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Research

In-Vitro Evaluation Of Anti-Oxidant Activity Of *Coccinia Indica* Leaves

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Abstract

Antioxidants are compounds that can inhibit or prevent the oxidation of the easily oxidized substrate. One of the plants as a potential source of bioactive compounds and antioxidant activity (*Coccinia indica*). This plant was commonly found in the India has been used by the society. This study aimed to determine the proximate compositions, bioactive compounds and antioxidant activity from large-leafed mangrove fruit which extracted by methanol. The phytochemical screening was carried on the both extracts of leaves of *Coccinia indica*, revealed the presence of some active ingredients such as Alkaloid, Flavonoids, Tannins, Saponins, Phenols. The aqueous and alcoholic leaves extract were also evaluated for their antioxidant activity using FRAP assay, Metal chelating assay, DPPH radical scavenging assay, superoxide-radical scavenging assay and Hydrogen peroxide scavenging assay. The result of the present study showed that the ethanolic leaves extract of *Coccinia indica* has shown the greatest anti-oxidant activity than aqueous extracts. The high scavenging property of may be due to hydroxyl groups existing in the phenolic compounds. Further work is needful to isolate the exact compound which is responsible for antioxidant activity and biophysical characterization can be done in the future.

Keywords: *Coccinia indica* and Antioxidant activity.

INTRODUCTION

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. They may protect cells from damage caused by unstable molecules known as free radicals. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. Free radicals are fundamentals to any biochemical process and represent an essential part of aerobic life and metabolism. Majority of the diseases are mainly linked to oxidative stress due to free radicals [1,2]. Our body is rich in endogenous antioxidants, the substances that have the ability to stop free radicals formation or to limit the damage they cause[3]. The effectiveness of current used exogenous antioxidants arises most probably from the increase of the endogenous free radical scavengers as enzymes (superoxide dismutase and selenium-dependent glutathione peroxidase), vitamins (alpha tocopherol and ascorbic acid). Many plants have been also found to posses free radical scavenging activity (Polyphenols, alkaloids and terpenoids). Low levels of one or more of the essential antioxidants have been shown to be associated with many disorders including cancer, inflammation,

atherosclerosis, coronary heart disease and diabetes. Thus, in such cases, the administration of exogenous antioxidants seems to be salutary. Nowadays, a great deal of effort being expended to find effective antioxidants for the treatment or prevention of free radical-mediated deleterious effects[4].

Oxidative stress is characterized as an imbalance between the production of reactive species and antioxidant defense activity, and its enhanced state has been associated with many of the chronic diseases such as cancer, diabetes, neurodegenerative and cardiovascular diseases[5]. Based on that, many research groups have driven efforts to assess the antioxidant properties of natural products. These properties have been investigated through either chemical (in vitro) or biological (in vivo) methods, or both [6]. The results of these researches have led some to suggest that the long-term consumption of food rich in antioxidants can retard or avoid the ocurrence of such diseases [7,8]. According to Brewer, the effectiveness of a large number of antioxidant agents is generally proportional to the number of hydroxyl (OH) groups present in their aromatic ring(s). Based on that, the natural compounds would seem to have better antioxidant activity than the currently used synthetic antioxidants, making them a particularly attractive ingredient for commercial foods. Despite the large number of natural products that are currently consumed as antioxidant agents, the search for new chemical entities with antioxidant activity still remains a burgeoning field. In this context, the lichens have played an important role as a source for new antioxidant agents. Lichens are symbiotic organisms consisting of a fungus and one or more photosynthetic partners, the latter usually being either a green alga or a cyanobacterium [11,12]. They are found in a wide variety of natural habitats or in places with low temperatures, prolonged darkness, drought and continuous light. Lichens produce characteristic and unique secondary metabolites, and most of them occur exclusively in these symbiotic organisms. The most common lichen compounds are aromatic polyketides, particularly depsides, depsidones, depsones, dibenzofurans, and chromones. Lichens have been used in the folk medicine for numerous purposes, among them as astringents, laxatives, anticonvulsives, antiemetics, antiasthmatics, anti-inflammatories, antibiotics, and also for the treatment of cardiovascular, respiratory, and gastric disorders. Furthermore, pharmacological and biotechnological studies have been carried out in order to test and to develop biomaterials containing lichen-isolated natural compounds for humans use [13,14].

Medicinal plants with antioxidant potential

1. Rhizophora mangle is a plant from Rhizophoraceae family. The bark extract of the plant showed scavenging activity of hydroxyl radicals and the extract contained polyphenols, carbohydrates and sterols[15].
2. Diospyros malabarica is a plant from Ebenaceae family. The bark is used for the treatment of fever and fruit juices for healing of wound ulcer5 . The stem extract of the plant competes with oxygen to react with nitric oxide and thus, inhibits the generation of anions. The main phytoconstituents in the extract are phenolic compounds[16].
3. Asparagus racemosus is a tree from Liliaceae family. It shows antioxidant activity through the free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, nitric oxide scavenging, metal chelation, reduction power and inhibition of lipid peroxidation in rats. The main phytoconstituents are saponins, alkaloids and flavonoids[17].
4. Auricularia auricular is a tree and known as _tree ear or wood ear‘ from Auriculaceae family: It has shown a potent hydroxyl radical scavenging and lipid per-oxidation inhibitory activities. The main phytoconstituents are flavonoids [18].
5. Eucalyptus globules is a tree and known as ―Karpura maram‖ from Myrtaceae family. The antioxidant activity of Eucalyptus oil was estimated by two in vitro assays namely diphenyl picryl hydrazyl radical scavenging activity and inhibition of ascorbate induced lipid peroxidation method[19].
6. Acacia arabica is a plant from Mimosae family. The antioxidant assays were carried out in vivo and in vitro experimental models. In vitro, lipid peroxidation was carried out by tertiary butyl hydroperoxide (TBH) induced lipid peroxidation. In vivo, experiments were carried out in CCl4-induced hepatotoxicity in rats. The bark of the plant contained quercetin, (+) catechin, (-) epicatechin and gallic acid. The polyphenol rich active fraction of Acacia arabica is a potent free radical scavenger and protects TBH induced lipid peroxidation and CCl4-induced hepatic damage. The bark is used in the treatment of asthma, bronchitis, diabetes, dysentery and skin diseases[20].
7. Ligustrum vulgare is a plant from Oleaceae family. The leaves antioxidant activity was evaluated using DPPH test. The main phytoconstituents are flavonoids, iridoids, coumarins and essential oil, where flavonoid aglycones are responsible for the antioxidant activity and it shows a potent free radical scavenging activity[21].
8. Terminalia chebula is a tree and known as Myrobalanus chebula. Combretaceae family. The main phytoconstituents are tannins, chebulinic and gallic acids. The extract was tested by studying the inhibition of radiation induced lipid peroxidation in rat liver microsomes. It shows free radical scavenging activity due to presence of tannins and also It inhibits the development of duodenal ulcer and so the extract has appeared to show a cytoprotective effect on the gastric mucosa [22].
9. Lobelia nicotianaefolia is a plant from Campanulaceae family. The chemical constituents are alkaloids as lobeline and also it contains volatile oil, resin, gum and fixed oil. It is mainly used in the treatment of asthma and as respiratory stimulant[19].

10. Citrus lemon is a tree from Rutaceae family. The antioxidant activity was estimated by two in vitro assays, DPPH radical scavenging activity and inhibition of ascorbate induced lipid peroxidation (LPO) method. The main phytoconstituents are citral and limonene. The antioxidant property is shown due to the presence of citral.

Ageratum conyzoides

Ageratum conyzoides (billygoat-weed, chick weed, goatweed, whiteweed) is native to Tropical America, especially Brazil, and considered an invasive weed in many other regions. It is an herb that is 0.5–1 m. high, with ovate leaves 2–6 cm long, and flowers are white to mauve. In Vietnamese, the plant is called cứt lợn (meaning "pig feces") due to its growth in dirty areas. As a medicinal plant, Ageratum conyzoides is widely used by many traditional cultures, against dysentery and diarrhea.[4] It is also an insecticide and nematicide.

Fig 1: Plant of Ageratum conyzoides

Now a days many antimicrobial drugs cannot destroy their pathogenic microorganisms as they are becoming resistant. Therefore researchers are finding some new drugs from mangrove species to control the pathogens due to presence of antimicrobial compounds.

Natural Antioxidants in Plant Sources

Natural and synthetic antioxidants can inhibit or delay the process of lipid oxidation. Antioxidants refer to any substances present at low concentration in foodstuffs and able to significantly prevent oxidation mediated by prooxidants. Prooxidants, considered as synonymous with reactive oxygen species, refer to any substances that when being with low concentration in foods can cause or promote an oxidative reaction. An antioxidant may play a role in antioxidation as a free radical scavenger, reducing agent, chelator, and/or singlet oxygen scavenger. Numerous synthetic antioxidants have been registered, but only a few species are permitted as food additives by the law because of toxicity effects and other side-effects. Typical antioxidants permitted as the food additives are butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), pueraria glycoside (PG), and tertiary-butylatedhydroquinone (TBHQ).

Recently, public's concern about the problems of human health caused by food additives has once more evoked food scientists to the enthusiasm of seeking natural antioxidants from the various sources. So far one of the understandings on this issue is that natural antioxidants are primarily plant phenolic compounds occurring in all parts of plant bodies. Common phenolic antioxidants from plant sources include flavonoid compounds, cinnamic acid derivatives, coumarins, tocopherols, carotenoids, and polyfunctional organic acids[5].

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Tocopherols

Tocopherols as mono-phenolic compounds have been used in the food industry for several decades due to the functionality of preventing oxidation of polyunsaturated fatty acids of foods containing fats or oils (Khan and Shahidi, 2001; Boskou, 1998). They are thus added to edible oils in storage, processing, and shipment. Tocopherols are found with high quantity in many plant sources typically including vegetable oils, the fresh leaves of vegetables, cereals, nuts, and oilseeds such as sesame, rice, corn, and soybean. Tocopherols consist of eight different compounds grouped into two families: tocols and tocotrienols.

Each family has four members: α-, β-, γ-, δ-tocols or tocotrienols, classified by the number and position of methyl groups attached to the chromane rings. The side-chains of tocols are saturated, whereas the side-chains of tocotrienols are unsaturated. There is an opinion believing that the antioxidant activity gradually decreases from δ to α [28,29]

The mechanism of antioxidation by tocopherols has been illustrated in detail, tocopherols acting to transfer phenolic hydrogen to the radical molecules and to scavenge singlet oxygen. The mechanism is elutriated on the basis of the tocopherol-tocopheryl quinine redox system. Tocopheols (AH2) as radical scavengers quench lipid radicals (R•) produced in free radical reactions by donating a hydrogen atom to the radical R•, regenerating RH molecules, and tocopheols themselves convert into a tocopheryl semiquinone radical (AH·). Two tocopheryl semiquinone radicals (AH•) may combine with one another to form a tocopheryl quinine (A) and a tocopherol molecule. Or it may be reduced by hydrogen atoms donated by ascorbic acids. Oxidized ascorbic acids are further reduced by other reducing agents, i.e., NADH. Such mechanism may be represented in figure 1. It is worthy to point out that tocopherols retard or delay a free radical reaction but do not fully stop the reaction. It has been known that soybean oil contains luxury tocopherols and tocotrienols as compared to other plant materials. During the process of manufacturing oils, however, roughly 30 to 40% of tocols and tocotrienols are lost, entering into sludge or gums in the stages of bleaching and deodorization of oils[9]. Tocopherols are lost in deep-fat frying, and particularly α-tocopherol is lost faster than β-, γ-, and δ-tocopherol. Additionally, the variously bioactive substances such as phytosterols, esters of phytosterols, phospholipids are lost in the defining of oil and deep-fat frying [29].

Flavonoids

Flavonoids are a collective term of polyphenolic compounds and ubiquitously exist in all parts of plants. They are categorized into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins, and chalcones in light of chemical structures. Over 4,000 flavonoids have been reported, many of which are believed to have antioxidant activities acting as scavengers of free radicals and active oxygen, chelators of iron ions, and/or an inactivation agent of lipoxygenase. Catechin and quercetin are believed to have high antioxidant activity and are thus used to stabilize lard in the food industry [30,31].

The antioxidant abilities of flavonoids are based upon the characteristics of their molecular structure. Hydroxylation (related to the position and numbers of hydroxyl groups) of the B ring is particularly important for such activity. Why quercetin plays a potent role in antioxidation is because it has all the right structural features for scavenging of free radicals. Because robinetin and myricetin have an additional hydroxyl group at their 5' position of the B ring, their antioxidant activities are thus enforced more than those of their corresponding flavones with no such 5' hydroxyl group. It has been elucidated that both hydroxyl groups in the 4' and 5' positions are necessary for significant antioxidant activity of an isofavone, i.e., genistein. Steric hindrance as a result of methyl groups attached the positions also benefits such properties of hydroxyl groups in the molecules [31].

Ascorbic Acids

An important antioxidant is ascorbic acid acting as a reducing agent and free radical scavenger. Based on its redox properties, ascorbic acid and its etherified derivatives act as antioxidants by donating a hydrogen atom to free-radical chain reactions and thus protect double bonds of substrate molecules. Ascorbic acid can also perform as a synergist for tocopherol by converting the oxidized tocopherols back to its reduced status. Furthermore, the dehydroascorbic acid is regenerated with a hydrogen atom stemming from reduced glutathione or NADH, where oxygen is reduced into water[31].

Carotenoids

Carotenoids are a category of natural, fat-soluble compounds ubiquitously existing in all parts of plants. They are mainly located in membrane systems of cells where one of the major functions of the compounds is to involve in photosynthesis, and are responsible for the red, orange, and yellow colors of plant leaves, fruits, and flowers. They are also found in algae, photosynthetic bacteria, non-photosynthetic bacteria, yeasts, and molds[14], carrying out a protective function against membrane damage induced by light and oxygen. The most common groups that is concerned by food scientists due to their antioxidant properties are carotenes (i.e., β-carotene) and xanthophylls (lutein). The later ones are the oxygenated derivatives of carotenes[32].

Carotenoids are isoprenoid compounds. The majority of molecules of carotenoids have a 40-carbon polyene chain regarded as the backbone of the molecule[14]. Like other compounds, the structural properties of carotenoid molecules ultimately determine their chemical characteristics and biological functions. The distinctive characteristics of carotenoids are the long system of conjugated polyene backbone rich in electrons, in which the π-electrons are effectively delocalized over the entire length of the polyene chain therefore allowing them to absorb excess energy from other molecules to quench singlet oxygen. The nature of the specific end groups account for the polarity of the molecules. Each double bond in the polyene chain of a carotenoid exists in either of two configurations: *trans* or *cis* geometrical isomers. Because *cis* isomers create greater steric hindrance between nearby hydrogen atoms and/or methyl groups, they are particularly thermodynamically unstable than are their *trans* counterparts[33]. Therefore, most carotenoids predominantly or entirely occur as the all-trans form in nature. Pure carotenoids in an organic solution are particularly unstable in the presence of oxygen, thus easily bleached and degraded; but carotenoids in vivo are much more stable[34].

MATERIALS AND METHODS

Reagents

Sodium hydroxide (Analytical grade, Fisher Chemicals Inc., Fair Lawn, NJ), citric acid (analytical grade), hexanes (HPLC grade, EMD Chemicals Inc., Gibbstown, NJ), methanol (HPLC grade, EMD Chemicals Inc., Gibbstown, NJ), ethyl acetate (HPLC grade, EMD Chemicals Inc., Gibbstown, NJ), BCL3-methanol (Supelco Inc., Belletonte, PA), 98% 2, 2- Dimethoxypropane (Sigma-Aldrich Inc., St. Louis, MO), Anhydrous sodium sulfate (10-60 mesh, Fisher Chemicals Inc., Fair Lawn, NJ), cholesterol (Aldrich Chem. Co., Milw., WI), 5α- cholestane (Sigma-Aldrich Co., St. Louis, MO), heptadecanoic acid (Sigma chemical Co., St.Louis, MO), DHA (cis-4, 7, 10, 13, 16, 19-Docosahexaenoic acid, Sigma-Aldrich Inc., St. Louis, MO). The solvents were stored at room temperature (20-25ºC) and other reagents were storedat -20ºC freezer. Sodium Hydroxide and citric acid were dissolved in distilled water. All of organic reagents were dissolved in hexanes, except for being particularly noted. Whatman filter papers (Whatman®, 150mm Dia × 100Circles, Cat No 1001 150, Whatman International Ltd, Maidstone, England).

Plant Material Collection

The leaves of *Coccinia indica* were collected and was identified and authenticated from Department. The plant material was cleaned, reduced to small fragments, air dried under shade at room temperature and coarsely powdered in a mixer. The powdered material was stored or taken up for extraction process.

Preparation of plant extracts

Preparation of Aqueous Extract

Fresh leaves of *Coccinia indica* were collected and washed under tap water. The leaves extract used was prepared by taking 20gms of finely cut leaves into 250ml beaker containing 200ml of water. The contents were mixed well and then the mixture was boiled upto 80-100^{0}C for 4-5hrs. Further the extract was filtered with whatmann filter paper. The filtrate was boiled until the concentrated residue is formed. The concentrated product was sealed in sample covers and stored under room temperature and used for further experiment to check the activities.

Preparation of Alcoholic Extract

Fresh leaves of *Coccinia indica* were collected and washed under tap water. The leaves extract used was prepared by taking 20gms of finely cut leaves into 250ml beaker containing 200ml of alcohol. The contents were mixed well and then the mixture was boiled up to 50-60^{0}C for 4-5hrs. Further the extract was filtered with whatmann filter paper. The filtrate was boiled until the concentrated residue is formed. The concentrated product was sealed in sample covers and stored under room temperature and used for further experiment to check the activities.

Phytochemical evalution

The powdered drug was extracted and subjected to qualitative chemical tests.

Detection of Carbohydrates

Small quantities of powdered drug and different extracts were dissolved in distilled water separately and filtered. The filtrates were taken for Molisch's Test, Fehling's Test, Benedict's Test, Barfoed's Test, Test for starch tests to detect the presence of carbohydrates.

Test for Gums and Mucilages
The powdered drug and extracts were treated with absolute alcohol stirred and filtered. The filtrate was dried and examined for its swelling properties.

Test for Proteins and Amino Acids: Small quantities of powdered drug and different extracts were dissolved in few ml of distilled water and subjected to Ninhydrin, Biuret, Million, Xanthoproteic test, test with tannic acid and heavy metals.

Test for Fixed Oils and Fats
The powdered drug and extracts were subjected for Spot Test, Saponification Test.

Test for Alkaloids
Small amount of powdered drug and solvent free various extracts were separately stirred with a few ml of dilute hydrochloric acid and filtered. The filtrates were tested with various alkaloidal reagents such as Mayer's, Dragendroff's, Wagner's and Hager's reagent and Tannic acid.

Test for Glycosides
A small amount of powdered drug and different extracts were dissolved separately in 5ml of distilled water and filtered. Another portion of the extracts were hydrolyzed with hydrochloric acid for one hour on a water bath and hydrolysate was subjected to Legal's, Baljet's, Borntrager's, KellerKilliani'stests and for the presence of Cyanogenetic glycosides.

Test for Phytosterols
The powdered drug and extracts were refluxed with 0.5N alcoholic potassium hydroxide until the saponification was complete. The saponification mixture was diluted with distilled water and extracted with petroleum ether. The ethereal extract was evaporated and unsaponification matter.

***In vitro* methods of anti-oxidant activity**

Antioxidant activity should not be concluded based on a single antioxidant test model. And in practice several *in vitro* test procedures are carried out for evaluating antioxidant activities with the samples of interest. Another aspect is that antioxidant test models vary in different respects. Therefore, it is difficult to compare fully one method to other one. Researcher has to critically verify methods of analysis before adopting that one for his/her research purpose. Generally *in vitro* antioxidant tests using free radical traps are relatively straightforward to perform. Among free radical scavenging methods, DPPH method is furthermore rapid, simple (i.e. not involved with many steps and reagents) and inexpensive in comparison to other test models. On the other hand ABTS decolonization assay is applicable for both hydrophilic and lipophilic antioxidants. In this work five *in vitro* methods are described and it is important to note that one may optimize logically the respective method to serve his/her experimental objective as no one method is absolute in nature rather than an example.

Ferric reducing-antioxidant power (FRAP) assay

The FRAP assay was done according to the method described by Benzie and Strain (1999) with some modification. This method is based on reduction of TPTZ-Fe3+ complex to TPTZ-Fe2+ form in the presence of antioxidants. The stock solutions included acetate buffer (300 mM, pH 3.6), 2, 4, 6-tripyridyl s-triazine (TPTZ) solution (10 mM in 40 mM HCl) and ferric chloride ($FeCl_3.6H_2O$) solution (20 mM). The fresh working FRAP solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution and 2.5 ml $FeCl_3$ solution. Extracts were made up to 2.0 ml with distilled water and 1.0 ml of FRAP solution was added. An intense blue color developed was measured at 593 nm, after an incubation period of 20 min. The absorbance was related to absorbance changes of a ferrous sulphate solution (0 – 100 NM) tested in parallel. All results were based on three separate experiments and antioxidant capacity was expressed as NM FeSO4/ g of dry extract. Quercetin and Butylated Hydroxy Toluene (BHT) were used as positive control.

Metal chelating activity

The chelating capacity of *Coccinia indica* extracts on Fe2+ ions was determined according to the method of Dinis et al (1994), wherein Fe2+ chelating potential of extracts was monitored by measuring ferrous iron – ferrozine complex at 562 nm. Briefly, extracts (0.05 – 1.0 mg/ml), quercetin, BHT and EDTA (10 – 250 Ng/ml) were made up to 4.7 ml with distilled water and then mixture was allowed to react with 0.1 ml of Ferrous chloride (2.0 mM) and 0.2 ml of ferrozine (5.0 mM) for 20 min. Absorbance of mixture was measured at 562 nm against a blank, which contained distilled water, instead of extracts/EDTA/standard antioxidants. The ability of extracts to chelate ferrous ion was calculated using the following equation:

Chelating effect (%) = [Ab control 562 – Ab sample 562/ Ab control 562] x 100.

Experiments were done in triplicate.

DPPH radical-scavenging activity

DPPH radical-scavenging activity of *Coccinia indica* extracts was determined as previously described (Burits and Bucar, 2000). The capacity of extracts to scavenge lipidsoluble 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, which results in bleaching of purple color exhibited by stable DPPH radical, is monitored at an absorbance of 517 nm. Briefly, 1.0 ml of extracts (0.05 – 1.0 mg/ml) and quercetin/BHT (10 – 250 Ng/ml) in ethanol were added to 4 ml of 0.004% methanolic solution of DPPH. After incubation for 30 min at room temperature in the dark, absorbance was read against a blank at 517 nm. Tests were carried out in triplicate. The ability of extracts and quercetin/BHT to scavenge DPPH radical was
calculated using the following equation:

Radical scavenging activity (%) = [A0 – A1/ A0] x 100.

Where A0 was absorbance of negative control (containing all reagents except test compounds) at 517 nm and A1 was absorbance of the extracts or quercetin/BHT at 517 nm. DPPH scavenging activity of extracts and standard was expressed as IC50, which was interpolated from a graph constructed, using percent inhibition (Y-axis) against concentration (X-axis) of extracts and standards.

Superoxide radical-scavenging activity

The ability of *Coccinia indica* extracts, Quercetin and BHT to quench generation of superoxide radicals was determined according to the method of Nishikimi et al (1972) with a slight change. Superoxide radicals were generated in PMS-NADH system by oxidation of NADH and analyzed by NBT reduction. 0.1 ml of extracts (0.05 – 1.0 mg/ml) and Quercetin/BHT (10 – 250 Ng/ml) were mixed with 2.9 ml of phosphate buffer (40 mM, pH 7.4), 1.0 ml of nitroblue tetrazolium (NBT) solution (150 NM in 40 mM phosphate buffer, pH 7.4) and 1.0 ml of NADH (468 NM in 40 mM phosphate buffer, pH 7.4). The reaction was initiated by addition of 1.0 ml of phenazine methosulphate (60 NM in 40 mM phosphate buffer, pH 7.4) to reaction mixture. After incubation at 25°C for 5 min, absorbance was measured at 560 nm. Negative control was subjected to the same procedures as extracts, except that only solvent was added for negative control. All measurements were made in triplicate. The ability of extracts and quercetin/BHT to scavenge superoxide radical was calculated using the following equation:

Superoxide radical scavenging activity (%) = [A0 – A1/ A0] x 100.

Where A0 was absorbance of negative control at 560 nm and A1 was absorbance of the extracts or quercetin/BHT at 560 nm. IC50 value, which represents concentration of extracts and standards that caused 50% inhibition, was determined by a linear regression analysis.

Hydrogen peroxide scavenging activity

The method of Sinha (1972) originally designed for the estimation of antioxidant enzyme, catalase, was adopted to evaluate hydrogen peroxide scavenging effect of *Coccinia indica* extracts. Extracts (0.05 – 1.0 mg/ml) and quercetin/BHT (10 – 250 Ng/ml) were incubated with 0.6 ml of H2O2 (40 mM in a phosphate buffer, 0.1 M pH 7.4) in the dark for 10 min. A negative control was set up in parallel with entire reagent except extract or standard. After incubation, remaining H2O2 was allowed to react with 1.0 ml of dichromate in acetic acid (5% potassium dichromate and glacial acetic acid in the ratio of 1:3) in a boiling water bath for 10 min. Absorbance of green color developed was determined at 620 nm. All experiments were performed in triplicate. The percentage scavenging of H2O2 by *Coccinia indica* extracts and standards were calculated using the following equation:

H_2O_2 scavenging activity (%) = [A0 – A1/ A0] x 100.

Where A0 was absorbance of negative control and A1 was absorbance of the extracts or standards. H2O2 scavenging activity of extracts and standards was expressed as IC50, which was interpolated from a graph constructed using percent inhibition (Y-axis) against concentration (X-axis) of extracts and standards.

RESULTS

Phytochemical screening of *Coccinia indica.*

The present investigation concluded that the isolated compounds from the plant *Coccinia indica* are pure and the plant *Coccinia indica* shows the various antibacterial effects against different bacteria and found that different phytochemical compounds. Further study is needed for the isolation of the constituents present in the plant and its individual pharmacological activity should need to consider and ultimately it should be implemented for the benefit to human beings.

Table 1: Phytochemical screening of *Helicteres Isora*

S.No.	Phytoconstituents	Aqueous	Alcoholic
1.	Alkaloid	-	+
2.	Flavonoids	+	-
3.	Tannins	+	+
4.	Saponins	-	+
5.	Phenols	+	+

Antioxidant properties of *coccinia indica*

Several mechanisms have been proposed to be involved in antioxidant activity such as hydrogen donation, termination of free radical mediated chain reaction, prevention of hydrogen abstraction, chelation of catalytic ions and elimination of peroxides (Gordon, 1990). Antioxidant activity is system- dependent and characteristic of a particular system can influence outcome of analysis. Hence, a single assay would not be representative of antioxidant potential of plant extracts. In this present study, different models of antioxidant assays were employed, which could provide a more consistent approach to assess antioxidant activity of leaves of *Coccinia indica.*

Ferric reducing ability of *Coccinia indica*

FRAP assay is based on a redox-linked reaction, whereby antioxidants present in plant extracts act as reductants while ferric ions in reagents serve as oxidants. Reduction of ferric-tripyridyltriazine to ferrous complex forms an intense blue color with maximum absorption at 593 nm, which is related to amount of antioxidants in the sample. The ferric reducing ability of leaves of *Coccinia indica* is shown in Table 4.6. Water and alcohol extract reduced ferric ions efficiently and had reducing activity in the range of 0.82 – 2.83 mM/g, which was greater than or comparable to synthetic antioxidant BHT (1.28 mM/g). Both extracts were less effective, when compared with reducing activity of Quercetin (15.61 mM/g).

Reduction of ferric to ferrous ion is frequently used as an indicator of electron donating activity, which is considered to be an important factor dictating antioxidant activity of plant. Figure 4.5 shows dose-response curves for reducing power of different extracts from *Coccinia indica* leaves. Leaves extracts showed significant ability to reduce ferric ions in a dose-dependent manner. Water and alcohol extract showed highest reducing power. Quercetin and BHT revealed potent reducing power, which were distinctly higher than that of any of *Coccinia indica* extracts.

Antioxidant activity has been reported to be concomitant with reducing power of plant extract (Gordon, 1990). Significant ferric reducing ability of *Coccinia indica* extracts observed in this study suggest that polyphenolics present in the extracts have the ability to donate electrons to free radicals by acting as reductones and thus could terminate free radical-mediated oxidative reactions. Catechin, sinapic acid, ferulic acid, quercetin and myricetin, which were identified in Coccinia indica have been shown to possess significant ferric reducing ability in their pure form, suggesting that ferric reducing ability of *Coccinia indica* could have been partly contributed by these phenolics (Pulido et al, 2000). Present findings are in line with those of other investigators, who have also reported that antioxidant properties are concomitant with development of reducing power (Chung et al, 2005).

Table 2: Ferric Reducing Ability - FRAP (expressed as mM FeSO4/g dry weight) of leaves of *Coccinia indica*

Group	Drugs	IC_{50} value µg/ml
I	Quercetin	15.17±0.075
II	Butylated Hydroxy Toluene(BHT)	4.63 ±1.115
III	AQECI	2.36±0.051
IV	ALECI	1.43±0.090

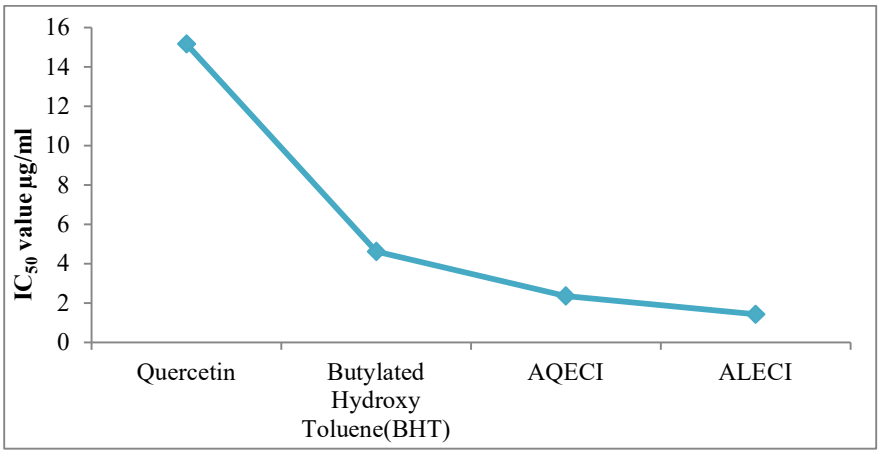

Fig 1: Reducing power of *Coccinia indica* Quercetin and BHT were used as reference antioxidant Values are means ± SD (n = 3).

Metal chelating activity of *Coccinia indica*

Coccinia indica extracts were evaluated for their ability to chelate ferrous ion by competing with ferrozine in free solution. All extracts displayed an ability to chelate ferrous ion in a dose-dependent manner (Figure 4.6). However, estimated IC50 was very high (more than 2.0 mg/ml); particularly, in comparison with positive control EDTA (7.75 Jg/ml). Quercetin and BHT showed moderate metal chelating activity when compared with EDTA with an IC50 of 134Jg/ml and 86Jg/ml respectively. Water and alcohol extract showed a chelating ability of 28.54 and 20.86% respectively at 1.0 mg/ml. In case of leaves extract, metal chelating activity varied from 2.15% to 30.83%. Alcoholic extracts were the highest, followed by water extract. EDTA, Quercetin and BHT exhibited 99.23%, 60.54% and 71.36% of chelating activity respectively, which were significantly higher than that of *Coccinia indica* extracts.

Transition metal ions gain utmost significance in biological system due to their ability to generate reactive free radicals. They can initiate Fenton type reaction with production of hydroxyl radicals or Haber-Weiss reactions with superoxide radicals (Kehrer, 2000; Wong and Kitts, 2001). They hasten peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate chain reaction of lipid peroxidation (Halliwell and Gutteridge, 1984; Halliwell, 1991). Metal chelating capacity is imperative as it decreases concentration of catalyzing transition metal ions in Fenton type reaction and protects system from oxidative damage through inhibition of metal-dependent processes. Chelating agents that form bonds with metals are effective as secondary antioxidants because they can reduce redox potential by stabilizing oxidized form of metal ion (Gordon, 1990). Regardless of reduced activity, *Coccinia indica* extracts did possess moderate iron binding capacity, suggesting their protective action against lipid peroxidation-mediated oxidative damage. This result is not surprising, as non-phenolic compounds are supposed to be better chelators of metal ions than polyphenols (Chan et al, 2007).

Table 3: Metal chelating activity of leaves of *Coccinia indica*

Group	Drugs	IC_{50} value µg/ml
I	EDTA	7.53
II	Quercetin	132
III	Butylated Hydroxy Toluene (BHT)	85
IV	AQECI	21.69
V	ALECI	33.41

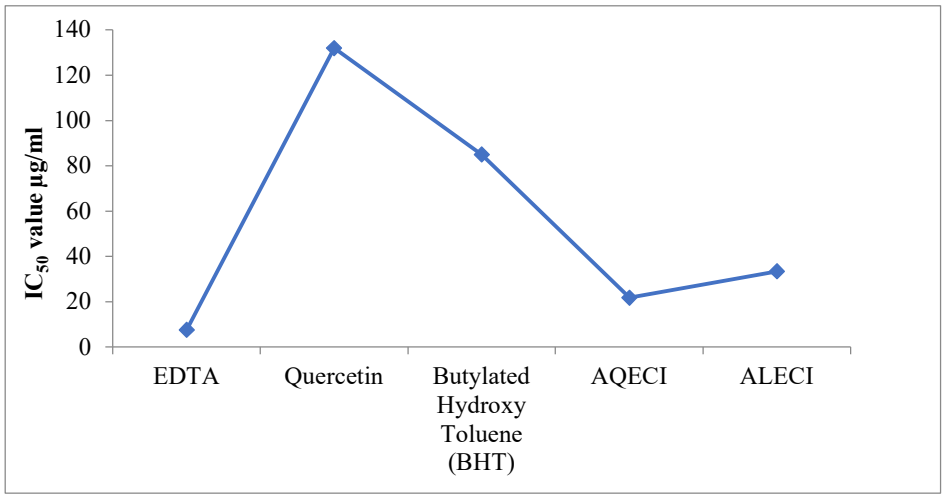

Fig 2: Metal chelating activity of *Coccinia indica* EDTA was used as positive control. Quercetin and BHT were used as reference antioxidants. Values are means ± SD (n = 3).

DPPH radical scavenging activity of *Coccinia indica*

Basic information on efficacy of compounds in *Coccinia indica* extracts to quench free radicals can be deduced from DPPH assay. The DPPH is a stable free radical, which is recognized as a tool for evaluating radical scavenging ability of compounds and antioxidant activity of foods (Sanchez-Moreno, 2002). It accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capacity of DPPH is determined by decrease in its absorbance at 517 nm, induced by antioxidants. It has also been used to quantify antioxidants in complex biological systems, because of its ease and convenience. Even though, DPPH radicals may not be biologically pertinent, it presents an indication of hydrogen/ electron-donating capacity of plants and provides a useful means to measure in vitro antioxidant activity. *Coccinia indica* extracts revealed a concentration-dependent scavenging of DPPH radicals, with leaves presenting strongest effect followed by leaves (Figure 4.8). of aqueous and alcoholic extracts showed strongest effect (IC50 at 31 Jg/ml for leaves), followed by water and alcohol extracts (Table 4.8.1). Comparison of DPPH radical scavenging activity with standard antioxidants showed that the most potent *Coccinia indica* extracts had scavenging ability higher than BHT (IC50 at 493 Jg/ml), but lower than quercetin (IC50 at 11 Jg/ml).

Effective DPPH radical scavenging activity exhibited by *Coccinia indica* extracts could be explained by the presence of polyphenolics in them, whose radical scavenging properties were reported previously in various model systems (Fukumoto and Mazza, 2000). Radical scavenging ability of polyphenolics is attributed to their ability to donate a hydrogen atom from a phenol to give DPPH-H and a phenoxyl radical. Alcoholic extracts contained more amounts of ferulic acid and sinapic acid, which could partially explain higher ability to scavenge DPPH (Kim et al, 2008), in comparison with water and alcohol extracts. Catechin, the major component of water extracts was found to be moderately active as an antioxidant in DPPH assay (Hwang et al, 2001). A comparison between DPPH radical scavenging activities of *Helicteres Isora*. *Coccinia indica* extracts were more potent in terms of radical scavenging activity whereby their IC50 values were comparatively much lower than these BHT (Lee et al, 2008; Koksal and Gulcin, 2008; Borowski et al, 2007), thus further demonstrating effectiveness of *Coccinia indica* leaves as natural antioxidants.

Table 4: Scavenging ability of root, stem and leaves of *Coccinia indica* and standard antioxidants on DPPH• as determined by their IC50, expressed as mg/ml.

Group	Drugs	IC_{50} value µg/ml
I	Quercetin	5.1 ±0.097
II	Butylated Hydroxy Toluene(BHT)	3.63 ±0.081
III	AQECI	1.82 ±0.057
IV	ALECI	1.8 ±0.031

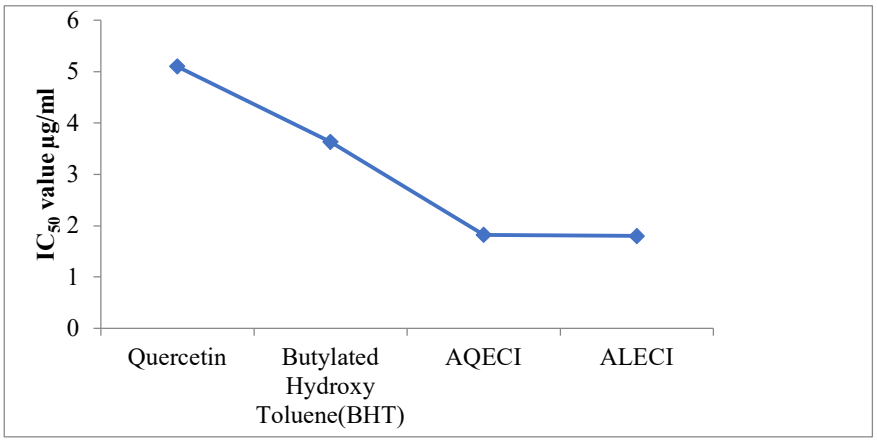

Fig 4: DPPH radical scavenging activity of *Coccinia indica* Quercetin and BHT were used as reference antioxidant. Values are means ± SD (n = 3).

Superoxide radical scavenging activity of *Coccinia indica*

Superoxide anion is a reduced form of molecular oxygen that is generated during normal metabolic processes. It is known to be destructive to cellular components as a precursor of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical or singlet oxygen (Stief, 2003), contributing to tissue damages and various chronic diseases (Halliwall, 1991). The scavenging activity of *Coccinia indica* extracts on superoxide radicals is shown in Figure 4.9. Extracts from different parts of *Coccinia indica* displayed concentration dependent protective activity against superoxide radicals of which leaves were the most effective. Alcoholic extracts of leaves (IC50 at 23 Jg/ml) showed potent scavenging activity. Aqueous extracts exhibited moderate activity with IC50 in the range of 131 – 841 Jg/ml. When radical scavenging activity of *Coccinia indica* extracts compared to IC50 values calculated for reference antioxidants BHT (IC50 at 19 Jg/ml), but less effective than Quercetin (IC50 at 10 Jg/ml).

Table 5: Scavenging ability of root, stem and leaves of *Coccinia indica* and standard antioxidants on superoxide radical (O2•) as determined by their IC50, expressed as mg/ml.

Group	Drugs	IC_{50} value µg/ml
I	Quercetin	0.018 ±0.005
II	Butylated Hydroxy Toluene(BHT)	0.012 ±0.001
III	AQECI	0.28 ±0.006
IV	ALECI	0.305 ±0.003

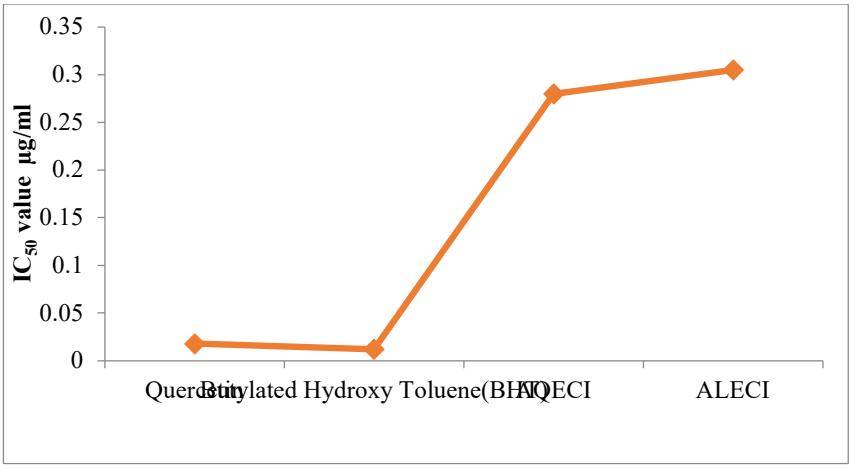

Fig 5: Superoxide radical scavenging activity of *Coccinia indica* Quercetin and BHT were used as reference antioxidant. Values are means ± SD (n = 3).

Hydrogen peroxide scavenging activity of *Coccinia indica*

Though hydrogen peroxide (H2O2) itself is not very reactive, it can occasionally be toxic to cells, since it may give rise to potentially reactive hydroxyl radicals (Halliwell, 1991). The scavenging activity of *Coccinia indica* extracts on H_2O_2 is shown in Figure and compared with Quercetin and BHT as standard antioxidants. *Coccinia indica* extracts were capable of scavenging H_2O_2 in a concentration-dependent manner. Of different extracts, alcoholic group showed strongest H_2O_2 scavenging activity. The aqueous extract of leaves displayed the most potent activity with IC50 at 67 Jg/ml, which was comparable to Quercetin (IC50 at 34 Jg/ml) and more effective than BHT (IC50 at 89 Jg/ml).

Table 6: Scavenging ability of leaves of *Coccinia indica* and standard antioxidants on hydrogen peroxide (H2O2) as determined by their IC50, expressed as mg/ml

Group	Drugs	IC_{50} value µg/ml
I	Quercetin	0.038±0.001
II	Butylated Hydroxy Toluene(BHT)	0.058±0.006
III	AQECI	0.070±0.017
IV	ALECI	0.486±0.031

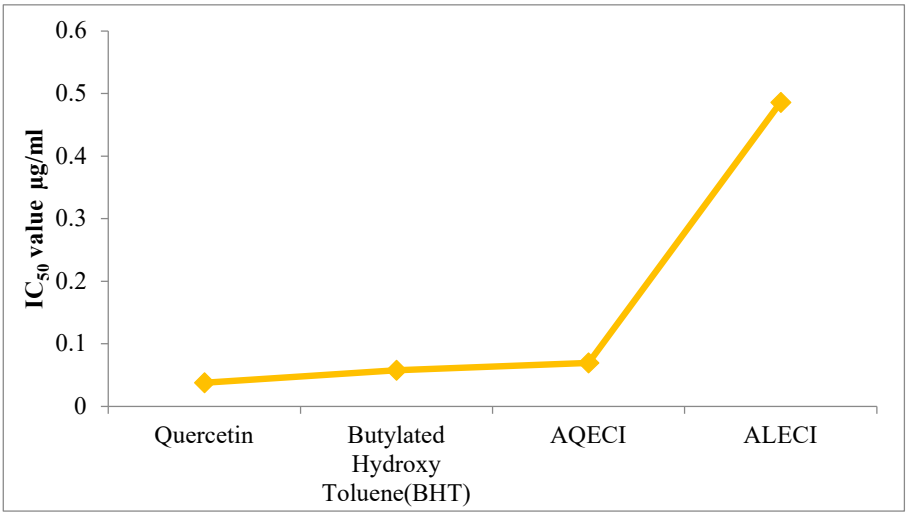

Fig 6: Hydrogen peroxide scavenging activity of *Coccinia indica* Quercetin and BHT were used as reference antioxidant. Values are means ± SD (n = 3)

CONCLUSION

The result of the present study showed that the aqueous and alcoholic extract of *Coccinia indica* plant, which contains phenolic and flavonoidal compounds, exhibited the great antioxidant activity. The high scavenging property of methanolic extract of *Coccinia indica* plant may be due to hydroxyl groups existing in the phenolic compounds' chemical structure that can provide the necessary component as a radical scavenger. Free radicals are often generated as byproducts of biological reactions or from exogenous factors. The involvements of free radicals in the pathogenesis of a large number of diseases are well documented. A potent scavenger of free radicals may serve as a possible preventative intervention for the diseases. Aqueous and alcoholic extracts of *Coccinia indica* plant in this research exhibited antioxidant. The antioxidant potential may be attributed to the presence of polyphenolic compounds.

In this study, all antioxidant methods (FRAP assay, Metal Chelating assay, DPPH radical-scavenging assay, Superoxide radical scavenging assay and Hydrogen peroxide scavenging assay) showed that the both aqueous and alcoholic extracts of *Coccinia indica* contain more antioxidant activities. More- over, this study demonstrated the important source of phenol compounds, which are a good source of antioxidant activity. The phenol component has a high inhibitory effect that prevents lipid peroxidation. However, the solvent type has an important role in detecting phenol compounds and antioxidant factors. Thus, we concluded that *Coccinia indica* act via its free radical scavenging to prevent lipidperoxidation. Therefore, natural antioxidants and phenol compounds in *Coccinia indica* have the capability to be used medically and in food systems to preserve food quality.

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