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PHYTOCHEMICAL CONSTITUENTS AND ANTIOXIDANT POTENTIAL IN FLORAL EXTRACTS OF *CALENDULA OFFICINALIS* LINN.

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ABSTRACT

Calendula officinalis Linn. (Compositae) commonly known as pot marigold is distributed throughout world as an ornamental plant. It also has long history of usage by folk systems because of rich ethanomedicinal values. Present study was aimed to determine phytochemical constituents endowed with antioxidant potential in floral extracts of *C. officinalis*. Ethanolic extract has significantly high ($P<0.05$) total phenolic, flavonoids, β-carotene, lycopene, tannin, chlorophylls contents as compared to aqueous extract of *C. officinalis*. Phytochemical ingredients present in ethanolic extract have high total antioxidant activity, superoxide, nitric oxide, hydroxyl, free radicals scavenging activity and reducing power. A dose dependent relationship has been observed between different concentrations of extract and radicals scavenging activity. Median effective concentrations of ethanolic extract for total antioxidant activity, free radical, superoxide, nitric oxide and hydroxyl radicals scavenging activities are 0.88, 1.20, 0.53, 0.68 and 0.79 mg ml^{-1} respectively. Ethanolic extract of *C. officinalis* has high phenolic, flavonoids, β-carotene, lycopene, tannin, chlorophylls contents endowed with high antioxidant potential as compared to aqueous extract. Thus, the dietary supplementation or addition of ethanolic extract of *C. officinalis* may provide protection against free radicals induced cellular damage besides improving the food quality by retarding oxidative degeneration of food lipids.

KEY WORDS : Total antioxidant activity, free radical, hydroxyl, *Calendula officinalis*.

INTRODUCTION

Calendula officinalis Linn. (Compositae) is widely distributed throughout world as an ornamental plant. The plant is commonly known as pot marigold and commercial scale cultivation occur mainly in North America, Eastern Europe and Germany. It has long history of usage by the folk systems because of its rich ethanomedicinal values. Flowers of Calendula is used in skin care products, as it has unique properties and assists the cell rejuvenation, wound healing, reducing inflammation, soothing and softening the skin. [1] Plant contains saponins, flavonoids, carotenes, mucilage, resin bitter glycosides and steroidal compounds. [2] Plant is also rich source of free and esterified triterpenic alcohols and polyunsaturated fatty acids, such as calendic acid. [1,3-4] Most of these ingredients in plant possess strong antioxidant potential either by its quenching ability to free radicals or by improving antioxidant defense of the mammalian body or both. Because of these phytochemical ingredients plant have potent anti-inflammatory [5], anti-tumor [6], antioxidant [7], anti-HIV [8], wound healing [9-10], immunomodulatory [11], hepatoprotective [12], etc. properties. These phytochemical ingredients have been found in various parts of the plants [13] and amounts of active ingredients vary with the plants maturity and the time of harvesting. [14] Various studies have been carried for maximum extraction of these phytochemical ingredients using various solvent to enhance the recovery of active ingredients having high antioxidant potential. [15]

The ability of man and animal to fight against large number of biological and environmental factors is important for maintenance of their health and productivity. Medical surveys suggested that diet rich in antioxidants (fruits and vegetables) have been reported to exert a protective effect against variety of diseases. [16] Free radicals and reactive oxygen species (ROS) like hydroxyl ($OH^{\cdot}$), superoxide ($O_2^{\cdot -}$), nitric oxide ($NO^{\cdot}$), hydrogen peroxide (H_2O_2) radicals are the commonly generated by auto-oxidation processes or enzymatic reactions in mammalian system from wide variety of sources are responsible for disturbance in oxidant and antioxidant status. [17-18] Antioxidant is important because they act as free radical scavengers, thus prevent and also repair damage done by free radicals. The studies suggested that polyphenolic compounds present in the plant extracts produce antioxidant action by scavenging these radicals. [19-20] Further generation of free radicals during processing of food is also responsible for food deterioration affecting the shelf life of different foods. Thus, there is a scope for identifying and developing effective antioxidants from natural sources to prevent the free radicals implicated diseases in mammals and also minimizes extent of lipid

peroxidation in foods during manufacturing processes. [21] Therefore, the present study was aimed to quantitative determination of phytochemical constituents endowed with antioxidant potential in different floral extracts of *Calendula officinalis* Linn.

MATERIAL AND METHODS

Collection and preparation of extracts

The flowers of *Calendula officinalis* Linn. were collected from Jammu, (INDIA). Plant sample was taxonomic identified by Taxonomist, Department of Botany, University of Jammu and voucher sample was deposited with the Curator of the museum. Sufficient fresh flowers were collected, air-dried in shade (temperature not exceeding 40°C) for 4-5 weeks. Air dried flowers were pre-crushed and later pulverized into fine powder using electric blender. Aqueous floral extract was prepared by soaking dry powder in 1:10 ratio in distilled water for 72 h with intermittent shaking. After 72 h of soaking the content was filtered through filter paper (0.45 µm) and filtrate was concentrated under reduced pressure using rotatory evaporator (temp 45-50°C, 10-15 rpm). Ethanolic extract was prepared by using ethyl alcohol in extract container of soxhlet apparatus according to method described.[22]

Phytochemical constituents of plant extracts

Total phenolic content in aqueous and ethanolic floral extracts was determined by Folin-Ciocalteu method. [23] Different concentrations of gallic acid (0.1-0.60 mg ml^{-1}) were prepared in methanol for preparation of standard curve. All determinations were analyzed in triplicate and result expressed in mg gallic acid equivalents (GAE) g^{-1} dried extract. Similarly the total flavonoid content of extracts was estimated according to method described by Zhishen et al. [24] Standard curve of quercetin (Sigma Aldrich, USA) was prepared in a concentration ranging from 0-12 mg ml^{-1} and the results were expressed as quercetin equivalents (mg quercetin equivalents g^{-1} dried extract). Tannin content in sample was determined using insoluble polyvinyl-polypirrolidone which binds tannins. [25] Non-tannin phenolics were determined in the same manner as the total phenolics. Tannin content was calculated as a difference between total and non-tannin phenolic content. [26] β-carotene, lycopene, chlorophyll (a and b) contents were determined using specific absorption coefficients according to the method used. [27-28]

In vitro Antioxidant Assay

To determine the *in vitro* antioxidant potential of plant extracts various antioxidant parameters viz. total antioxidant activity, free radical, superoxide anion, nitric oxide and

hydroxyl radicals scavenging activity and reducing power of extract were determined. The total antioxidant activity of plant extracts was determined according to the method of Re et al [29] using 2, 2, Azonobis 3, ethylene benzothiazoline-6-sulphonic acid (ABTS) (Sigma Aldrich, USA). Free Radicals scavenging activity of extracts was determined using 1,1-diphenyl l-2-picrylhydrazyl (α,α-diphenyl-β-picrylhydrazyl) (DPPH, Sigma Aldrich, USA). The capacity of the extracts to scavenge the stable free radical DPPH was monitored according to the procedure described by Amarowicz et al [30] using UV-visible spectrophotometer (U-1800, Spectrophotometer, Hitachi, Japan). The superoxide anion radical scavenging ability of fractions was determined as per method described earlier. [31] The potential of different concentrations of plant extract to scavenge the hydroxyl radicals generated by the Fenton reaction was measured.[32-33] Nitric oxide radical scavenging activity was measured by using Griess reagent [34] and reducing power of plant extracts was determined according to method described by Oyaizu. [35] The samples were run in triplicate and mean value were plotted in graph in order to calculate the median effective concentration (EC_{50}) of different radical scavenging activity.

Statistical analysis

All determinations were made in triplicate for all assays. The results were subjected to analysis of variance (ANOVA) with statistical significance at $P < 0.05$ being tested using the Duncan's Test and Pearson correlation.

RESULTS

Extractability and quantitative analysis of phytochemical ingredients

In the present study aqueous and ethanolic floral extracts of *C. officinalis* Linn. have 23.62 and 17.61 per cent extractability respectively. Table 1 shows different phytochemical constituents like total phenolic, flavonoids, β-carotene, lycopene, tannin, non-tannin and chlorophylls contents were determined in aqueous and ethanolic flower extracts of *C. officinalis*. Total phenolic, flavonoids, β-carotene, lycopene, tannin, chlorophylls contents were significantly ($P<0.05$) higher in ethanolic extract as compare to aqueous extract. However, non-tannin content differs non-significantly in different floral extracts of *C. officinalis.*

Table 1: The major phytochemical ingredients present in aqueous and ethanolic floral extracts *C. officinalis*.

Parameters (unit)	Flower extracts of *C. officinalis*	
	Aqueous	Ethanolic
Total phenolic content (mg of GAE g^{-1} extract)	$1.97^{a} \pm 0.23$	$5.56^{b} \pm 0.71$
Total flavonoids content (mg Quercetin g^{-1} extract)	$40.67^{a} \pm 5.92$	$79.57^{b} \pm 8.93$
Non-tannin content (mg of GAE g^{-1} extract)	$0.37^{a} \pm 0.27$	$0.35^{a} \pm 0.27$
Tannin content (mg of GAE g^{-1} extract)	$1.53^{a} \pm 0.48$	$5.22^{b} \pm 0.98$
β-Carotene (mg g^{-1} extract)	$0.74^{a} \pm 0.17$	$10.17^{b} \pm 2.50$
Lycopene (mg g^{-1} extract)	$0.34^{a} \pm 0.01$	$7.57^{b} \pm 0.39$
Chlorophyll a (µg g^{-1} extract)	$0.62^{a} \pm 0.44$	$6.44^{b} \pm 1.73$
Chlorophyll b (µg g^{-1} extract)	$1.21^{a} \pm 0.38$	$14.87^{b} \pm 3.93$
Values are expressed as mean ± SD of three replicates. The different superscripted (a, b) values differ significantly ($P<0.05$) from the other extract of same plant. (GAE-Gallic Acid Equivalent)		

Total antioxidant activity

Total antioxidant activity of aqueous and ethanolic floral extracts of *C. officinalis* was determined using ABTS radical cation ($ABTS^{+}$). ABTS on reacting with potassium persulphate produces ABTS radical cation ($ABTS^{+}$), a blue green chromogen. Effect of antioxidant or reductant on $ABTS^{+}$ radical cations is due to its hydrogen donating ability which is visually observed by a change in colour of $ABTS^{+}$ cation to colorless ABTS. The extent of decolorization is an indicator of antioxidant activity present in the extract. Different concentrations of aqueous and ethanolic floral extracts of *C. officinalis* produces dose dependent scavenging activity of $ABTS^{+}$ radical cation upto concentration of 3.0 mg ml^{-1} (Figure 1A). Ethanolic extract has more potent $ABTS^{+}$ radicals scavenging activity than the aqueous extract of *C. officinalis*. EC_{50} value was calculated for the ascorbic acid (48.69 µg ml^{-1}), aqueous (1.61 mg ml^{-1}) and ethanolic (0.88 mg ml^{-1}) extracts of *C. officinalis* by plotting per cent inhibition of ABTS radicals versus different concentrations of extract.

Free radical scavenging activity

Free radical scavenging activity of the different plant extract was determined by using DPPH molecule. DPPH is a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecule do not dimerize, as would be the case with most other free radicals. The effect of antioxidant on DPPH radical scavenging is primarily due to their hydrogen donating availability. Exposure of different concentrations of aqueous and ethanolic extracts of *C. officinalis* produces dose dependent scavenging activity of DPPH radical (Figure 1B). EC_{50} values were calculated for the ascorbic acid (21.51 µg ml^{-1}), aqueous (1.30 mg ml^{-1}) and ethanolic (1.20 mg ml^{-1}) extracts of *C. officinalis* by plotting per cent inhibition of DPPH radical versus different concentrations of extract.

Superoxide anion radical scavenging assay

Superoxide anion radicals were generated in PMS–NADH systems by oxidation of NADH are assayed by the reduction of NBT. The decrease in absorbance of the reaction mixture on addition of different concentrations of standard or extract indicated the superoxide anion scavenging activity. The EC_{50} values for aqueous and ethanolic floral extracts of *C. officinalis* were 2.07 mg ml^{-1} and 0.53 mg ml^{-1} respectively. Per cent inhibition of superoxide anion radicals versus different concentrations of floral extracts of *C. officinalis* are presented in Figure 1C. Observations of present study show EC_{50} values of superoxide radical scavenging activity are lowest for ascorbic acid and highest for aqueous extract of *C. officinalis* (AA < EECO < AECO).

Nitric oxide ($NO^{\cdot}$) **scavenging assay**

Different concentrations were incubated with $NO^{\cdot}$ radical generator e.g. sodium nitroprusside to determine the $NO^{\cdot}$ radical scavenging potential of the extract. Dose dependant $NO^{\cdot}$ radical scavenging activity of aqueous and ethanolic floral extracts of *C. officinalis* was observed in the medium. Per cent inhibition of $NO^{\cdot}$ radicals versus different concentrations of aqueous and ethanolic extract are depicted in Figure 2A. In present study EC_{50} values of NO radicals scavenging activity was lowest for ascorbic acid and highest for aqueous extract of *C. officinalis* (AA < EECO < AECO).

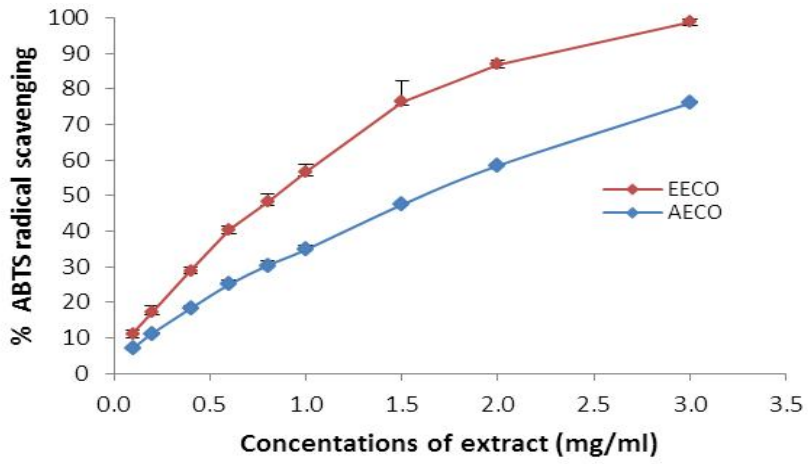
Hydroxyl radical ($OH^{\cdot}$) scavenging activity

Hydroxyl radical generated by Fenton reaction in the solution was used for determining the $OH^{\cdot}$ radical scavenging activity. Addition of different concentrations of extract produces a dose dependent $OH^{\cdot}$ radical scavenging activity upto the concentration of 2.0 mg ml^{-1} (Figure

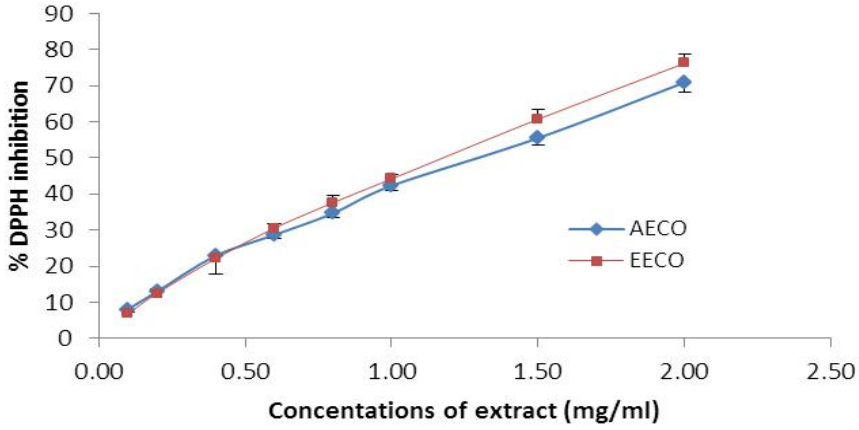
2B). Ethanolic extract has more potential to scavenge $OH^{\cdot}$ radical as compared to aqueous extract of *C. officinalis*. EC_{50} values of ascorbic acid, aqueous and ethanolic extracts of *C. officinalis* were 64.24 µg ml^{-1}, 1.06 mg ml^{-1} and 0.79 mg ml^{-1} respectively.

Reducing power assay

The reductive ability of extract was measured by its ability to transform the ferric (Fe^{3+}) to ferrous (Fe^{2+}) form. The presences of reductants such as antioxidant in the sample cause the reduction of Fe^{3+}/ferricyanide complex to the Fe^{2+} form. The Fe^{2+} concentration was measured by monitoring the formation of Perl's Prussian blue. Reducing power of different floral extracts of *C. officinalis* increased as the increases in the concentration of extract as indicated in increasing optical density. Further, reducing power of ethanolic extract has more reducing potential than the aqueous extract of *C. officinalis* at same dose level. The relationship between optical density and different concentrations of the extract are presented in figure 2C**.**



(A)



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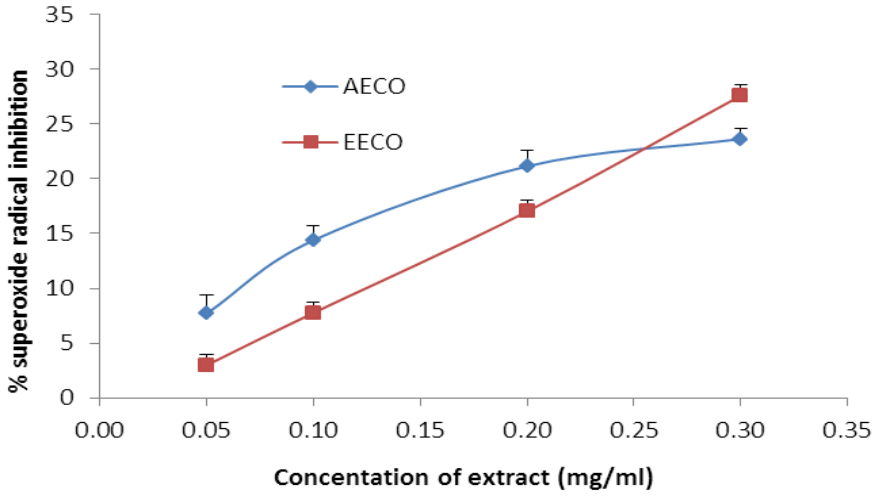
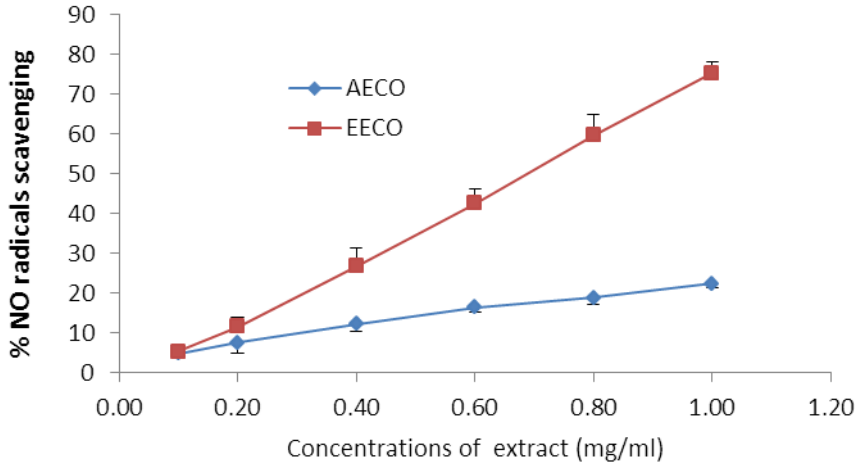
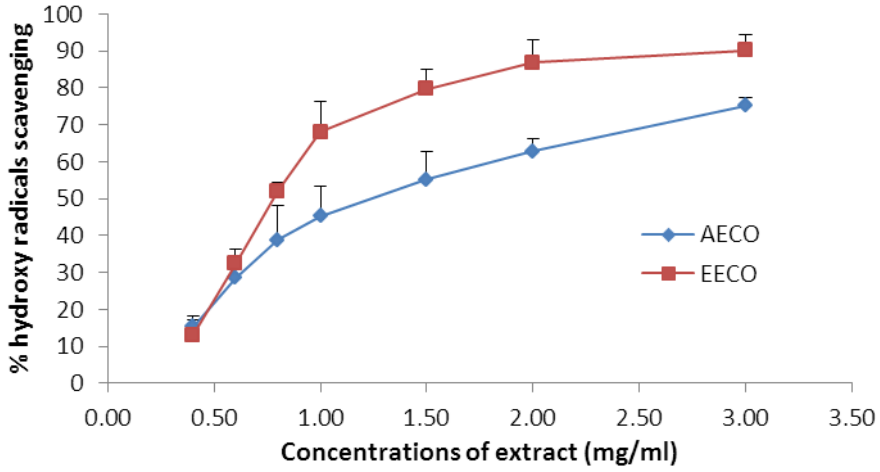


(C)

Fig. 1: Total antioxidant capacity (A), Free radical (B) and Superoxide radicals scavenging (C) activities of aqueous (AECO) and ethanolic (EECO) extracts of *C. officinalis*.



(A)



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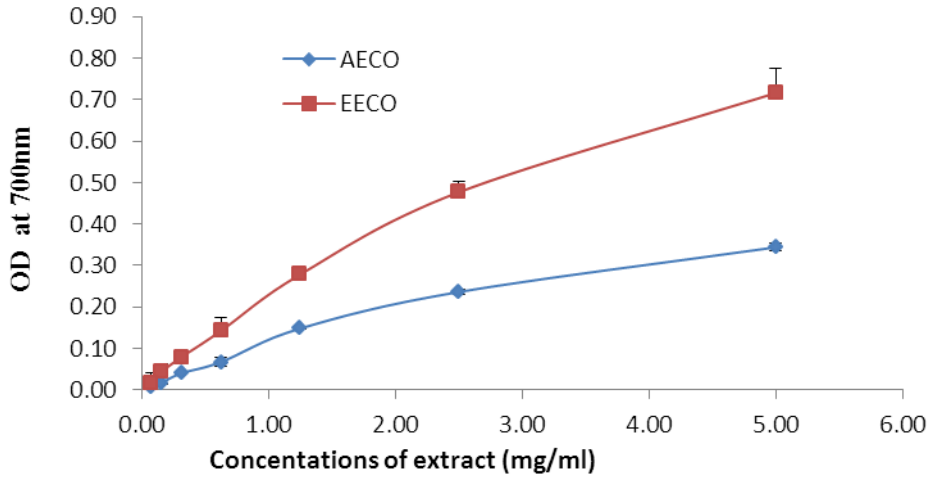

(C)

Fig. 2: Nitric oxide (A), hydroxyl (B) radicals scavenging activities and reducing power (C) of aqueous (AECO) and ethanolic (EECO) extracts of *C. officinalis*.

DISCUSSION

Plant kingdom is a huge reservoir of phytochemical ingredients, many of which have been explored for various pharmaceutical applications. Various epidemiological studies have shown a consistent relationship between a diet rich in polyphenolic phytochemical ingredients and a lower risk for many chronic diseases including cancer, [34] heart disease, [37] obesity and type 2 diabetes [38-39] due to strong free radicals and ROS scavenging properties of these phytochemical ingredients. A number of synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) have been extensively added to foodstuffs, although their use has begun to be questioned because of their toxicity [40], so there is considerable interest in preventive medicine and in the food industry in the development of natural antioxidants obtained from botanical sources. [21] In the present study ethanolic floral extract has significantly high total phenolics, flavonoids tannin, β-carotene lycopene and chlorophylls contents as compared to aqueous extract of *C. officinalis*. High contents of phenolic, flavonoids and tannin in ethanolic extract in comparison to aqueous medium, make this organic solvent an ideal and selective to extract a great number of bioactive polyphenolic compounds may be due to increased solubility of these ingredients. [41] Similar observations have also reported that plant produces contains high polyphenolic contents have well established health beneficial activities in mammals. [42-43] Fruits such as blueberry, cranberry and pomegranate have been proven to be rich in flavonoids and other antioxidant properties that protect endothelial cells from oxidation, a key factor in the

development of cardiovascular diseases. Different pharmacological potential of ethanolic extract of *C. officinalis* may be due to presence of high phenolic, flavonoid, tannin, β-carotene and lycopene contents in the extract. [9-12]

Total antioxidant activity

Testing for antioxidant activity of new synthesized chemical compounds or extracts from natural sources, is a rational method of screening for future products with potential beneficial impact on human health. In this system, radical cations ($ABTS^{+}$) are formed prior to the addition of antioxidant test system, rather than generation of radical taking place continually in the presence of antioxidant. By the virtue to its excellent spectral properties, solubility in both organic and aqueous media, stability in wide range of pH, ABTS assay in considered as to be more reliable and accurate. Compounds with one OH group in the aromatic ring which is found in inactive towards the DPPH molecule are significantly active towards ABTS. [44-45] The method is used to screen the activity of both lipophilic and hydrophilic antioxidants. [46] In the present study, ethanolic extract have significant high total antioxidant activity as compared to aqueous extract of *C. officinalis*. The chemical diversity of phenolic antioxidants makes it difficult to separate and quantify individual antioxidant from the extract. Moreover, the total antioxidant activity as an integrated parameter of antioxidants present in a sample is often more meaningful to evaluate health beneficial effects because of the cooperative action of antioxidants. [47] The result of the analysis shows that there is a positive correlation between phenolic content and total antioxidant activity of extracts. Similarly *in vitro* ABTS radical scavenging potential of floral extract of *C. officinalis* and their relation with total polyphenolic contents have been also reported. [42,43,48-49]

Free radical scavenging activity

DPPH is relatively stable nitrogen centered free radical used to measure antioxidant activity of different phenolic compounds. Free radical scavenging activity of DPPH is based on the exchange of hydrogen atoms between the antioxidant and DPPH radicals. [50] This model is best for the scavenging of lipophilic radicals by reducing DPPH radicals to the corresponding hydrazine. [51-52] DPPH radicals show primarily active reaction with glutathione, aromatic amines (such as p-phenylene diamine and p-aminophenol), and α-tocopherol (Vitamin E) and polyhydroxy aromatic compounds (such as hydroquinone). On the other hand, monohydric phenols (such as tyrosine), simple sugars (such as glucose), purines and pyrimidines, do not react, while proteins are precipitated. [50] Thus, external supplementation or addition of *C.*

officinalis extracts may overcome the adverse effects induced by free radicals on the body and also minimizes extent of lipid peroxidation in foods during manufacturing processes. [21,53] Lower EC_{50} value of ethanolic extract higher is the antioxidant potential and increasing the dose the free radicals scavenging activity get enhanced. [50] Similarly, potent DPPH radical scavenging potential of floral extract of *C. officinalis* has also been reported. [49]

Superoxide radical scavenging activity

Superoxide anions are the most common free radicals *in vivo* whose concentration increases under the conditions of oxidative stress in both aerobic and anaerobic organisms. [18] Scavenging potential of different plant extracts may provide protection of cellular damage induced by generation of excessive superoxide radicals. Ethanolic extract has high superoxide anion scavenging activity as compared to aqueous floral extract of *C. officinalis.* Similarly *in vitro* studies also shown superoxide radical scavenging potential of floral extract of *C. officinalis.*[10,49] Superoxide anion is an important factor in the killing of bacteria by phagocytes and produced excessively in activated phagocytes such as monocytes, macrophages, eosinophils and neutrophils. [54] The oral administration of floral extract of *C. officinalis* inhibited superoxide generation in macrophages *in vivo* by 12.6 % and 38.7 % at doses of 100 and 250 mg kg^{-1} body weight in mice. [49] In biological system, superoxide radicals are generated in variety of auto-oxidation processes or enzymatic reactions and also responsible for production of other cell damaging free radicals and oxidizing agents.[55] In different pathophysiological processes superoxide radical anions transform into more reactive species such as hydroxyl radical that initiate lipid peroxidation and oxidative reactions associated with aging. [51,56]

Nitric oxide scavenging activity

Nitric oxide (NO) is a gaseous free radical relatively less reactive but highly potent neurotransmitter for some important physiological processes such as smooth muscle relaxation, neuronal signaling, regulation of cell mediated toxicity, etc in mammals. Instead of possible benefit of NO radical its contribution to oxidative damage is increasingly becoming evident on excessive production. This may be due its interaction with superoxide to produce reactive peroxynitrite anions ($ONOO^{-}$), which is a strong oxidant that can further decompose to produce hydroxyl and nitric oxide radicals. [57] NO radicals are also implicated in inflammation, cancer and other pathological conditions in addition to ROS. [58] Thus, regulation of NO production level in biological tissue is important for avoiding harmful effect

produced by excessive NO radical generation. [59] The plant extract may have the property to counteract the formation of NO radicals and in turn may be of considerable interest in preventing the harmful effect of excessive NO generation in the body. Nitric oxide formation by macrophages in culture was also scavenged by incubation with *C. officinalis* floral extract between 3.2 % and 32.6 % at concentration of 10 to 150 mg of extract. [49]

Hydroxyl radical scavenging activity

Among the major active oxygen species the hydroxyl radical ($OH^{\cdot}$) is the most reactive and severely damages adjacent macromolecules such as proteins, lipids, nucleic acids and almost every macromolecule in vicinity. [21] Therefore, the removal of hydroxyl radical is probably one of the most effective defenses of living body against various diseases. These radicals can be formed from the superoxide anion ($O^{2-\cdot}$) and hydrogen peroxide (H_2O_2) in the presence of metal ions such as copper and iron. [18,58] Observations of the present study suggested that by increasing concentration of either extracts produces dose dependent inhibition of $OH^{\cdot}$ radicals scavenging. In present study, ethanolic floral extract has maximum $OH^{\cdot}$ radicals scavenging activity as compared to aqueous extract of *C. officinalis.* Similarly *in vitro* studies also reported hydroxyl radical scavenging potential floral extract of *C. officinalis.* [49] *In vitro* assessment of these radicals scavenging potential of extracts help in determining the possible mechanisms of protection during *in vivo* studies.

Reducing power

The reducing capacity of an extract may serve as a significant indicator of its antioxidant potential. In the present study, reducing power of the different extracts increased with increasing concentration of extract. Similarly different parts of *C. officinalis* also reported to have potent reducing power in vitro studies. [49] The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activity of an antioxidant compound has been attributed to various mechanisms among which is prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, radical scavenging, etc. [60]

CONCLUSION

Observations of the study suggested that ethanolic floral extract has high phenolic, flavonoids, β-carotene, lycopene, tannin, chlorophylls contents as compared to aqueous extract of *C. officinalis*. Further phytochemical constituents in ethanolic floral extract endowed with high total antioxidant activity, free, superoxide anion, nitric oxide, hydroxyl

radicals scavenging activity and reducing power, though the activity was low as compared to ascorbic acid. Thus, the dietary supplementation ethanolic extract of *C. officinalis* may provide protection against degenerative changes associated with free radicals induced damage (aging, cancer, alzheimer disease, etc) besides improve the food quality by retarding oxidative degeneration of food lipids.

Conflict of Interest: Nil

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