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Research Article

SCREENING OF SOME INDIAN HOUSEHOLD SPICES FOR COMPARATIVE STUDIES OF ANTIOXIDANT AND ANTIRADICAL ACTIVITIES BY USING *IN-VITRO* MODELS

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ABSTRACT

Background: India is endowed with enormous varieties of spices grown in the majority of the country and one of the largest exporters of spices in the world. Many household spices are being used integrally in the Indian foods. Spices are normally added in the food to impart flavor. They are naturally occurring antioxidants, which have the potential capacity to counteract aging process in the body, to stabilize the cell membrane by scavenging free radicals in small doses.

Objectives: The current study was designed to determine antioxidant potential of some selected spices and their mixture based on the established scientific evidences and oxygen radical absorbance capacity values using different *in vitro* models and correlating.

Methods: The powder of assigned spices and their mixture were alcoholically extracted by a simple maceration method. They were evaluated for their total phenolic and flavonoid contents. Antioxidative abilities of the extracts of spices individually and their mixtures extracts were analyzed by phosphomolybdenum assay, cupric ions reducing antioxidant capacity (CUPRAC) and ferric ions reducing ability power methods. The free radical scavenging activities such as hydrogen peroxide, ABTS, anti-peroxidation like TBARS (thiobarbituric acid reactive substance), crocin bleaching and metal chelation capacity were assayed through *in vitro* models.

Results and Discussion: Antioxidant and antiradical effects of the selected spices extracts and spices mixture extract (SME) was ascertained through different *in vitro* models. Results of individual tests demonstrated good correlation among themselves. CUPRAC has strong correlation with all other assays except to diphenyl-1-picrylhydrazyl (DPPH) and thiobarbituric acid reactive substances (TBARS). DPPH and TBARS were correlated with each other however they demonstrated no relationship with other assays.

Conclusion: Extracts of selected Indian spices extracts and SME have shown dissimilarity to each other. Study for the 1st time establishes aggregate index for evaluation of Indian spices. On basis of aggregate index, nutmeg has highest value among the selected spices and no significant synergistic effect has been found in SME. CUPRAC as antioxidant assay and hydroxyl free radical scavenging assay by using Smirnoff reagent are most appropriate assays to ascertain antioxidative and free scavenger properties of natural products.

Keywords: Antioxidants, Antiradical, Oxygen Radical Absorbance Capacity, Spices, Spices Mixture Extract.

INTRODUCTION

Oxidative stress

Oxidative stress is a common element involved in the aging process as well as in the pathological conditions such as damage of biopolymers such as nucleic acids, proteins, polyunsaturated fatty acids and carbohydrates, serious cell damage leading to a variety of human diseases like Alzheimer's disease, Parkinson's disease, atherosclerosis, cancer, arthritis, neurodegenerative disorders, etc., [1]. Different sources of reactive oxygen species (ROS) can lead to the formation of toxic compounds within organisms. Cellular respiration, interaction of biomolecules with ionizing radiation, and cellular pathways for ROS formation as a protective mechanism ensure chronic exposure of living organisms to ROS. A proper balance must be maintained between oxidants and antioxidants to ensure the ubiquitous ROS do not become deleterious to the organism [2]. Antioxidants play an important role to reduce oxidative stress and disorders implicated by them, by minimizing excess free radicals induced by oxidative stress in the body [3].

Need and importance of natural antioxidants in food

Spices are defined as dry plant material to be used as flavor/additive in foods [4]. India is endowed with enormous varieties of spices grown in major land area of the country and one of the largest exporters of spices in the world. Many house hold 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 [5] even when they are consumed in small doses. Usually, antioxidants have been classified into two types: Natural and synthetic. Natural antioxidants are presumed to be safe for consumption. Recently, several studies have recommended for replacing synthetic antioxidants by natural ones due to carcinogenicity and toxicity properties associated in the synthetic antioxidants when used as an additive in food [6]. Now-a-days people and medical practitioners are keenly interested in the antioxidants and they prefer naturally available antioxidants rather than synthetic antioxidants in the anti-aging treatment or as additives in the food. Tirzitis *et al.* have clearly explained about the definitions of antiradical and antioxidant activity [7]. Hence, there is need to study distinctively between antioxidant and antiradical activities.

Many *in-vitro* methods are available to test antioxidant activity in the food stuffs. There is need for ascertaining antioxidant potential of the selected spices in different *in-vitro* models. The current study was therefore designed with an objective of comparative analysis of different Indian spices using different *in vitro* models and further to ascertain synergistic antioxidant influence of spices mixture.

Table 1: Household spices selected as per USDA database for the ORAC of selected foods

Sr. no.	Household spices	Label	Common names in marathi language	Biological source	Family	ORAC per 100 g
1	Cloves	S1	*Lavang*	Dried unopened flower buds of *E. caryophyllata*	Myrtaceae	2,90,283
2	Cinnamon	S2	*Dalchini*	Dried barks of *C. cassia*	Lauraceae	1,31,420
3	Turmeric	S3	*Halad*	Dried rhizomes of *C. longa*	Zingiberaceae	1,27,068
4	Nutmeg	S4	*Jayaphal*	Dried kernels of seeds of *M. fragrans*	Myristicaceae	69,640
5	Holy Basil	S5	*Tulas*	Dried aerial parts of *O. sanctum*	Lamiaceae	61,083
6	Cumin	S6	*Jeera*	Dried seeds of *C. cyminum*	Apiaceae	50,372
7	Curry leaves	S7	*Kadhilimb*	Dried leaves of *M. koenigii*	Rutaceae	48,504
8	Ginger	S8	*Ala*	Dried rhizomes of *Z. officinale*	Zingiberaceae	39,041
9	Black pepper	S9	*Kalimeeri*	Dried fruits of *P. nigrum*	Piperaceae	34,053
10	Mustard	S10	*Mohari*	Dried seeds of *B. nigra*	Brassicaceae	29,257
11	SME	S11	S11 = (S1+S2+S3+S4+S5+S6+S7+S8+S9+S10)			

B. nigra: Brassica nigra, P. nigrum: Piper nigrum, Z. officinale: Zingiber officinale, M. koenigii: Murraya koenigii, C. cyminum: Cuminum cyminum, O. sanctum: Ocimum sanctum, M. fragrans: Myristica fragrans, E. caryophyllata: Eugenia caryophyllata, C. cassia: Cinnamomum cassia, ORAC: Oxygen radical absorbance capacity

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.

Selection based on the documentation of some spices and its oxygen radical absorbance capacity (ORAC) values

Based on the scientific evidences reported by studies ten commonly used edible spices in Indian cooking were selected for *in vitro* models studies. Ten edible spices were tabulated and ranked as per highest antioxidant potency as per the specified value of US Department of Agriculture (USDA) Database for the oxygen radical absorbance capacity (ORAC) of Selected Foods [8] as given in the Table 1.

Chemicals and reagents

Ammonium persulphate, thiobarbituric acid, Folin and 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 sulfate, 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.

Ethanolic extraction of individual spices extract and SME

All selected species were labeled as S1-S10 and weighed 10 g each. All species (S1 + S2 + S3 + S4 + S5 + S6 + S7 + S8 + S9+ S10) were weighed 2 g each and mixed as spices mixture with label S11. All selected spices extracts and SME were extracted by ethanol using a simple maceration method. Filtrates were concentrated. Percentage yield was thereby calculated.

Phytochemical estimation assays

Phenolic content estimation

Folin–Ciocalteu method was used to determine the total phenolics content of extract [9].

Flavonoid estimation

Aluminum chloride colorimetric method was used for flavonoids determination with slight modification [10].

In-vitro antioxidant activity

Phosphomolybdenum assay (PMA)

Total antioxidant activity was estimated by phosphomolybdenum assay as described by PMA [11].

Cupric ion reducing antioxidant capacity assay (CUPRAC)

Cupric ion reducing capacity was measured in accordance to the method of CUPRAC [12].

Ferric reducing ability power (FRAP)

Ferric ions reducing power was measured according to the method of FRAP [13] with a slight modification.

FREE RADICAL SCAVENGING ACTIVITY (FRSA)

Free radical scavenging activity (FRSA)

Hydrogen peroxide (HP-FRSA)

Hydrogen peroxide scavenging activity was assayed by the method of HP-FRSA [14] with a slight modification. 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 FRSA (NO-FRSA)

The method was based on using Griess reagent [15]. 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 FRSA (OH-FRSA)

The scavenging ability of the extracts on hydroxyl radicals was determined according to the method described by OH-FRSA [16]. 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.

DPPH-FRSA

The capacity of extracts to scavenge the stable DPPH free radical was measured in the method of DPPH-FRSA [17]. 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 the extract.

Superoxide FRSA (S-FRSA)

Superoxide radical scavenging activity was estimated by the nitro blue tetrazolium reduction method [18]. 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.

ABTS FRSA

The scavenging activity of ABTS radical was measured by the method of ATBS-FRSA [19]. The percentage inhibition of the samples was

calculated as: Scavenged ABTS % = $[(A_c-A_e)/A_c \times 100]$ where, A_c = absorbance of control and A_e = absorbance of extract.

Thiobarbituric acid reactive substance assay (TBARS)

Lipid peroxidation assay was performed according to modified protocol of TBARS [20] 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 following method [21]. Percentage of lipid peroxidation inhibition was calculated by following formula. Antioxidant index (AI) was calculated using the following equation: AI = (1-E/C) × 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 provides a degree of improvement, indicated as % protection.

Crocin bleaching capacity assay (CBC)

Crocin bleaching assay is method for measuring oxidation of crocin induced by azo-initiator, AAPH/ABPH (2,2'-azobis [2-amidinopropane] dihydrochloride) by producing peroxyl radicals [22].

Extraction of crocin from dried stigmas of saffron

Crocin was extracted from dried stigmas of saffron as per method [23]. With slight modifications. To determine maximum absorbance of crocin extract as control, different series of volume 600 µl, 650 µl, 700 µl, 750 ml, and 800 µl were added to tubes containing 75 µl of 0.5 M ABPH and 5 ml of ethanol respectively. 775µl of crocin extract shown highest absorbance at 443 nm. 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 capacity assay (MICC)

Chelating activity on Fe^{2+} ions

The chelating ability of the extracts on ferrous ions was determined according to the method of MICC [24]. 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.

Statistical analysis

All experiments were performed in triplicates and the results were expressed as mean ± standard deviation. Data were analyzed using Student's t-test for two sets while one-way Analysis of Variance for more than two sets. Significant differences were considered when means of compared sets differed at $p<0.05$. Data were carried out using SPSS v.16.0 (Statistical Program for Social Sciences) software.

Correlation analysis

Pearson's correlation method was used to analyze correlation between the results of different assays against phenolics along with flavonoids. All results of assays were correlated in the same method.

Aggregate index

Aggregate Index was calculated by total summation of all values of each assay in order to know highest score.

RESULTS

Phytochemical characterization

Extraction yield (EY), total phenolics content (TPC) and total flavonoids content (TFC) of selected SME.

Table 2 depicts the EY, TPC and TPC of selected spices extracts and SME.

***In-vitro* antioxidant assays of selected spices extract SME**

Antioxidant assay measures the capacity which is directly proportional to the absorbance value.

Table 2: EY, TPC and TFC of selected spices extracts and SME

Spices	Label	EY%	TPC*	TFC*
Tulsi	S1	05.6	0.16±0.08	0.89±0.44
Clove	S2	27.4	0.32±0.23	1.21±0.65
Curry leaves	S3	06.5	0.21±0.07	1.10±0.53
Ginger	S4	03.9	0.18±0.09	0.39±0.19
Cinnamon	S5	09.6	0.21±0.12	0.80±0.56
Turmeric	S6	06.9	0.17±0.06	3.87±0.11
Mustard	S7	06.1	0.14±0.08	0.14±0.16
Black pepper	S8	07.1	0.13±0.06	0.62±0.29
Nutmeg	S9	24.0	0.13±0.06	0.22±0.15
Cumin	S10	15.7	0.13±0.06	0.53±0.41
SME	S11	25.6	0.15±0.07	2.18±1.31

*Values are mean±SD (n=3), SD: Standard deviation, EY: Extraction yield, TPC: Total phenolics content, TFC: Total flavonoids content, SME: Spices and mixture extracts

PMA

Phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and subsequent formation of a bluish green colored phosphate/Mo (V) complex with absorbance at 695 nm. It provides reduction capacity quantitatively through the reduction reaction rate among antioxidant, oxidant and molybdenum ligand by thermally generating auto-oxidation during prolonged incubation period at higher temperature. The order of phosphomolybdenum activity was found to as follows: Turmeric > SME > ginger, cumin > curry leaves > nutmeg > tulsi > mustard > clove > cinnamon > black pepper.

CUPRAC

CUPRAC assay is based on the complexometric and redox reaction between copper (II)-neocuproine [Cu (II)-Nc] reagent as the chromogenic oxidizing agent. CUPRAC evaluation of spices extracts and SME were found in the order of: Clove > cinnamon > nutmeg > ginger > SME > turmeric > curry leaves > tulsi > mustard > black pepper > cumin.

FRAP

FRAP assay includes the simultaneous use of ferricyanide and ferric ions as chromogenic oxidants. High absorbance indicates the more reducing power of spices extracts and SME. FRAP descended in the order of: SME > clove > ginger, nutmeg > turmeric > black pepper > tulsi > cinnamon > mustard > curry leaves > cumin.

Free radical scavenging assay of the selected spices extracts and SME

FRSA measures the antiradical capacity which is inversely proportional to the absorbance value. Antiradical activities of spices and SMEs were assessed through different assays such as hydrogen peroxide, nitric oxide, hydroxyl, DPPH, superoxide and ABTS.

HP-FRSA

H_2O_2 has only a weak activity to initiate lipid peroxidation, but its activity as an active oxygen species comes from its potential to produce the highly reactive hydroxyl radical through the Fenton reaction. Hydrogen peroxide FRSA of spices and their SME was found in the order: Mustard > cumin > cinnamon > nutmeg > tulsi > ginger > black pepper > curry leaves > SME, turmeric and clove.

NO-FRSA

Sodium nitroprusside in aqueous medium at physiological pH spontaneously produces nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated by Griess reagent. Selected spices and SMEs were estimated for nitric oxide free radical scavenging capacity and arranged in descending order: Cinnamon > nutmeg > tulsi > turmeric > mustard > black pepper > cumin > clove > ginger > curry leaves > SME.

OH-FRSA

Hydroxyl radical is the most reactive among the oxygen radicals which induces severe damage to proteins, DNA and lipids by crossing cell membranes and leads to lipid peroxidation. Hydroxyl FRSA of the selected spices and spices mixture were found in the order: Nutmeg > mustard > cinnamon > ginger > cumin > tulsi > black pepper > curry leaves > clove> SME > turmeric.

DPPH-FRSA

DPPH is a stable free radical, which has been excessively used for assessment of scavenging activity of natural products. The ability of the investigated extracts to act as donors of hydrogen atoms or electrons in transformation of DPPH radical into its reduced form DPPH-H was measured. DPPH free scavenging activity of spices and SMEs were found to follow order of: Clove > cinnamon > nutmeg > SME > turmeric > curry leaves > ginger > tulsi > mustard > black pepper > cumin.

S-FRSA

Superoxide radical, known to be very harmful to cellular components as a precursor of the more ROS, contributes to tissue damage and various diseases. The ranking results tagged here as: Mustard > black pepper > clove, ginger, turmeric > nutmeg > cumin > SME > curry leaves > tulsi > cinnamon.

ABTS-FRSA

Extracts of spices and spices mixture are added to the blue green chromophore $ABTS^{+}$ (2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) is measured by changing color. ABTS FRSA of selected spices extracts and SME was found in the order: Mustard, SME > ginger > turmeric, nutmeg > black pepper > tulsi > clove > cumin, curry leaves, cinnamon.

TBARS assay

Egg yolk lipids undergo rapid non-enzymatic peroxidation when hatched in the presence of ferrous sulfate. MDA is the end product in the egg-lipid peroxidation process. During oxidative degeneration by free oxygen free radicals which give pink color as indicator in the presence of thiobarbituric acid. Lipid peroxides inhibited by spices and spices mixture was arranged in the order: Nutmeg > curry leaves > ginger > turmeric > clove > spices SME > Tulsi > cinnamon > mustard > cumin > black pepper.

CBC

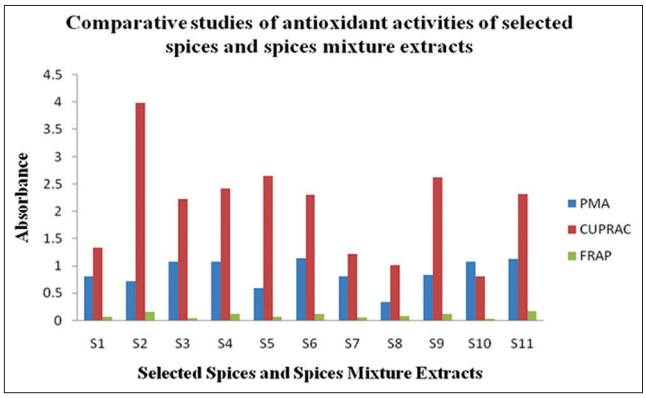
Decrease in peroxyl radicals oxidized crocin by extracts of spices and spices mixture were measured in the crocin bleaching capacity. Amount of crocin bleached by spices and SMEs were denoted in the order of: Cumin > mustard > nutmeg, curry leaves > ginger > tulsi > black pepper > cinnamon > clove > SME > turmeric.

MICC

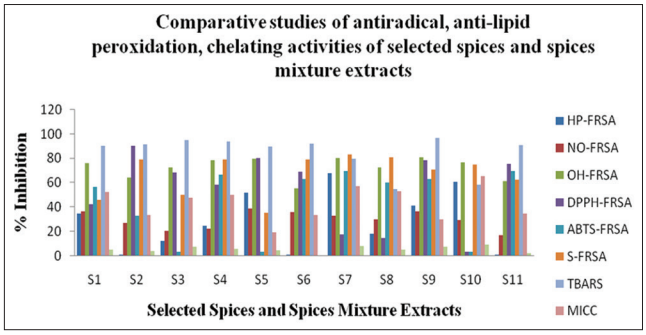
Metal ions catalyze the rate of free radicals formation. Ferrozine chelates with Fe^{+2}. In the presence of chelating properties of spices, the complex formation is disrupted, leading to a decrease in the red color of ferrous ion and ferrozine complex. Ability of selected spices extracts and SME to chelate the free metal ions was found to be in the order of: Cumin > mustard > black pepper > tulsi > ginger > curry leaves > SME > clove > turmeric > nutmeg > cinnamon.

Table 3 specifies the values of all parameters of spices extracts and SME.

Table 4 describes the ranking of spices extracts and SME.



Graph 1: Depicts comparative studies of antioxidant activities of selected spices extracts and spices and mixture extracts



Graph 2: Depicts the comparative studies of antiradical, anti-lipid peroxidation, chelating activities of selected spices and mixture extracts

Table 3: Values of all parameters of spices extracts and SME

Values	Absorbance*			Percentage inhibition								
Spices	PMA	CUPRAC	FRAP	HP-FRSA	NO-FRSA	OH-FRSA	DPPH-FRSA	S-FRSA	ABTS-FRSA	TBARS	CBC	MICC
S1	0.80±0.10	1.33±0.15	0.07±0.03	35.15	36.48	76.27	42.55	45.83	56.66	90.36	5.24	52.6
S2	0.71±0.17	3.99±0.00	0.15±0.05	1.10	27.03	64.27	90.22	79.16	33.33	91.78	3.89	33.9
S3	1.07±0.04	2.23±0.14	0.04±0.005	12.52	20.44	72.65	68.50	50.00	03.33	95.46	7.92	47.7
S4	1.08±0.10	2.42±0.02	0.12±0.08	24.75	22.36	78.69	58.39	79.16	66.66	94.05	6.03	50.4
S5	0.59±0.03	2.64±0.56	0.06±0.06	52.05	38.86	80.06	80.26	35.41	03.33	89.80	4.97	19.7
S6	1.44±0.08	2.30±0.16	0.10±0.05	1.10	35.91	55.60	68.92	79.16	63.33	92.06	0.86	33.4
S7	0.81±0.11	1.21±0.05	0.05±0.01	67.82	33.00	80.18	17.74	83.33	70.00	79.88	8.06	57.6
S8	0.33±0.29	1.01±0.25	0.08±0.01	18.30	30.04	72.60	14.75	81.25	60.00	54.95	5.13	53.3
S9	0.83±0.12	2.62±0.27	0.12±0.03	41.27	36.68	81.16	78.74	70.83	63.33	97.16	7.92	30.4
S10	1.08±0.15	0.81±0.10	0.03±0.01	61.07	29.37	77.08	3.79	75.00	03.33	58.35	9.60	65.9
S11	1.13±0.04	2.32±0.42	0.17±0.07	1.10	36.48	61.22	75.61	62.50	70.00	91.21	2.21	35.1

*Mean±SD, SD: Standard deviation, PMA: Phosphomolybdenum assay, CUPRAC: Cupric ion reducing antioxidant capacity, FRAP: Ferric reducing ability power, HP-FRSA: Hydrogen peroxide free radical scavenging activity, NO-FRSA: Nitric oxide free radical scavenging activity, OH-FRSA: Hydroxyl free radical scavenging activity, DPPH-FRSA: 2, 2-Diphenyl-1-picrylhydrazyl free radical scavenging activity, S-FRSA: Superoxide free radical scavenging activity, ABTS-FRSA: 2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt free radical scavenging activity, TBARS: Thiobarbituric Acid Reactive Substance, CBC Crocin bleaching capacity, MICC: Metal ion chelating capacity, SME: Spices and mixture extracts

Table 4: Ranking of all parameters

Spices	PMA	CUPRAC	FRAP	HP-FRSA	NO-FRSA	OH-FRSA	DPPH-FRSA	S-FRSA	ABTS-FRSA	TBARS	CBC	MICC
S1	7	8	7	5	3	6	8	10	7	7	6	4
S2	9	1	2	9	9	9	1	3	8	5	9	8
S3	5	7	10	8	11	8	6	9	9	2	3	6
S4	3	4	3	6	10	4	7	4	3	3	5	5
S5	10	2	8	3	1	3	2	11	9	8	8	11
S6	1	6	5	9	5	11	5	5	4	4	11	9
S7	8	9	9	1	6	2	9	1	1	9	2	2
S8	11	10	6	7	7	7	10	2	6	11	7	3
S9	6	3	4	4	2	1	3	6	4	1	3	10
S10	4	11	11	2	8	5	11	7	9	10	1	1
S11	2	5	1	9	3	10	4	8	1	6	10	7

PMA: Phosphomolybdenum assay, CUPRAC: Cupric ion reducing antioxidant capacity, FRAP: Ferric reducing ability power, HP-FRSA: Hydrogen peroxide free radical scavenging activity, NO-FRSA: Nitric oxide free radical scavenging activity, OH-FRSA: Hydroxyl free radical scavenging activity, DPPH-FRSA: 2, 2-Diphenyl-1-picrylhydrazyl free radical scavenging activity, S-FRSA: Superoxide free radical scavenging activity, ABTS-FRSA: 2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt free radical scavenging activity, TBARS: Thiobarbituric Acid Reactive Substance, CBC Crocin bleaching capacity, MICC: Metal ion chelating capacity, SME: Spices and mixture extracts

Table 5: Correlation between the results of different assays

R2	TPC	TFC	PMA	CUPRAC	FRAP	DPPH-FRSA	OH-FRSA	NO-FRSA	HP-FRSA	S-FRSA	CBC	TBARS	MICC	ABTS
TFC	0.139	1												
PMA	−0.086	0.618[a]	1											
CUPRAC	0.804[b]	0.242	0.079	1										
FRAP	0.291	0.330	0.122	0.648[a]	1									
DPPH-FRSA	−0.617[a]	−0.370	−0.190	−0.917[b]	−0.617[a]	1								
OH-FRSA	0.299	0.909[b]	0.469	0.332	0.509	−0.350	1							
NO-FRSA	0.163	0.064	0.294	0.142	0.354	−0.102	0.304	1						
HP-FRSA	0.433	0.667[a]	0.270	0.521	0.651[a]	−0.550	0.821[b]	0.497	1					
S-FRSA	0.134	−0.028	−0.120	0.084	−0.252	−0.344	−0.214	−0.150	−0.098	1				
CBC	0.282	0.815[b]	0.212	0.414	0.626[a]	−0.497	0.813[b]	0.042	0.730[a]	0.009	1			
TBARS	−0.433	−0.231	−0.368	−0.727[a]	−0.463	0.841[b]	−0.124	−0.050	−0.307	−0.308	−0.306	1		
MICC	0.417	0.379	−0.023	0.759[b]	0.503	−0.873[b]	0.282	−0.221	0.349	0.375	0.557	−0.621[a]	1	
ABTS	0.368	−0.154	−0.079	0.069	−0.547	−0.034	−0.194	−0.009	−0.212	0.503	−0.379	0.171	0.029	1

PMA: Phosphomolybdenum assay, CUPRAC: Cupric ion reducing antioxidant capacity, FRAP: Ferric reducing ability power, HP-FRSA: Hydrogen peroxide free radical scavenging activity, NO-FRSA: Nitric oxide free radical scavenging activity, OH-FRSA: Hydroxyl free radical scavenging activity, DPPH-FRSA: 2, 2-Diphenyl-1-picrylhydrazyl free radical scavenging activity, S-FRSA: Superoxide free radical scavenging activity, ABTS-FRSA: 2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt free radical scavenging activity, TBARS: Thiobarbituric Acid Reactive Substance, CBC Crocin bleaching capacity, MICC: Metal ion chelating capacity, SME: Spices and mixture extracts, [a]p<0.05, [b]p<0.01

Correlation analysis

Pearson correlation among the results of overall assays is specified in following Table 5.

Correlation analysis between different assays in relation with phenolics and flavonoids

CUPRAC assay was high positively significant correlated with TPC (R=0.804, p<0.01). CUPRAC was found to be an ideal assay among these assays to screen the total phenolics of spices and condiments. Reducing capacity was specified upon the content of phenolics rather than flavonoids. On other hand, hydroxyl assay was highly positive significant correlated with TFC (R=0.908, p<0.01). Flavonoids impart the quenching capacity to chelate free radicals generated in either *in-vitro* assays or *in-vivo*. PMA assay revealed negative correlation with phenolics content. PMA assay was only exceptional correlation with TFC therefore it indicated for flavonoids. ABTS was fine correlated with TPC. Superoxide assay was lowly correlated with phenolics content while negatively with flavonoids content similarly. DPPH assay and TBARS assay were negatively correlated with both of phenolics and flavonoid contents. Other assays were good correlated relatively to the phenolics and flavonoids content.

Correlation analysis among all assays

CUPRAC assay was positively significant correlation with FRAP (R=0.648, p<0.05), highly positive significant correlation with MICC (R=0.759, p<0.01) whereas was negatively significant correlation with TBARS assay (R=−0.727, p<0.05), strongly negative significant correlation with DPPH assay (R=−0.917, p<0.01). The reason behind strong correlation was between CUPRAC and metal chelating activity that free metal ions were responsible to play as catalyst for oxidation. This was obviously concluded that inhibition of oxidation reaction involved in CUPRAC assay and metal chelation is interrelated.

FRAP were positively correlated with hydrogen peroxide assay (R=0.651, p<0.05) and crocin bleaching assay (R=0.626, p<0.05) while negatively correlated with DPPH assay (R=−0.617, p<0.05). DPPH assay was highly positive correlation with TBARS assay (R=0.841, p<0.01) and highly negative correlation with metal chelating activity (R=−0.873, p<0.01). TBARS was significantly negative correlated

Table 6: Aggregate index of spices extracts and SME

1	S1 Tulsi	443.34
2	S2 Clove	429.53
3	S3 Curry leaves	381.86
4	S4 Ginger	484.11
5	S5 Cinnamon	407.73
6	S6 Turmeric	434.18
7	S7 Mustard	499.68
8	S8 Black pepper	391.74
9	S9 Nutmeg	511.06
10	S10 Cumin	385.41
11	S11 SME	439.05

SME: Spices and mixture extracts

with metal chelating activity. Therefore, DPPH and TBARS assays were closely interrelated to each other as both of them were highly negative correlated with metal chelating activity respectively. TBARS was exclusively negative correlated with all assays except to DPPH and ABTS.

Hydroxyl assay were excellent correlated with hydrogen peroxide assay($R=0.821$, $p<0.01$) and crocin bleaching assay ($R=0.813$, $p<0.01$). Crocin bleaching assay was good correlated with hydrogen peroxide assay ($R=0.730$, $p<0.05$) while negatively correlated with DPPH and ABTS.

Hydrogen peroxide assay was good correlated with all assays with the exception of superoxide, TBARS and DPPH. Apart from DPPH, superoxide, TBARS and metal chelating ability assays, nitric oxide assay was good correlated with all assays. Superoxide assay was especially good correlation with metal chelating activity, CUPRAC and poorly correlated with crocin bleaching assay and negatively with other assays. Except to PMA, DPPH, nitric oxide and TBARS, metal chelating activity was good correlated with all parameters.

Aggregate index

Aggregate index of selected spices extracts and SME is displayed in Table 6. Aggregate index is the sum of all results of all antioxidant and antiradical assays spice-wise to provide the highest value and lowest value of spices. Nutmeg > mustard > ginger > tulsi > SME > turmeric > clove > cinnamon > black pepper > cumin > curry leaves are arranged in the following order of aggregate index.

DISCUSSION

Scientific data based on certification of spices

We have compiled some spices documented in several literatures for their medicinal significance.

Tulsi

Tulsi has been used as traditional medicine since it possesses anti-fertility, hypoglycemic, hypolipidemic, antioxidant, anti-ulcerative, anticancer, antifungal, antimicrobial, hepatoprotective, cardioprotective, analgesic, adaptogenic activities and miscellaneous properties [25]. It has been well explored on account of its therapeutic potentials described in ayurveda [26].

Clove

Clove flower buds contains essential oil which acts as a powerful source of antioxidant [27]. It has several potent therapeutic qualities such as antimicrobial, antinociceptive, antiviral, antioxidant, larvicidal activities [28].

Cinnamon

Cinnamon is widely used in traditional system of medicine to treat diabetes in India. It's bark contains cinnamaldehyde [29] and cinnamon oil [30] which has potent anti-diabetic, antibacterial, antioxidant and anti-obesity activities [31].

Turmeric

Curcumin longa has been a well explored spice with multiple medicinal uses such as hypoglycemic, hypolipidemic, antioxidant, anti-ulcerative, anticancer, anti-inflammatory, anti-microbial, hepatoprotective, cardioprotective, analgesic, adaptogenic and etc. [32]. Turmeric contains 28 kDa glyco protein named "BGS-Haridrin" has antioxidant potency [33].

Ginger

Several pungent essentials present in ginger possess potent antioxidant and anti-inflammatory activities [34], and exhibit cancer preventive activity in experimental carcinogenesis [35].

Curry leaves

Curry leaves comprise polyphenolics with high antioxidant potencies [36]. Different activities of curry leaves have been reported as vasodilator, antidiabetic, hypocholesterolemic, antidiarrheal, cytotoxic, analgesic, antioxidant, protective and other diverse properties [37].

Mustard

Experimental reports on mustard seeds highlight their antioxidant, hypoglycemic, anticancer antimicrobial and anti-epileptic effects [38].

Cumin

Cumin seeds possess carminative, stimulant, diuretic, antispasmodic and astringent properties [39]. Its essential oil has antimicrobial and cytotoxicy potencies [40].

Black pepper

Essential oil present in black pepper is responsible for antioxidant, anti-inflammatory and analgesic properties [41]. Piperine has also been used to lower lipid peroxidation *in vivo* and has been proved to significant influence on cellular antioxidant status in experimental oxidative stress condition [42].

Nutmeg

Nutmeg is widely used as alternative medicine due to aphrodisiac, memory enhancer, antidiarrheal, anti-inflammatory and anti-cancer properties, antioxidant and antimicrobial agent [43].

Assessment of different antioxidant and antiradical activities

Antioxidant activities involve in the reduction in the redox reaction between oxidant and substrate and antiradical activities involve in sequestering of free radicals created by oxidant and substrate. Ethanolic extract of clove demonstrated highest phenolics content while the highest flavonoids content obtained in the ethanolic extract of spices mixture. In phosphomolybdenum method, the spices extracts such as curry leaves, ginger, turmeric, cumin and spices mixture exhibit higher grade of reduction capacity. Clove has the highest reducing capacity for cupric ions of all selected spices while spices mixture held second rank in the reducing capacity by CUPRAC assay. SME exhibited ferric ion reducing activity as compared to the individual spices as found by FRAP assay. Mustard extract showed highest value in hydrogen peroxide and superoxide Free radical scavenging activities; turmeric extract exhibited highest nitric oxide free scavenging activity. Nutmeg extract demonstrated the highest value in hydroxyl free radicals scavenging activity along with highest inhibition percentage in TBARS. Clove extract exhibited the highest rate of DPPH free radicals scavenging activity while cumin extract showed the higher chelation capacity and higher crocin bleaching activity in among all spices extracts and SMEs.

Limitations of ORAC and lack of "gold standard" for antioxidant assays

ORAC assay is one of the antioxidant methods developed recently to assess the total antioxidant activity of a biological sample or extract. However, this method has severe drawbacks: Showing only antioxidant activity against peroxyl radicals particularly; lack of explaining the nature of the damaging reaction; no evidence of free radicals involving in this reaction; and ORAC values have not any biological significance following consumption of any food [8]. It has underlined that ORAC value should be used as a complementary analytical tool in the investigation process. Without ORAC value, it is difficult to analyze the *in-vitro* assay of spices and herbs according to Ronald prior [44]. As there are no gold standard methods for measuring antioxidant capacity of sample or extract by either of *in-vivo* or *in-vitro* methods [45], we therefore have put efforts to analyze the different antioxidant methods with special attending in case of free radical scavenging assays. It would be better to establish the validity of results by correlating all maximum different antioxidant as well as antiradical assays with phenolics content and flavonoid contents.

Until date, researchers have screened some edible plants for antioxidant activity evaluation through three methods like low density lipoprotein oxidation assay, DPPH radical scavenging [46]. 50 traditional Chinese medicinal materials have been screened for antioxidant parameters like FRAP, hydroxyl radical scavenging assay, DPPH radical scavenging assay along with total phenolics content [47]. 32 spices extracts have been surveyed in the different assays like ABTS, FRAP and DPPH radical scavenging assays in terms of trolox equivalent antioxidant capacities jointly with total phenolics [48]. Only hydroxyl radical scavenging activity has been used for assay of some commonly used spices in India [49]. Three selected spices in the six antioxidant methods like DPPH, FRAP, NO, SO, H_2O_2 radical scavenging assays have been analyzed in conjunction with total phenolics, total flavonoids and other methods [50]. Some ginger species have been compared in terms of DPPH antioxidant capacity and total phenolics [51].

In the present study, top ten highest antioxidant potential spices and their mixture recognized by ORAC as per USDA data with maximum different antioxidant and antiradical assays were analyzed to know the exact nature of spices and spices mixture. In several studies, spices and herbs vary in the results in the antioxidant and antiradical assays. For that reason we have cross checked the different antioxidant and antiradical assays of ten spices and SME based on mechanisms of the electron transfer reaction and hydrogen transfer reaction.

Correlation analysis

A variety of tests expressing antioxidant potency of spices components has been suggested in several literatures. These have been categorized into two groups: Antioxidant activity and anti-radical activity. In more than a few studies related *in-vitro* antioxidant studies are lacking in the evaluation of correlation analysis. Antioxidant and FRSA reflect upon the content of phenolics and flavonoids present in the natural products. Therefore it is significant to correlate their assays with antioxidant and antiradical assays for better understanding the role of each assay and combinations of the followed results. Assessing the correlation coefficient analysis is useful to provide the comprehensive evaluation of natural products like spices and herbs.

The results of different assays used in the present investigation of ten spices and their mixture extracts were correlated with phenolics and flavonoids. Total phenolic and flavonoid contents have been reported to be responsible for the antioxidant activities of botanical extracts. DPPH, hydroxyl radical scavenging activity, and superoxide anion radical scavenging activity have been used to measure antiradical activity and these results should correlate with those of total phenolic and flavonoids content.

To our knowledge, this is the first study wherein the CUPRAC method was used to investigate household spices in terms of total antioxidant capacity. Apart from total phenolics and total flavonoid contents, CUPRAC has considerably correlated with other assays except to DPPH and TBARS assays. Both of TBARS and DPPH are having positively correlation with each other while negatively correlation with all other assays except to each other.

Negative correlation between TBARS and TPC indicate that non-phenolics may contribute to the lipid peroxidation reaction [52]. Thus the resulting discrepancy observed in correlation among TBARS with TFC together with TPC indicates the essential oils, as main components present in the species which are responsible for their scavenging activity. TBARS has no statistically significant relationship with either phenolics or flavonoids [53]. There is a strong association between TBARS and DPPH, which has shown contrast to the results reported [54]. Hydrophilic antioxidants are better estimated by ABTS when compared with that of DPPH [55]. The findings suggest some bioactive components like essential or volatile oils may also contribute to antioxidative potentials of the spices. Correlation analysis helps to provide better understanding the actual relation between different activities and phenolics and flavonoids. CUPRAC, OH, PMA, DPPH, and TBARS would be suggested as combined assays plainly to explain the comprehensible nature of spices in terms of anti-oxidant and anti-radical potencies. Not only depending on the phenolics and flavonoids contents for analysis of antioxidant or FRSA, but also it should be studied bioactive components other than phenolics and flavonoids, which could be accountable for antioxidant potentials. It has anticipated that essential oils present in spices may be an important contributing factor to establish the direct relationship with anti-oxidative potencies of spices.

The experimental variations established in correlation analysis among different anti-oxidant and antiradical methods indicate that only a single assay taken as a whole may not ensure to evaluate either total antioxidant activity or FRSA [56]. Cross checking among different assays related with antioxidant and antiradical activities is more preferable rather than depending only two or three assays.

CONCLUSION

Selected Indian spices extracts and SME have shown dissimilarity to each other therefore the study thus 1st time establishes aggregate index for evaluation of Indian spices. On basis of aggregate index, nutmeg has highest value among the selected spices. There is no significant synergistic effect found in SME.

CUPRAC as antioxidant assay and hydroxyl free radical scavenging assay by using Smirnoff reagent are most appropriate assays to ascertain anti-oxidative and free scavenger properties of natural products.

Aggregate index helps to provide the highest score and to outline overall results in the comparative studies.

Spices with the highest ORAC were screened for representing the potential sources of effective natural antioxidants for commercial exploitation. This study provides direct comparative data on total antioxidant capacity, free radical quenching capacity and other activities of the ten spices extracts and SME. It is a need of further investigation for comparing SME with standard synthetic antioxidant in a dose-dependent manner study.

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