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COMPARATIVE FREE RADICAL SCAVENGING ACTIVITY OF DIFFERENT FRACTIONS OF CINNAMOMUM TAMALA LEAVES IN CHEMICAL SYSTEM

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ABSTRACT

The leaves of Cinnamomum tamala (Lauraceae), known as tejpatta used as spices for flavour purposes in Indian houses. The antioxidant property of the leaves is known, but here, we compare free radical scavenging activity of various fractions extracted in different organic solvents such as hexane (CTH) Ethyl acetate (CTEA), and methanol (CTE) sequentially and total methanolic fractions (CTT) and compared with their reducing capacity. For this purpose, dried powder of CT leaves was extracted with different solvents, and solvent-free extracts were prepared and dissolved in desirable solvents. Their free radical scavenging activity was determined at different parameters. Total antioxidant capacity was determined using an $ABTS^{+}$ assay, Lipid peroxidation was assessed in terms of thiobarbituric acid-reactive substances by using egg-yolk homogenates as lipid-rich media, Superoxide radical scavenging was measured using riboflavin-light-nitro blue tetrazolium (NBT) assay and Hydroxyl radical trapping potential was determined by evaluating hydroxyl radical-induced deoxyribose degradation using the thiobarbituric acid method. All the fractions show antioxidant properties but not polar fraction (CTH) was found to be more potent than others. The activity is related to their reducing potential.

KEYWORDS: Free radicals, Cinnamomum tamala, antioxidant, Lipid peroxidation, Superoxide radicals, Reducing potential.

INTRODUCTION

Reactive oxygen species (ROS) are important for a cell's survival and death.[1] These ROS include superoxide ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), and also hydrogen peroxide (H_2O_2),[2] lipid peroxyl ($LO^{\bullet}$) ,[3] etc. The mitochondrial respiratory chain is responsible for most oxygen reduction and energy produced in cells. Most common radical derivatives of oxygen like superoxide free radical anion ($O_2^{\bullet}$), hydroxy free radical ($^{\bullet}OH$), lipid peroxyl ($LO^{\bullet}$), lipid alkoxyl ($LOO^{\bullet}$) and lipid peroxide (LOOH) as well as non-radical derivatives such as hydrogen peroxide (H_2O_2) and singlet oxygen ($O_2^{\bullet}$) are collectively known as reactive oxygen species (ROS). In biological systems, these radicals were managed by enzymes such as superoxide dismutase (SOD), Catalase (CAT), Glutathione (GSH) etc. In normal physiology, the enzyme quenches radicals produced by respiration, but additional antioxidants are needed when they are extra-produced by any means.

Many herbs show significant antioxidant activity due to the presence of various secondary metabolites such as terpenes, flavones, anthocyanins, and many more. In the Indian system of medicines, Cinnamomum tamala leaves were used to treat diabetes and others.[4] Previous reports have shown its antioxidant and antidiabetic,[5] antifungal[6], and antidiarrheal[7] properties. Previously, we have reported its immunosuppressive properties.[8] Here, we prepare the various polar and non-polar fractions of the leaves, and their antioxidant potential was compared among them. The property is further correlated with the reducing potential of various fractions.

MATERIALS AND METHODS

2,2-Azino-bis(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) and deoxyribose were purchased from Sigma Ltd. Nitro blue tetrazolium (NBT), riboflavin, L-methionine, thiobarbituric acid and ethylenediaminetetraacetic acid (EDTA) were purchased from Hi-Media Ltd., ferric chloride anhydrous ($FeCl_3$), ascorbic acid, trichloroacetic acid and potassium persulphate were purchased from Merck Ltd. All reagents were of analytical grade.

Preparation of different fractions of CT leaves

The dried leaves were purchased from the local market and their authenticity was checked on pharmacogenetic parameters. Its coarse powder was extracted separately with methanol (CTT) and also successively with hexane, Ethyl acetate, and Methanol in a separate extractor by continuous Soxhlet extraction method. These four extracts were distilled under reduced

pressure and desiccated until constant weight was attained. Their % yield (w/w) was calculated with the original amount of coarse powder used for extraction. They were dissolved in respective solvents @ 100 mg/mL and further diluted with phosphate-buffered saline (PBS) for the study.

ABTS assay

ABTS radical scavenging activity of different fractions of CT leaves was determined by earlier.[13,14] ABTS radical was freshly prepared by adding 5 ml of a 4.9 mM potassium persulfate solution to 5 ml of a 14 mM ABTS solution and kept for 16 h in the dark. 950µl of prepared ABTS radicals (diluted with distilled water to yield an absorbance of 0.70 at 734 nm) was mixed with 50 µl of different concentrations of CT extract, and after 6 minutes; absorbance was recorded at 734 nm against distilled water by using an ELICO (SL-150) UV–vis spectrophotometer. In the control group, distilled water was used in place of test samples. Ascorbic acid was used as a reference drug as a free radical scavenger.

Lipid peroxidation assay

It was measured in terms of thiobarbituric acid-reactive substances (TBARS) by using egg yolk homogenates as lipid-rich media.[15,16] In brief, 1 ml reaction mixture containing 0.5 ml Egg yolk homogenate (10 % in distilled water, v/v), 0.1 ml of CT extracts was mixed with 0.05 ml $FeSO_4$ (0.07 M) and incubated for 30 min to induce lipid peroxidation. Thereafter, generated lipid peroxides were estimated by developing a pink color with 0.8 % TBA (2-thiobarbituric acid w/v) solution and heating for 60 min in a boiling water bath. The mixture was cooled, and extracted with 5.0 ml of butan-1-ol, and absorbance was recorded at 532 nm against butanol as blank. The standard curve drawn by TEP (Tetra ethoxy propane) was used as the standard and ascorbic acid was used as a positive control. The results were expressed in nM MDA and are calculated as -

MDA in NM = (Conc. Of standard × OD of test)/OD of standard

Superoxide radical-scavenging property

Superoxide radical scavenging potential of different fractions of CT leaves was reported in terms of its capacity to inhibit the formazan formation upon photochemical reduction of nitroblue tetrazolium (NBT).[17,18] In brief, each 3 ml reaction mixture (0.01M phosphate buffer (pH 7.8), 130 mM methionine, 60 µM riboflavin, 0.5mM EDTA, NBT (0.75mM) with 0.5ml CT extract ($CuSO_4$ solution is used as positive control) was kept in front of fluorescent

light for 6 minutes and absorbance was taken at 560 nm. Identical tubes were kept in the dark and served as blanks.[19] The results were expressed in percent inhibition as compared to control.

Hydroxyl radical-scavenging assay

The reaction mixtures containing 150µl ascorbic acid (200µM), 30 µl $FeCl_3$ (200 µM), 90 µl EDTA (200 □M), 30 µl H_2O_2 (20mM), 100 µl different concentration of test samples and then 150 µl deoxyribose (5mM) and incubated for 1 hr at 37°C. The control group received the same volume of buffer in place of test samples and the blank received the same volume of drug vector. The reaction was stopped by adding 1 ml of 2.8% TCA (w/v in water) and then 1 ml of 1% thiobarbituric acid (TBA) (w/v). The mixture was heated in a boiling water bath for 30 min, cooled and absorbance was taken at 532 nm. Thiourea was taken as positive control and PBS as blank.[20,21] The results were expressed in percent inhibition as compared to control.

Reducing potential

This was determined according to the method of Afolabi.[22] Different concentrations of CT extracts were mixed with 0.2M phosphate buffer (2.3ml, PH 6.6) and potassium ferricyanide (2.5ml, 1%). The mixture was incubated at 37^0C for 20 min. Trichhloroacetic acid (2.5 ml, 10%) was added to the mixture and centrifuged for 10 min at 1000 rpm. The upper layer (2.5ml) was mixed with 2.5ml of distilled water and 0.5ml of 0.1% $FeCl_3$. After standing for 10 min, the absorbance was measured at 700 nm using a UV/VIS spectrophotometer. High absorbance of the reaction mixture indicates high reducing power.

Statistics

All data are expressed as means ± SD. Pearson's correlation analysis (SPSS 7.5 for Windows, SPSS Inc.) was used to test for the significance of the relationship between the concentration and percentage inhibition. The single star (*) value shows significance and the double star (**) value shows a high significance value.

RESULTS

ABTS assay

For this assay, different extracts (2-100µg/ml) were added into 1 ml of ABTS solution, and free radical scavenging activity was analyzed based on their absorbance at 734nm as detailed in the method section. The result was compared with the control (only ABTS solution)

having an absorbance of 0.712±0.032. Ascorbic acid was used as a reference compound. The result showed that different fractions of CT leaves showed different degrees of scavenging potential for $ABTS^{\cdot+}$ radicals, but the response in all the cases was concentration-dependent. CTH was the most active among all, following the order as CTH>CTEA>CTT>CTE. When CTH was compared with the trapping potential of ascorbic acid, it was found to be 1.14 fold lower than ascorbic acid (w/w) and when compared to other extracts it was 1.31 fold higher than CTEA; 1.03 fold higher than CTT and 1.60 fold higher than CTE (Table 1).

Table 1: Effect of different fractions of CT leaves on $ABTS^{\cdot+}$ radicals.

Extract concentration (µg/ml)	**trapping potential for $ABTS^{\cdot+}$ radicals (% change with control value) mean ± SD**			
Fractions	CTH	CTEA	CTE	CTT
2	2.6±1.0	1.5±0.8	0.8±0.8	5.5±1.4
5	11.2±1.2*	5.6±1.2*	3.4±1.4	14.8±0.8*
10	21.5±1.5**	14.0±1.5*	9.1±1.6*	26.6±2.1**
20	47.2±2.2**	22.0±1.0**	14.2±2.0*	34.3±1.1**
50	70.6±2.5**	51.1±1.4**	33.1±1.2**	61.3±1.6**
70	92.4±3.1**	72.2±1.6**	63.2±1.7**	91.8±1.8**
90	95.2±2.1**	84.6±1.5**	72.4±2.1**	93.6±1.6**
100	96.8±1.4**	92.6±1.8**	78.4±1.7**	95.4±1.5**
IC_{50} (in µg/ml)	38.86±1.64	50.91±2.56	62.37±2.80	40.37±3.22

Level of significance: *P<0.05, **P<0.001

Lipid peroxidation assay

For this assay, egg yolk homogenate was used as lipid source, and free radicals were produced by Fenton reagent ($FeSO_4/H_2O_2$). Free radical rupture the lipid bilayer to form malonaldehyde as a secondary product. Two molecules of Thiobarbituric acid react with one molecule of MDA to form pink colored product showing maximum absorbance at 532 nm called TBARS. When the reaction mixture was mixed with different concentrations of extracts of CT leaves, it reduced the formation of TBARS product in a concentration-dependent manner compared to control (reaction mixture without antioxidant having absorbance 0.672±0.033 at 532 nm). Ascorbic acid was used as reference compound which showed significant reduction in lipid peroxidation with IC_{50} 276.7±2.24 µg/ml. CTH was found to be most active among all fractions, with IC_{50} 430±3.2 µg/m, but its inhibitory activity was 1.5-fold lower than Ascorbic acid (Table 2)

Table 2: Effect of different fractions of CT leaves on FeSO4-induced lipid peroxidation in egg yolk homogenate.

Concentration In µg/ml	% Inhibition (Mean±SD)			
	CTH	CTTEA	CTE	CTT
50	6.8±2.1	6.7±1.6	1.4±1.5	5.5±1.8
100	15.5±2.6*	13.7±1.0*	4.5±1.7	8.7±2.1*
250	28.7±2.3**	18.8±1.5*	8.4±1.3*	14.8±1.6*
500	44.9±2.2**	33.5±2.3**	18.7±1.6*	28.5±2.3**
750	62.8±2.3**	46.1±1.5**	32.6±1.1**	44.1±1.5**
1000	67.2±2.1**	63.4±2.2**	49.2±1.7**	62.4±1.5**
IC50 (µg/ml)	430±3.2	699±3.8	907±4.2	610±3.4

Level of significance: *P<0.05, **P<0.001

Superoxide scavenging assay

This assay is based on the capacity of extract to inhibit formazan formation by decreasing the reduction of nitro blue tetrazolium (NBT) in presence of riboflavin. Here superoxide is instantly generated by a photochemical reaction, which is trapped by added scavenger in that reaction system. The formation of formazan is highest in absence of any SO trapper (antioxidant) and that is considered as experimental control and used for comparison of trapping potential. Here copper sulphate solution (50 µg/ml), has been used as reference compound (positive control) along with the experiment. Different concentrations of extracts/ reference compound were added to the reaction system and the reaction was initiated by adding riboflavin solution and keeping the tubes in front of fluorescent light as detailed in the method section. Different fractions of CT leaves showed a concentration-dependent reduction in formazan formation. CTH showed the highest trapping potential, among all the fractions, with IC50 value at 187.48±2.0 µg/ml, but it was 4.5-fold lower than CuSO4. The inhibition order of different fractions of CT was CTH>CTEA>CTT>CTE (Table 3).

Table 3: Effect of different fractions of CT leaves on Superoxide (SO) radicals.

Drug dose in µg/ml	% Inhibition (Mean±SD)			
	CTH	CTEA	CTE	CTT
10	8.15±1.9*	5.35±1.0	9.75±1.0*	13.25±1.0*
30	14.2±2.4*	12.25±1.3*	13.45±1.1*	14.5±2.1*
50	36.3±2.8**	25.25±1.1**	18.2±1.9*	23.45±1.4**
80	51.1±1.7**	35.3±1.3**	22.85±1.8**	28.8±2.5**
100	59.85±1.7**	43.8±1.5**	24.8±1.5**	31.5±2.5**
300	66.15±2.6**	52.95±1.4**	36.15±1.3**	40.4±1.9**
500	70.55±2.0**	57.6±1.3**	42.5±2.1**	58.95±1.5**
IC50 in µg/ml	187.48±2.0	280±1.5	530.56±1.2	370.3±2.4

Level of significance: *P<0.05, **P<0.001

Hydroxyl radical-scavenging assay

Deoxyribose method was used for the determination of hydroxyl radicals scavenging capacity of different fractions of CT leaves. Different concentration of different extracts of CT leaves (50-500µg/ml) was added to the reaction mixture and finally, absorbance was recorded at 532 nm. Thiourea was used as a standard as a hydroxyl radical scavenger. Results showed that different fractions of CT leaves scavenged hydroxyl radicals in a concentration-dependent manner. CTH was most active in trapping hydroxyl radicals with IC_{50} 493±3.2µg/ml, but it was 2-fold lower than Thiourea with IC_{50} 242.17±2.56 µg/ml (Table 4).

Table 4: Effect of different fractions of CT leaves on hydroxyl radicals.

Conc in µg/ml	% Inhibition (Mean±SD)			
	CTH	CTEA	CTE	CTT
25	12.8±2.2*	7.6±1.8*	3.5±2.0	8.2±2.1*
50	32.6±2.1**	30.9±2.0**	33.5±1.9**	25.4±1.9**
100	39.0±2.2**	41.5±2.3**	37.4±1.1**	36.5±1.9**
200	55.33±2.6**	43.3±1.8**	45.3±1.4**	54.4±1.2**
500	60.4±1.3**	52.7±1.2**	53.7±1.9**	55.7±1.6**
700	72.8±1.6**	65.5±1.4**	57.2±1.7**	65.3±1.5**
1000	74.2±2.1**	69.1±1.6**	60.0±1.5**	67.9±1.1**
IC_{50} in µg/ml	493±3.2	753±2.8	798±2.1	585±3.5

Level of significance: $^{*}P<0.05$, $^{**}P<0.001$

Reducing potential of different fractions of CT leaves

Different fractions of CT leaves (CTH-Successive hexane fraction, CTEA-Successive ethyl acetate, CTE-Successive methanol fraction and CTT-total methanol fraction) were dissolved in suitable solvent of fixed concentration. Their reducing potential was assayed by the potassium ferricyanide method and absorbance was recorded at 700 nm as described in the method section. The highest absorbance of CTH among the same concentration of other fractions showed its higher reducing potential. Quercetin was used as a standard phenolic compound. An increase in absorbance shows increased reducing potential and Quercetin is used as standard (Table 5).

Table 5: Reducing potential of different fractions of CT leaves.

Concentration (mg/ml)	Absorbance at 700nm (mean ± SD) n=6				
	CTH	CTEA	CTE	CTT	Quercetin
0.1 mg/ml	0.207±0.015	0.157±0.017	0.108±0.012	0.188±0.012	0.257±0.013
0.3 mg/ml	0.311±0.011	0.302±0.014	0.111±0.016	0.298±0.010	0.356±0.018
0.5 mg/ml	0.591±0.014	0.567±0.018	0.214±0.011	0.354±0.018	0.627±0.011
1 mg/ml	0.755±0.024	0.737±0.018	0.402±0.019	0.515±0.015	0.836±0.022

3 mg/ml	1.116±0.015	0.989±0.016	0.512±0.012	0.677±0.017	1.156±0.020
5 mg/ml	1.216±0.012	1.010±0.018	0.717±0.022	0.919±0.018	1.326±0.016

DISCUSSION

Free radicals of oxygen and nitrogen species (RONS) have several normal physiological roles, but their overproduction may cause many diseases such as diabetes, arthritis, aging, etc. RONS are natural and physiological modulators of the cellular redox milieu and are thereby involved in the signaling cascade and control of a wide range of known and unknown physiological and pathophysiological processes. Despite the multi-line antioxidant systems, the level of RONS generation can exceed the capability of the defense network, leading to oxidative stress.[9] It is generally assumed that an increase in aerobic metabolism or hyperoxia easily generates increased level of RONS and causes oxidative damage which is initiated by the production of superoxide radicals, hydrogen peroxides and lipid peroxidation which further cause degradation of proteins and DNA. The increased level of RONS production is not only due to mitochondrial respiration, anaerobic exercise also could cause oxidative damage.[10] In normal respiration, molecular oxygen is released and a membrane-bound protein diffuses molecular oxygen into a radical form called super oxide radicals. These SO radicals further produce many other radicals such as hydroxyl radicals, nitric oxide, peroxy nitrile, etc by different mechanisms Phytomolecules act as scavengers of free radicals by rapidly donating hydrogen atoms.[11] In addition to having antioxidant properties, polyphenols have several other specific biological actions and modulate the activity of a wide range of enzymes and cell receptors.[12]

In a recent experiment, CT fractions were tested for their total antioxidant capacity using ABTS radicals. Ascorbic acid was used as a reference compound as an antioxidant. Different fractions of CT leaves decolorized the ABTS solution in a concentration-dependent manner, similar to how ascorbic acid showed its antioxidant nature.

We generated SO radicals instantly in the chemical system by NBT, methionine, and riboflavin by fluorescent light. Different fractions of CT leaves scavenged these free radicals (SO radicals) to different extents in a concentration-dependent manner but CTH was the most active among all, whereas CTE showed the least scavenging capacity, because of its low phenolic content which was reported earlier.

The hydrogen peroxide formed after the neutralization of SO radicals and produced hydroxyl

radicals which cause lipid peroxidation. In our experiment thiourea was used as a standard hydroxyl radical trapper which initially binds with C=S and produces radicals on thiourea which further neutralizes. When different fractions of CT leaves were used in parallel to thiourea in the reaction system, they showed a similar effect as shown by thiourea and the effect was in a concentration-dependent manner showing its activity towards hydroxyl radicals.

For lipid peroxidation, egg yolk homogenate was used as a lipid source and their per-oxidation was carried out by the Fenton reaction. As a result of this reaction, lipid breaks and forms a secondary product called malonaldehyde (MDA) which is measured by thiobarbituric acid (TBA). Two molecules of TBA react with one molecule of MDA to form a pink coloured substance soluble in organic solvents called Thiobarbituric acid reactive substance (TBARS) with maximum absorbance at 532nm. Different fractions of CT leaves were mixed with the reaction mixture then reduced lipid peroxidation was observed in different fractions in a concentration-dependent manner. CTH showed maximum inhibition followed by successive ethyl acetate and alcoholic fraction.

Thus, on the basis of the behaviour of different fractions of CT leaves with different species of free radicals was found to be same as shown by their known scavengers and concluded that CT leaves have potent antioxidant capacity. Their IC_{50} values are compared with each other and found that CTH have lowest IC_{50} values, and CTH was concluded as the most active fraction in free radical scavenging.

The antioxidant potential of various fractions was correlated with their reducing potential. The greater the reducing potential, the greater be antioxidant potential, and vice- versa. Non-polar fraction of the CT leaves (CTH) showed the highest free radical scavenging property as well as reducing potential.

CONCLUSION

Based on the above, it was concluded that hexane fraction is most active towards free radical scavenging activity.

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