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Evaluation of antioxidant and free radical scavenging activities of spices mixture extract as additive with reference to synthetic antioxidant

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ABSTRACT

Background: Spices are normally added in the food to impart flavour. They are naturally occurring antioxidants which have potential capacity to counteract aging process in the body, to stabilize the cell membrane by scavenging free radicals in small doses. Objectives: to ascertain synergistic antioxidant influence of spices mixture in comparison with synthetic antioxidant substance by using different in vitro models. Material & Methods: The powder of assigned spices were mixed and alcoholically extracted by a simple maceration method. It was evaluated for its total phenolic and flavonoid contents. Antioxidative abilities of the extracts of spices individually and their mixtures extracts were analyzed by PMA (Phosphomolybdenum Assay), CUPRAC (Cupric ions Reducing Antioxidant Capacity) and FRAP (Ferric ions Reducing Ability Power) methods. The free radical scavenging activities such as hydrogen peroxide, nitric oxide, hydroxyl, DPPH, superoxide, ABTS, anti-peroxidation like TBARS(Thiobarbituric Acid Reactive Substance), crocin bleaching and metal chelation capacity were assayed with synthetic antioxidant through in-vitro models. Results and Discussion: Antioxidant and antiradical effects of spices mixture extract (SME) was ascertained through different in-vitro models. Conclusion: Spices mixture exhibited better antioxidative potency with effective free radical scavenging and could be safer as additive than synthetic antioxidant for consumption and useful as natural preservative.

Keywords: Antioxidant, Antiradical, Spices Mixture Extract

INTRODUCTION

Spices are defined as dry plant material to be used as flavour/additive in foods [1]. Generally spices consist of different phytochemicals and active principles such as flavonoids, essential oils, volatile oils, phenolics and polyphenolics. Recently several studies have reported synthetic antioxidant substances have carcinogenicity and toxicity properties if it is used as an additive in food [2]. Natural antioxidants are presumed to be safe for consumption. Nowadays medical practitioners prefer natural occurring antioxidants rather than synthetic antioxidants in the anti-aging treatment or as additives in the food.

Cui *et al* [3] reported that the spices have used in the food products with a point of view for inhibiting growth of microorganisms including health risk pathogen *Clostridium botulinum*. Therefore spice mixture is commonly used as natural preservative in the preparation of processed food and canned food products. Many household spices are being used regularly in the Indian foods. They have been shown to impart many anti-oxidative effects. They are

naturally occurring antioxidants which have potential capacity to counteract the aging process in body and to stabilize the cell membrane by scavenging free radicals [4].

Spices mixture extract is a nutraceutical formulation consisting of different selected Indian spices blend. High antioxidant profile spices such as cloves, cinnamon, turmeric, nutmeg, tulsi, cumin, curry leaves, ginger, black pepper, and mustard were selected with reference to ORAC values [5] and their combination contributes synergistic capacity in the antioxidant status and also provide enhancement of quenching free radicals generated in the body. The current study was therefore designed with an objective to ascertain synergistic antioxidant influence of spices mixture in comparison with synthetic antioxidant substance by using different *in vitro* models

MATERIALS AND METHODS

Spices were purchased from the local market in the city of Karad (Western Maharashtra) and validated them from the Dept of Botany, Yashwantrao Chavan College of Sciences, Karad.

Formulation of spices-mixture-extract (SME):

Selected species like cloves, cinnamon, turmeric, nutmeg, tulsi, cumin, curry leaves, ginger, black pepper, and mustard were weighed 2gm each and mixed as spices mixture. Spices mixture extract (SME) was extracted by ethanol using a simple maceration method. Filtrates were concentrated. Percentage yield was thereby calculated.

Chemicals and Reagents:

Ammonium persulphate, Thiobarbituric acid, Folin & Ciocateu's phenol reagent, Aluminum chloride, Gallic acid, Neocuproine, Cupric chloride, Ferric chloride, Nitro B.T., Ammonium molybdate, Hydrogen peroxide, Trichloroacetic acid, Ferrous chloride, Ferrous Sulphate, Potassium phosphate, Sodium phosphate, Potassium ferricyanide, Sodium carbonate, Sodium nitroprusside, Sodium acetate, Sodium salicylate, Butan-1-ol were purchased from Loba chemicals. Griess reagent, 2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2, 2′-Azobis (2-methylpropionamidine) (ABPH) dihydrochloride Potassium persulfate were purchased from Sigma Aldrich. Riboflavin was given as a gift sample from Nes Ltd, Mumbai. Ferrozine SP was purchased from Hi-Media.

Phytochemical estimation assays and *in-vitro* antioxidant and free radical scavenging methods:

SME, Ascorbic Acid (AA), Gallic Acid (GA) and Rutin (RT) were prepared in the varied concentration range of 100µl-500µl.

Phenolic Content Estimation

Folin-Ciocalteu method was used to determine the total phenolics content of SME and GA [6]. SME and GA were added to each test tube individually containing 3 ml of ethanol; 100µl of distilled water and 100µl of Folin-Ciocalteu reagent solution. 100µl of 100mg/ml sodium carbonate was added after 5min. These tubes kept aside for 2hrs. Absorbance was measured at 765nm.

Flavonoid Estimation

Aluminum chloride colorimetric method was used for flavonoids determination of SME and RT with slight modification [6]. SME and RT were added separately to each test tube containing 3 ml of ethanol; 100µl of 20% aluminum chloride in ethanol; 100µl of 5% sodium acetate and 800µl of distilled water. These tubes kept incubated at room temperature for 30min. Absorbance was measured at 415nm.

Phosphomolybdenum Assay (PMA)

Total antioxidant activity was estimated by phosphomolybdenum assay as described by Prieto *et al* [7]. This assay is based on the reduction of Mo (VI) to Mo (V) by the sample and subsequent formation of a bluish green colored phosphate/Mo (V) complex at acid pH. 1ml each of 0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate were added in 20ml of distilled water and made up volume to 50ml by adding distilled water. SME and AA were mixed with 1ml of Molybdate reagent solution and incubated at 95°C for 90min. After cooling the absorbance of the reaction mixture was measured against at 695nm.

Cupric Ion Reducing Antioxidant Capacity (CUPRAC)

Cupric ion reducing capacity was measured in accordance to the method of Apak [8]. 1ml 10mM cupric chloride ($CuCl_2$), 1ml 7.5mM neocuprione and 1ml 1M ammonium acetate buffer (pH 7) solutions were added to test tubes. SME and AA were mixed with reaction mixture independently. These reaction mixtures were incubated for half hour at room temperature and measured against blank at 450nm.

Ferric Reducing Ability Power (FRAP)

Ferric ions reducing power was measured according to the method of Oyaizu with a slightest modification [9]. Higher absorbance of the reaction mixture indicated greater reducing power. SME and AA were mixed with 1ml of 20mM phosphate buffer and 1ml potassium ferricyanide (1%, w/v) and incubated at 50°C for 30 min. 1ml of TCA (10%, w/v) and 0.5ml ferric chloride (0.1%, w/v) were added to the reaction mixture and absorbance was measured at 700nm.

Hydrogen Peroxide Free Radical Scavenging Activity (HP-FRSA)

Hydrogen peroxide scavenging activity was assayed by the method of Ruch [10] with a slightest modification. SME and AA were added separately to each test tube containing 2ml of 20mM phosphate buffer, 1ml of 43mM hydrogen peroxide solution and 1ml of distilled water. Absorbances of phosphate buffer solution and hydrogen peroxide solution without phosphate buffer were used as blank and control respectively. After incubating at room temperature for 10min, the absorbance of all extracts were measured at 230nm against blank phosphate buffer solution. Control was prepared by measuring absorbance of 4ml of 43mM hydrogen peroxide solution only. The percentage of scavenged hydrogen peroxide of extract was calculated using the following formula: Scavenged H_2O_2 % = $[(A_c-A_e)/A_c \times 100]$ where, A_c = absorbance of hydrogen peroxide solution without phosphate buffer and A_e = absorbance of extract.

Nitric Oxide Free Radical Scavenging Activity (NO-FRSA)

The procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent [11]. SME and AA were mixed with 3ml of PBS and 1ml of 0.1M sodium nitroprusside and incubated at normal temperature for 30min. 1ml of Griess reagent was added and optical density was measured at 517nm. Control was prepared by mixing 10µl of PBS in place of extract with 3ml of PBS, 1ml of 0.1M sodium nitroprusside, 1ml of Griess reagent and its absorbance was determined immediately. The percentage of scavenged NO^- of extract was calculated using the following formula: Scavenged NO^- % = $[(A_c-A_e)/A_c \times 100]$ where, A_c = absorbance of control and A_e = absorbance of extract.

Hydroxyl Free Radical Scavenging Activity (OH-FRSA)

The scavenging ability of the extracts on hydroxyl radicals was determined according to the method described by Smirnoff and Cumbes [12]. 0.041gm of $FeSO_4$ and 0.32gm of sodium salicylate was mixed to 100ml of distilled water. 4µl of $H_2O_2$was dropped to it, vortexed for uniform mixing and labeled as "Smirnoff Reagent". SME and AA were mixed with 1ml of Smirnoff reagent and incubated about 30min at 37°C. Absorbance of the reaction mixtures was read at 562nm. The scavenging ability on hydroxyl radicals was calculated by use of given equation. The percentage of scavenged OH^- of extract was calculated using the following formula: Scavenged OH^- % = $[(A_c-A_e)/A_c \times 100]$ where, A_c = absorbance of control and A_e = absorbance of extract.

Superoxide Free Radical Scavenging Activity (S-FRSA)

Superoxide radical scavenging activity was estimated by the nitro blue tetrazolium reduction method [13]. 100 µl of 20 µg Riboflavin solution, 200 µl 12mM EDTA solution, 200 µl methanol and 100 µl of 0.1mg NBT solution (Nitro-blue tetrazolium) were mixed in test tube and reaction mixture was diluted up to 3 ml with 50mM phosphate buffer. The absorbance of solution was measured at 590nm using phosphate buffer as blank after illumination for 5 min. This was taken as control. To each of these reaction mixtures, SME and AA were mixed and its absorbance was measured after illumination for 5 min at 590 nm. The percentage inhibition of the samples was calculated as: Scavenged superoxide % = $[(A_c-A_e)/A_c \times 100]$ where, A_c = absorbance of control and A_e = absorbance of extract.

DPPH Free Radical Scavenging Activity (DPPH-FRSA)

The capacity of extracts to scavenge the stable DPPH [2, 2'-diphenyl-2-picrylhydrazyl] free radical was measured in the method of Duan [14]. SME and AA were mixed with 1ml of 0.1mM DPPH and kept incubated in dark room at normal temperature for 30min. After incubation, optical density of these incubated tubes was measured at 517nm.

Control was prepared by mixing 10µl of ethanol in place of extract with 3ml of ethanol and 1ml of 0.1mM DPPH and absorbance was determined immediately. The percentage of scavenged DPPH of extract was calculated using the following formula: Scavenged DPPH % = $[(A_c - A_e)/A_c \times 100]$ where, A_c = absorbance of control and A_e = absorbance of extract.

Lipid Peroxidation Assay: Thiobarbituric Acid Reactive Substance (TBARS)
Lipid peroxidation assay was performed according to modified protocol of Banerjee [15] to measure the lipid peroxide formed using egg yolk homogenates as lipid-rich media. In the assay of lipid peroxidation, malondialdehyde (MDA) was detected by presence of pink color. Egg homogenate was prepared by either of two following methods.

(a) Egg Yolk Homogenate Preparation:
One egg was broken by piercing hole for removing outer layer surrounding yellow colored yolk which is albumin rich media. After removing all protein layers, yellow yolk as lipoprotein rich media was transferred to a beaker consisting of hexane and isopropyl alcohol mixture in ratio of 3:2 as suggested in Shigi Peng Zhao *et al* [16]. Protein was isolated from lipoprotein by precipitating down as yellow colored mass while phospholipids dissolved in hexane-isopropyl alcohol. It was filtered through Whatmann filter paper. The filtrate was known as egg yolk homogenate which stored in a close tightly container and kept in refrigerator for further analysis. 100µl of egg homogenate filtrate was diluted in 5ml of distilled water in test tube and vortexed well while micropipetting.

(b) Egg Yolk Homogenate Preparation:
In accordance of Vasudewa N S *et al* [17], egg yolk was separated from the albumen and the yolk membrane was removed. 10ml of egg yolk solution was added in 1.15 gm of KCl or NaCl in 100ml of distilled water. The solution was homogenized for 30 seconds and ultrasonicated for 5 min. In (A+TBA) set, each test tube containing 1ml of sample, 100µl of diluted egg homogenate was transferred. To induce lipid peroxidation, 50µl of 0.07M $FeSO_4$ was added. These mixture tubes were kept for 30min for incubation. To stop lipid peroxidation, 50µl of 1.2M trichloroacetic acid (TCA) was added and following 0.8% thiobarbituric acid (TBA) and 3.5M acetic acid in amount of 0.5ml each were added to it and vortexed well. These resultant tubes were placed in the incubator at 95°C for 60min. To eliminate this non-MDA interference, another (B–TBA) set of extracts was treated in the same way as above mentioned set (A+TBA) by excluding TBA. The absorbance of (B–TBA) was subtracted to the absorbance of (A+TBA) for yielding the absorbance for extract (E). After cooling it, 5ml of butan-1-ol was added to each tube and vortexed for 5min. The absorbance of upper organic layer was measured at 532nm.

Percentage of lipid peroxidation inhibition was calculated by following formula. Antioxidant index (AI) was calculated using the following equation: $AI = (1 - E/C) \times 100$ where, E = absorbance of extract [E= (A + TBA) – (B – TBA)], C = absorbance of fully oxidized control. All values are based on the anti-oxidant index whereby the control is completely peroxidized and each extract providing a degree of improvement, indicated as % protection.

Crocin Bleaching Assay (CBA)
Crocin bleaching assay is based as result of oxidation of crocin induced by peroxyl radicals produced from thermal decomposition of azo-initiator, AAPH/ABPH [2, 2'-azobis (2-amidinopropane) dihydrochloride] [18]. Crocin was extracted from dried stigmas of saffron in the method of Lussignoli [19] with slight modifications.

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Extraction of Crocin from Dried Stigmas of Saffron
10.5gm of dried stigmas of saffron was suspended in a 500ml of beaker containing 200ml of 80% methanol. It was placed in refrigerator at 20°C in two days for maceration. On third day, it was filtered through Whatmann filter paper and the filtrate was collected in a wide mouthed brown shaded container. Residue was placed on a mortar containing a little quantity of 80% methanol and it was crushed with help of pestle to maximize extraction of crocin content from stigma of saffron. It was placed again for maceration in 1 day at 20°C. On fourth day, it was filtered. 100ml of 80% methanol was added to the residue with shaking for 10min then filtered. This step was repeated five times more till residue become colorless from dark orange. The total volume of filtrate as saffron stigma extract was

made approximately 550ml in the wide mouthed large plastic container. This container was well-sealed and kept in darkness at -5°C.

Selection of control absorbance of crocin extract

To determine maximum absorbance of crocin extract as control, different series of volume 600µl, 650µl, 700µl, 750µl, and 800µl were added to tubes containing 75µl of 0.5M ABPH and 5ml of ethanol respectively. Absorbances of these non-incubated tubes were measured at 443nm. 775µl of crocin extract was selected.

SME and AA were added to each test tube individually containing 5ml of ethanol, 75µl 0.5M ABPH and 775µl crocin extract. These tubes kept incubated at room temperature for 60min. After incubation, absorbance of these reaction mixture tubes was measured at 443nm. Control was prepared by mixing 10µl of ethanol in place of extract with 5ml of ethanol, 75µl 0.5M ABPH and 775µl crocin extract and absorbance was determined immediately.
The percentage of crocin bleached by extract was calculated using the following formula: Bleached crocin % = $[(A_c-A_e)/A_c \times 100]$ where, A_c = absorbance of control and A_e = absorbance of extract.

Metal Ion Chelating Activity (MICA)

The chelating ability of the extracts on ferrous ions was determined according to the method described by Dinis [20]. The extracts were assessed for their ability to compete with Ferrozine for iron (II) ions in free solution. SME and AA were mixed with 50µl of solution of 2mM $FeCl_2.4H_2O$ and incubated about 30min at 37°C. The reaction was initiated by the addition of 200µl of 5mM ferrozine. The mixture was shaken vigorously and left standing at room temperature for 10min. The chelating activity measured by measuring the disappearance of purple color in absorbance of solution at 562 nm. Different concentration range of EDTA as standard was prepared in correspondence to the sample. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the following formula: Chelating % = $[(A_c-A_e)/A_c \times 100]$ where, A_c = absorbance of control and A_e = absorbance of extract.

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Statistical Analysis:

Results were taken of minimum triplicates and expressed as mean ± standard deviation. Data were analyzed using student't' test for two sets while one-way analysis of variance (ANOVA) for more than two sets. Significant differences were considered when means of compared sets differed at $P<0.05$. Data was carried out using SPSS v.16.0 (Statistical Program for Social Sciences) software.

RESULTS AND DISCUSSION

Summary of results

Percentage yield:

Percentage yield of SME is 0.2%

Total phenolics content and total flavonoids content of SME, GA and RT:

Different concentration range of SME and gallic acid were assayed in the Folin Ciocalteu Reagent (FCR) assay for estimating phenolics content. The gallic acid standard line has equation, y=0.155x-0.003, (R^2=0.994). Total Phenolic Content was calculated from the following equation: TPC=C x V/M where, T=Total Phenolic Content (mg/g) of extract as GAE, C=Concentration of GA established from the calibration curve in (mg/ml), V=Volume of the extract solution in ml {0.1-0.5ml} & M= weight of extract in g {0.2-1.0g}. So we have used the reversed formula; x=(y + 0.003)/0.155.

Different concentration range of SME and rutin were assayed in the aluminum chloride method for estimating total flavonoid contents. The rutin standard line has equation, y=0.014x, (R^2=0.982). Total Flavonoid Content was calculated from the following equation: TFC=C x V/M where, T= Total Flavonoid Content (mg/g) of extract as RT, C= Concentration of RT established from the calibration curve in (mg/ml), V= Volume of the extract solution in ml {0.1-0.5ml} & M= weight of extract in g {0.2-1.0g}. So we have used the reversed formula; x=y/0.014.

Table 1: Phytochemical Characterization

	Total Phenolics Content			Total Flavonoids Content		
(µl)	SME	GA	GAE	SME	RT	RTE
100	0.134 ± 0.07	0.163 ± 0.01	0.441	0.027 ± 0.005	0.019 ± 0.005	1.357
200	0.212 ± 0.11	0.314 ± 0.01	0.693	0.099 ± 0.005	0.029 ± 0.02	2.071
300	0.320 ± 0.14	0.433 ± 0.04	1.041	0.174 ± 0.007	0.044 ± 0.01	3.142
400	0.404 ± 0.13	0.610 ± 0.03	1.312	0.325 ± 0.0005	0.063 ± 0.01	4.500
500	0.514 ± 0.17	0.792 ± 0.03	1.667	0.662 ± 0.02	0.072 ± 0.01	5.142

Values are Mean ± SD (n=3); Spices Mixture Extract - SME; Gallic Acid- GA, RT- Rutin, GAE-Gallic Acid Equivalents, RTE- Rutin Equivalents

Total antioxidant capacity of SME and AA:

Total antioxidant capacity of SME and ascorbic acid in the various concentration range were estimated in the different methods such as PMA, CUPRAC and FRAP. It measures the antioxidant power directly proportional to the absorbance value.

Table 2: Total Antioxidant Capacity (TAC)

	PMA		CUPRAC		FRAP	
(µl)	SME	AA	SME	AA	SME	AA
100	0.406 ± 0.04	0.275 ± 0.08	0.431 ± 0.08	0.420 ± 0.04	0.543 ± 0.06	0.487 ± 0.01
200	0.572 ± 0.03	0.512 ± 0.05	0.763 ± 0.13	0.608 ± 0.07	0.705 ± 0.03	0.804 ± 0.01
300	0.752 ± 0.02	0.892 ± 0.10	1.042 ± 0.17	0.744 ± 0.05	0.816 ± 0.04	0.866 ± 0.01
400	0.921 ± 0.04	1.278 ± 0.24	1.321 ± 0.20	0.851 ± 0.06	0.898 ± 0.03	0.890 ± 0.01
500	1.040 ± 0.03	1.660 ± 0.04	1.563 ± 0.20	0.953 ± 0.09	1.030 ± 0.06	0.912 ± 0.02

Values are Mean ± SD (n=3); Spices Mixture Extract - SME; Ascorbic Acid – AA

Free radical scavenging activities of SME and AA:

Free radical scavenging activity measures the antiradical capacity which is inversely proportional to the absorbance value. Antiradical activities of SME were assessed to scavenge free radicals through different assays such as hydrogen peroxide, nitric oxide, hydroxyl, DPPH, superoxide and ABTS as specified in (Table 3 & 4).

Table 3: Free Radical Scavenging Activity Related to Patho/Physiological Free Radicals

	HP-FRSA		NO-FRSA		OH-FRSA		S-FRSA	
(µl)	SME	AA	SME	AA	SME	AA	SME	AA
100	2.683±0.06	2.666±0.02	0.490±0.02	0.489±0.02	0.490±0.02	0.489±0.02	0.020±0.001	0.004±0.001
200	2.653±0.03	2.665±0.02	0.613±0.03	0.585±0.02	0.613±0.03	0.585±0.02	0.041±0.003	0.005±0.0005
300	2.694±0.02	2.690±0.01	0.698±0.02	0.631±0.03	0.698±0.02	0.631±0.03	0.081±0.01	0.006±0.0005
400	2.689±0.009	2.688±0.01	0.821±0.06	0.594±0.02	0.821±0.06	0.594±0.02	0.145±0.03	0.004±0.0005
500	2.689±0.02	2.699±0.02	0.990±0.04	0.566±0.02	0.990±0.04	0.566±0.02	0.209±0.03	0.003±0.0005

Values are Mean ± SD (n=3); Spices Mixture Extract - SME; Ascorbic Acid – AA

Table 4: Synthetic Free Radical Scavenging Activity

	DPPH-FRSA	
(µl)	SME	AA
100	0.559 ± 0.008	2.447 ± 0.03
200	0.424 ± 0.065	2.016 ± 0.03
300	0.247 ± 0.065	1.591 ± 0.16
400	0.145 ± 0.005	1.144 ± 0.01
500	0.115 ± 0.008	0.813 ± 0.05

Values are Mean ± SD (n=3); Spices Mixture Extract - SME; Ascorbic Acid – AA, Trolox –TLX

Table 5: Associated Free Radical Scavenging Activity

	TBARS		CBC		MICA	
µl	SME	AA	SME	AA	SME	EDTA
100	0.221 ± 0.040	0.308 ± 0.106	0.334 ± 0.02	0.450 ± 0.012	0.636 ± 0.08	0.380 ± 0.03
200	0.146 ± 0.014	0.193 ± 0.022	0.415 ± 0.02	0.480 ± 0.002	0.530 ± 0.06	0.195 ± 0.05
300	0.124 ± 0.051	0.172 ± 0.007	0.450 ± 0.001	0.479 ± 0.003	0.482 ± 0.06	0.099 ± 0.04
400	0.168 ± 0.012	0.178 ± 0.023	0.612 ± 0.03	0.470 ± 0.012	0.500 ± 0.04	0.036 ± 0.007
500	0.181 ± 0.015	0.175 ± 0.016	0.846 ± 0.06	0.456 ± 0.002	0.487 ± 0.04	0.027 ± 0.01

Values are Mean ± SD (n=3); Spices Mixture Extract - SME; Ascorbic Acid – AA

Anti-lipid peroxidation, bleaching and chelating capacities of SME, AA and EDTA:

Anti-peroxidation method measures the inhibition of lipid peroxidation made by spices mixture extracts through TBARS assay. Decrease in peroxyl radicals oxidized crocin by extracts of spices and spices mixture were measured

in the crocin bleaching capacity. Free metal ions catalyze the increased rate of free radicals formation. Metal chelating capacity measures the chelation of free metal ions by spices and spices mixture extracts. Lower optical density indicates higher capacity with respect to anti-peroxidation, bleaching and chelating activities as mentioned in (Table 5).

Percentage of anti-radical activities, anti-peroxidation, bleaching, chelating activities of selected spices and mixture extracts were calculated by formula and charted in the data (Table 6).

Need of analysis by using combined different assays
Mostly antioxidant assays are based on colorimetric and spectrophotometric methods. They measure the antioxidant power deciding upon the increase or decrease in absorbance. Tirzitis G et al have clearly explained about the definitions of antiradical and antioxidant activity [21]. Antiradical activities of spices and spices mixture were assessed through different assays such as hydrogen peroxide, nitric oxide, hydroxyl and superoxide free radicals generated by *in-vitro* models which can correlate with *in-vivo* models in the physiological system of the body. DPPH and ABTS are synthetic antiradical assays which generate synthetic free radicals in the simple chemical reaction. These assays give a direct measure of quenching potency towards free radicals *in-vitro* purely. These models do not correlate with *in-vivo* models. These methods have a good point for understandable obviously the exact nature of antiradical activity by visualizing the disappearance in colour. However sometimes other free radical scavenging assays like hydrogen peroxide, nitric oxide, superoxide and crocin bleaching assays are more difficult to predict antiradical power of extract compared to DPPH, ABTS, TBARS and hydroxyl assays due to lack of decolouration process. Pigmentation of some extract i.e. cinnamon, spice mixture and turmeric may perhaps give false negative results by showing higher absorbance value in the hydrogen peroxide, nitric oxide, superoxide and crocin bleaching assays in turn which shows lower inhibition percentage in spite of these extracts having naturally superior capacity to sequester free radicals while spice mixture extract, being having combination of different spice extracts which must have synergistic antiradical activities. Pigmentation of extracts itself may interfere in the reading of absorbance in case of free quenching assays like hydrogen peroxide, nitric oxide, superoxide and crocin bleaching lacking colour disappearance process. It is the need of removing interference of pigment colouration; we have tried to have cross checking with other antiradical assays like DPPH, ABTS, TBARS and OH.

Total antioxidant assays like FRAP, CUPRAC and PMA are easier and rapid assays which gives the direct value by measuring absorbance irrespective of any affects of colour pigmentation. These assays give measure of the reduction ability of extract. Combination of studies of these assays gives a better estimation, reproducible results and exact antioxidant nature of extract in terms of reduction ability. MCC acts as a perfect coordination activity with antioxidant assays. Metal ions play an important role as a catalyst in accelerating in the oxidation reaction process as well as in the formation of free radicals. TBARS plays an important role in the assessment of lipid peroxides reduction in percent. TBARS has its uniqueness in the assay by presence of pink colour due to formation of malondialdehyde so it remains unaffected regardless of colour pigmentation of extracts. Yet, there are a few limitations in some of antioxidant assays explained many authors like Oboh G *et al* [22] explored in colour interference limitation in the DPPH method. Yvette Porter [23] has suggested more assays than one assay needs to establish the validity of results by giving an illustration of addition of ABTS assay to overcoming the drawbacks of DPPH assay. FRAP has too limitation.

CONCLUSION

Spices mixture exhibited better antioxidative potency with effective free radical scavenging and could be safer as additive than synthetic antioxidant for consumption and useful as natural preservative.

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