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Anti-oxidant activities and phytochemical content of methanol extracts of *Capsicum frutescens*

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Abstract
Free radicals and related species such as reactive oxygen species/ROS and reactive nitrogen species/RNS were generated by various endogenously, physicochemical exposure or pathophysiological states able to alter lipids, proteins and DNA and have been implicated in aging and several human diseases. Plants are the cheapest source of anti-oxidants with desirable nutrients. In this context, sequential solvent extracts of *Capsicum frutescens* (Gandhari Menasu) fruit, a common Indian spices was subjected to estimation phytochemical and evaluate antioxidant property. Among the extracts, methanolic extract of *Capsicum frutescens* (MCF) exhibit higher levels of Phenolics (1.609 ± 0.35g/100g), tannins (0.904 ± 0.26g/100g), flavonoids (0.509 ± 0.31g/100g), alkaloids (0.516 ± 0.13g/100g) and saponins (0.182 ± 0.2g/100g) followed by ethanol and water extract. The DPPH, nitric oxide, superoxide free radical scavenging activity and reducing power capacity of MCF was found to be 17.25 to 53.20% at 50 µg/ml concentrations with IC50 values ranged from 94.43µg/mg to 246.4µg/mg.

Keywords: *Capsicum frutescens*, DPPH, antioxidants, phytochemicals, free radicals, spices

1. Introduction
Antioxidant therapy has gained an immense importance in treatment of several oxidative diseases originated by oxidative stress due to free radicals [1]. Antioxidants are radical scavengers; protect the human body against free radicals that may cause pathological conditions such as ischemia, anaemia, neuro-degeneration, Parkinson's diseases, ageing process, asthma, arthritis, and inflammation [2]. Free radicals such as superoxide anion, hydroxyl radicals and non radical species such as hydrogen peroxide, singlet oxygen are different forms of activated oxygen are main type of Reactive oxygen species (ROS) [3-4]. Overproduction ROS from activated neutrophil and macrophages leads to tissue injury by damaging the macromolecule and lipid peroxidation of membranes. ROS propagate inflammation by stimulating the release of the cytokines such as tumour necrosis factor α, interleukins [5], which stimulate recruitment of additional neutrophil and macrophages. Thus free radicals are important mediators that provoke or sustain inflammatory processes and consequently, their neutralization by antioxidants and radical scavengers can attenuate inflammation [6]. Inflammation is a complex process is associated with pain and involves occurrences such as, the increase of vascular permeability, increase of protein denaturation and membrane alteration [7].
Phytochemicals with antioxidant property are great interest due to their beneficial effects on human health as they provide protection against oxidative deterioration [5]. Many researchers indicated that flavonoids are mainly responsible for the antioxidant activity [8-9]. The antioxidant activity of dietary polyphenols is considered to be much greater than that of the essential vitamins [10]. *Capsicum frutescens* (Gandhari menasu in Kannada) fruit was commonly consumes as spice in India. *C. frutescens* reported to be good plasma glucose reducing capacity and anti-microbial activity [11]. Even though limited research was done on anti-oxidant activity of *C. frutescens* [12]. In this context, the present study has been undertaken to investigate the comparative profile of antioxidant activity and phytochemical properties of the *C. frutescens* by sequential extraction.

2. Materials and Methods
2.1 Materials
Diphenyl Picryl Hydrazyl Radical (DPPH˙), Thiobarbituric acid, Quercetin, Gallic acid, were purchased from Sigma-Aldrich Chemical Laboratories, St. Louis, MO, USA. Alu chrosep silica Gel 60/UV254 TLC plates were obtained from S D FINE CHEM. Limited, Mumbai. Dimethyl sulfoxide (DMSO) and other chemicals purchased from Merck. All other chemicals and reagents used in this study were of analytical grade or better.

2.2 Collection of samples and preparation of extracts
Capsicum fruits were collected from Kodagu in April, 2018 and Samples were washed thoroughly under running tap water and then dried at 45°C for 48 h upon arrival at the laboratory. The 10g of pulverized sample was shaken separately in selected solvent in polarity wise for 72 hrs on an orbital shaker at room temperature. Extracts were filtered through Whatman No 1 filter paper, concentrated under reduced pressure at 40ºC using a rotary evaporator. The solid powder obtained was weighed to calculate the percentage yield from the initial weight. For the further studies the extracts were suspended in respective solvent to make 50 mg/ml stock solution.

The dried extracts were weighed to deter-mine the percentage yield of the soluble constituents using the formula:

% Yield: Weight of dry extract/ sample Weight taken for extraction × 100 ... eq.1

2.3 Phytochemical estimation

2.3.1 Thin-layer Chromatographic Studies (TLC)

Thin-layer chromatography was carried out on all the fractions using TLC pre-coated plates (silica 60/UV254) by using one way ascending technique [13]. The plates were marked with pencil about 1cm from the bottom of the plate. Each sample was faintly dissolved in methanol and capillary tubes were used to uniformly apply the dissolved samples on the plates and allowed to dry. The plates were developed in a chromatographic tank using solvent system 1) chloroform: methanol (30:1, 15:1), 2) hexane: ethyl acetate (8:2) and 3) n-butanol: acetic acid: water (4: 1: 5). The plates were dried and visualized under normal day light, ultraviolet light (254nm & 366nm). The retention factor (Rf) for each active compound was calculated using the following formula:

Rf = Distance moved by the solute/ Distanced moved by the solvent (solvent front) ... eq.2

2.3.2 Estimation of total polyphenolic content

The total polyphenolic content of extracts was determined using folin ciocalteu's by colorimetrically [14]. Aliquotes (25-100µg) of Gallic acid was taken in test tubes and made up to 1ml with distilled water. Extract was also diluted accordingly; 1 ml of 1:1 FC reagent and 2ml of 10% sodium carbonate was added to each of the tubes. After 30 minutes absorbance was read at 760 nm against a blank. Concentration of polyphenols in extract was calculated using standard curve and expressed as gallic acid equivalents (mg of gallic acid/ g of dried extract).

2.3.3 Total flavonoid content

Total flavonoid content was determined using aluminium chloride ($AlCl_3$) according to previous method [15]. The reaction mixture contains 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water was incubated at room temperature for 30 min. The absorbance was measured at 415 nm. The aluminium chloride (10%) was substituted with distilled water in blank. Flavonoids in extracts reacted with aluminium chloride was determined as described above (Labman UV-Vis spectrophotometer). Total flavonoid content was calculated as quercetin equivalents (QE).

2.3.4 Estimation of total alkaloids

The total alkaloid contents in *C. frutescens* extracts were measured using 1, 10-phenanthroline method [16] with slight modifications. 100mg sample powder was extracted in 10ml 80% chloroform, filtered through muslin cloth and centrifuged at 5000rpm for 10 min. Supernatant obtained was used for the further estimation total alkaloids. The reaction mixture contained 1ml seed extract, 1ml of 0.025M $FeCl_3$ in 0.5M HCl and 1ml of 0.05M of 1, 10-phenanthroline in chloroform. The mixture was incubated for 30 min in hot water bath with maintaining a temperature of 70 ± 2ºC. The absorbance of the red coloured complex was measured at 510nm against the reagent blank. Alkaloid contents were estimated and it was calculated with the help of standard curve of colchicines (0.1mg/ml, 10mg dissolved in 10ml chloroform and diluted to 100ml with distilled water). The values were expressed as g.100g-1of dry weight (CE)

2.3.5 Estimation of total saponins

The *C. frutescens* extracts total saponins content were studied by previously established procedure [17]. 20 g of powdered sample was treated with 100 ml of 20% aqueous solution of chloroform, heated over a hot water bath for 4 h at about 55°C with continuous stirring. The mixture was filtered and the residue re-extracted. 20 ml of diethyl ether was added with vigorous shaking after above mixtures were reduced to 40 ml in water bath at 90°C. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and 60 ml of n-butanol was added to the combined extracts and washed twice with 10 ml of 5% aqueous NaCl. The excess water was removed by heating in a water bath, dried in an oven to a constant weight and the saponin content was calculated as g/ 100g.

2.4 Anti-oxidant activity

2.4.1 DPPH Free Radical Scavenging Assay (DPPH Assay)

Radical scavenging activity of *C. frutescens* extracts was measured according to the method of Blois with slight modification [15]. Briefly, 25-100µg Extracts of *C. frutescens* were mixed with 5 ml of 0.1 mM methanolic solution of DPPH and incubated at 20°C for 20 min in complete dark. The DPPH alone serves as control and methanol were used for the base line correction. Gallic acid was used as standard. The absorbance of the samples was measured at 517 nm and radical scavenging activity was expressed as percentage activity using the following formula.

% I = {(Ao – A1)/Ao} X 100 .... .eq.3

Where,

A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/standard.

Then % inhibitions were plotted against concentration.

2.4.2 NO scavenging activity

The scavenging effect of MCF on NO was measured according to published (18). Briefly, sodium nitroprusside (5 mM) in phosphate-buffered saline (PBS) (pH 7.4) was mixed with different concentrations of the test sample (25-100 µg/ml) and incubated at 25^0C for 150 minutes. After incubation, nitrite produced from sodium nitroprusside was measured by Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% 1-naphthylethylenediamine dihydrochloride in water). The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with 1-naphthylethyelenediamine dihydrochloride was measured at 570 nm. Catechin was used as a positive control. The

percentage of NO scavenging activity was calculated using the following formula:

$$\{(Ao - A1)/Ao\} X\ 100 \quad \text{.... Eq.4}$$

Where
A_0 is the absorbance of the control and A_1 is the absorbance of NJE or the standard sample.

2.4.3 Superoxide radical scavenging activity
The reduction of NBT was assayed according to previously described method [15]. Superoxide radicals generated by non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system often reduce nitro blue tetrazolium (NBT) to a purple formazan. The 1 ml reaction mixture contains phosphate buffer (20 mM, pH 7.4), NBT (50 µM), NADH (73 µM), PMS (15 µM) and *C. frutescens* extracts (25-100µg/ml). Incubate for 5 min at room temperature and the absorbance was measured at 562 nm against an appropriate blank to determine the quantity of formazan generated. Quercetin was used as positive control

$$\text{Percent inhibition (\%)} = (A_{control}-A_{test})/A_{control}\times 100$$

Where,
$A_{control}$ is the absorbance of the control reaction at 560 nm and A_{test} represents the absorbance of a test reaction at 560 nm.

2.4.4 Determination of reducing power assay
The reducing power ability of extracts was determined by the method as described in previous method [15]. The reaction mixture contains extracts (25- 100µg/ml) with equal volume of 0.2M phosphate buffer pH 6.6 and potassium ferricyanide was incubated at 50o C for 20 min. Centrifuged at 3000 rpm for 10 min by adding equal volume of 10% TCA to the mixture. To the upper layer, distilled water and 0.1% ferric chloride in a ratio of 1:1:2(v/v/v) were added. The absorbance was measured at 700 nm and increased absorbance of the reaction mixture compare to blank indicates increased reducing power activity. BHT was used as standard.

2.5 Statistical analysis
All the experiments were conducted with at least 3 independent repeats and the results were expressed as mean ± SD. Student's t-test was used to analyze the results and p value below 0.05 was considered as significant. For IC_{50} calculation and Graphs were plotted using Graph Pad Prism software 5.0 (San Diego, CA, USA).

3. Result and Discussion
The damage of tissues and bio molecules leading to several disease conditions, mainly degenerative diseases and extensive lyses mainly mediated by free radicals that are constantly generated in living systems. The antioxidants work in repairing oxidative cell damages and thus prevent a lot of physical ailment [21]. Many synthetic drugs like non steroidal anti-inflammatory drugs (NSAIDs) protect against oxidative damage, but they have adverse side effects. Consumption of natural antioxidants from food supplements and traditional medicine is the best way to overcome this problem. Photochemical/ biomolecules present in aromatic, medicinal, spices, vegetable, fruits and other plants contain exhibits antioxidant properties [22]. Various researchers also reported high antioxidant activities of different plant extracts using different assaying methods [23].

3.1 Phytochemical content
The antioxidant and phytochemical contents of *C. frutescens* extracts were determined. Among the phytochemicals, total phenolics and total flavonoids contents are the widest secondary metabolite in plant kingdom. Because of ability of radical scavenging and metal chelating activities, these diverse groups of compounds have received much attention as potential natural antioxidant. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers [19]. Flavonoids are a class of secondary plant phenolics with powerful antioxidant properties [20]. Total phenolic and flavonoid contents of *C. frutescens* extracts were estimated to be equivalents to gallic acid and quercetin. The total phenolics were found to be 1.609± 0.35, 1.020± 0.11 and 1.005± 0.17 mg/g and flavonoid contents were 904± 0.26 0.832± 0.21 0.604± 0.42 for MCF, ECF and AQCF respectively. Phytochemical content of all six extracts was found to decrease in the order MCF > ECF > AQCF > CCF > ACF>HCF (TAB.1). Several Activity in vitro model systems have been used for assessing the scavenging activity of all extracts at 50µg concentration (Fig.1). MCF exhibited the strongest radical scavenging activity compared to other extracts. Further, MCF is subjected to check antioxidant activity by concentration dependent manner.

3.2 DPPH free radical
DPPH free radical is stable nitrogen centred free radical commonly used for testing radical scavenging activity of the compound or plant extracts. The violet colour of the DPPH radical was reduced to yellow colored diphenylpicrylhydrazine radical after accepting an electron from the antioxidant compound, which was measured spectrophotometrically. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers [24]. DPPH radical scavenging activity of MCF was denoted in Fig. 2 over the range of 25–100 µg/mL concentration and the IC_{50} value was found to be 94.43µg/mL. Gallic acid was used as standards and the IC_{50} value was found 46 g/mL.

3.3 Superoxide
Lot of research reveals that superoxide anions damage biomolecules directly or indirectly by forming H_2O_2, ^{-}OH, per-oxy nitrite or singlet oxygen during aging and pathological events such as ischemic reperfusion injury. Superoxide has also been observed to directly initiate lipid peroxidation [27-28]. The superoxide anion radical scavenging activity of MCF was assayed by PMS-NADH system. The superoxide scavenging activity of MCF was increased markedly with the increase in concentrations (Fig.2). The result suggested that MCF has a potent superoxide radical scavenging effect the IC_{50} value was found to be 246.4 µg/mL.

3.4 NO radical

NO is responsible for many physiologic and pathologic events because of its toxic nature with a free radical character [29]. Incubation of a sodium nitroprusside solution in PBS at 25^0C for 150 minutes resulted in linear time-dependent nitrite production, which was reduced by MCF in a concentration-dependent manner (Fig.2). The moderate NO scavenging activity was observed by MCF is 61.28% at 100µg/ml (Tab.2)

3.5 Reducing power

Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of antioxidant action [25]. The reducing ability of a phytochemical generally depends on the presence of reductones (antioxidants), which exert the antioxidant activity by breaking the free radical chain by donating a hydrogen atom [26]. Reducing power of the extracts increases with the increase in concentration and showed good reducing power ability in a dose dependent manner (Fig.2) with IC_{50} of 240.5µg/ml (Tab.2). The antioxidant principles present in MCF caused the reduction of $Fe3^+$/ ferricyanide complex to the ferrous form and thus proved the reducing power ability.

3.6 Thin layer chromatographic studies

TLC analysis of all the fractions using different solvent systems revealed the presence of promising spots as shown in (Table 3). A phytochemical constituent in MCF gives different Rf values in different solvent system. This variation in Rf values provides a very important clue in understanding of their polarity and also helps in selection of appropriate solvent system for separation of pure compounds by column chromatography. Variable polarity solvents mixture in a different ratio can be used for separation of pure compound from plant extract. The selection of suitable solvent system for a particular plant extract can only be achieved by analyzing the Rf values of compounds in different solvent system [30-31]. Thin-layer chromatographic analysis carried out for MCF revealed to have complex spots as there was no separation in solvent system 1 and 3. Whereas solvent system 2 (Hexane: Ethyl acetate) shows three promising spots with Rf values 5.34, 6.47 & 8.23 (fig 4).

Table 1: Quantitative analysis of phytochemical compounds of different solvent extracts of *Capsicum frutescens*. All the values are mean of triplicate determinations and expressed in g /100 g; GAE- Gallic acid equivalent; QE-Quercetin equivalent; CE- colchicine equivalent.

Phytochemicals	Hex	Acetone	Chloroform	Ethanol	Methanol	Water
Phenolics (GAE)	0.170± 0.15	0.316± 0.11	0.342± 0.26	1.020± 0.17	1.609± 0.35	1.005±0.11
Flavanoids (QE)	0.094± 0.22	0.091± 0.03	0.132± 0.32	0.832± 0.21	0.904± 0.26	0.604± 0.42
Alkaloids (CE)	0.042± 0.05	0.018± 0.27	0.071± 0.02	0.302± 0.13	0.509± 0.31	0.181± 0.50
Saponins	0.034± 0.11	0.016± 0.02	0.070± 0.16	0.434± 0.20	0.516± 0.13	0.320± 0.11

Table 2: IC_{50} values of MCF for DPPH, Nitric oxide, superoxide and reducing power activity.

S. No.	Scavenging activity	IC_{50} values of MEC µg/ml
1	DPPH	94.43
2	Nitric oxide	221.9
3	Superoxide	246.4
4	Reducing Power	240.5

Table. 3: The Rf values of TLC solvent systems for different fractions of methanolic extract of *C. frutescens* fruit. SS: solvent system.

Solvent system	No. of spots	Rf values
SS1	2	3.25& 9.36
SS2	3	5.34,6.47 & 8.23
SS3	4	4.75, 5.21,8.21 & 9.30

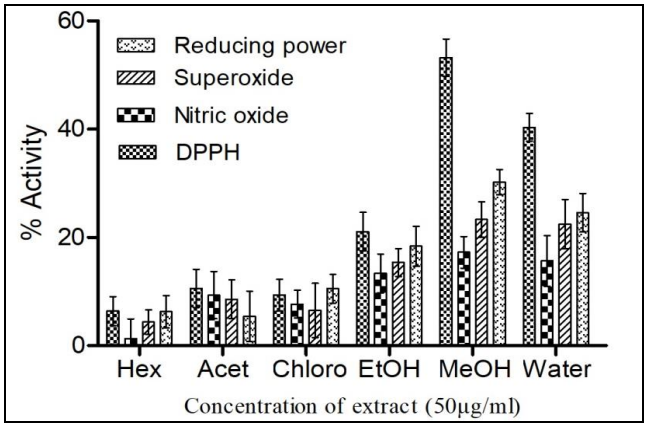

Fig 1: Anti-oxidant activities- DPPH, Superoxide, Nitric oxide radical scavenging and reducing power activity of C. *frutescens* fruit extracts (50µg/ml).

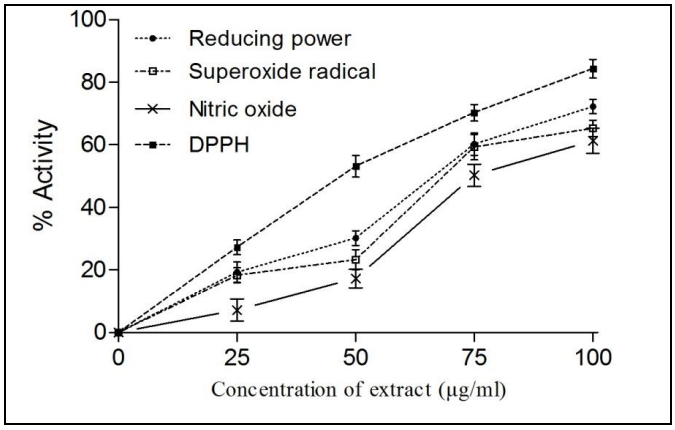

Fig 2: DPPH, Nitric oxide, superoxide scavenging and reducing power activity of methanolic *C. frutescens* fruit (MCF) extract at concentration dependent manner (µg/ml). Data values are mean ± SD with significantly different from each other ($p \leq 0.05$.

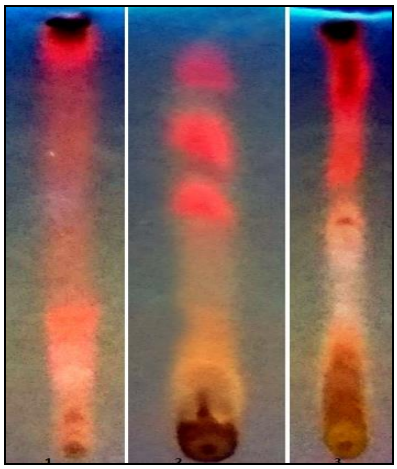

Fig 3: Thin layer chromatography of MCF at different solvent system. 1) Chloroform: methanol (30:1, 15:1), 2) hexane: ethyl acetate (8:2) and 3) n-butanol: acetic acid: water (4: 1: 5). The Rf values were calculated and tabulated.

4. Conclusion

The measurement of the antioxidant capacity of spices is important to quantify the intake of natural antioxidants in a dietary supplement. Additionally, these studies will help in the selection of natural source of antioxidant and in the use of health relevant food industry. Although we have very rich history of their medicinal use in 'Ayurveda' limited antioxidant containing drugs and cosmetics was manufactured. Antioxidant can be used to produce medicines and cosmetics so that, the drug and cosmetic industries in our county can have their own herb based formulation. Hence, MCF contains high levels of total phenolics and flavonoids with good free radicals scavenging capacity may contribute a protective effect against oxidative damage induced to cellular macromolecules. The MCF seems to be the most promising source of natural antioxidant compounds further isolation of bioactive compounds is required for identifying the unknown compounds to establish their pharmacological properties.

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