Research Paper

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Antioxidant and Free Radical Scavenging Potential of Ethanolic Extract of Garcinia Indica Linn. Fruits.

Biochemistry

KEYWORDS : Antioxidant, free radicals, total phenolics, Garcinia indica, Garcinol, DPPH.

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ABSTRACT

Objective: To evaluate the in vitro antioxidant potentials of ethanolic extract of Garcinia indica Linn fruits. Methods:The ethanolic extract of fruit rind of G.indica was subjected tothe analysis of phytochemicals, total phenolic, tannin and flavonoid contents and free radical scavenging activities such asDPPH,Nitric oxide radical,Superoxide radical, Hydroxylradical radical,ABTS+radical,reducingpower and Metalion-chelatingactivities were determined.Results: The ethanolic extract of G.indica fruit rinds were demonstrated a notable antioxidant potential dose dependently with best activity at 500µg/ml. conclusion:Traditionally claimed medicinal benefits of G.indica might be due to its potent anti-oxidant nature.

1. Introduction

Free radicals are highly reactive oxygen species such as superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), peroxyl ($ROO^{\cdot}$), peroxinitrite ($^{\cdot}ONOO^-$), and nitric oxide ($NO^{\cdot}$) radicals produced via oxidative process within the mammalian body (Abdel-Hameed, 2009). Free radicals attacks lipids, sugar, protein, DNA and induce their oxidation, which may results in oxidative damage such as membrane dysfunction, protein modification, enzyme inactivation and breakage of DNA strands and modification of its bases(Sang *et al*., 2001). The human body defence mechanism against oxidative stress, including enzymatic and non-enzymaticantioxidant compounds like superoxide dismutase, catalase, glutathione peroxidase, Vitamin-C, Vitamin-E and Reduced glutathione in this natural antioxidant mammalian mechanism become insufficient and then excess of free radicals can damage both the structure and function of cell membrane in a chain reaction leading to degenerative disease and a condition such as Alzhemer, acute liver toxicity, cataracts, cardiovascular disease, arteriosclerosis, neural disorders, diabetes mellitus, rheumatism inflammation process, nephritis and DNA damage that can lead to carcinogenesis (Mosquera *et al*., 2007; Wong et al., 2006).Recent trends in nutrition towards development of healthy foods in the form of 'Functional foods', one of the desirable properties in a dietary component is considered to be its antioxidant effect.

G. indica belonging to the family of clusiaceae commonly called as 'kokam' is found in Maharashtra and particular in konkan, Goa and the western region of India. The fruits of *G. indica* have been suggested in the Indian system of medicine for number of diseases. Indian spice used in many parts of the country for making several vegetarian and non-vegetarian 'curry' preparation including the popular 'solkadhi'. The fruits are steeped in sugar syrup to make 'amrutkokam', a healthy soft drink to relieve sunstroke, which is popular drink summer. It is a traditional home remedy in case of flatulence, heatstroke and infections. Many therapeutic effects of the fruit have been described in traditional medicine based on Ayurveda. This include its usefulness as an infusion, in skin aliments such as rashes caused by allergies; treatment of burns, scalds and chaffed skin; to relieve sunstroke; remedy dysentery and mucous diarrhea; an appetizer and a good liver tonic; to improve appetite and to allay thirst as cardio tonic and for bleeding, piles, dysentery, Tumors and heart diseases (Mishra *et al*., 2006). The fruit rind contains polyisoprenylated benzophenones, garcinol, its isomer isogarcinol, xanthochymol and isoxanthochymol(Chattopadhyay and kumar., 2006).

Based on traditional uses we selected this plant for the study. The purpose of present study was to evaluate antioxidant and free radical scavenging activities of ethanolic extract of *G.indica* fruits.

2. Materials and methods

2:1Chemicals

Potassium ferricyanide, ferric chloride, 2,2- diphenyl-1-picrylhydrazyl (DPPH), potassium persulfate, 2,2'-azinobis(3-ethylbenzothiozoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine(Ferrozine), linoleic acid, ferrous chloride, ammonium thiocyanate,hydrogen peroxide, ferrous ammonium sulfate,e thylenediaminetetracetic acid (EDTA) disodium salt, trichloroaceticacid (TCA), naphthylethylenediaminedihydrochloride (NEED),sodium nitroprusside and sulphanilamide were obtained from Himedia,Merck or Sigma. All other reagents used were of analytical grade.

2:2 Collection of plant material

G. inidica fruits were collected in and around Goa. The fruits samples were authenticated by Dr.K.Arumugasamy, Associate Professor, Department of Botany, Kongunadu Arts and Science College, Coimbatore, Tamilnadu. Fruits were cut open and the seeds were separated from the pulp. Then the fruit rinds were allowed to dry in the shade. The fruit rinds were cut into pieces and shade dried at room temperature. The dried fruit rinds were subjected to size reduction to coarse powder by using mixer grinder. The coarsely powdered sample was kept under refrigerator at 4°C.

2:3 Preparation of extract

30 gram of *G. inidica* fruit rinds powder was extracted with 250ml of ethanol in a soxhlet apparatus. The extract was dried at room temperature till semisolid mass was obtained, The sweet scented, chocolate colored semisolid residue formed after the complete dryness.

2:4 PreliminaryPhytochemical screening

Preliminary phytochemical screening of *Garcinia indica*was carried out for the detection of phytoconstituents such as alkaloids, flavonoids, phenols, steroids, tannins and glycosides) using standard protocols (Peach and Tracey, 1955).

2:5Determinations of total phenolics and tannin contents

The total phenolic content ofethanolic extract of *G. indica* was determined by Folin-ciocalteu method. Using the same extract, the tannins were estimated after treatment with polyvinylpolypyrrolidone (PVPP). The amount of total phenolics and tannins were calculated as the tannic acid equivalents (TAE) via, (Siddhuraju and Becker, 2003) and (Siddhuraju and Manian, 2007).

2:6Determination of total flavonoid content

The total flavonoid content was determined according to the

method described by (Zhishen *et al.*, 1999). 0.5 ml aliquot of the extract (2 mg/2mL) was mixed with 2 mL of distilled water and subsequently with 0.15 mL of a 5% $NaNO_2$ solution. After 6 min, 0.15 mL of 10% $AlCl_3$solution was added and allowed to stand for 6 min, and then 2 mL of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 5 mL, and then the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was determined at 510 nm versus water blank. The analysis was performed in triplicate and the results were expressed as rutin equivalent.

2:7Total antioxidant activities

2:7:1 Free radical scavenging activity on DPPH•

The DPPH radical scavenging activity of ethanolic extracts of *G. indica*wasmeasured according to the method of Blois (1958). IC_{50} values of the extract i.e., concentration of extract necessary to decrease the initial concentration of DPPH by 50% was calculated.

2:7:2 Nitric oxide radical scavenging activity

The nitric oxide scavenging activity of ethanolic extracts along with the reference standard ascorbic acid was measured according to the method of Sreejayan and Rao,(1997). The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

2:7:3 Superoxide radical scavenging activity

Superoxide radicals were generated by a modified method of Beauchamp and Fridovich (1971).The assay was based on the capacity of the sample to inhibit farmazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system. Each 3 ml reaction mixturecontained 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, 12 mM EDTA, 0.1 mgNBT and various concentrations (100 - 500µg) of sample extracts. Reaction was started byilluminating the reaction mixture with sample extract for 90 seconds. Immediately afterillumination the absorbance was measured at 590 nm. The entire reaction assembly was enclosedin a box lined with aluminium foil. Identical tubes with reaction mixture kept in dark served asblank. The percentage inhibition of superoxide anion generation was calculated as:

% Inhibition = (control OD-sample OD / control OD) x 100.

2:7:4 Hydroxyl radical scavenging activity

The scavenging activity of ethanolic extracts on hydroxyl radical was measured according to the method of Klein *et al* (1991). Different concentrations of the extract (100 - 500µg) were addedwith 1 mL of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%), and 1 mL of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 80-90° C for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1 mL of ice-cold TCA (17.5% w/v). Three millilitres of Nash reagent (75.0g of ammonium acetate, 3.0 mL of glacial acetic acid, and 2 mL of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the color formed was measured spectroscopically at 412 nm against reagent blank. The % hydroxyl radical scavenging activity wascalculated as follows:

% HRSA=1-(difference in absorbance of sample/difference in absorbance of blank) × 100.

2:7:5 Antioxidant activity by radical cation (ABTS•+)

The total antioxidant activity of the samples was measured by ABTS radical cation decolorization assay according to the method of Re *et al.* (1999) described by Siddhuraju and Manian (2007). ABTS•+ was produced by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium per sulfate in the dark for 12-16 h at room temperature. Prior to assay, this solution was diluted in ethanol (about 1: 89 v/v) and equilibrated at 30°C to give an absorbance at 734 nm of 0.700 ± 0.02. The stock solution of the sample extracts were diluted such that after introduction of 10µL aliquots into the assay, they produced between 20-80% inhibitions of the blank absorbance. After the addition of 1 mL of diluted ABTS•+ solution to 10µL of sample or Trolox standards (final concentration 0- 15µM) in ethanol, absorbance was measured at 30°C exactly 30 min after the initial mixing. Appropriate solvent blanks were also run in each assay. Triplicate determinations were made at each dilution of the standard, and the percentage inhibition was calculated of the blank absorbance at 734 nm and then was plotted as a function of Trolox concentration. The unit of total antioxidant activity (TAA) is defined as the concentration of Trolox having equivalent antioxidant activity expressed as µg/ml sample extracts on dry matter.

2:7:6 Reducing power

The reducing power of ethanolicextracts of *G. indica*was determined by the method reported by Siddhuraju *et al* (2002). 100-500µg of extracts was taken in 1 mL of phosphate buffer and 5 mL of 0.2M phosphate buffer (pH 6.6) was added. To this, 5 mL of 1% potassium ferricyanide solution was added and the mixture was incubated at 50°C for 20min. After the incubation, 5 mL of 10% TCA was added. The content was then centrifuged at 1000 rpm for 10min. The upper layer of the supernatant (5 mL) was mixed with 5mL of distilled water and 0.5 mL of 0.1% ferric chloride. Then the absorbance of the reaction mixture was read spectroscopically at 700 nm.

2:7:7 Chelating capacity

Chelating property of ethanolic extracts was assessed by bipyridyl assay (Yamaguchi*et al.*, 2000). The reaction mixture contained 0.25 mL of extracts having the concentration of 1 mg, 0.25 mL of 1mM FeSO4 solution, 1 mL of 0.2 M Tris-HCl buffer (pH 7.4), 1 mL of 2,2' bipyridyl solution (0.1% in 0.2 M Tris-HCl), 0.4 mL of 10% hydroxylamine-HCl and 2.5 mL of ethanol. The final volume was made up to 5 mL with deionized water and the absorbance was determined at 522 nm. The chelating activity of the extracts was evaluated using EDTA as standard. The results were expressed as mg EDTA equivalent / g sample extracts.

2:7:8Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range test ($P<0.05$) using Statistica (Statsoft Inc., Tulsa, USA). Values expressed are means of three replicate determinations ± standard deviation.

Results

Preliminary phytochemical analysis

Qualitative phytochemical evaluation is one of the tools for the quality assessment, which includes preliminary phytochemical screening and marker compound analysis using modern analytical techniques. Preliminaryphytochemical analysis of *Garcina indica* fruit extract was done and results are represented in the table 1.

Table 1: Phytochemical evaluation of fruit extract of Garcinia indica.

Sl.NO	Test	Ethanolic fruit extract of Garcinia indica
1.	Alkaloids	+++

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2.	Flavanoids	+++
3.	Saponins	+++
4.	Phenols	+++
5.	Sterols	+++
6.	Glycosides	-
7.	Resins	-
8.	Thiols	-
9.	Tannins	+++

(+) presence **(-) absence**

Preliminaryphytochemical analysis of ethanolic fruit extract of Garcinia indica revealed the presence of alkaloids, flavonoids, phenols, saponins, sterols and tannins. The flavonoids and polyphenols were abundant in Garcinia indica may be responsible for its cardioprotective and antioxidant activities.

Total phenol and tannin content

Total phenolic and tannin content of the ethanolic extract of *G.indica*fruits were estimated and expressed as milligrams of Tannic acid equivalents (TAE) are shown in the Table 2, the total phenolic content ofin *G.indica*was found to be 49.1 and tannin was4.55 mg/g extract.

Table 2: Total phenol and tannin contents of ethanolic extract of *G. indica* fruits.

Phytochemicals	**(mg TAE/g extract)**
Total phenol	49.14 ± 0.34
Tannin	4.55 ± 0.13

Values are mean of three independent analysis ± SD

TAE – Tannic acid equivalent

Total flavonoid content

The total flavonoid contents in ethanolic fruit extract of *G. indica* was present in the table 3.The flavonoid distribution in the *G. indica* fruits as presented in the table 3 that the total flavonoid content is found to be 2.39 mg/g extract.

Table 3: Total Flavonoid content of ethanolic extract of *G. indica* fruits.

Phytochemicals	**(mg RE/g extract)**
Flavonoid	2.39 ± 0.03

Values are mean of three independent analysis ± SD

RE – Rutin equivalent

In vitro Free Radical Scavenging Activities

Inhibition of DPPH radical

DPPH radical scavenging activities of extract of *G. indica* exhibited a 50% inhibition (IC_{50}) value of 133.04µg/ml and standard ascorbic acid 4.42µg/ml.The data obtained in this study reveal that the tested extract was significant free radical scavenger which reacts with DPPH radical owing to its electron donating ability. The DPPH radical scavenging activity of the ethanol extract of *G.indica* and the standard ascorbic acid are debited in the fig.1. The % activity of ethanolic extract of *G.indica*and standard at the concentration range of 100 to 500µg/ml showed an inhibition of about 31.56 to 56.92% where as the standard ascorbic acid at a concentration range of 4 to 20 µg/ml registered an inhibition of 29.56 to 88.00%.

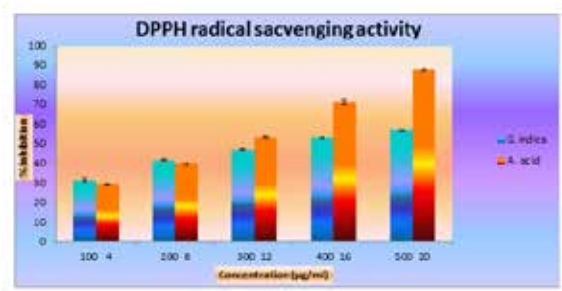

Fig. 1: DPPH radical scavenging activity of ethanolic extract of *G. indica* fruits

Values are mean of three independent analysis ± SD

Nitric oxide radical scavenging activity

Nitric oxide scavenging activity of *G.indica* were studied and compared with ascorbic acid. The percentage activity of *G.indica* shows the minimum inhibition of about 33.65% at 100µg and the maximum inhibition of 50.17% at 500µg, whereas the percentage activity of standard shows minimum inhibition at 18.95%at 10µg and the maximum inhibition of 77.75% at 50µg concentrations. IC_{50} value of*G.indica*was 145.85µg/ml and the standard ascorbic acid 11.32µg/ml.

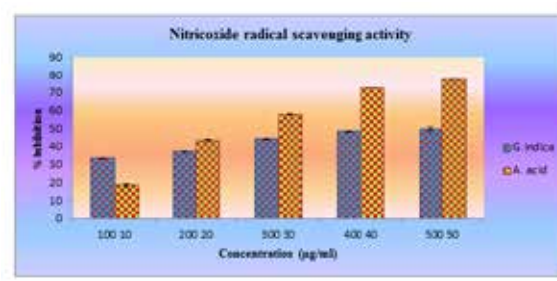

Fig. 2: Nitric oxide radical scavenging activity of ethanolic extract of *G. indica* fruits

Values are mean of three independent analysis ± SD

Superoxide radical scavenging activity

Ethanolic extract of *G.indica* fruit showed a dose dependent inhibition of superoxide radicals. The superoxide anion scavenging ability of extract has been presented in Fig.3. The minimum *in vitro* superoxide radical scavenging activity act of *G.indica*was 27.09 % at 100µg, and the maximum inhibition *in vitro* ability as 36.47% at 500µg concentration standard ascorbic acid showed minimum inhibition of 14.12% at 10µg and the maximum concentration of 58.84% at 50µg.

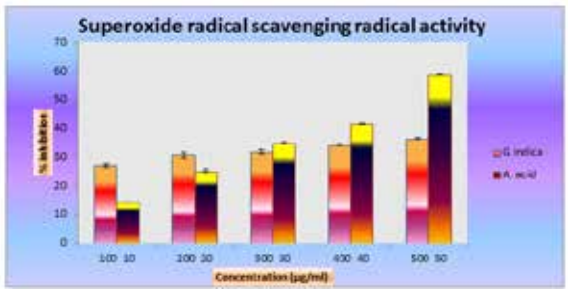

Fig. 3: Super oxide radical scavenging activity of ethanolic extract of *G. indica* fruit rind
Values are mean of three independent analysis ± SD of triplicates (n=3)

Hydroxyl radical scavenging activity
The ethanolic extract of *G.indica* was capable of protecting 2-deoxy-D-ribose from oxidative degradation by scavenging hydroxyl radicals and did so in a concentration-dependent fashion (Fig. 4). The most effective hydroxyl radical scavenger was BHA followed by fruit extract.The percentage inhibition of ethanolic extract showed that an IC_{50} values of200.26 ± 19.26µg/ml as compared to the standard BHA 6.26 ± 0.18µg/ml.

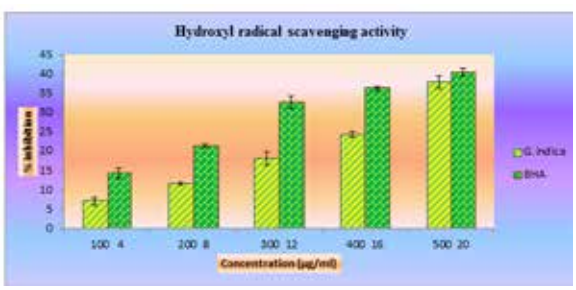

Fig. 4: Hydroxyl radical scavenging activity of ethanolic extract of *G. indica* fruit rind
Values are mean of three independent analysis ± SD of triplicates (n=3)

Antioxidant activity by radical cation (ABTS•+)

In this assay, total antioxidant activity (TAA) reflects the ability of hydrogen donating antioxidants to scavenge ABTS•+, comparable with that of Trolox.The G. indica fruit extract were subjected to theABTS•+ scavenging activity and the results were shown in figure 5. The percentage inhibition of ethanolic extract showed that IC50 values 28.56 ± 0.96µg/ml as compared to the standard values of Ascorbic acid10.28 ± 0.08µg/ml.

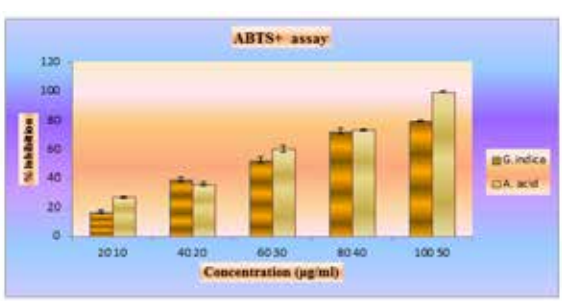

Fig. 5: ABTS+ radical scavenging activity of ethanolic extract of *G. indica* fruit rind
Values are mean of three independent analysis ± SD

Reducing power assay
The reducing power activity of ethanolic extract of *G. indica*were studied and compared with ascorbic acid is shown in the fig.6. The reducing power of extract (absorbance at 700nm) correlated well with increasing concentrations. The figure shows that the plant exhibit maximum absorbance of 0.19 at concentration 500µg and the minimum absorbance of 0.06 at 100µg, whereas standard shows the minimum absorbance of 0.08 at 100µg and maximum absorbance of 0.23 at 500µg.

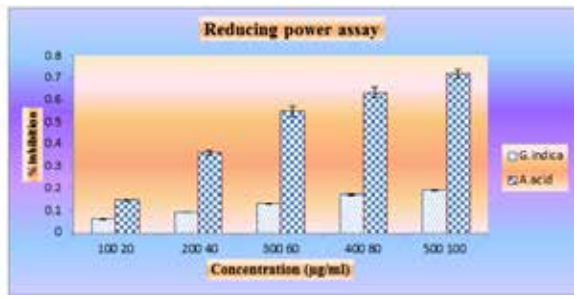

Fig. 6: Reducing power of ethanolic extract of *G. indica* fruit rind
Values are mean of three independent analysis ± SD of triplicates(n=3)

Metal Chelating capacity

The chelating effects of ethanolic extracts of *G. indica* fruit are shown in Table 4. The final reaction mixture contained 1 mg ofextract sample and the values are expressed as mg EDTA/g extract equivalent. The extract demonstrated marked ability to chelate ions.The sample tested was revealed as 54.66 mg EDTA/gas compared to the standardAscorbic acid which showed a value of11.2.

Table 4: Metal chelating activity of ethanolic extract of*G.indica* fruit extract

Sample	Metal chelating activity (mg EDTA E/g extract)
G.indica	54.66 ± 0.34
Ascorbic acid	11.20 ± 0.50

Values are mean of three independent analysis ± SD of triplicates

EDTA E –Ethylene diamine tetra acetic acid equivalent.

Discussion
Free radicals are known to play a definite role in a wide variety of pathological manifestations. Their broad range effects in biological systems have drawn the attention of many experimental works. It has been proven that these mechanisms may be important in the pathogenesis of certain diseases and aging. There are many reports that support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases. They exert their action either by scavenging the reactive oxygen speciesor protecting the antioxidant defense mechanisms (Umamaheswari*et al.*, 2008).

The ethanolic extract of *G. indica* contained appreciable amount of phenolics, tannins and flavonoids. The presence of phenolics compounds may exert antioxidant effects and free radical scavengers, as hydrogen donating sources or as singlet oxygen quenchers and metal ion chelators(Hossain *et al.*, 2012).DPPH is a commercially available nitrogen centre free radical which produced a deep purple color when dissolved in methanol. This purple color decreased with increasing concentrations of the *G.*

indica fruit extract whose absorbance was read at 517nm. The *G. indica* fruit extract scavengers the free radical by functioning as a proton radical or hydrogen donor (Mishra *et al*., 2012).

The nitric oxide scavenging assay is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interact with oxygen to produce nitrite ions that can be estimated using Griess reagent. In the present study, the ethanolic extract scavenged the nitric oxide radical and/or inhibited the nitrite formation but at much lower levels when compared with standard antioxidant Ascorbic acid. The extract inhibits nitric oxide formation by competing with oxygen to react with nitric oxidedirectly and also to inhibit its synthesis. The polyphenolic compounds presentin *G.indica* fruit mightbe responsible for the observed scavenging activity. NO• scavenging activity of flavonoids and phenolic compounds are already well known (Madson*et al*., 2000).

Superoxide radical is known to be a very harmfulspecies to cellular components as a precursor of more reactive oxygen species. Photochemical reduction of flavins generates $O2^{-}$, whichreduces NBT, resulting in the formation of formazan (Manjunath *et al*., 2011). The ethanol extract of*G.indica* were found to be efficient scavengers of superoxide radical generated in PMS–NADH–NBTsystem *in vitro* and their activities are incomparable to that of ascorbic acid. From this experiment using ethanolextract of*G.indica*; it is noted that the inhibition of the formation of formazan and also the percentage inhibition are directly proportional to the concentration of the plant extracts.

The highly reactive hydroxyl radical can cause oxidative damage to DNA, lipids and proteins. Hydroxyl radicals are highly strong reactive oxygen species and there is no specific enzyme to defence against them in human body. Therefore, it is important to discover some chemicals with good scavenging capacity on these reactive oxygen species. In this study, the hydroxyl radical scavenging ability of ethanolic extract was compared with BHA showed more pronounced hydroxyl radical scavenging activity in a dose dependent manner (Suresh and Suriyavanthana, 2012).

The ethanol extract of *G.indica*showed a moderate antioxidant activity, the trolox equivalent, which can be explained by the fact that the ABTS test shows the best results when in the presence of hydrophilic antioxidants. Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals (Mathew and Abraham, 2004).

The reducing power increases with increasing concentration. Among the extract; however, their reducing power was inferior to standard. Polyphenolic contents of the sample extract appear to function as electron and hydrogen atom donors and therefore should be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products. Similar observation in terms of dose dependant activity between the polyphenolic constituents in terms of dose dependent and reducing power has been reported for several plant extract. (Manian *et al*., 2008).

The metal ion chelating activity increased with increasing concentration of *G. indica*extract. This chelating agent may serve as secondary antioxidant because it reduces the redox potential there by stabilizing the oxidized form of the metal ions(Anusya *et al*., 2012). Though the extract of *G. indica* did not exhibit a strong antioxidant effect as that of the standard antioxidant Ascorbic acid, it did have an activity that reveals *G.indica* as a potential electron donor which can react with the free radicals and convert them to a stable product.

Conclusion:

The finding of this study supports the view, that theethanolic extract of *G. indica* fruit rind are promising sources of potential antioxidant and may be efficient as preventive agents in some diseases and can be considered as a natural herbal source in pharmaceutical industry. Further investigations on the isolation and characterization of bioactive compound (Garcinol) will be elucidating their different antioxidant mechanism.

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