In vitro antimutagenic, antioxidant activities and total phenolics of clove (*Syzygium aromaticum* L.) seed extracts

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Abstract: The present work explores antimutagenic and antioxidant potential as well as total phenolics of aqueous and acidified methanol extractable components from clove (*Syzygium aromaticum* L.) seed. The magnitude of antimutagenic activity of clove seed extracts (CSE) against two mutant bacterial strains: *S. typhimurium* TA98 and *S. typhimurium* TA100 (Ames bacterial test) ranged from 34.11-79.74%. Antioxidant activity in terms of measurement of DPPH radical scavenging capacity and inhibition of linoleic acid peroxidation was noted to be 71.16-94.58% and 54.96-86.89%, respectively. CSE also exhibited an appreciable amount of total phenolics with contribution between 22.80 and 115.33 GAE mg/100g. A strong correlation between total phenolics and tested biological activities were recorded. The results of this study advocate that clove seed can be explored as a viable source of bioactives for the development of chemotherapeutic drugs against cancer in addition to acting as nutraceutical and functional food ingredient.

Keywords: Clove seed, effective extraction, TPC, antimutagenic potential, radical scavenging.

INTRODUCTION

Nature has blessed us with wide array of medicinally and/ or economically important flora providing food, feed and phytomedice (Biglari *et al*., 2008; Dawara *et al*., 2012). Medicinal plants are of great value due to their potential uses as ingredients of folk medicine and functional foods (Giorgi *et al*., 2009; Siahsar *et al*., 2011). The multifarious physiological functions of medicinal plants might be attributed to the presence of bioactives and natural antioxidants *e.g.* polyphenols having multiple biological activities (Pandey *et al*., 2006; Adnan *et al*., 2010; DeGrandi-Hoffman *et al*., 2010).

Clove (*Syzygium aromaticum* L.) belonging to family *Myrtaceae*, is an evergreen plant with height ranging from 8-12 m, large square leaves and sanguine flowers in numerous groups of terminal clusters. Cloves, the dried flower buds of *S. aromaticum*, have been used as a spice in cuisines all over the world. More importantly these have been employed as folk medicine over the centuries to treat indigestion, atherosclerotic, asthma, cough, skin disorders, headache, tooth infections and gum disease, acne, wounds, scabies, insect bites and male sexual disorders (Shukri *et al*., 2005; Nassar, 2006; Santoro *et al*., 2007; Saeed and Tariq, 2008; Jin and Cho, 2011; Pawar and Patil, 2011; Mushtaq *et al*., 2012).

The most frequently reported biological activities such as antimicrobial (Giordani *et al*., 2004; Pawar and Thaker, 2006; Karuppiah and Rajaram, 2012), anti-inflammatory (Park *et al*., 2007), antioxidant (Kim *et al*., 1998), antiulcerogenic (Bae *et al*., 1998; Chaie *et al*., 2007), antithrombotic (Li *et al*., 2005), antiparasitic (Srivastava and Malhotra, 1991), antiseptic, antispasmodic, carminative, expectorant, germicidal, rubefacient, stomachic and stimulant of clove are ascribed to the presence of biologically active constituents (Yang *et al*., 2003; Cai and Wu, 1996; Arina and Iqbal, 2002; Lopez *et al*., 2005; Betoni*et al*., 2006; Chaieb *et al*., 2007).

Recently, an extensive research is being focussed on extraction and isolation of natural dietary antioxidant components, especially plant polyphenols for the development of chemo preventive drugs, anticancer agents, and other nutraceuticals to supplement and fortify the physiological defence mechanisms of human body (Fu *et al*., 2007; Yoshimura *et al*., 2011). The yield of bioactive extracts and their *in vitro* or *in vivo* biological activities is influenced by the choice of extraction solvent (Siddhuraju and Becker, 2003; Sultana *et al*., 2007). Thus, it would be important to investigate antimutagenic and antioxidant potential as well as total phenolic contents of clove seeds as a function of different extraction media so as to explore their potential therapeutic and functional food uses on scientific basis.

MATERIALS AND METHODS

Collection of sample

The seeds of clove (*S. aromaticum* L.) were purchased from the local market of Faisalabad, Pakistan. Mature and healthy clove seeds were screened, air-dried, and ground into a fine powder using a commercial grinder. The material that passed through 100-mesh sieve was used for extraction purposes.

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Reagents and standards

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, butylated-hydroxytoluene (BHT), ascorbic acid, trichloroacetic acid, gallic acid, Folin-Ciocalteu reagent, sodium nitrite, aluminium chloride, ferric chloride, potassium ferricyanate, linoleic acid and reference chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The culture media and mutant strains *S. typhimurium* TA98 and *S. typhimurium* TA100 were obtained from Oxoid Ltd. (Hampshire, UK). All other chemicals like anhydrous sodium carbonate, ferrous chloride, ammonium thiocyanate, ethanol and methanol, used were of analytical grade and purchased from Merck (Darmstadt, Germany).

Extraction of bioactive

10 grams of finely ground seeds of clove were separately extracted by shaking in an orbital shaker (Gallenkamp, UK) with 100 mL of each of the four extraction solvents (70% methanol; 70:30 methanol: water v/v, 30% methanol; 30:70 methanol: water v/v, 1N acidified methanol; HCl: Methanol 9:91 v/v, 0.5 acidified methanol; HCl: methanol 4.5:95.5 v/v) for 24 hours at room temperature. The residues, recovered after separation from the extracts through filtration, were re-extracted twice with the fresh solvents. The combined extracts were freed from solvent at 45°C in a vacuum rotary evaporator (Rotary Evaporator N-1001, EYELA, Tokyo, Japan). The solvent-free, crude concentrated extracts (CCE) were weighed to calculate the yield and stored at -4ºC, for further analyses.

Determination of total phenolic contents (TPC)

The total phenolic contents in the extracts were determined using Folin-Ciocalteu reagent and were expressed as gallic acid as equivalents. Briefly, 0.5 mL sample (0.1mg/mL) and 2mL of sodium carbonate (7.5%) were mixed with 2.5mL of 10 % (v/v) Folin–Ciocalteu reagent (Albano and Miguel, 2011) in a test tube. After 30 min of incubation at room temperature, the absorbance of the final reaction mixture was read at 765 nm using a Shimadzu 160-UV spectrophotometer and the amounts of phenolics calculated.

DPPH free radical scavenging assay

Free radical scavenging potential of clove extracts was assessed by their capacity to scavenge purple colored 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals in methanol using spectrophotometric assay as described by Shivhare *et al*. (2010). Different concentrations of clove extracts were prepared in methanol and 3mL of each solution was mixed with 1mL of 0.1mM methanolic DPPH solution. After 30 min incubation period at room temperature, absorbance (A) was recorded at 517 nm. Inhibition percentage of DPPH (I %) was calculated as follows:

$$\text{Inhibition \%} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where $A_{control}$ indicates the absorbance of solution containing only the DPPH$^{\circ}$ whereas A_{sample} is the absorbance of the sample reaction. The effective dose of 50% inhibition (IC_{50}) was also obtained from a plot of percentage inhibition verses extract concentration. All the run were triplicated and mean values thus were calculated against ascorbic acid and butylated hydroxyl toluene (BHT) as positive control.

Inhibition of linoleic acid peroxidation

The antioxidant activity in terms of inhibition of peroxidation of linoleic acid by extracts of clove was measured using the thiocyanide method (Shi *et al*., 2011).

Anti-mutagenic assay

The assessment of anti-mutagenic potential of clove extracts was made following Ames bacterial reverse mutation test using mutant strains *S. typhimurium* TA98 and *S. typhimurium* TA100 with some modification as described by Razak *et al.* (2007). The test was carried out in liquid culture. Briefly, Davis Mingioli salt (21.62 mL, 5.5 time concentrated), D-glucose (4.5 mL, 40 w/v), bromocresol purple (2.3 mL, 0.2% w/v), D-biotin (1.19 mL, 0.01% w/v), and L-histidene (0.01% w/v) were mixed aseptically in a sterile bottle to get "reagent mixture". Then the reaction mixture was prepared in five sterile bottles labelled as: Blank: having reagent mixture (2.5mL) and deionized distilled water (17.5mL), Background: reagent mixture (2.5 mL), distilled water (17.5 mL) and bacterial strain (5µL), Standard Mutagen: reagent mixture (2.5mL), distilled water (17.4mL), standard mutagens (0.1mL) and bacterial strain (5µL) and sample: reagent mixture (2.5mL), distilled water (17.5mL), bacterial strain (5µl) and clove extract (5µl, 1 mg/mL in DMSO). The standard mutagen sodium azide (0.5%) was used with *S. typhimurium* TA100 and potassium dichromate (30%) for with *S. typhimurium* TA98. After incubating at 37ºC for 2h, standard mutagen (0.1mL) was also added.

The contents of each bottle were transferred into reagent boat and 200 µL aliquot mixture was transferred into each well of 96-well ELISA plate reader using a multichannel pipette. These plates were tightly packed with aluminium foil and incubated at 37ºC for 4 days. The blank plate was observed first and the purple coloration of all wells in this plate showed that no contamination was present. The antimutaganic effect was measured as %age inhibition of mutagenicity by the following formula:

$$\text{Anti mutagenicity (\%age)} = 1 - \frac{\text{No. of positive well in test sample} - \text{m SM}}{\text{No. of positive well in positive control} - \text{SM}} \times 100$$

Whereas SM stands for Spontaneous Mutation. The antimutaganic effect was considered as strong, moderate, and weak' when %age inhibition of mutagenicity was more than 40%, 25-40% and less than 25%, respectively (Mosovska *et al*., 2010).

STATISTICAL ANALYSIS

The data obtained were analysed by one way analysis of variance (ANOVA) using Minitab 2000 Version 13.2 statistical software (Minitab Inc. Pennsylvania, USA) at 95% significance level.

RESULTS

Yield of extracts

The yields (g/100g of dry material) of extractable components from clove seeds extracted by four solvents 70% methanol, 30% methanol, 0.5 *N* acidified methanol, and 1.0 *N* acidified methanol, varied over a wide range of 6.9-44.0g/100g of dry material (Table 1).

Total phenolic contents (TPC)

The results observed regarding TPC in aqueous (30 and 70 %) and acidified methanol (0.5 and 1*N*) have been incorporated in Table 1. The assimilated date reveals that 1*N* acidified methanol produced extract with substantial levels of total phenolic compounds (115.33 ± 2.98 mg GAE/100 g of dry matter) as compared to other solvents tested.

Inhibition of peroxidation

Inhibition of linoleic acid of different extracts was found to be varied in relation to extraction solvents (Table 1). Overall, all the clove seed extracts exhibited appreciable % age inhibition of peroxidation ranging from 54.96% to 86.89%.

DPPH radical scavenging assay

The results obtained from the present analysis showed the free radical scavenging activity of seeds extracts of clove to be ranged from 17.69-94.58% (fig. 1). The highest scavenging activity (94.58%) was recorded for 0.5*N* acidified methanol extract whereas the lowest (17.69%) for 30% methanol extract.

Antimutagenic activity

The extracts of clove seed produced in 1*N* acidified, 0.5*N* acidified, 70% aqueous and 30% aqueous methanol were tested for their antimutagenic potential against direct mutagens using two bacterial strains of *Salmonella* (TA100 and TA98). The strongest anti-mutagenic activity 79.74% was observed when clove seeds were extracted using 1*N* acidified methanol while the lowest 49.41% by those extracted with 30% aqueous methanol (table 2).

DISCUSSION

The variation in extract yield indicated that solvent nature and polarity strongly affected the recovery of bioactives (table 1). Maximum extract yield (44.0%) was observed with 1*N* acidified methanol while the minimum (6.9%) using 30% methanol. The highest extraction yield as achieved with 1*N* acidified methanol revealed greater efficacy of this solvent media to extract bioactive components from clove.

A review of previously documented literature reveals that methanol is usually recognized as an effective solvent for the extraction of antioxidant compounds because of its appropriate polarity and ability to solubilize and recover optimum amounts of phenolics from plant matrices (Park *et al.*, 2007; Albano and Miguel, 2011; Shi *et al.*, 2011). The effectiveness of methanol to extract plant bioactive scan further be improved by using water as a co-solvent (Hsu *et al.*, 2006). But in the present experiments, we observed that acidifying the methanol enhances the availability of extractable components from clove with over all efficacy order: 1*N* acidified methanol> 0.5*N* acidified methanol>70% methanol> 30% methanol.

Mostly a direct relationship has been found between total phenolics and antioxidant activity of plant extracts and fruits indicating that the phenolic compounds are the major contributor towards imparting antioxidant attributes to plants. The multiple biological activities of medicinal plants indicate their potential as a source of functional foods and nutraceuticals (Han *et al.*, 2007; Singh *et al.*, 2009). In the present study, total phenolic compounds (TPC) in different clove seed extracts ranged from 22.81-115.33 mg GAE/ l00 g of dry matter (table 1). The maximum TPC (115.33 mg GAE/g), determined in 1*N*

Table 1: Percentage Yield, TPC and antioxidant activity of extracts from clove seeds

Solvent	Yield of extract[M]	TPC[N]	Antioxidant activity
			% age inhibition[O]
30% methanol	6.90±1.76[d]	22.80 ± 0.08 [d]	54.96±0.18[b]
70% methanol	16.62±0.08[c]	81.04 ± 0.78 [c]	83.77±0.5[a]
0.5*N* acidified Methanol	29.61±0.78[b]	105.33 ± 1.76 [b]	85.79±0.07[a]
1*N* acidified Methanol	44.05±2.98 [a]	115.33 ± 2.98 [a]	86.89±0.15[a]

[M]Values are (g /100g dry matter) mean±SD, [N]Values are given as mg/ 100 g of dry matter, calculated as gallic acid equivalents, [O]Inhibition of linoleic acid peroxidation compared against positive control BHT (92.01±0.21), Different letters in superscript within the same column indicate significant differences (p<0.05) among different solvents used for extraction.

Table 2: Antimutagenic potential of extracts from clove seeds

	Salmonella TA 98			*Salmonella* TA 100		
	Number of positive wells /96 wells			Number of positive wells /96 wells		
	Clove seed	% antimutagenic activity	Effect	Clove seed	% antimutagenic activity	Effect
[L]Background	8		NA	14	NA	NA
[M]$K_2Cr_2O_7$	93	--	--	NA	NA	NA
[N]NaN_3	NA	NA	NA	93	0	--
70% Methanol	32	71.76	++	30	79.74	++
1*N* acidified Methanol	36	67.05	+++	34	74.68	+++
0.5 *N* acidified methanol	51	49.41	+++	42	64.55	++
30% methanol	64	34.11	+++	54	49.36	+++
BHT	28	76.47	++	21	91.13	++

[L]Negative control, [M]Postive control, [N]postive control, +++ strong antimutagenic, ++ moderate antimutagenic, + week antimutagenic - non-mutagenic and -- indicate mutagenic response.

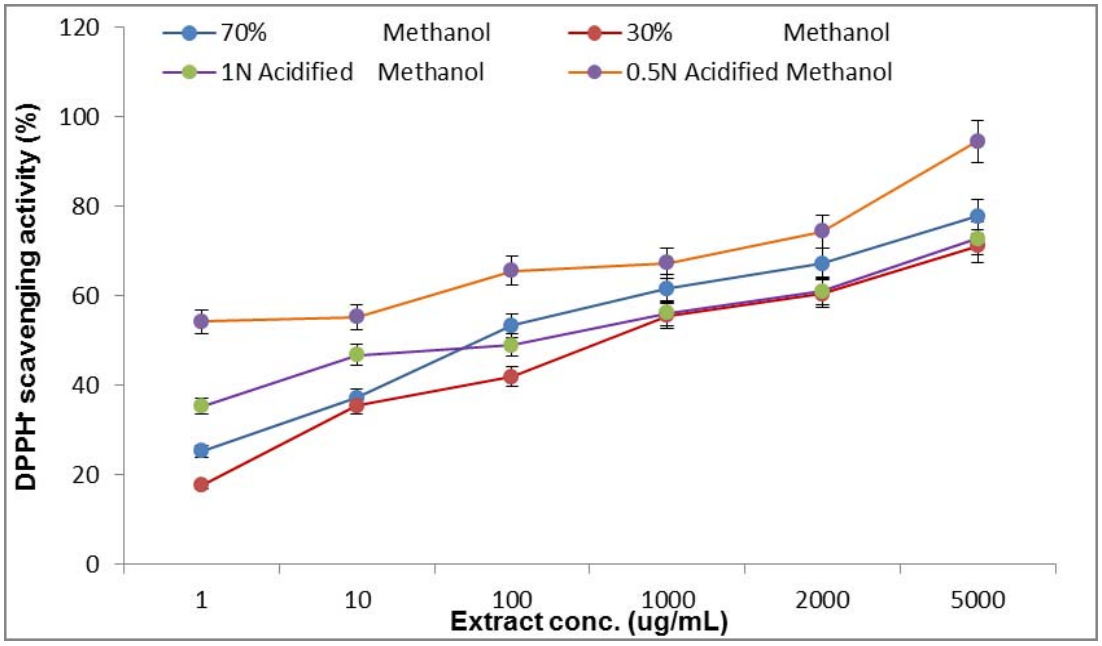

Fig. 1: DPPH° scavenging activity (%) of clove seed extract in 1N acidified, 0.5N acidified, 70% and 30% methanol, respectively.

acidified methanol extract of clove (*S. aromaticum)* was found to be greater than those in certain vegetables (Sultana *et al*., 2013) and spices (Han *et al*. 2007; Singh *et al*., 2009). Furthermore, overall order of TPC in different solvent extracts decreased in the following order: 1*N* acidified methanol > 0.5*N* acidified methanol > 70% methanol > 30% methanol

The antioxidant activity of extracts of clove seed was also determined by assessing their ability to prevent oxidation of linoleic acid. In this test peroxides formed as result of linoleic acid oxidation oxidize ferrous (Fe^{+2}) to ferric (Fe^{+3}), the later forms complex with SCN the concentration of which is estimated colorimetrically (Iqbal *et al*., 2005). Considerable inhibition of peroxidation (86.89%) observed for the 1*N* acidified methanol during the present research could be linked to the presence of polyphenols such as xanthones, flavans, flavonols in the extracts (Sultana *et al*., 2007). 1*N* acidified methanolic extract offered the highest inhibition of peroxidation (86.89%) among others showing that acidification enhances the amount of extractable antioxidants. This data is well in line with the trends of TPC indicating that per cent inhibition is strongly

correlated with the phenolics. The maximum %age inhibition (86.89%) offered by clove seed extract produced by using 1*N* acidified methanol was found to be quite comparable with synthetic antioxidant butylated hydroxy toluene (BHT) which inhibited 92.01% of peroxidation in linoleic acid system under same conditions.

Most of the biological activities of plants such as antioxidant, antiapoptosis, anti-aging, anticarcinogenic, anti-inflammatory, anti-artherosclerosis, cardiovascular protection, improvement of the endothelial function as well as inhibition of angiogenesis and cell proliferation can be attributed to their intrinsic reducing capabilities (Srivastava and Malhotra, 1991; Karuppiah *et al*., 2012).

In contradiction to TPC trends, the decrease in DPPH$^{\bullet}$ scavenging activity observed in the case of 1*N* acidified methanol might be ascribed to decreased degree of hydroxylation associated with protons transfer due to more acidic extraction media used (fig. 1). A significant correlation was observed between the amount of extractable total phenolic components and DPPH$^{\bullet}$ scavenging potential of clove extracts. All extracts offered antiradical activity comparable with the synthetic antioxidant BHT (97.02%). The substantial DPPH$^{\bullet}$ scavenging capacity of clove extracts could be correlated with phenolic components (Siddhuraju and Becker, 2003). The overall trend for DPPH$^{\bullet}$ of the solvent extracts were observed to be 30% methanol < 1*N* acidified methanol < 70%methanol < 0.5 *N* methanol.

The results (49.41-79.74%) regarding antimutagenic potential as expressed by Table 2 are well in line with the trends of TPC data. The higher antimutagenic activity by 1 *N* acidified methanol extract might be attributed to the availability of larger concentration of antioxidant components in acidified methanol. A strong correlation (0.976), observed between total phenolics and antimutagenic activity (Table 3), indicates that the antimutagenic activity of clove seed is directly related with the availability of phenolic antioxidants. Furthermore, our present findings regarding the antimutagenic potential of clove seeds could be supported by similar previous investigation by Chughtai *et al*. (1998) from Pakistan, Balaji and Chempakam (2008) from India and Mosovska *et al*. (2010) from Iran.

CONCLUSIONS

In the present study antioxidant components (TPC) antiradical capacity (scavenging DPPH$^{\circ}$), inhibition of linoleic oxidation, and antimutagenic activities of different solvent extracts from clove seeds were appraised. The clove seed extracts produced by the tested four extraction media exhibited considerable antioxidant and antimutagenic activities. Specifically, 1*N* acidified methanol extracts offered the best antioxidant and antimutagenic potential and total phenolics concentration advocating the use of clove for the development of target and specific anticancer drugs and neutraceuticals to treating related health disorders.

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