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COMPARATIVE EVALUATION OF ANTIOXIDANT AND FREE-RADICAL SCAVENGING ACTIVITY OF AQUEOUS AND METHANOLIC SPICE EXTRACTS

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ABSTRACT

The aqueous and crude methanolic extracts from five traditionally used spices *Cinnamomum cassia, Myristica fragrans, Murraya koenigii, Piper nigrum* and *Trachyspermum ammi* were investigated for their in-vitro antioxidant and free-radical scavenging activity by using standard methods namely reducing power assay and 2,2-Diphenly-1-picryl hydrazyl (DPPH) free-radical scavenging activity, the values are comparable to ascorbic acid.The results revealed that the tested spices shows antioxidant potential in concentration dependent manner. The methanolic extracts shows the higher percentage of free-radical scavenging activity than the aqueous extract of spices. The percent increase in reducing power assay of both the extractsof these spices was in order ascorbic acid> *C.cassia> P.nigrum> M.koenigii>T.ammi>M.fragrans* and the DPPH free-radical scavenging potential was found in order ascorbic acid>*C.cassia>M.koenigii>P.nigrum>T.ammi>M.fragrans*. Earlier studies by other workers on this aspect showed that the majority of antioxidants are phenolic compounds and their structural variants, therefore the estimation of total phenolic content will be determined by using Folin-ciocalteau method.

Keywords: methanolic spice extracts, antioxidant**,** *Cinnamomum cassia, Myristica fragrans, Murraya koenigii, Piper nigrum* and *Trachyspermum ammi*

1] INTRODUCTION

Since oxygen is very crucial for existence of life on earth but it is also a highly reactive molecule that damages living organisms by producing reactive oxygen species (ROS). These oxidative and free-radical stress is thought to be contribute to the development of wide range of diseases including aging, cancer, coronary heart disease, diabetes, liver disease and certain neurodegenerative diseases [Nagabeppu Y.et al 2006, Valko M. et al 2007, Kamleshiya P. et al 2011]. Consequently, organisms contain a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components such as DNA, Protein and lipid [Kamleshiya P. et al 2011].

Recently there has been upsurge of interest in the therapeutic potential of medicinal plants as antioxidants in reducing such free-radical induced cell injury [Nikhat et al 2009, Kamleshiya P. et al 2011]. It is widely known fact that spices and herbs have been part of traditional diet around the world for years. They not only add flavor to food but also pack a power full punch when it come to their bio-power rich in antioxidants and other phytonutrients [Davis J. 2006]. Their general benefits include supporting the immune system, aiding digestion and

promoting cardiovascular health. The natural antimicrobial properties are also believed to reduce the risk of bacterial contamination [Davis J. 2006, Kamleshiya P. et al 2010].

While still in the early stages of research, studies are now showing positive results therefore the antioxidant and free-radical scavenging properties of spices have been the subject of considerable study. The aim of the present study is to assess the antioxidant potential of given spices *Cinnamomum cassia,Myristica fragrans, Murraya koenigii, Piper nigrum* and *Trachyspermum ammi* which are traditionally used in Indian diet.

2] MATERIAL AND METHODS

2.1] Chemicals

The required chemicals were purchased from Hi-media Mumbai, India. Potassium ferricyanide was purchased from E. Merck Pvt. Ltd. Mumbai, India and 2,2-Diphenyl-1-picryl hydrazyl (DPPH) was obtained from Sigma Alderich(USA). All chemicals used were of analytical grade.

2.2] Solvents

Absolute methanol (99.9% analytical grade) was diluted with distilled water to produce 50%(v\v) solution of methanol for extraction and also used for reconstitution and dilution in the DPPH assay. Distilled water was used for extraction, reconstitution where appropriate [Chan L. et al 2008].

2.3] Extraction and Preparation of crude extracts

All the spices under study including *Cinnamomum cassia*, *Myristica fragrans, Murraya koenigii*, *Piper nigrum* and *Trachyspermum ammi* were purchased at retail from local market of Nagpur and authenticated by a Botanist. The spices were inspected for any visible dirt and damaged parts were removed [Kaur G, Arora D. 2009].They were shade dried and milled in to fine powder using mortar and pestle. The aqueous and methanolic extracts were made by steeping 50 gm powder spices in 500 ml of water/ 50% methanol for two days with intermittent shaking after which it was filter with the aid of whatman filter paper No.1 before evapouration.

The spice extracts were evaporated to dryness on hot plate set at 40^0C for methanol extracts and 60^0C for aqueous extracts. The obtained solid content of the extracts were weighed. The dried extracts were stored in a freezer at -20^0C for further use [Chan L.et al.2008].

2.4] Characterization of Plant extracts

The crude and dried aqueous & methanolic extracts were characterized by their odor, appearance and texture. The weight of the dried extracts was also determined [Chan L. et al, Chua M. et al 2008].

3] IN-VITRO ANTIOXIDANT ACTIVITY DETERMINATION

Both the type of spice extracts were tested for in-vitro antioxidant activity using standard methods. The final concentration of the extracts and standard solutions used were 50, 100, 150, 200 and 250 ug/ml. The absorbance was measured colorimetrically against corresponding blank solution. The percent inhibition was calculated by using standard formula [Nikhat et al 2009, Jinesh V.K et al 2010].

3.1] Reducing power assay [Oyaizu M.1986, Oktay M. 2003, S. Lakshmidevi et al 2007]

The free-radical scavenging activity of reducing power of aqueous and methanolic extract of spices was evaluated by the method of Oyaizu. Substances which have reduction potential can react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}). Later, in the reaction mixture when ferric-chloride ($FeCl_2$) is added, the potassium ferrocyanide (Fe^{2+}) is reacts with it and produces a ferric-ferrous complex which can be colorimetrically measured at 700nm. [Singh R.et al] Various concentration of the aqueous and methanolic extracts powder (50-250ug/ml) in distilled water were taken in test-tubes, mixed with 2.5ml of phosphate buffer (0.2M, P^H 6.6) and 2.5ml of potassium ferricyanide (1%w/v). The mixture was incubated at 50^0C for 20 minute then 1.5ml of 10% TCA was added and centrifuged at 3000g for 10 minute. From all the test-tubes, 0.5 ml of the

supernatant was mixed with 1ml of distilled water and 0.5 ml of ferric-chloride (0.1% w/v). The absorbance was measured at 700nm using colorimeter (ELICO Ltd. SL. No.07060, Hyderabad, India), against a blank which was prepared without adding extract. Increased absorbance of the reaction mixture indicates increased reducing potential. 1% ascorbic acid was used as standard. All the tests were carried out in triplicates. The percent increased in reducing power was calculated by using following formula:-

$$\% \text{ increase in reducing power} = \left[A_{test}/A_{Blank}-1\right] x100$$

Where, A_{test} is optical density if test solution and A_{Blank} is the optical density of blank solution.

3.2] DPPH (2, 2-Diphenyl-1-picrylhydrazyl) assay [Oktay M et al 2003, Chan L. et al 2008, Nikhat et al 2009]

Antioxidant activities of the extracts were determined with 2, 2-Diphenyl-1-picrylhydrazyl assay. The free-radical DPPH, serve as the model oxidizing agent to be reduced by the antioxidants present in the extracts. The DPPH free radical is reduced to corresponding hydrazine when it reacts with hydrogen donors. The DPPH radical is purple in color and upon reaction with hydrogen donor changes to yellow color. It is a discoloration assay which is evaluated by the addition of the antioxidant to DPPH solution in methanol and the degree of discoloration indicates the scavenging potential of the antioxidant compound in terms of hydrogen donating ability. 0.1mM solution of DPPH (39.4 mg of DPPH in 1000ml of analytical grade methanol) was prepared and 1ml of this solution was added to 3ml of (aqueous and methanolic powder extract) extract solution of different concentration (50-250ug/ml) prepared with methanol. The mixture was shaken vigorously and allowed to stand at room temperature in dark for 10 minute. Later, the decrease in absorbance of the resulting solution was measured using colorimeter (ELICO Ltd. SL. No.07060, Hyderabad, India) at 517 nm. 1% ascorbic acid at various concentrations (50- 250 ug/ml) was used as standard. Lower absorbance of the reaction mixture indicates higher free-radical scavenging activity. The tests were carried out in triplicates. The DPPH radical scavenging activity was calculated with the following formula

$$\text{DPPH radical scavenging activity (\%)} = \left[A_0 - (A_1 - A_s)\right]/A_0 \times 100$$

Where A_0 is the absorbance of the control solution containing only DPPH after incubation, A_1 is the absorbance in the presence of spice extract in DPPH solution after incubation and A_s is the absorbance of sample extract solution without DPPH for base line correction arising from unequal color of the sample solution (optical blank for A_1).

4] RESULTS AND DISCUSSIONS

4.1] Reducing power assay

The reduction potential of aqueous and methanolic extracts of spices at various concentrations is illustrated in Table 1&2 and Fig. 1&2. The reducing capacity of both kind of spice extracts were compared with ascorbic acid for the reduction of Fe^{3+} - Fe^{2+}. The reducing capacity of a compound indicates its power to serve as an antioxidant [S. Lakshmidevi et al 2007, Nikhat 2009]. However, different types of antioxidant possess different mechanism of action to prevent oxidation of any compound viz. prevention of chain initiation, decomposition of peroxidases [Singh R et al 2008] , binding of transition metal ion catalysts, inhibition of diene conjugates [Gacche R.N.et al 2010], Prevention of continued hydrogen abstraction and radical scavenging antioxidant activity.

For determination of reducing capability of aqueous and methanolic spice extracts the Fe^{3+} – Fe^{2+} color transition was measured. On the basis of this investigation it was reported that there was a concentration dependent increase in reducing power of extracts. It was found that methanolic extract of spices have higher percentage of reduction potential as compared to aqueous extract of spices. The percent increase in reducing power of methanolic extract was in order Ascorbic acid >*C. cassia*>*P. nigrum*>*M. koenigii*>*T. ammi*>*M.fragrans*. The methanolic extract of *Cinnamomumcassia* (150-200ug/ml) and *Piper nigrum* (200-250ug/ml) showed significant activity (i.e. >50%)

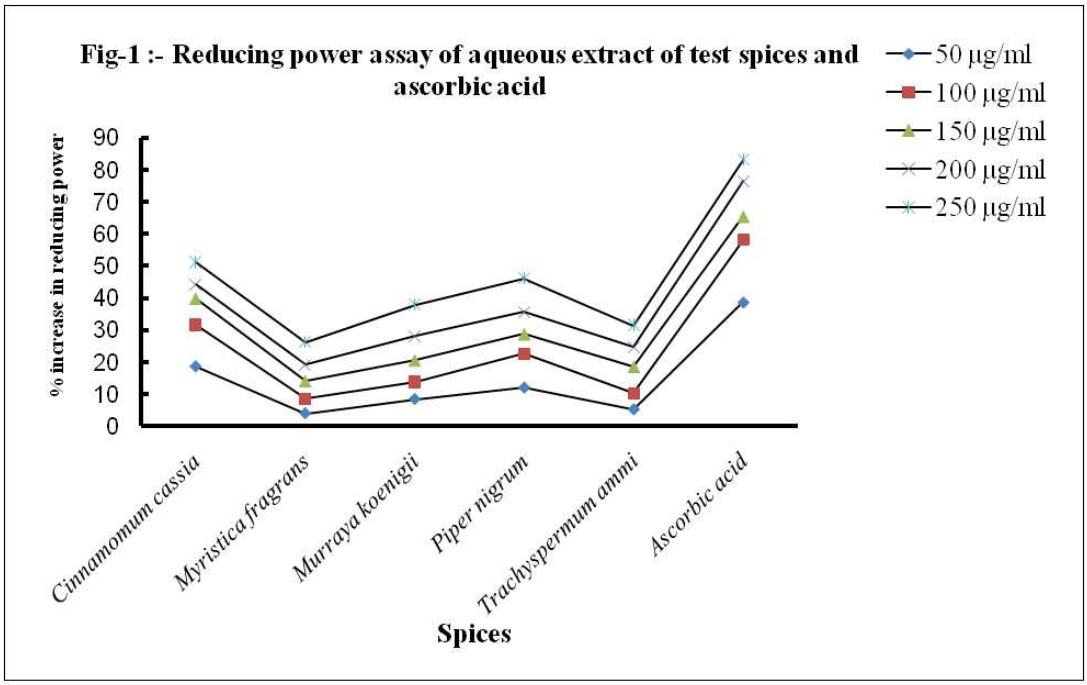
Table-1 :- ***Reducing power assay of aqueous extract of test spices and ascorbic acid***

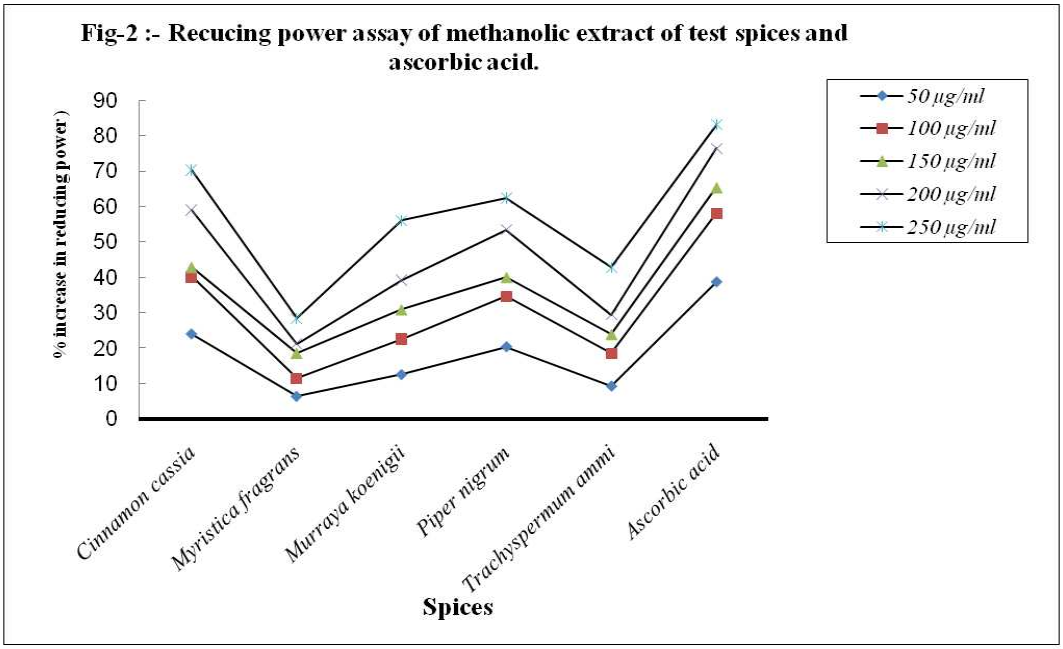
Quantity of the spice extract used in µg/ml	% increase in reducing power ± SEM					
	Cinnamomum cassia	***Myristica fragrans***	***Murraya koenigii***	***Piper nigrum***	***Trachyspermum ammi***	**Ascorbic acid**
50 µg/ml	18.63 ±0.38	3.94 ±0.12	8.39 ±0.23	11.96 ±0.54	5.16 ±0.26	38.62 ±0.15
100 µg/ml	31.68 ±0.30	8.57 ±0.22	13.86 ±0.15	22.53 ±0.41	10.23 ±0.18	58.07 ±0.80
150 µg/ml	39.87 ±0.48	14.18 ±0.60	20.63 ±0.46	28.78 ±0.47	18.64 ±0.91	65.36 ±0.25
200 µg/ml	44.15 ±0.52	19.27 ±0.43	28.12 ±0.62	35.62 ±0.71	24.68 ±0.89	76.41 ±0.33
250 µg/ml	51.09 ±0.42	26.23 ±0.37	37.78 ±0.17	46.02 ±0.32	31.53 ±0.63	83.12 ±0.28

Table-2 :- ***Recucing power assay of methanolic extract of test spices and ascorbic acid.***

Quantity of the spice extract used in µg/ml	**% increase in reducing power ± SEM**					
	Cinnamomum cassia	***Myristica fragrans***	***Murraya koenigii***	***Piper nigrum***	***Trachyspermum ammi***	**Ascorbic acid**
50 µg/ml	23.94 ±0.34	6.33 ±0.32	12.48 ±0.47	20.33 ±0.36	9.24 ±0.51	38.62 ±0.15
100 µg/ml	40.18 ±0.52	11.58 ±0.98	22.63 ±0.55	34.61 ±0.64	18.53 ±0.34	58.07 ±0.80
150 µg/ml	42.86 ±0.55	18.56 ±0.79	30.86 ±0.82	39.98 ±0.58	23.86 ±0.46	65.36 ±0.25
200 µg/ml	59.02 ±0.22	21.24 ±0.25	39.16 ±0.29	53.46 ±0.49	29.32 ±0.52	76.41 ±0.33
250 µg/ml	70.26 ±0.16	28.37 ±0.15	56.08 ±0.43	62.43 ±0.28	42.79 ±0.65	83.12 ±0.28

± SEM – Standard arithmetic mean





4.2] DPPH assay

The 2, 2-Diphenyl-1-picryl hydrazyl radical is widely used as the model system to investigate the scavenging activity of several natural compounds. The assay is based on the measurement of the scavenging activity of antioxidant toward DPPH which reacts with suitable reducing agent. The electron become paired off and solution losses color stochiometrically depending upon the number of electron paired taken up and when this reaction takes place the color changes from purple to yellow which shows a characteristic absorbance at 517nm [S. Lakshmidevi et al 2007, Chua M, et al2008, Nikhat et al 2009]. From the present result summarized in Table 3 &4 and Fig. 3 &4 indicates that the selected samples were found to interact with DPPH radicals and there by stabilize their hyperactivity. The free-radical activities of aqueous

and methanolic extract, of spices were compared with standard ascorbic acid for transformation of DPPH – $DPPH_2$. The measurements showed that antioxidant activities of spice extracts increased with increasing amount of sample concentration. When compared, the methanolic extract of investigated spices had more potent antioxidant activity than aqueous spice extracts. The percent increase in DPPH radical scavenging activity of methanolic extract was in order Ascorbic acid>*C.cassia*>*M. koenigii*>*P. nigrum*>*T.ammi*>*M. fragrans*. The scavenging activity of all the methanolic spice concentration was lower than ascorbic acid. The methanolic extract concentrations 150-250ug/ml of *C.cassia*, *M.koenigii* and P.*nigrum* possess significant DPPH radical scavenging activity. The lower activity of aqueous extract of spices in both the type of assay might be due to the fact that active principles of plant material are generally located in the conduit structures called the apoplast (cytoplasm) and symplast (cell wall) the aqueous maceration alone is not sufficient to extract these compounds from the structures whereas methanol may partially solubilize the membrane of the plant cells and storage organs, helping leach the chemicalaway. Results are mean of ± SD of the three measurements.

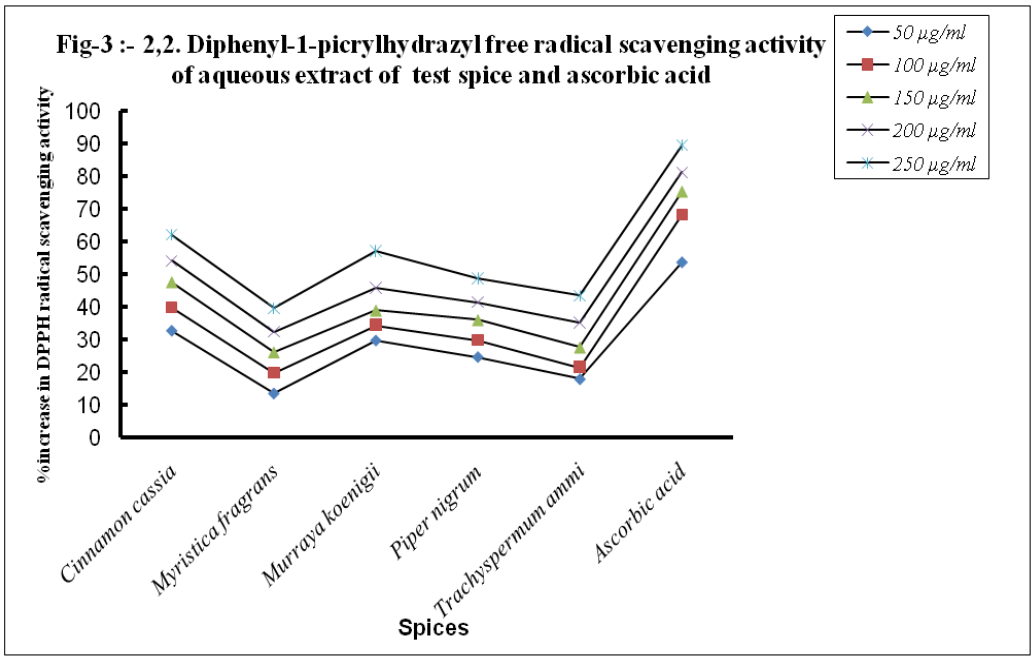
Table-3 :- ***2,2-Diphenyl-1-picrylhydrazyl free radical scavenging activityof aqueous extract of test spice and ascorbic acid***

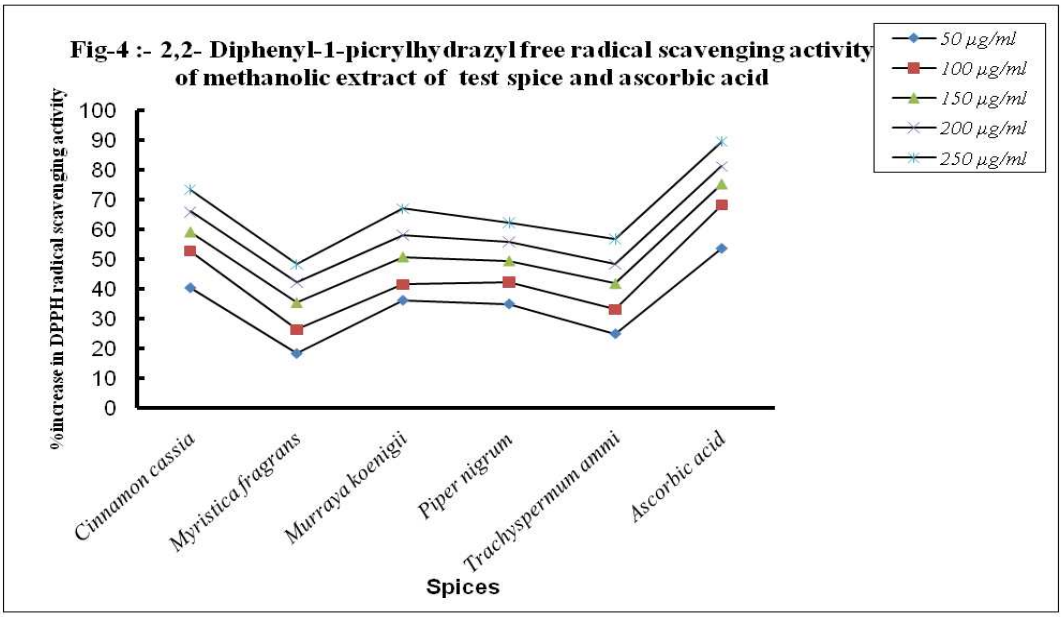
Quantity of the spice extract used in µg/ml	%increase in DPPH radical scavenging activity ± SEM					
	Cinnamomum cassia	***Myristica fragrans***	***Murraya koenigii***	***Piper nigrum***	***Trachyspermum ammi***	**Ascorbic acid**
50 µg/ml	32.69 ±0.08	13.52±0.28	29.72 ±0.68	24.66 ±0.22	17.89 ±0.57	53.59 ±0.56
100 µg/ml	39.82 ±0.22	19.89±0.45	34.43 ±0.51	29.83 ±0.53	21.55 ±0.60	68.10 ±0.89
150 µg/ml	47.53 ±0.10	26.16 ±0.35	38.9 ±0.43	36.02 ±0.19	27.63 ±0.28	75.18 ±0.44
200 µg/ml	54.13 ±0.32	32.43 ±0.62	45.84 ±0.66	41.54 ±0.63	35.17 ±0.81	81.22 ±0.69
250 µg/ml	62.08 ±0.37	39.68 ±0.48	57.13 ±0.39	48.76 ±0.42	43.52 ±0.54	89.48 ±0.74

Table-4 :- 2,2-*Diphenyl-1-picrylhydrazyl free radical scavenging activity of methanolic extract of test spice and ascorbic acid*

Quantity of the spice extract used in µg/ml	%increase in DPPH radical scavenging activity ± SEM					
	Cinnamomum cassia	***Myristica fragrans***	***Murraya koenigii***	***Piper nigrum***	***Trachyspermum ammi***	**Ascorbic acid**
50 µg/ml	40.38 ±0.72	18.33 ±0.68	36.19 ±0.19	34.96 ±0.37	24.96 ±0.48	53.59 ±0.56
100 µg/ml	52.72 ±0.59	26.58 ±0.86	41.63 ±0.27	42.35 ±0.29	33.18 ±0.83	68.10 ±0.89
150 µg/ml	59.19 ±0.83	35.56 ±0.94	50.86 ±0.49	49.52 ±0.57	41.94 ±0.69	75.18 ±0.44
200 µg/ml	65.94 ±0.52	42.23 ±0.42	58.16 ±0.38	55.86 ±0.39	48.29 ±0.57	81.22 ±0.69
250 µg/ml	73.45 ±0.96	48.37 ±0.59	67.08 ±0.61	62.38 ±0.61	56.78 ±0.64	89.48 ±0.74

± SEM – Standard arithmetic mean





CONCLUSION

This study supports the hypothesis that the dietary intake of these spices can be useful in the management of oxidative stress and could be used in medicine to treat the various ailments.This preliminary study indicates a need for further assessment on chemical analysis of spices under investigation. Since the antioxidant activities are mainly due to presence of phenolic compounds therefore further study will be carried out to determine the total phenolic content.

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