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Effect of Extraction Techniques and Solvents on Various Phytochemicals and Antioxidant Activity of Clove (*Syzygium aromaticum* L.) Buds

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The selection of appropriate extraction technique and solvent for extraction of phytoconstituents is an important aspect to achieve the maximum concentration of desired phytoconstituents in plant extracts. The present study was undertaken to study the effect of extraction techniques (mechanical shaking, refluxing, Soxhlet extraction, centrifugation) and solvents (acetone, ethanol, water) on phytoconstituents and antioxidant activities of clove buds. In clove buds, total phenols (127.26 mg GAE/g) and flavonoids (26.72 mg CE/g) contents, DPPH free radical scavenging activity (range of IC_{50}: 89.9-125.3 µg/mL) and antioxidant activity (85.86 %) were found to be highest in extracts obtained by Soxhlet technique followed by refluxing, mechanical shaking/centrifugation. Amongst solvents, total phenols (140.29 mg GAE/g), DPPH free radical scavenging activity (range of IC_{50}: 89.9-118.5 µg/mL) and antioxidant activity (84.17 %) were highest in water extracts whereas flavonoids (29.71 mg CE/g) were highest in acetone extracts. Thus, the phytochemical contents and antioxidant activity of clove depend on the type of extraction technique and solvent polarity.

Keywords: *Syzygium aromaticum*, Extraction techniques, Total phenols, Flavonoids, Antioxidant activity, Clove.

INTRODUCTION

Plants secondary metabolites such as phenolic acids, flavonoids, tannins, tocopherols, stilbenes, lignans, tocotrienols and hydroxycinnamic acid derivatives have recently aroused considerable interest because of their potential antioxidant effects on human health. The extraction of phytoconstituents is challenging due to their complex chemical structure and their interaction with other food components. The extraction technique must allow complete extraction of the compounds of interest and it must avoid their chemical modification [1]. Efficiency of extraction techniques and solvents are strongly dependent on plant matrix used [2,3]. Most of the bioactive components present in plant matrix are medium sized molecules, which are highly polarizable due to the presence of aromatic delocalized µ-electrons. Their high polarizability makes the molecules liable to a variety of specific interactions with polar solvents *e.g.* protonation, hydrogen bonding and specific solvation [4]. During extraction of plant material, it is important to minimize interference from compounds that may co-extract with the chemicals. The extract yields and resulting antioxidant activities of plant materials are strongly dependent on the nature of extracting solvent, due to the presence of different antioxidant compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent.

Cloves (*Syzygium aromaticum* L.) are aromatic buds of medium sized evergreen tree belonging to family Myrtaceae. Clove buds have deep brown colour and powerful odour that is warm, strongly sweet and slightly astringent. Dried clove buds contain carbohydrates (61 %), steam-volatile oil (15-20 %), proteins (5 %), total sugars (2.36 %), mineral matter (5.2 %) and moisture (6-7 %) [5]. Clove has a few non-volatile constituents, which include tannins, sterols, triterpenes and flavonoids [6]. Survey of the literature reveals that no systematic work has been done on the comparative study of various extraction techniques and solvents on total phenols, flavonoids and antioxidant activity of clove buds. Thus, the objective of the present study was to evaluate the efficacy of different extraction techniques and solvents towards the extraction of total phenols, flavonoids as well as the antioxidant activity of the extracts produced from clove buds.

EXPERIMENTAL

Commercially available and highest purity chemicals were used for various experimental procedures. Germplasm material of dried clove buds was procured to study its chemical profile.

Healthy clove buds were selected and ground in warring blender to obtain a fine powder. Powdered samples were extracted by using following four extraction techniques:

Maceration followed by mechanical shaking: Two gram of powdered samples of clove buds were extracted with 60 mL of solvents (acetone, ethyl alcohol and distilled water) in conical flasks by maceration followed by shaking on a mechanical shaker for 2 h. Extracts were filtered and residues were again extracted twice (each maceration and shaking time 1 h) with 40 and 30 mL of respective solvents. Filtrates of each solvent from three extraction steps were pooled and their volumes were noted.

Refluxing: Two gram of powdered samples of clove buds were placed in a round bottom flask containing 75 mL solvents (acetone, ethyl alcohol and distilled water) fitted with condenser. The extraction was carried out at boiling temperature of the respective solvent for 5 h. The extract was allowed to cool, filtered and residue was again extracted twice (each refluxing time 2 h and 1 h, respectively) with 75 mL of respective solvents. Filtrates of each solvent from three extraction steps were pooled and their volumes were noted.

Soxhlet extraction: Four gram of powdered samples of clove buds were placed in a filter paper (Whatman No. 1) thimble in a classical Soxhlet apparatus fitted with a 250 mL round bottom flask. The solvents (acetone, ethyl alcohol and distilled water) were added up to one and a half siphons that is approximately 150 mL. Extraction was performed at boiling temperature of respective solvent for 5 h with completion of up to seven to eight cycles through siphon mechanism in case of acetone and ethanol as solvent. In case of water as solvent, time required for completion of one cycle was significantly more hence, with water as a solvent extraction was carried out for longer time with the completion of up to seven to eight cycles through siphon mechanism. After the completion of first extraction step, residue in thimble was again extracted twice (each extraction time 2 h and 1 h, respectively) with suitable amount of respective solvents. Filtrates of each solvent from three extraction steps were pooled and their volumes were noted.

Centrifugation: Two gram of powdered samples of clove buds were extracted with 60 mL of solvents (acetone, ethyl alcohol and distilled water) in centrifuge tubes by centrifugation at 6000 rpm for 10 min. Extracts were decanted and residues were again extracted twice (each centrifugation time 10 min at 6000 rpm) with 40 and 30 mL of respective solvents. Filtrates of each solvent from three extraction steps were pooled and their volumes were noted.

All the samples extracted by using above-mentioned techniques were performed in triplicate. Extracts were used for the estimation of total phenols, flavonoids and evaluation of DPPH free radical scavenging activity and antioxidant activity.

Estimation of total phenols content: Total phenols were determined by the Folin-Ciocalteu method [7] 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 1.0 mL of 1 mol/L Folin-Ciocalteu reagent followed by addition of 2 mL 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 phenols present in the extracts was calculated from the calibration curve and the results were expressed as milligrams of gallic acid equivalent per gram (mg GAE/g).

Estimation of flavonoids content: Flavonoids content of extracts was estimated according to the colorimetric assay [8] 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 and 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 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 [9]. Extracts were dried up completely and the weight of dry mass was noted. The dry mass of acetone and ethanol extracts was redissolved in appropriate amount of methanol to make the stock solution (500 µg/mL). Since, the dry mass of water extract 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 (25-500 µg/mL) were made by appropriate dilutions with methanol. For evaluation of antioxidant activity, in 0.2 mL of extracts (various concentrations), 3.0 mL of 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH; 0.1 mM in 100 % methanol) was added and mixed thoroughly for 5 min. 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 per cent DPPH free radical scavenging activity (y-axis) against extract concentration (x-axis). Then using Microsoft Excel Software, quadratic regression equation ($y = ax^2 + bx + c$) was obtained and using the quadratic equation IC_{50} was calculated. 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.

Antioxidant activity: Antioxidant activity was measured by β-carotene bleaching method [10]. 1.0 mg of crystalline β-carotene was dissolved in 5 mL of $CHCl_3$ and 0.1 mL of linoleic acid and 0.9 mL of Tween 20 (200 mg) were added. The solvent was removed at 40 °C using a vacuum evaporator and the mixture was immediately diluted with 250 mL of double distilled water. To 0.2 mL of test sample, 4 mL of this emulsion was added. A control containing 0.2 mL of solvent and 4.0 mL of emulsion was also used. The test tubes were placed in a water bath at 50 °C after covering with aluminium foil. The absorbance at 470 nm was recorded with a UV-visible double beam Spectrophotometer Model 2203 (Systronics Co.) at intervals of 30 min, until the colour of β-carotene disappeared from the control tubes. The above mixture without β-carotene served as blank. All determinations were carried out in triplicates. The antioxidant activity was calculated using the following equation:

$$A_A(\%) = \frac{[(A_o)_{control} - (A_t)_{control}] - [(A_o)_{sample} - (A_t)_{sample}]}{[(A_o)_{control} - (A_t)_{sample}]} \times 100$$

where, $(A_0)_{control}$ and $(A_0)_{sample}$ are the absorbance values measured at zero time of incubation for the control and sample, respectively and $(A_t)_{control}$ and $(A_t)_{sample}$ are the corresponding values at the end of the reaction time.

RESULTS AND DISCUSSION

Total phenols content: Total phenols content (mg GAE/g) in various extracts of clove buds varied widely. Amongst extraction techniques, total phenols content in extracts obtained by Soxhlet technique was highest *i.e.* 127.26 followed by refluxing (116.83), centrifugation (97.44) and mechanical shaking (96.53). Amongst solvents, total phenols content was highest (140.29) in water extracts followed by ethanol (101.95) and acetone (86.32) extracts (Table-1). Higher content of total phenols in extracts obtained by Soxhlet technique and refluxing may be due to higher solubility of phenols in hot solvents as compared to solvents used at room temperature in mechanical shaking and centrifugation techniques. The probable reason for higher amount of total phenols in water may be due to presence of phenols having more solubility in water and ethanol in comparison to acetone. A review of previously documented literature also revealed that Soxhlet extraction is an effective technique for the extraction of phenols from plant materials. *G. mollugo* L. extracts obtained by refluxing contained higher (295.83 mg GAE/g) amount of total phenols in comparison to those obtained by maceration (243.30 mg GAE/g) and this is due to the increased solubility of phenols in the extracting solvent at higher extraction temperatures [11]. Water extract of *Annona squamosa* showed highest (208.70 mg GAE/g) total phenols content followed by ethanol (171.58 mg GAE/g) and acetone (29.95 mg GAE/g) extracts [12]. Total phenols content in 80 % methanolic extract of clove buds procured from Hong Kong local market was 143.8 mg GAE/g [13].

Flavonoids content: Flavonoids content (mg CE/g) in various extracts of clove buds varied widely. Amongst extraction techniques, flavonoids content in extracts obtained by Soxhlet technique was highest *i.e.* 26.72 followed by refluxing (24.04), mechanical shaking (21.03) and centrifugation (20.26). Amongst solvents, flavonoids content was highest (29.71) in acetone extracts followed by ethanol (23.69) and water (15.64) extracts (Table-2). Flavonoids content was higher in extracts obtained by Soxhlet technique and refluxing in comparison to mechanical shaking and centrifugation, which may be due to more efficiency of hot solvent to extract more flavonoids in comparison to solvent at room temperature. Flavonoids present in clove buds are mainly kaempferol, quercetin, rhamnetin and myricetin, which are flavonol aglycones. Flavonol aglycones being non-polar in nature are more soluble in organic solvents like acetone in comparison to water. The present findings are

TABLE-1
TOTAL PHENOLS (mg GAE/g) IN CLOVE BUDS EXTRACTS OBTAINED BY USING DIFFERENT EXTRACTION TECHNIQUES

Extraction technique	Total phenols (mg GAE/g)				
	Acetone	Ethanol	Water	Mean	Increase over T_1 (%)
Mechanical shaking	76.98 ± 0.06	88.60 ± 0.09	124.02 ± 0.05	96.53	–
Refluxing	88.42 ± 0.07	112.18 ± 0.10	149.89 ± 0.07	116.83	21.0
Soxhlet	97.07 ± 0.05	118.89 ± 0.09	165.83 ± 0.08	127.26	31.8
Centrifugation	82.79 ± 0.04	88.13 ± 0.05	121.40 ± 0.07	97.44	0.9
Mean	86.32	101.95	140.29		

SE(m): Extraction technique = 0.04; Solvent = 0.03; Extraction technique × Solvent = 0.07
CD at 5 %: Extraction technique = 0.12; Solvent = 0.10; Extraction technique × Solvent = 0.21
CV %: 0.11

TABLE-2
FLAVONOIDS (mg CE/g) IN CLOVE BUDS EXTRACTS OBTAINED BY USING DIFFERENT EXTRACTION TECHNIQUES

Extraction technique	Flavonoids (mg CE/g)				
	Acetone	Ethanol	Water	Mean	Increase over T_4 (%)
Mechanical shaking	27.28 ± 0.18	21.95 ± 0.12	13.85 ± 0.05	21.03	3.8
Refluxing	30.44 ± 0.09	24.77 ± 0.12	16.90 ± 0.04	24.04	18.7
Soxhlet	34.46 ± 0.04	26.95 ± 0.12	18.76 ± 0.05	26.72	31.9
Centrifugation	26.66 ± 0.21	21.09 ± 0.33	13.03 ± 0.02	20.26	–
Mean	29.71	23.69	15.64		

SE(m): Extraction technique = 0.08; Solvent = 0.07; Extraction technique × Solvent = 0.14
CD at 5 %: Extraction technique = 0.24; Solvent = 0.21; Extraction technique × Solvent = 0.42
CV %: 1.07

in agreement with the studies on flowers and leaves of *C. angustifolia* that the flavonoids content was higher in Soxhlet extracts (27.35 and 22.52 mg/g, respectively) than extracts prepared by refluxing (18.07 and 19.91 mg/g, respectively) [14]. Acetone (23.17 mg RE/g) was the best solvent for extracting flavonoids from bitter melon followed by ethanol (5.38 mg RE/g) and water (0.66 mg RE/g) [15].

DPPH free radical scavenging activity: DPPH free radical scavenging activity was measured by the decrease in absorbance of sample with respect to control as the DPPH radical accept an electron or hydrogen radical from an anti-oxidant compound to become a stable diamagnetic molecule [16]. DPPH free radical scavenging activity (%) of clove buds was concentration dependent. It increases with the increase in concentration level from 25 to 500 µg/mL. DPPH free radical scavenging activity (%) of various extracts at different concentration levels are shown in Figs. 1-3. IC_{50} values (Fig. 4) were calculated from the quadratic regression equations (Table-3). Amongst extraction techniques, IC_{50} values (µg/mL) of extracts obtained by Soxhlet technique were lowest ranging from 89.9 to 125.3, followed by refluxing (103.2 to 142.0), mechanical shaking (117.0 to 154.5) and centrifugation (118.5 to 158.0) showing that extracts obtained by Soxhlet technique has highest DPPH free radical scavenging activity followed by refluxing, mechanical shaking and centrifugation. Amongst solvents, IC_{50} values (µg/mL) of water extracts were lowest ranging from 89.9 to 118.5 followed by ethanol (100.8 to 138.8) and acetone (125.3 to 158.0) extracts showing that water extracts has highest DPPH free radical scavenging activity followed by ethanol and acetone extracts. The probable reason for highest DPPH free radical scavenging activity (*i.e.* lower value of IC_{50}) in the extracts obtained by Soxhlet technique may be due to the presence of higher amount of antioxidant compounds *i.e.* total phenols and flavonoids in the extracts obtained by Soxhlet technique. Some other research workers have also worked on various other crops and evaluated DPPH free radical scavenging activity of extracts obtained by using different extraction techniques and solvents. IC_{50} values calculated from DPPH free radical scavenging activity of flowers and leaves of *C. angustifolia* were lowest (3.4 mg/L and 4.2 mg/L) in extracts obtained by Soxhlet in comparison to extracts obtained by refluxing (6.5 mg/L and 6.2 mg/L) [14]. Water and hot water extracts of 20 commonly consumed edible mushrooms had much higher DPPH free radical scavenging activities than acetone and ethanol extracts [17]. Ethanolic

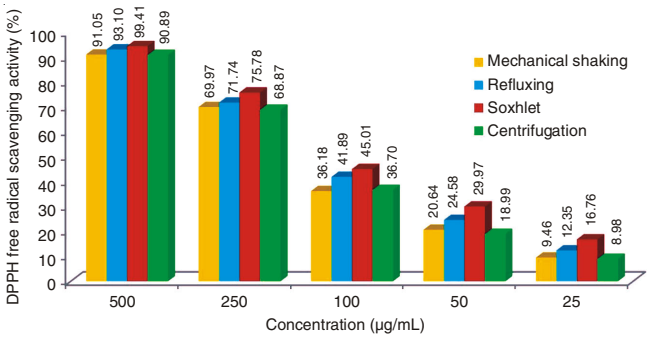

Fig. 1. DPPH free radical scavenging activity of acetone extracts of clove buds obtained by using different extraction techniques

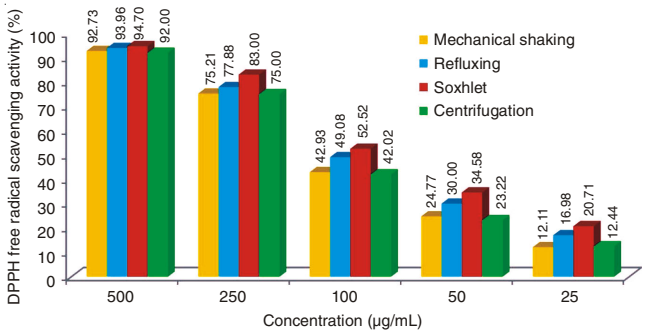

Fig. 2. DPPH free radical scavenging activity of ethanol extracts of clove buds obtained by using different extraction techniques

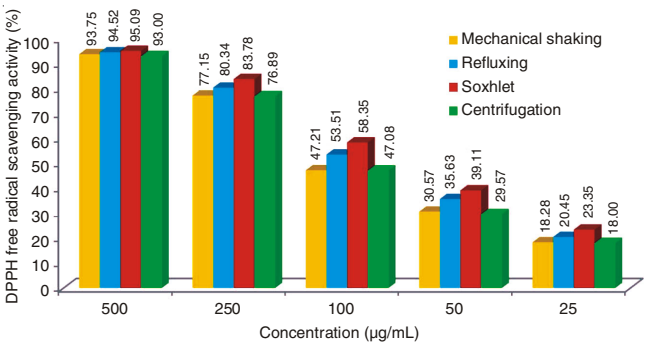

Fig. 3. DPPH free radical scavenging activity of water extracts of clove buds obtained by using different extraction techniques

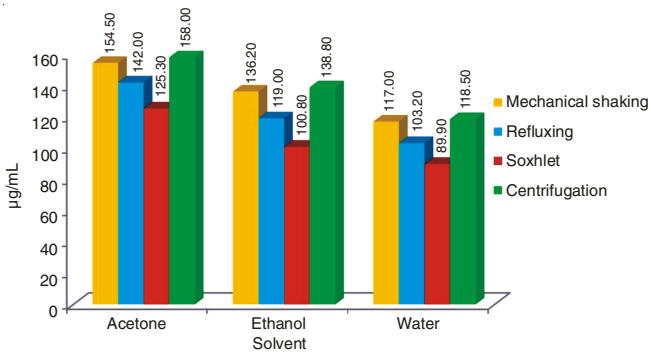

Fig. 4. IC_{50} values of clove buds extracts obtained by using different extraction techniques

extract of clove (from Egypt) exhibited 93 % DPPH free radical scavenging activity at 400 mg/mL concentration level which is comparable to that of BHT (synthetic antioxidant) having 95 % DPPH free radical scavenging activity at 400 µg/mL concentration level [18].

Antioxidant activity: Antioxidant activity of clove buds was evaluated by β-carotene bleaching method based on the inhibition of lipid peroxidation. Amongst extraction techniques, antioxidant activity (%) of clove buds extracts obtained by Soxhlet technique was highest *i.e.* 85.86 followed by refluxing (84.04), mechanical shaking (81.38) and centrifugation (78.35) at 500 µg/mL concentration level. Amongst solvents, anti-oxidant activity (%) of clove buds extracts at 500 µg/mL concentration level was highest (84.17) in water extracts followed by ethanol (82.43) and acetone (80.63) extracts (Table-4). The probable reason for highest antioxidant activity of the extracts obtained by Soxhlet technique may be due to presence of higher amount of antioxidant compounds *i.e.* total phenols

TABLE-3
QUADRATIC REGRESSION EQUATIONS FOR DPPH FREE RADICAL SCAVENGING ACTIVITY OF CLOVE BY USING DIFFERENT EXTRACTION TECHNIQUES

Extraction technique	Acetone	Ethanol	Water
Mechanical shaking	$y = -0.0004x^2 + 0.3726x + 1.8633$ $R^2 = 0.9990$	$y = -0.0005x^2 + 0.4012x + 4.7420$ $R^2 = 0.9965$	$y = -0.0004x^2 + 0.3726x + 11.6873$ $R^2 = 0.9959$
Refluxing	$y = -0.0004x^2 + 0.3666x + 6.0777$ $R^2 = 0.9953$	$y = -0.0005x^2 + 0.3910x + 10.5510$ $R^2 = 0.9924$	$y = -0.0005x^2 + 0.3842x + 15.3666$ $R^2 = 0.9871$
Soxhlet	$y = -0.0004x^2 + 0.3652x + 10.6376$ $R^2 = 0.9959$	$y = -0.0005x^2 + 0.4073x + 13.8256$ $R^2 = 0.9940$	$y = -0.0005x^2 + 0.3961x + 18.4281$ $R^2 = 0.9822$
Centrifugation	$y = -0.0004x^2 + 0.3733x + 1.0238$ $R^2 = 0.9981$	$y = -0.0005x^2 + 0.4018x + 4.0930$ $R^2 = 0.9981$	$y = -0.0004x^2 + 0.3762x + 11.0330$ $R^2 = 0.9962$

TABLE-4
ANTIOXIDANT ACTIVITY (%) OF CLOVE BUDS EXTRACTS OBTAINED BY USING DIFFERENT EXTRACTION TECHNIQUES

Extraction technique	Antioxidant activity (%)				
	Acetone	Ethanol	Water	Mean	Increase over T_4 (%)
Mechanical shaking (T_1)	79.48 ± 0.12	81.56 ± 0.17	83.11 ± 0.09	81.38	3.9
Refluxing (T_2)	82.33 ± 0.14	84.25 ± 0.06	85.54 ± 0.08	84.04	7.3
Soxhlet (T_3)	84.68 ± 0.15	85.50 ± 0.10	87.39 ± 0.12	85.86	9.6
Centrifugation (T_4)	76.02 ± 0.16	78.41 ± 0.08	80.62 ± 0.21	78.35	–
Mean	80.63	82.43	84.17		

SE(m): Extraction technique = 0.07; Solvent = 0.06; Extraction technique × Solvent = 0.13.
CD at 5 %: Extraction technique = 0.22; Solvent = 0.19; Extraction technique × Solvent = 0.38
CV %: 0.27

and flavonoids in the extracts obtained by Soxhlet technique. The findings are in agreement with the study that water extracts of various spices (cumin, chilli, pepper, garlic and coriander) of Egypt exhibited higher (81.80, 76.75, 64.38, 82.69 and 81.77 %, respectively) antioxidant activity by β-carotene bleaching method in comparison to methanol extracts (56.13, 69.58, 60.32, 64.36 and 66.60 %, respectively) [19]. Antioxidant activity of marine edible seaweeds *E. cottonii* and *Padina* species evaluated by using β-carotene bleaching method showing that extracts prepared by Soxhlet extraction technique has higher antioxidant activity (34.72 and 28.67 %) in comparison to extracts prepared by shaking (27.86 and 21.45 %) [20].

Relationship between total phenols and flavonoids and their antioxidant activities: The relationship between polyphenolic compounds *i.e.* total phenols and flavonoids and DPPH free radical scavenging activity and antioxidant activity (by β-carotene bleaching method) can be explained by finding the correlation between the polyphenolic compounds and activities. There was a positive and highly significant correlation (at 1 % level of significance) between total phenols content of clove buds and DPPH free radical scavenging activity (R = 0.784) and antioxidant activity evaluated by β-carotene bleaching method (R = 0.756). This data is in accordance with the study of other research workers on different crops, who have reported that higher total phenols content increases the antioxidant activity [21-23]. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [24]. The correlation between flavonoids content of clove buds and DPPH free radical scavenging activity (R = -0.134) and antioxidant activity evaluated by β-carotene bleaching method (R = 0.275) was not significant. The probable reason for non-significant correlation may be due to higher flavonoids content in acetone extracts of clove buds followed by ethanol and water extracts whereas, DPPH free radical scavenging activity and antioxidant activity was highest in water extracts followed by ethanol and acetone extracts. Some research workers who investigated different crops also reported that there might not be a significant correlation between flavonoids content and antioxidant activity [22,23].

Conclusion

The results of present study showed that phytochemical constituents and antioxidant activity of clove were dependent on the extraction technique and solvent polarity. Extracts obtained from Soxhlet extraction technique has highest amount of phytochemicals and exhibited highest antioxidant activity. Water extracts were found to have highest total phenols content and possessed highest antioxidant activity. The high content of total phenols is responsible for high antioxidant activity of clove buds.

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