Asian Journal of Chemistry; Vol. 23, No. 2 (2011), 819-822

ASIAN JOURNAL OF CHEMISTRY

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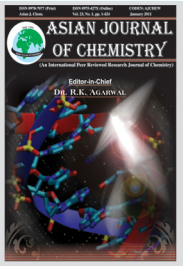

Comparison of Radical Scavenging Capacity of Different Extracts of Barks and Needles of *Pinus roxburghii* and *Pinus wallichiana*

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(*Received*: 7 May 2010; *Accepted*: 10 October 2010) AJC-9172

In this paper, the comparative study of antioxidant activities exhibited by different extracts of bark and niddles of *Pinus roxburghii* and *Pinus wallichiana* were evaluated. Polar and non-polar extracts were obtained by fractionating the crude methanolic extracts of bark and needles of each plants with *n*-hexane, dichloromethane, ethylacetate and butanol, respectively leaving aqueous fractions behind. Activity of each sample was evaluatd using 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging assay. The total phenolic and total flavonid contents of each sample were assessed. Correlation between DPPH scavenging potential (IC_{50}) and total phenolic content is not so strong and is found to be positive only in case of PrB ($r^2 = 0.3602$) fractions while correlation between scavenging percentage and total flavonoids is positive in PrN, PwN and PrB fractions. However, fractions exhibiting best antioxidant potential are rich in both flavonoids and phenolics and these fractions are polar *i.e.* ethyl acetate, butanol and water fractions.

Key Words: DPPH radical scavenging activity, *Pinus roxburghii*, *Pinus wallichiana*, Oxidative stress.

INTRODUCTION

Main contributer of the oxidative stress that opens way to several diseases like cancer, atherosclerosis and arthritis is the overproduction of various forms of reactive oxygen species in the form of free radicals[1]. These reactive oxygen species (ROSs) may be the result of endogenous activity like aerobic respiration and external factors like ionizing radiation, organic solvents, smoke of vehicles and tobacco, many pollutants and pesticides effect additively[2]. Presence of free radicals is strongly corelated with disease, toxicity and aging process[3,4].

Against the hazardous effects of free radicals, living organisms have efficient defence system[5]. But this oxidative stress is caused by the imbalanced generation and neutralization of ROS as a result of disturbed redox homoeostasis[6]. The cause of this disturbed redox may be an increased production of free radicals along with weakened antioxidant defence system of the body. Although self supporting system of the body consisting of quenching enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (Gred) *etc.* and antioxidant molecules can capture these ROS but prolonged exposure to unfavourable conditions slowers down the system and cause irreversible damage to body[7]. So an exogenous supply of antioxidants is necessary to keep the system functional.

There are few reports about the antioxidant activity of conifers in general but one genus Pinus of the family Pinaceae is worth mentioning in this regard. Many species of the Pinus have been reported for antioxidant activity in their barks and needles extracts.

The procyanidins rich maritime pine bark extract Pycnogenol has well documented antioxidant and antiinflammatory activity. It is the patent product of *Pinus pinaster* and is found to be effective in inhibiting the matrix metaloprotienases[8] and posseses antiproliferative effect in melanoma cells[9]. Antioxidant activity of maritime pine *Pinus pinaster* bark extracts towards free radicals has been studied for use in skin cosmetics and on lung carcinoma cells[10,11]. The antioxidant effect of pycnogenol in protecting DNA against Fenton reaction radicals and I/R-induced oxidative renal damage has been reported[12,13]. Pycnogenol also show interactive antioxidative action with nicotine, coenzyme Q 10(CoQ) and phytoestrogens[14].

Antioxidant potential of needles of *Pinus densiflora* against hydroxyl radicals and inhibiting total reactive oxygen species generation in kidney homogenates has been reported[15].

In another Pine species *Pinus radiata*, hot water extracts ontaining monomeric, oligomeric and polymeric fractions exhibit antioxidant activities[16] and these activities has been compared with that of *Pinus pinaster*[17]. Results indicates that *Pinus radiata* bark is richer in total phenols and procyanidins.

Based on the evidences about antioxidant potential of different Pine species, this article is aimed to explore the antioxidant activity of different fractions of barks and needles extracts of *Pinus wallichiana* and *Pinus roxburghii*. Northern hilly area of Pakistan is enriched with the two species. These are approximately 2500 years old trees. These forests are rightly spoken as wealth of Pakistan. There is need to do meaningful research to uplift the use of this gift of nature.

EXPERIMENTAL

Fresh specimens of *Pinus roxburghii* and *Pinus wallichiana* were collected from Sunny Bank and Ghora gali, Murree Hills, Pakistan in May 2009. Mir Ajab Ali Khan, Professor Department of Biological Sciences, Quaid-e-Azam University, Islamabad identified the plants and specimen deposited in the Prem Madan Herbarium of Lahore College For Women University, Lahore.

All the solvents used were of analytical grade (Merk, Germany) available commercially 1,1-dephenyl-2-picryl hydrazyl radical (DPPH)was purchased from Sigma Aldrich.

UV/Vis double beam spectrophotometer (Hitachi, U2800) and 1 cm quartz cells were used for all absorbance measurements

Extraction and fractionation: Barks and needles of the two species were taken for analysis. Fresh material with weight of 1 Kg each was air-dried at room temperature and then ground with machine to powder. Each material was dipped in 100 % CH_3OH for 15 days. Then materials were filtered and filterates condensed at low pressure on rotary evaporater to have crude methanol extract. Crude extract was dissolved in deionized water and then treated successively with *n*-hexane, dichloromethane, ethyl acetate and butanol to obtain fractions with respect to increasing polarity including aqueous fractions at the end. All fractions were concentrated at low pressure and kept in refrigerator for further use.

Determination of total phenolics: For the determination of total phenolics, Cliffe's[18] method involving the use of Folin-Ciocalteu reagent was applied. Simply, 20 µL of the sample was mixed with 1.58 mL deionized water and 100 µL of Folin-Ciocalteu reagent was added to this mixture. After 10 min at room temperature, 300 µL of 25 % sodium carbonate solution (w/v) was added. Following the incubation at 40 ºC and cooling for 0.5 h, absorbance was measured at 765 nm and results expressed as gallic acid equivalent (mg g^{-1} dry wt.) using calibration curve of gallic acid.

Determination of total flavonoids: Colorimetric method[19] was used to determine total flavonoids contents. 0.25 mL of the extract was diluted with 0.5 mL of the deionized water and 90 µL of sodium nitrate solution (5 %) was added. After 6 min, 180 µL of 10 % $AlCl_3$ solution was added and allowed to stand for another 5 min accompanied by the addition of 0.6 mL of 1 M NaOH solution and making the final volume upto 3 mL with deionized water. Finally absorbance measured at 510 nm against blank. Using quercetin calibration curve, results implicated as quercetin equivalent (mg g^{-1} dry wt.)

DPPH Radical scavenging assay: DPPH was used as stable free radical to assess the free radical-scavenging ability of different samples using modified method of blois[20]. The reaction mixture contained 0.5 mL of sample solution (in methanol) and 2.5 mL DPPH radical solution (1×10^{-4} M) (Fig. 1). After incubation at 37 ºC for 0.5 h, absorbance was recorded at 517 nm by UV-Vis spectrophotometer (Hitachi, U2800). Applying following equation, the scavenging % of DPPH was calculated.

$$\text{Scavenging}\,(\%) = \left(1 - \frac{(A_1 - A_2)}{A_0}\right) \times 100$$

where A_0 is the absorbance of the control (blank, without extract) and A_1 is the absorbance in the presence of the extract, A_2 is the absorbance without DPPH.

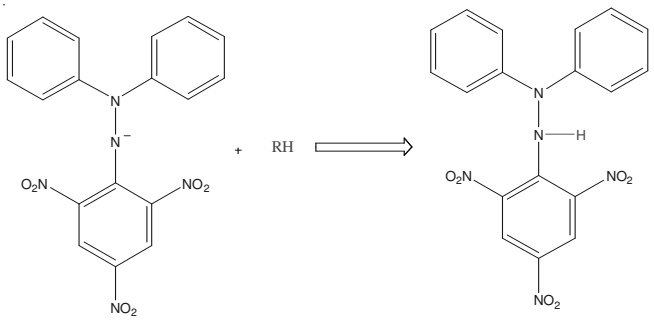

Fig. 1. DPPH radical scavenging activity

RESULTS AND DISCUSSION

Radical scavenging capacity of the extracts in terms of scavenging percentage (%) and IC_{50} is shown in Table-1 and total flavonoids and phenolics of each extract are listed in Table-2. With few exceptions, most of the extracts have flavonoids and phenolics to varying extent but it is difficult to find a clear relationship between free radical scavenging activity and the concentration of flavonoids present in the extracts. Infact free radical scavenging activity of these extracts is not exclusively due to flavonoids but is dependent on the specific phenolic content present in each extract[21]. This relationship is quite evident in case of PrB (*n*-hexane) extract in which flavonoid content is only 2.57 ± 0.30 mg/g but owing to phenolic content that is 13.31 ± 0.38, it showed 92.3 % scavenging ability at a concentration of 200 µg/mL with IC_{50} of 10.494 µg/mL. Similarly PrB (methanol) extract is the best radical scavenger with scavenging percentage of 88.2 and IC_{50} 5.224 µg/mL at a concentration of 150 µg/mL and it posseses flavonoids and phenols upto 17.0 ± 0.01 mg/g and 38.45 ± 1.32 mg/g, respectively. All the extracts except PrB (aq) fraction which was not soluble in methanol exhibited scavenging ability [Table-1(a)] while Table-1b shows the amount of each extract required for 50 % inhibition (IC_{50}). Total phenolics *versus* reciprocal values of IC_{50} of flavonoid extracts gave a positive correlation in case of PrB fractions only ($r^2 = 0.3602$) and remaining showed negative correlation. This may be due to medium used *i.e.* methanol which is slightly acidic (pH = 5.1) and it is reported[21] that total phenols *versus* reciprocal values of IC_{50} of flavonoid extracts gave better correlation in basic medium ($r^2 = 0.7829$) as compared to distilled water (r^2

TABLE-1
RADICAL (DPPH) SCAVENGING CAPACITY IN TERMS OF SCAVENGING (%) AND IC_{50} OF DIFFERENT EXTRACTS OF TWO PINE SPECIES

No.	Extract fraction	Radical scavenging capacity		
		Scavenging conc. (µg/mL)	Scavenging (%)	IC
1	PwB(*n*-Hexane)	200	75.60	94.41
2	PwB(Dichloromethane)	200	87.10	18.55
3	PwB(Ethylacetate)	200	90.70	2.831
4	PwB(Butanol)	200	92.70	5.516
5	PwB(Aqueous)	200	86.00	4.935
6	PwN(*n*-Hexane)	200	15.00	nd
7	PwN(Dichloromethane)	200	27.30	nd
8	PwN(Ethyl acetate)	200	96.50	8.403
9	PwN(Butanol)	200	90.10	85.90
10	PwN(Aqueous)	200	20.60	nd
11	PrB(*n*-Hexane)	200	92.30	10.49
12	PrB(Dichloromethane)	200	93.10	9.52
13	PrB(Ethylacetate)	200	93.45	43.96
14	PrB(Butanol)	10	83.10	2.93
15	PrB(Aqueous)	nd	nd	nd
16	PrN(*n*-Hexane)	200	24.70	nd
17	PrN(Dichloromethane)	200	59.30	163.45
18	PrN(Ethylacetate)	50	86.10	11.62
19	PrN(Butanol)	100	64.80	3.283
20	PrN(Aqueous)	200	85.10	120
21	PwB(Methanol)	200	23.50	nd
22	PrB(Methanol)	150	88.20	5.224

TABLE-2
(a) TOTAL FLAVONOIDS AND PHENOLICS IN BARK EXTRACTS OF TWO PINE SPECIES

No.	Extraction fraction	Total flavonoids (mg/g)	
		PrB	PwB
1	*n*-Hexane	2.57 ± 0.30	nd
2	Dichloromethane	7.40 ± 0.15	9.43 ± 0.01
3	Ethyl acetate	3.34 ± 0.36	5.22 ± 0.12
4	Butanol	2.76 ± 0.02	2.53 ± 0.18
5	Aqueous fraction	0.97 ± 0.05	1.95 ± 0.37
6	Methanol	17.0 ± 0.01	19.3 ± 0.23
No.	Extraction fraction	Total phenolics (mg/g)	
		PrB	PwB
1	*n*-Hexane	13.31 ± 0.38	nd
2	Dichloromethane	8.10 ± 0.62	5.15 ± 1.00
3	Ethyl acetate	8.91 ± 0.84	12.61 ± 2.3
4	Butanol	8.93 ± 0.25	5.91 ± 0.13
5	Aqueous fraction	nd	5.10 ± 0.08
6	Methanol	38.45 ± 1.32	28.77 ± 2.39

(b) TOTAL FLAVONOIDS AND PHENOLICS IN NEEDLES EXTRACTS OF THE TWO PINE SPECIES

No.	Fraction extraction	Total flavonoids (mg/g)	
		PrN	PwN
1	*n*-Hexane	1.08 ± 0.24	0.77 ± 0.01
2	Dichloromethane	1.60 ± 0.15	4.17 ± 1.05
3	Ethyl acetate	4.28 ± 0.18	3.95 ± 0.21
4	Butanol	3.91 ± 0.34	2.59 ± 0.07
5	Aqueous fraction	1.01 ± 0.04	nd
No.	Fraction extraction	Total phenolics (mg/g)	
		PrN	PwN
1	*n*-Hexane	9.47 ± 0.70	nd
2	Dichloromethane	9.42 ± 1.3	5.10 ± 0.17
3	Ethyl acetate	10.08 ± 0.09	4.09 ± 0.08
4	Butanol	8.55 ± 0.21	4.06 ± 0.12
5	Aqueous fraction	3.94 ± 0.45	nd

= 0.6012) and acidic medium (r^2 = 0.1242). Total flavonoids *versus* scavenging percentage gave positive correlation with r^2 = 0.4339, 0.5605 and 0.4210 for PrN, PwN and PrB, respectively. Antioxidant nature of flavonoids and phenolics and their beneficial effects regarding human nutrition and health have been recognized. Action mechanism of both is different. Flavonoids behave as scavenger or chelators of the free radicals[22,23]. While phenolics belong to a class of antioxidants which acts by terminating the free radicals[24].

In this study, different fractions of barks and needles of the two Pine species are found to have both falvonoids and phenolics. Of the total fractions, six samples *i.e.* PwN(*n*-hexane), PwN(CH_2Cl_2), PwN(Aq), PrB(Aq), PrN(*n*-hexane) and PwB-(methanolic) show negligible inhibition at a concentration of 200 µg/mL in initial screening. So IC_{50} of these samples is not calculated. Ten fractions show good potential towards DPPH radical scavenging ability with IC_{50} value less than 12 µg/mL shown in Fig. 2. With the exception of two fractions PrB-(dichloro methane) and PrB (*n*-hexane), polar extracts showed good activity and PrB(butanol fraction) is excellent in scavenging DPPH radical (scavenging 83.1 %) with IC_{50} value of 2.83 at a conc. of 10 µg/mL. While PrN(ethyl acetate), PrN(butanol), and PrB(methanolic) scavenge radical (86.1, 64.8 and 88.2 %) with IC_{50} values of 11.62, 3.283 and 5.22 µg/mL at conc. of 50, 100 and 150 µg/mL, respectively. It is clear from Fig. 2 that scavenging capacity is somehow related with total flavonoid and phenolic content.

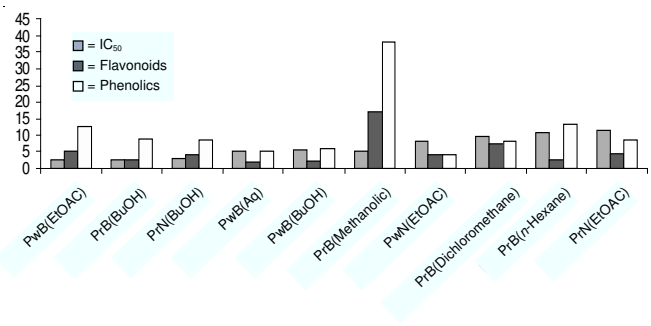

Fig. 2. Fractions with IC_{50} less than 12 µg/mL along with their flavonoids and phenolic content

Conclusion

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. All the extracts including in this study have antioxidant activity to certain extent but 10 fractions have high antioxidant potential with respect to their ability to scavenge the free radicals. Ethyl acetate, butanol and aqueous fractions of bark and needles of *Pinus roxburghii* and *Pinus wallichiana* are found to be more potent.

ACKNOWLEDGEMENTS

The authors would like to thank Higher Education Commission, Pakistan for financial support.

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