.3 ± 0.01
ChF	1.5 ± 0.9	4.2 ± 0.3	0.04 ± 0.04
DEF	1.3 ± 1.1	8.5 ± 0.1	0.1 ± 0.04
EAF	1.8 ± 0.2	5.4 ± 0.3	0.1 ± 0.1
BF	6.5 ± 0.9	5.4 ± 0.1	0.05 ± 0.02

[a]calculated as (DW ext*100)/DW plant (DW ext and DW plant: The weight of extract after evaporation and powdered plant, respectively); [b](TPC)expressed as mg gallic acid equivalents/g dry weight [c](TFC) expressed as mg quercetin equivalents/g dry weight, Values are expressed as means of three replicates ± SD

Antioxidant activity

The total antioxidant capacity (TAC) assay is a quantitative method expressed as numbers of equivalents of gallic acid. Table IV shows that polar solvents had the higher antioxidant capacity, 144.4 ± 0.5 mg/g dry weight was observed in HME of *P. coulteri*. DEF exhibited weaker activity (15.1 ± 0.4 mg/g dry weight) when compared to different other solvent fractions. Excellent r correlation coefficients (0.9 and 0.8) were observed between the total antioxidant capacity (TAC) and the total phenolic and flavonoid contents (TPC and TFC respectively).

DPPH radical scavenging activity had been used extensively to test the ability of compounds and extracts from the plants or food materials to act as free radical scavengers or hydrogen donors (Raghavendra et al., 2013). As a kind of stable free radical, DPPH can accept an electron or hydrogen radical from antioxidant (A-H) to become a stable diamagnetic molecule as described in the following equation: (DPPH·)+(A-H)→DPPH-H + (A·). In our investigation, all extracts were found to be effective scavengers against DPPH radical. As shown in Table IV, the ethyl acetate fraction had the highest potent DPPH radical scavenger activity (3.2 ± 0.3 µg/mL) and the free radical-scavenging activity of crude extract was seven times less important than that of ethyl acetate fraction, which might result from the active components through enrichment effects during the solvent–solvent partitioning processes. As it can be seen, ascorbic acid was used as the standard in this

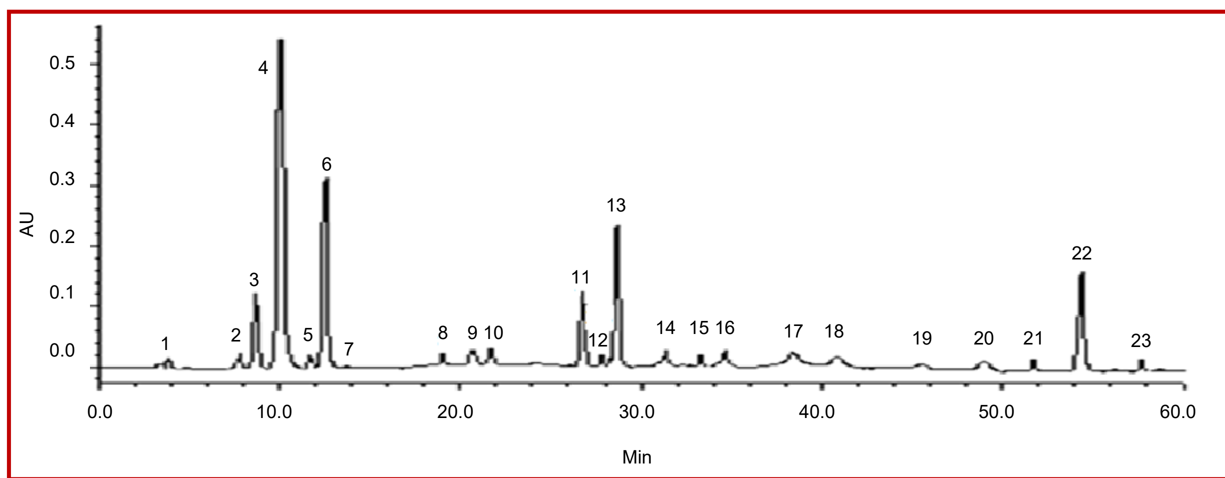

Figure 1: HPLC profile of hydromethanolic extract of *P. coulteri* needles

Table III

Phenolic compounds identified by HPLC-DAD in a hydromethanolic extract of *P. coulteri* needles

SL	Identified compounds	Retention time (min)	Area (%)	λ UV max (nm)
1	Nd	4.6	0.8	-
2	Nd	7.8	1.0	-
3	Gallic acid	8.3	4.2	220.6; 272.8
4	Protocatechuic acid	10.1	41.1	219.1; 264.5; 293.8
5	*p*-Hydroxybenzoic acid	11.4	0.9	252.1
6	Catechin	12.1	3.3	220.0 ; 277.7
7	Nd	14.9	0.8	-
8	Vanillic acid	18.3	10.4	231.0; 261.4; 292.6
9	Elagic acid	20.8	0.7	263.6; 367.3
10	Nd	21.8	1.5	-
11	Syringic acid	26.8	5.9	218.3; 274.5
12	Chlorogenic acid	27.9	1.0	218.3; 241.4; 326.9
13	*p*-Coumaric acid	28.4	10.5	261.1; 342.3
14	Rosmarinic acid	31.1	0.8	290.1; 328.6
15	Sinapic acid	33.2	0.8	243.2; 322.0
16	Hisperidin	34.3	0.9	242.2 ; 281.3 ; 363.7
17	luteolin 7 glucoside luteolin 4 glucoside	38.4	1.0	256.1; 267.0; 350.3
18	Myricetin	41 .1	0.7	253.5 ; 370.0
19	Naringenin-7-glycoside	45.8	0.1	286.1 ; 332.4
20	Quercetin	49.2	1.2	254.2; 365.1
21	Naringenin	50.9	0.03	213.1; 283.2; 330.3
22	Kaempferol	54.0	2.8	254.3 ; 368.1
23	Apigenin	59.2	0.1	267. 4; 336.2

Nd: Non identified compounds

Table IV

Anti-oxidant capacities of extracts from *P. coulteri* and standards measured by different assays

Extracts/fractions /standard	TAC[a] (mg GAE/g DW)	IC_{50}/DPPH[a] (µg/mL)	EC_{50}/Reducing power[a] (µg/mL)	β-Carotene/linoleic acid[a] (%)
HME	144.4 ± 0.5	22.7 ± 0.6	1006.6 ± 0.3	61.3 ± 0.01
ChF	42.9 ± 0.1	24.7 ± 0.2	462.9 ± 0.9	62.7 ± 0.05
DEF	15.1 ± 0.4	16.7 ± 1.1	67.0 ± 0.4	71.5 ± 0.2
EAF	40.1 ± 0.1	3.2 ± 0.3	324.4 ± 0.6	50.9 ± 0.2
BF	43.0 ± 0.4	58.2 ± 0.4	787.5 ± 0.7	66.6 ± 0.5
Ascorbic acid	-	3.7 ± 0.3	50.0 ± 0.6	-
α-Tocopherol	-	9.1 ± 0.4	506.5 ± 1.0	-
BHT	-	67.9 ± 0.4	613.6 ± 0.6	77.8 ± 0.4

[a]Each value is expressed as the mean ± standard deviation (n = 3); TAC = total anti-oxidant capacity; IC_{50} = inhibition concentration 50%; EC_{50} = effective concentration at which the absorbance was 0.5

Table V

After RAA treatment

	HME		ChF		DEF		EAF		BF		Standards[c]	
Microorganisms	ID[a]	MIC[b]	ID	MIC	ID	MIC	ID	MIC	ID	MIC	ID	MIC
Gram negative bacteria												
P. aeruginosa	–	–	–	–	–	–	–	–	–	–	24 ± 2.1	0.02
E. coli	–	–	–	–	–	–	–	–	–	–	39 ± 1.0	0.02
K. pneumonia	7.5 ± 0.1	3.1	7.0 ± 0.1	3.1	10.0 ± 0.1	3.1	7.5 ± 0.1	1.5	8.5 ± 0.2	1.5	25.0 ± 1.8	0.09
E. cloacae	–	–	6.5 ± 0.1	<0.09	7.0 ± 0.2	3.1	–	–	–	–	27 ± 1.5	0.05
S. typhimurium	–	–	–	–	–	–	–	–	7.0 ± 0.2	1.5	35.5 ± 0.5	0.05
Gram positive bacteria												
B. subtilis	6.0 ± 0.2	3.1	7.0 ± 0.1	3.1	6.0 ± 0.1	1.5	7.0 ± 0.1	0.7	–	–	39 ± 0.0	0.01
S. aureus	10.8 ± 0.2	6.2	13.5 ± 0.2	3.1	10.0 ± 0.0	3.1	9.8 ± 0.3	1.5	8.3 ± 0.3	3.1	25.3 ± 1.2	0.01
L. monocytogenes	10.8 ± 0.2	0.3	13.3 ± 0.5	<0.09	18.3 ± 1.5	0.3	11.0 ± 0.0	0.2	11.5 ± 0.5	1.5	31.3 ± 1.1	0.01
Yeast												
C. albicans	–	–	–	–	–	–	–	–	–	–	20 ± 0.0	0.12

[a]Inhibition zone in diameter (mm) around the impregnated discs and each value is presented as mean ± SD (n = 3); [b]Minimal inhibition concentrations(mg/mL); [c]Levofloxacin for bacteria, Nystatin for yeast. (–): no antimicrobial activity

study, had a higher IC_{50} value than that the ethyle acetate fraction (3.7 ± 0.3 µg/mL). This indicates potent free radical scavenging activity of EAF of *P. coulteri*.

For the determination of the reductive ability, the Fe^{3+}-Fe^{2+} transformation was investigated in presence of *P. coulteri* extracts. Samples with different concentration were used for this assay and all of them exhibited the dose-dependent activity. As shown in Table IV, the DEF revealed a good reducing power (EC_{50}= 67.0 ± 0.4 µg/ mL). While, the EC_{50} values of ethyl acetate and chloroform fractions (324.4 ± 0.6 and 462.9 ± 0.9 µg/mL, respectively) were higher to that of positive controls α-tocopherol and BHT (506.5 ± 1.0 and 613.6 ± 0.6 µg/mL, respectively). The reducing power of all fractions was presented in the following order: DEF > EAF >ChF> BF.

The β-carotene bleaching assay measures the ability of a plant extract to prevent the loss of the yellow color of *β*-carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsified aqueous system (Parejo et al., 2002). The percentage of inhibition from the crude extract and its different fractions was found to be ranged from 50.9 to 71.5% (Table IV). It can be also noticed that the highest *β*-carotene bleaching effect was observed of DEF with 71% and the lowest one was observed of EAF with 50%. The *β*-carotene bleaching effect of the synthetic antioxidant BHT was found to be higher than the extracts of *P. coulteri.*

The mutual correlations among the four methods shown that high correlation among the total anti-oxidant capacity and reducing power was found (0.8), whereas DPPH/reducing power exhibited moderate correlations (0.5). Furthermore, no correlations were obtained with β-carotene-linoleic acid bleaching and other methods.

Antimicrobial activity

The *in vitro* antimicrobial activities of *P. coulteri* extracts were active against the bulk microorganisms tested with different levels of inhibition: Weak (inhibition zone diameter <12) and moderate (inhibition zone diameter 12-20 mm) (Table V). The chloroform fraction showed MIC ranged between 3.1–0.1 mg/mL and it exhibited a higher degree of antimicrobial activity (<0.1 mg/mL) against *E. cloacae* and *L. monocytogenes* as compared with the other fractions. These results were in conformity with the result of disc diffusion method that shows that the activity may be associated with active constituents.

Cytotoxic activity

In the current study by using brine shrimp lethality test, we evaluated the cytotoxic effect of hydromethanolic extract from *P. coulteri*. This test is rapid, inexpensive and simple bioassay for testing plant extracts bio-activity which in most cases correlates with cytotoxic and antitumor properties. Findings from this study revealed that needles extracts of *P. coulteri* was LC_{50}= 15.2 µg/mL.

Discussion

The present work is the first report of the biological activities of *P. coulteri* needles extract. Our findings showed the richness of phenolic content. For that, the *in vitro* estimation of antioxidant activities was determined. It is extremely important to point out that the most antioxidant activities from plant sources are correlated with phenolic compounds. The high content of total phenols in extracts and synergistic interactions might explain the strong antioxidant capacities of this plant (Cai et al., 2004; Huang et al., 2010; Erkan et al., 2008). These compounds are known to have the latest activity not only because of their ability to donate hydrogen or electrons but also to by stable radical intermediates (Wong et al., 2006).

On the other hand, a moderate correlation was found between phenolic compounds and the antioxidant efficiencies of the crude extract/fractions, suggesting that phenolic compounds are not the only contributors to the antioxidant activities of *P. coulteri*.

Comparing the obtained results with the previously published data of pine part extract, we have found that the scavenging activity against DPPH is comparable with studies of Ustun et al. (2012). It has exhibited weak to moderate activity in the needles extract. Whereas pycnogenol (the most common commercially available pine bark extract (*P. pinaster*)) was highly active in DPPH radical scavenging assay (88.3%). Our results were in accordance with the same previous published data showing the high reducing power of the ethanol and acetone needles extracts of *P. sylvestris* and *P. halepensis,* respectively (Ustun et al., 2012). The results of this study showed that the very good antioxidant activity is exhibited by the extracts containing higher amount of flavonoid and phenolic compounds. These may be the hydroxyl groups existing in the phenolic and flavonoid compounds which prove them good radical scavenger (Cai et al., 2004)

On the other hand the *P. coulteri* needles extracts exhibited antibacterial activity against the majority strains tested. The inhibitory effect of theirs phenolics could be explained by the mechanism of toxicity of flavonoids against microorganisms, for example, non-specific interactions such as the establishment of a hydrogen bond with proteins or enzymes of the cell wall, the chelation of inhibition of bacterial metabolism, or sequestration of substances necessary for the growth of bacteria (Vaquero et al., 2007). The reaction of bacteria against the phenolic compounds is related with the permeability of the substances to the cell. Gram negative are resistant because they have an outer

phospholipidic membrane, which inhibit the passage of the molecules. Despite this, the gram positive bacteria have only an outer peptidoglycan layer which is not an effective permeability barrier (Abirami et al., 2012).

Furthermore, the cytotoxic activity demonstrated that plant extracts with LC_{50} value greater than 100 µg/mL are considered as non-toxic, while LC_{50} value less than 100 µg/mL as toxic (Meyer et al., 1982). However, the hydromethanolic extract from *P. coulteri* is 15.2 µg/mL, this lethality indicates the presence of potent cytotoxic and probably antitumor components of this plant.

Despite that, published reports on the cytotoxic activity with brine shrimp lethality test are not available. Other studies reported that the use of natural antioxidants can reduce the risk of various cancers, an effect seemingly mediated by phenolic compounds (Bencherchar et al., 2017). Thus, the presence of cytotoxic agents in plants belonging to Pinaceae family is demonstrated (Cui et al., 2005; Huseini et al., 2015).

Indeed, in agreement with the results of the present study, many of the phenolic compounds produced by the pine species have shown possess an inhibition of growth of human gastric and breast cells, cytotoxic and antiproliferative effects (Kwak et al., 2006; Amalinei et al., 2014).

As a result, the biological activities exhibited by *P. coulteri* needles extracts are attributed to the presence of many active substances such as rosmarinic acid, chlorogenic acid, coumaric acid, gallic acid, catechin, myricetin, quercetin, naringenin, kaempferol and apigenin which are known as powerful antioxidant, antimicrobial and cytotoxic agents.

Conclusion

P. coulteri possess high biological activities and, therefore could represent an interesting alternative for natural and preventive therapies. Importantly, EAF of this plant was as strong as pure ascorbic acid in DPPH scavenging activity and HME confirmed to be cytotoxic.

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Conflict of Interest

Authors declare no conflict of interest

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