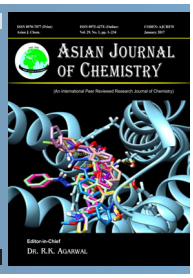
Asian Journal of Chemistry; Vol. 29, No. 3 (2017), 641-646

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2017.20291



Variation in Total Phenolics, Flavonoids and Antioxidant Activity Among Various Solvent Fractions of Bark of Babul (*Acacia nilotica*) Using Different Extraction Techniques

PRAVESH[1], V.K. MADAN[1,*] and SUSHILA SINGH[2]

[1]Medicinal, Aromatic and Potential Crops Section, Old IATTE Building, Chaudhary Charan Singh Haryana Agricultural University, Hisar-125 004, India
[2]Department of Chemistry and Biochemistry, Chaudhary Charan Singh Haryana Agricultural University, Hisar-125 004, India

*Corresponding author: E-mail: vikku60@gmail.com

Received: 6 September 2016; *Accepted*: 29 November 2016; *Published online*: 30 December 2016; AJC-18218

In present study, extraction techniques such as cold (mechanical shaking) and hot (Soxhlet) extraction were compared to evaluate their efficiency towards extraction of total phenolics and flavonoids. The bark of babul was extracted with methanol and further fractionated into various solvent fractions (*viz.* hexane, dichloromethane, chloroform, ethyl acetate and butanol). The methanol extract and its fractions were used for the estimation of total phenolics & flavonoids contents and for the evaluation of antioxidant activity by 2,2'-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity method. The methanol extract obtained by hot extraction technique possessed higher total phenolics and flavonoids contents *i.e.* 49.380 and 18.895 mg CE/g, respectively and also exhibited higher activity with IC_{50} value 67.7 µg/mL. Among various solvent fractions using both extraction techniques, ethyl acetate fraction contained highest amount of total phenolics (9.369 and 9.508 mg GAE/g) and flavonoids (3.229 and 3.786 mg CE/g) followed by butanol, residual aqueous, dichloromethane, chloroform and hexane fractions. Similarly, ethyl acetate fraction exhibited highest antioxidant activity (IC_{50} 24.9 and 35.2 µg/mL) followed by butanol, residual aqueous/dichloromethane, chloroform and hexane fractions.

Keywords: ***Acacia nilotica,*** **Extraction techniques, Solvent fractions, Total phenolics, Flavonoids, Antioxidant activity.**

INTRODUCTION

Medicinal plant parts (roots, leaves, branches/stems, barks, flowers and fruits) are commonly rich in phenolic compounds, such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and lignins [1-4]. They have multiple biological effects including antioxidant activity [5]. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [6,7]. They also have metal chelation properties. Flavonoids, which are partly responsible for the pigmentation of flowers, fruits and leaves are subdivided into flavanols, flavonols, flavones, flavanones and anthocyanins based on the saturation of the flavan ring and also their hydroxylation. Extraction is an important step involved in the discovery of bioactive components from plant material. Selection of the proper analytical strategy for extracting secondary metabolites *viz.* phenolics and flavonoids in plant materials depends on the purpose of the study as well as the nature of the sample and the analyte [8]. Solvent extractions are the most commonly used procedures to prepare extracts from plant materials due to their ease of use, efficiency and wide applicability. Solvents such as methanol, ethanol, acetone, ethyl acetate and their combinations have been used for the extraction of phenolics from plant materials, often with different proportions of water. Selecting the right solvent affects the amount and rate of polyphenols extracted [9].

Acacia nilotica (L.) Willd. Ex Delile commonly known as babul, kikar or Indian gum Arabic tree, belongs to family Fabaceae. *A. nilotica* can be grown in both moist and arid regions because of the fact that it can withstand to extremes of temperatures (> 50 °C) and moisture stress. It contains significant amount of polyphenols, saponins, terpenoids, proteins and polypeptides [10,11] which strengthen its ranking in medicinal plants. Various parts of this plant are known to be important source of secondary metabolities as alkaloids, cynogenic glycosides, fluoroacetate, gums, terpenes (including essential oils, diterpenes, phytosterol and triterpene genins and saponins), hydrolyzable tannins, flavonoids and condensed tannins. Many flavonol and flavones glycosides, aglycones, flavan-3-ols and flavan-3,4-diols have been found in various plant parts lacking 5-hydroxy group, a characteristic of family Fabaceae [12]. Its bark contains tannin (12-20 %), terpenoids, saponins, glyco-

sides, gallic acid, protocatechuic acid, pyrocatechol and (+)-catechin [13]. Plant extracts containing high amounts of bioactive compounds especially antioxidants, have potential of being used in food, agriculture, nutraceuticals, cosmetics and pharmaceuticals products [14]. Survey of literature reveals that less work has been done on the variation in total phenolics and flavonoids as well as on antioxidant activity among various solvent fractions of bark of babul. Therefore, the present study was initiated to study the variation in total phenolics, flavonoids and antioxidant activity among various solvent fractions of bark of babul (*Acacia nilotica*) using cold and hot extraction techniques.

EXPERIMENTAL

The commercially available chemicals from Sigma-Aldrich, Qualigens, Merck and Hi-Media of highest purity, were used for various experimental procedures. The bark sample of babul (*Acacia nilotica*) was procured from the local areas of Hisar. Babul bark were dried and ground in grinding machine to obtain a fine powder. Powdered samples were extracted by using following two extraction techniques:

Cold (mechanical shaking): 25 g of powdered samples of bark of babul were extracted with 175 mL of methanol in 500 mL conical flasks by shaking on a mechanical shaker for 2 h. In each set, total eight conical flasks were placed on mechanical shaker. Extracts were filtered and residues were again extracted twice (shaking time 1 h) with 125 and 100 mL methanol taken in each conical flask. Filtrates from three extraction steps were pooled and their volumes were noted. Several steps of mechanical shaking were repeated to have sufficient amount of extract required for various studies.

Hot (Soxhlet extraction): 200 g of powdered samples of bark of babul were placed in a filter paper (Whatman No. 1) thimble in a classical Soxhlet apparatus fitted with a 3 L round bottom flask. The methanol was added up to one and a half siphons that is approximately 1400 mL. Extraction was performed at boiling temperature of methanol, solvent vapours move up to the column and after getting condensed in the condenser part, floods into the chamber housing thimble filled with babul bark samples. When this chamber was filled completely with solvent, the siphon mechanism operates and the solvent containing some part of phytochemicals that got dissolved in solvent; empties this extract into round bottom flask containing solvent. Process was continued for 14h with completion of up to seven to eight cycles through siphon mechanism. After the completion of extraction step, residue in thimble was again extracted twice (each extraction time 10 and 8 h, respectively) with suitable amount of methanol. Filtrates from three extraction steps were pooled and their volumes were noted. Several steps of Soxhlet extraction were repeated to have sufficient amount of extract required for various studies.

All the samples extracted by using above mentioned techniques were performed in triplicate. All extracts were bottled properly and stored in refrigerator at 4 °C.

Liquid-liquid partitioning/extraction: 500 mL of each extract obtained by using cold or hot extraction technique was concentrated on rotary vacuum evaporator under reduced pressure followed by manifold evaporator till viscous mass was obtained. Viscous mass was defatted by washing 3-4 times with petroleum ether (60-80 °C). Defatted viscous mass was dissolved in 100 mL of 10 % methanol in distilled water, precipitation occurred and it was allowed to separate into aqueous layer and non-aqueous layer (precipitates). Both of these layers were separated by filtration. Further the aqueous layer was partitioned into various solvent fractions. Liquid-liquid partitioning/extraction was achieved by shaking the aqueous layer and solvent in a separating funnel *i.e.* successively partitioned with hexane (30, 20, 20 mL), dichloromethane (30, 20, 20 mL), chloroform (30, 20, 20 mL), ethyl acetate (60, 40, 40 mL) and butanol (40, 30, 30 mL) in sequence. However, occasionally large amount of emulsions were formed (except in partitioning with butanol) and it was difficult to separate out the solvent from the aqueous layer even after keeping it long time, then emulsion was broken down by adding a small amount (2-3 mL) of ethanol. All the volumes of each solvent fraction from three partitioning steps were pooled and their volumes were noted. Non-aqueous layer (precipitates) was redissolved in suitable amount of methanol (30-60 mL) and volume was noted.

Methanolic extracts of bark of babul and its various solvent fractions were used for estimation of total phenolics & flavonoids content and for evaluation of antioxidant activity by DPPH free radical scavenging method.

Estimation of total phenolics: Total phenolics were determined by the Folin-Ciocalteu method [15] using gallic acid as standard for which a calibration curve was obtained. Extracts were diluted to adjust the absorbance within calibration limits. Aliquots of 0.2 mL of each extract was added to 1 mL of 1 mol/L Folin-Ciocalteu reagent followed by 2 mL of Na_2CO_3 (20 %, w/v). The solution was mixed and volume was made up to 10 mL with distilled water. After 8 min, the mixture was centrifuged at 6000 rpm for 10 min. Then the absorbance of supernatant solution was measured at 730 nm using UV-visible double beam spectrophotometer Model 2203 (Systronics Co.) against a blank prepared similarly but containing respective solvent instead of extracts. The amount of total phenolics present in the extracts was calculated from the standard curve and the results were expressed as milligrams of gallic acid equivalent per gram (mg GAE/g).

Estimation of total flavonoids: Total flavonoids were determined by aluminium chloride colorimetric assay [16] using catechin as standard for which a calibration curve was obtained. Extracts were diluted to adjust the absorbance within calibration limits. 1 mL of each extract was added to test tubes containing 4 mL of double distilled water and 0.3 mL of $NaNO_2$ (5 %, w/v) was added. After 5 min, 0.3 mL of $AlCl_3$ (10 %, w/v) was added. 2 mL of 1 M NaOH was added immediately. The solution was mixed and total volume was made up to 10 mL with double distilled water. The solution was mixed thoroughly and the absorbance was measured at 510 nm using UV-visible double beam spectrophotometer Model 2203 (Systronics Co.) against a blank prepared similarly but containing respective solvent instead of extracts. The amount of total flavonoids present in extracts was calculated from the calibration curve and results were expressed as mg catechin equivalents per gram (mg CE/g).

DPPH free radical scavenging activity: The antioxidant activity of the extracts was evaluated by 2,2'-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method [17]. Extracts were dried up completely and the weight of dry mass was noted. The dry mass of methanolic extract and various solvent fractions *viz.* hexane, dichloromethane, chloroform, ethyl acetate and butanol were redissolved in appropriate amount of methanol to make the stock solution (5000 µg/mL). Since, the dry mass of water extract (residual aqueous fraction) was not soluble in pure methanol, hence, it was redissolved in 50 % (v/v) methanol:water to make the stock solution. From stock solution, different concentrations (25 to 5000 µg/mL) were made by appropriate dilutions with methanol for various solvent fractions and 50 % (v/v) methanol:water for residual aqueous fraction. For evaluation of antioxidant activity, in 0.2 mL extract of each concentration, 3 mL of 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH; 0.1 mM in 100 % methanol) was added and mixed thoroughly for 5 min. For antioxidant activity in residual aqueous fraction, DPPH stock solution was prepared in 50 % (v/v) methanol:water and remaining procedure was same. A control was also made containing 0.2 mL of methanol instead of extract. The absorbance of the sample as well as control was measured at 517 nm after 30 min of incubation in dark at room temperature using the UV-visible double beam spectrophotometer Model 2203 (Systronics Co.) against a blank containing methanol. A graph was drawn by plotting percent DPPH free radical scavenging activity (y-axis) against extract concentration (x-axis). Then using the Microsoft Excel Software, a quadratic regression equation ($y = ax^2 + bx + c$) was obtained. By putting y = 50 (for IC_{50}) in the equation $y = ax^2 + bx + c$; it was converted to the form $ax^2 + bx + c = 0$. IC_{50} was calculated from the equation $ax^2 + bx + c = 0$ by using the formula:

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

where, $x = IC_{50}$ (µg/mL).

Calculation: The percentage of DPPH scavenged (% $DPPH^*_{sc}$) was calculated using:

$$DPPH^*_{sc}\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where, $A_{control}$ is the absorbance of control and A_{sample} is the absorbance of the sample.

RESULTS AND DISCUSSION

Extract yield: Extract yield (g/100 g) of various extracts of babul bark varied widely. Amongst extraction techniques, extract yield was higher in methanolic extract obtained by hot extraction technique (10.889 g/100 g) in comparison to cold extraction technique (7.396 g/100 g) as shown in Table-1. Similarly, extract yields were higher in aqueous and non-aqueous layers of methanolic extract obtained by hot extraction technique (5.252 and 5.637 g/100 g, respectively) in comparison to cold extraction technique (4.364 and 3.032 g/100 g, respectively). Other research workers have also reported that burdock datura (*Xanthium strumarium*) extract obtained by Soxhlet method has highest extract yield followed by static maceration and dynamic maceration (shaking) methods and this difference might be due to the higher temperature, which may have increased the strength of solvation [18]. Methanolic extract of stem bark of *A. nilotica* obtained by using Soxhlet method was found to be 10.6 g/100 g [19].

Extract yield (g/100 g) of various solvent fractions varied widely. Amongst various solvent fractions of methanolic extract obtained by cold extraction technique, polar fractions *viz.* residual aqueous, butanol and ethyl acetate have higher extract yield (2.066, 1.171 and 1.049 g/100 g, respectively) whereas non-polar fractions *viz.* chloroform, dichloromethane and hexane have lesser extract yield (0.034, 0.033 and 0.011 g/100 g, respectively) as shown in Table-2. In case of various solvent fractions of methanolic extract obtained by hot extraction technique, polar fractions *viz.* residual aqueous, ethyl acetate and butanol have higher extract yield (2.177, 1.746 and 1.199

TABLE-1
EXTRACT YIELD, TOTAL PHENOLICS AND FLAVONOIDS IN METHANOLIC EXTRACTS OF BARK OF BABUL AND IN ITS AQUEOUS AND NON-AQUEOUS LAYERS

Extract/layers	Cold extraction technique			Hot extraction technique		
	Extract yield (g/100 g)	Total phenolics (mg GAE/g)	Total flavonoids (mg CE/g)	Extract yield (g/100 g)	Total phenolics (mg GAE/g)	Total flavonoids (mg CE/g)
Methanol	7.396 ± 0.043	32.926 ± 0.255	10.752 ± 0.086	10.889 ± 0.173	49.380 ± 0.378	18.895 ± 0.130
Aqueous	4.364 ± 0.052	17.241 ± 0.082	5.190 ± 0.017	5.252 ± 0.035	19.584 ± 0.103	6.351 ± 0.059
Non-aqueous	3.032 ± 0.042	15.685 ± 0.052	5.562 ± 0.193	5.637 ± 0.037	29.796 ± 0.086	12.544 ± 0.095

TABLE-2
EXTRACT YIELD, TOTAL PHENOLICS AND FLAVONOIDS IN VARIOUS SOLVENT FRACTIONS OF METHANOLIC EXTRACTS OF BARK OF BABUL OBTAINED BY HOT AND COLD EXTRACTION TECHNIQUES

Extract/layers	Cold extraction technique			Hot extraction technique		
	Extract yield (g/100 g)	Total phenolics (mg GAE/g)	Total flavonoids (mg CE/g)	Extract yield (g/100 g)	Total phenolics (mg GAE/g)	Total flavonoids (mg CE/g)
Hexane	0.011 ± 0.001	0.017 ± 0.001	0.002 ± 0.000	0.027 ± 0.001	0.031 ± 0.000	0.010 ± 0.000
Dichloromethane	0.033 ± 0.002	0.081 ± 0.001	0.030 ± 0.001	0.047 ± 0.003	0.117 ± 0.002	0.050 ± 0.000
Chloroform	0.034 ± 0.002	0.048 ± 0.002	0.018 ± 0.000	0.056 ± 0.001	0.056 ± 0.002	0.037 ± 0.001
Ethyl acetate	1.049 ± 0.023	9.362 ± 0.013	3.229 ± 0.026	1.746 ± 0.025	9.508 ± 0.011	3.786 ± 0.009
Butanol	1.171 ± 0.024	4.942 ± 0.025	1.279 ± 0.010	1.199 ± 0.017	5.845 ± 0.068	1.608 ± 0.013
Residual aqueous	2.066 ± 0.018	2.791 ± 0.033	0.632 ± 0.006	2.177 ± 0.034	4.027 ± 0.023	0.860 ± 0.004

g/100 g, respectively) whereas non-polar fractions *viz.* chloroform, dichloromethane and hexane have lesser extract yield (0.056, 0.047 and 0.027 g/100 g, respectively). A review of previously documented literature also revealed that residual aqueous fraction of wild parsley (*Torilis leptophylla*) has highest extract yield *i.e.* 8.2 g/100 g followed by ethyl acetate (6.1 g/100 g), butanol (4.8 g/100 g) and chloroform (4.3 g/100 g) fractions [20].

Total phenolics content: Folin-Ciocalteau phenol method was used for the determination of total phenolics content by using gallic acid as a standard phenolic compound. The Folin-Ciocalteau method involves the transport of electron from phenolic complexes to phosphomolybdic acid or phosphotungestic acid complexes, which are examined spectrometrically at 730 nm. Total phenolics content (mg GAE/g) in various extracts/fractions of babul bark varied widely. Amongst extraction techniques, total phenolics content was higher in methanolic extract obtained by hot extraction technique (49.380 mg GAE/g) in comparison to cold extraction technique (32.926 mg GAE/g) as shown in Table-1. Similarly, total phenolic contents were also higher in aqueous and non-aqueous layers of methanolic extract obtained by hot extraction technique (19.584 and 29.796 mg GAE/g, respectively) in comparison to cold extraction technique (17.241 and 15.684 mg GAE/g, respectively). Higher total phenolics was observed in clove buds extracts obtained by using Soxhlet extraction technique in comparison to extracts obtained by mechanical shaking extraction technique and this is due to the increased solubility of phenols in the extracting solvent had higher extraction temperature [21].

Amongst various solvent fractions of methanolic extract obtained by cold extraction technique, polar fractions *viz.* ethyl acetate, butanol and residual aqueous contained higher amount of total phenolics contents (9.362, 4.942 and 2.791 mg GAE/g, respectively) whereas non-polar fractions *viz.* dichloromethane, chloroform and hexane contained lesser amount of total phenolics (0.081, 0.048 and 0.017 mg GAE/g, respectively) as shown in Table-2. Similarly, in case of various solvent fractions of methanolic extract obtained by hot extraction technique, polar fractions *viz.* ethyl acetate, butanol and residual aqueous contained higher amount of total phenolics contents (9.508, 5.845 and 4.027 mg GAE/g, respectively) whereas non-polar fractions *viz.* dichloromethane, chloroform and hexane fractions contained lesser amount of total phenolics contents (0.117, 0.056 and 0.031 mg GAE/g, respectively). Quercetin, gallic acid, dicatechin are the main phenolic compounds present in bark of babul which are polar in nature whereas α-amyrin and β-sitosterol are non-polar compounds. Hence, higher amount of total phenolics in polar solvents may be due to presence of more polar phenolic compounds. Ethyl acetate fraction of garden mint (*Mentha spicata*) had highest total phenolics content (mg GAE/g) *i.e.* 54 followed by chloroform (30) and hexane (14) fractions [22]. Among different solvent fractions of African cabbage (*Cleome gynandra*), the total phenolics (mg GAE/g of fraction) content was highest in butanol (133.02) fraction followed by ethyl acetate (97.90), dichloromethane (37.380) and hexane (20.72) fractions and similarly, in different solvent fractions of bead bean (*Maerua angolensis*), the total phenolics (mg GAE/g of fraction) content was highest in butanol (107.55) fraction followed by ethyl acetate (69.51), dichloromethane (40.81) and hexane (13.43) fractions [23].

Total flavonoids content: Total flavonoids content (mg CE/g) in various extracts/fractions of bark of babul varied widely. Amongst extraction techniques, total flavonoids content was higher in methanolic extract obtained by hot extraction technique (18.895 mg CE/g) in comparison to cold extraction technique (10.752 mg CE/g) as shown in Table-1. Similarly, total flavonoid contents were higher in aqueous and non-aqueous layers of methanolic extract obtained by hot extraction technique (6.351 and 12.544 mg CE/g, respectively) in comparison to cold extraction technique (5.190 and 5.562 mg CE/g, respectively). Our finding is in agreement with previous investigation which reported that clove buds extracts obtained by using Soxhlet extraction technique had higher total flavonoids in comparison to extracts obtained by mechanical shaking extraction technique [21].

Amongst various solvent fractions of methanolic extract obtained by cold extraction technique (Table-2), polar fractions *viz.* ethyl acetate, butanol and residual aqueous contained higher amount of total flavonoids contents (3.229, 1.279 and 0.632 mg CE/g, respectively) whereas non-polar fractions *viz.* dichloromethane, chloroform and hexane contained lesser amount of total flavonoids contents (0.030, 0.018 and 0.002 mg CE/g, respectively). Similarly, amongst various solvent fractions of methanolic extract obtained by hot extraction technique, polar fractions *viz.* ethyl acetate, butanol and residual aqueous contained higher amount of total flavonoids contents (3.786, 1.608 and 0.860 mg CE/g, respectively) whereas non-polar fractions *viz.* dichloromethane, chloroform and hexane contained lesser amount of total flavonoids contents (0.050, 0.037 and 0.010 mg CE/g, respectively). Flavonoids present in bark of babul are mainly rutin, kaempferol, catechin which are polar in nature and leucocyanadin, a low polarity compound. Hence, higher amounts of total flavonoids in polar solvents may be due to presence of more polar flavonoids compounds. Similar findings were also reported by other research workers that among various solvent fractions of *Torilis leptophylla*, highest flavonoids content (mg CE/g) was found in ethyl acetate (60.9) fraction followed by butanol (55.0), chloroform (26.0) and hexane (15.8) fractions [20]. Similarly, among various solvent fractions of punchberry (*Myrcia splendens*), ethyl acetate fraction had highest total flavonoids content (mg QE/g) *i.e.* 85.75 followed by butanol (78.87), aqueous (68.05) and hexane (51.16) fractions [24].

DPPH free radical scavenging activity: DPPH is a stable and nitrogen centred violet coloured free radical that reacts with an antioxidant compound, which can donate hydrogen and reduce DPPH. The change in colour (from deep violet to light yellow) was measured at 517 nm on a UV visible light spectrophotometer [25]. The DPPH radical scavenging activity was recorded in terms of % inhibition as shown in Tables 3 and 4. DPPH free radical scavenging activity (%) of bark of babul was concentration dependent. It increases with the increase in concentration level from 25 to 5000 mg/mL. Amongst extraction techniques, it ranged from 19.08 to 95.21 % (hot extraction technique) and from 16.30 to 94.81 % (cold extraction tech-

TABLE-3
DPPH FREE RADICAL SCAVENGING ACTIVITY (%) OF METHANOLIC EXTRACT OF BARK OF BABUL OBTAINED BY USING COLD EXTRACTION TECHNIQUE AND OF VARIOUS SOLVENT FRACTIONS

Conc. (µg/mL)	DPPH free radical scavenging activity (%): Extract/Fractions						
	Methanol	Hexane	Dichloromethane	Chloroform	Ethyl acetate	Butanol	Residual aqueous
5000	94.81	–*	88.21	87.52	91.31	87.28	83.44
2500	94.78	48.30	87.57	86.34	90.95	87.14	82.97
1000	94.15	19.06	84.50	67.52	90.88	86.68	82.11
500	93.85	10.78	60.18	40.00	90.73	86.64	81.28
250	93.38	5.79	33.15	22.38	90.48	86.05	47.62
100	61.48	3.39	17.48	7.72	90.18	85.85	22.29
50	32.19	1.00	10.09	5.90	71.88	51.08	10.06
25	16.30	0.60	0.72	0.99	39.29	25.15	2.01

*Dry mass of hexane fraction was not sufficient to prepare solution of 5000 µg/mL.

TABLE-4
DPPH FREE RADICAL SCAVENGING ACTIVITY (%) OF METHANOLIC EXTRACT OF BARK OF BABUL OBTAINED BY USING HOT EXTRACTION TECHNIQUE AND OF VARIOUS SOLVENT FRACTIONS

Conc. (µg/mL)	DPPH free radical scavenging activity (%): Extract/Fractions						
	Methanol	Hexane	Dichloromethane	Chloroform	Ethyl acetate	Butanol	Residual aqueous
5000	95.21	----- *	87.98	88.57	92.52	88.31	85.06
2500	95.04	52.87	87.76	88.38	92.31	88.23	84.85
1000	94.66	22.70	87.32	76.95	91.41	87.73	83.98
500	93.92	14.35	79.17	52.00	91.04	87.29	83.31
250	93.02	8.36	50.18	25.90	90.57	87.29	50.29
100	67.59	5.56	22.83	12.76	90.41	86.92	25.77
50	37.52	1.98	9.24	10.53	75.43	52.15	12.48
25	19.08	1.19	4.17	1.71	45.13	27.48	1.50

*Dry mass of hexane fraction was not sufficient to prepare solution of 5000 µg/mL.

nique). Lower the IC_{50} values of extract, more effective it will be for inhibition of DPPH free radicals. IC_{50} values (Fig. 1) were calculated from the quadratic regression equations (Table-5). The IC_{50} values of methanolic extract obtained by hot extraction technique was lower *i.e.* 67.7 µg/mL in comparison to 71.7 µg/mL of cold extraction technique thereby showing that methanolic extract obtained by hot extraction technique has higher activity in comparison to cold extraction technique (Fig. 1). Clove buds extracts obtained by Soxhlet technique showed highest antioxidant activity followed by refluxing, mechanical shaking and centrifugation due to the presence of higher amount of antioxidants compounds *i.e.* total phenolics and flavonoids in the extracts obtained by Soxhlet technique [21]. 80 % aqueous methanol extract of *A. nilotica* bark showed maximum activity of 73.83 % at 1000 µg/mL [26].

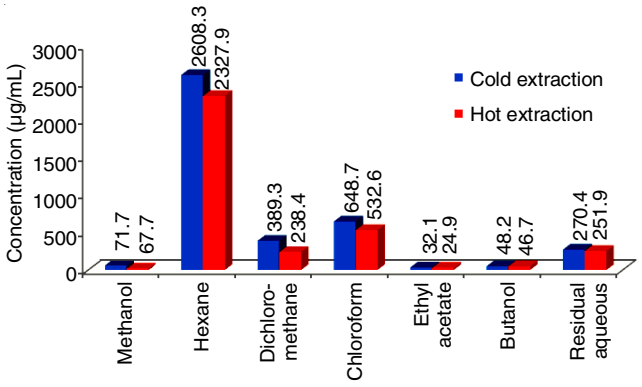


Fig. 1. IC_{50} values of methanolic extracts of bark of babul obtained by using cold and hot extraction techniques and various solvent fractions

Amongst various solvent fractions of methanolic extract obtained by cold extraction technique (Table-3), highest DPPH free radical scavenging activity was showed by ethyl acetate fraction which ranged from 39.29 to 91.31 % followed by butanol (25.15 to 87.28 %), residual aqueous (2.01 to 83.44 %), dichloromethane (0.72 to 88.21 %), chloroform (0.99 to 87.52 %) and hexane (0.60 to 48.30 %) fractions. The IC_{50} value of ethyl acetate fraction was lowest *i.e.* 32.1 µg/mL followed by 48.2

TABLE-5
QUADRATIC REGRESSION EQUATIONS FOR DPPH FREE RADICAL SCAVENGING ACTIVITY OF METHANOLIC EXTRACTS OF BARK OF BABUL OBTAINED BY USING COLD AND HOT EXTRACTION TECHNIQUES AND VARIOUS SOLVENT FRACTIONS

Extract/Fractions	Quadratic regression equations	
	Cold extraction	Hot extraction
Methanol	$y = -0.001x^2 + 0.82x - 3.631$; $R^2 = 0.999$	$y = -0.002x^2 + 0.912x - 2.547$; $R^2 = 1.00$
Hexane	$y = -0.004 \times 10^{-4} x^2 + 0.020x + 0.555$; $R^2 = 0.999$	$y = -0.001 \times 10^{-3} x^2 + 0.023x + 1.878$; $R^2 = 0.996$
Dichloromethane	$y = -0.007 \times 10^{-2}x^2 + 0.155x + 0.265$; $R^2 = 0.995$	$y = -0.001 \times 10^{-1}x^2 + 0.238x - 1.047$; $R^2 = 0.999$
Chloroform	$y = -0.002 \times 10^{-2}x^2 + 0.090x + 0.034$; $R^2 = 0.999$	$y = -0.003 \times 10^{-2}x^2 + 0.105x + 2.585$; $R^2 = 0.994$
Ethyl acetate	$y = -0.003x^2 + 1.044x + 19.62$; $R^2 = 0.947$	$y = -0.002x^2 + 0.928x + 28.14$; $R^2 = 0.933$
Butanol	$y = -0.003x^2 + 1.257x - 3.571$; $R^2 = 0.999$	$y = -0.003x^2 + 1.231x - 0.922$; $R^2 = 0.999$
Residual aqueous	$y = -0.002 \times 10^{-1}x^2 + 0.249x - 2.710$; $R^2 = 0.999$	$y = -0.002 \times 10^{-1}x^2 + 0.254x - 1.287$; $R^2 = 0.996$

µg/mL of butanol, 270.4 µg/mL of residual aqueous, 389.3 µg/mL of dichloromethane, 648.7 µg/mL of chloroform and 2608.3 µg/mL of hexane fractions thereby showing that ethyl acetate fraction has highest activity followed by butanol, residual aqueous, dichloromethane, chloroform and hexane fractions.

Amongst various solvent fractions of methanolic extract obtained by hot extraction technique (Table-4), highest DPPH free radical scavenging activity was showed by ethyl acetate fraction which ranged from 45.13 to 92.52 % followed by butanol (27.48 to 88.31 %), dichloromethane (4.17 to 87.98 %), residual aqueous (1.50 to 85.06 %), chloroform (1.71 to 88.57 %) and hexane (1.19 to 52.87 %) fractions. The IC_{50} value (µg/mL) of ethyl acetate fraction was lowest *i.e.* 24.9 µg/mL followed by 46.7 µg/mL of butanol, 238.4 µg/mL of dichloromethane, 251.9 µg/mL of residual aqueous, 532.6 µg/mL of chloroform and 2327.9 µg/mL of hexane fractions thereby showing that ethyl acetate fraction has highest activity followed by butanol, dichloromethane, residual aqueous, chloroform and hexane fractions. In our results, polar solvents *viz.* ethyl acetate, butanol, residual aqueous have higher DPPH free radical scavenging activity in comparison to non-polar solvents *viz.* chloroform, dichloromethane and hexane. The distinct scavenging activities of different extracts can be due to the diverse chemical nature of various phytochemicals that may react with different types of free radicals in unique ways [27]. Among various solvent fractions of punchberry (*Myrcia splendens*), ethyl acetate fraction exhibited highest DPPH free radical scavenging activity with EC_{50} 8.44 µg/mL followed by butanol (9.35 µg/mL), aqueous (16.99 µg/mL) and hexane (117.47 µg/mL) fractions [24].

Conclusion

Results of present study show that extraction techniques play a vital role in the extraction of the plant constituents. Methanolic extract and its various solvent fractions obtained from hot extraction technique contained higher amount of total phenolics, flavonoids and exhibited better DPPH free radical scavenging activity in comparison to cold extraction technique.

REFERENCES

1. R.A. Larson, *Phytochemistry*, **27**, 969 (1988).
2. M.J. Kahkonen, A.I. Hopia, H.J. Vuorela, J.P. Rauha, K. Pihlaja, T.S. Kujala and M. Heinonen, *J. Agric. Food Chem.*, **47**, 3954 (1999).
3. Y.Z. Cai, Q. Luo, M. Sun and H. Corke, *Life Sci.*, **74**, 2157 (2004).
4. S. Surveswaran, Y.Z. Cai, H. Corke and M. Sun, *Food Chem.*, **102**, 938 (2007).
5. H. Tapiero, K.D. Tew, G. Nguyen Ba and G. Mathé, *Biomed. Pharmacother.*, **56**, 200 (2002).
6. T. Osawa, Postharvest Biochemistry of Plant Food Materials in the Tropics, Japan Scientific Societies Press, Tokyo, Japan, pp. 241-251 (1994).
7. P.G. Pietta, *J. Nat. Prod.*, **63**, 1035 (2000).
8. K. Robards, *J. Chromatogr. A*, **1000**, 657 (2003).
9. B.J. Xu and S.K. Chang, *J. Food Sci.*, **72**, S159 (2007).
10. K. Kaur, H. Michael, S. Arora, P. Härkönen and S. Kumar, *J. Ethnopharmacol.*, **99**, 353 (2005).
11. A. Ali, N. Akhtar, B.A. Khan, M.S. Khan, A. Rasul, N. Khalid, K. Wassem, T. Mahmood and L. Ali, *J. Med. Plants Res.*, **6**, 1492 (2012).
12. D.S. Seigler, *Biochem. Syst. Ecol.*, **31**, 845 (2003).
13. R. Chaubal and A. Tambe, *Indian J. Chem.*, **45B**, 1231 (2006).
14. N.B. Tuncel and N. Yilmaz, *J. Food Sci. Technol.*, **52**, 141 (2015).
15. V.L. Singleton and J.A. Rossi, *J. Enol. Vitic.*, **16**, 144 (1965).
16. D. Marinova, F. Ribarova and M. Atanassova, *J. Univ. Chem. Technol. Metallurgy*, **40**, 255 (2005).
17. T. Hatano, H. Kagawa, T. Yasuhara and T. Okuda, *Chem. Pharm. Bull. (Tokyo)*, **36**, 2090 (1988).
18. R. Scherer and H.T. Godoy, *Rev. Brasil. Plantas Med.*, **16**, 41 (2014).
19. S. Agarwal, G.T. Kulkarni and V.N. Sharma, *Adv. Nat. App. Sci.*, **4**, 78 (2010).
20. A.A.R. Saeed, M.R. Khan and M. Shabbir, *BMC Complement. Altern. Med.*, **12**, 1 (2012).
21. I. Singh, V.K. Madan, S.S. Jangra and S. Singh, *Asian J. Chem.*, **28**, 801 (2016).
22. P. Arumugam, P. Ramamurthy, S.T. Santhiya and A. Ramesh, *Asia Pac. J. Clin. Nutr.*, **15**, 119 (2006).
23. N.T.R. Meda, M.J. Bangou, S. Bakasso, J. Millogo-Rasolodimby and O.G. Nacoulma, *J. Appl. Pharm. Sci.*, **3**, 36 (2013).
24. H.H. Moresco, M. Pereira, L.C. Bretanha, G.A. Micke, M.G. Pizzolatti and I.M.C. Brighente, *J. App. Pharm. Sci.*, **4**, 1 (2014).
25. P. Padmanabhan and S.N. Jangle, *Int. J. Pharm. Sci. Drug Res.*, **4**, 143 (2012).
26. R.K. Choudhary, A.E. Saroha and P.L. Swarnkar, *J. Pharm. Res.*, **4**, 712 (2011).
27. O.K. Chun, D.O. Kim and C.Y. Lee, *J. Agric. Food Chem.*, **51**, 8067 (2003).