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Determination of Antioxidant Activity of Rubia Cordifolia

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A B S T R A C T

The aim of this study was to investigate the antioxidant activity and phytochemical analysis of the leaves extracts of Rubia Cordifolia. The phytochemical screening was carried on the both extracts of leaves of Rubia Cordifolia, revealed the presence of some active ingredients such as Alkaloids, Carbohydrates, Phytosterols, saponins, phenolic , fixed oil and fats, proteins , free aminoacids and lignins. 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 Rubia Cordifolia 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.

Our findings suggest the use of Rubia Cordifolia leaves in functional foods and food supplements designed for prevention of various chronic diseases including cancer. However, further studies are needed to prove that the protective effects observed in vitro do indeed translate in vivo.

Keywords: *Rubia Cordifolia*, anti-oxidant activity, cancer

INTRODUCTION

The plants have been used as natural medicines. The use of plants as medicines has been in existence since prehistoric times. The different ways by which plants have been found useful as medicines such as crude extract of plants has been used directly due to the presence of natural chemical components such as berberine, morphine, psilocin, vincristine etc.[1] and natural compounds for the synthesis of drugs such as tubocurarine, colchicine, nicotine, quinine etc. for therapeutic purpose by general people. the modern medicines such as digitalis, vinblastine, aspirin, quinine and paracetamol had their origin from the natural compounds of medicinal plants viz., foxglove (digitalis purpurea), madagascar periwinkle (vinca rosea), willow bark (salix spp.), quinine bark (cinchona officinalis), respectively[2] . ayurvedic pharmacopoeia has recorded more than 300 medicinal plants that are commonly used for medicinal purpose. The knowledge of chemical constituents present in plants helps the scientists to understand the mechanism of drug action . It has been observed that the use of crude drugs obtained from different geographical regions showed large dissimilarity and variations in clinical results4 . Rubia cordifolia is a perennial, spiny climber with a stem, growing up to 12 m long. Leaves are ovate lanceolate, nerved, 2-10 cm long and 2-5 cm broad, occur in whorls of. Flowers with fragrance, minute, whitish or greenish yellow in colour. Fruit is minute, glabrous, 1-2 seeded, dark purplish or blackish at maturity. The plant carries fruits and flowers in the months of august to October5 . Rubia cordifolia is having red rhizomatous base and roots. It is an important raw material for the traditional herbal compositions such as aswagandharistam, gulguluthikthkarishtam, jaatyaadi ghrita, madhookasavam, majishthaadi taila, useerasavam etc. It is a member of Rubiaceae family, distributed in hilly areas of India up to 3750m.[3]

Rubia cordifolia is described to be protective against a various panel of cancer cell lines, such as P388, L1210, L5178Y, B16 melanoma, sarcoma180 and Lewis lung carcinoma . The leaves of Rubia cordifolia also possess antiviral and in-vitro free radical scavenging activity8 . Rubia cordifolia show powerful antioxidant activity against lead nitrate and radiation induced toxicity. Free radicals are formed in our body due to the biological oxidation. Oxidation is a natural method in an organism for the manufacture of energy to fuel biological cycles. On the other hand, free radicals cause damage to the body and create oxidative stress11-12. The normal metabolism produce oxidation byproducts which cause severe damage to DNA[4], protein and lipids, contribute to ageing and also to degenerative diseases including cancer, coronary artery disease, hypertension, diabetes etc . The secondary plant metabolities like phenolics and flavonoids present in food substances of plants are natural antioxidants can entrap few free radicals directly or through a sequence of reactions with antioxidant enzymes16 and also show various biological effects, including antimutagenicity, antiageing and protective effects on oxidative stress. Keeping in view the significance of medicinal plants and secondary metabolites, this research was carried out to evaluate the Rubia cordifolia, its phytochemical investigation along with its in vitro antioxidant activity[5-6].

MATERIALS AND METHODS

Plant Material

The leaves of Rubia Cordifolia was collected from the local Market. 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.

Chemicals

Sodium hydroxide (Analytical grade, FisherChemicals 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)

Extraction of plant material

Preparation of Aqueous Extract:

Fresh leaves of *Rubia Cordifolia* 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 up to 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 Rubia Cordifolia 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 upto 50-600C 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 like Detection of Carbohydrates, Test for Gums, Mucilages, Test for Proteins and Amino Acids, Test for Fixed Oils and Fats, Alkaloids, Glycosides and Phytosterols

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 decolorization 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 *Rubia Cordifolia* 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 *Rubia Cordifolia* 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.

Superoxide radical-scavenging activity

The ability of *Rubia Cordifolia* 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.

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 R.sativus 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 *Rubia Cordifolia* extracts and standards were calculated using the following equation:

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

RESULTS AND DISCUSSIONS

Phytochemical screening of *Rubia Cordifolia*.

The present investigation concluded that the isolated compounds from the plant *Rubia Cordifolia* are pure and the plant *Rubia Cordifolia* shows the various antibacterial effects against different bacteria and found that different phytochemical compunds. 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 *Rubia Cordifolia*.

S. No.	Phytoconstituents	Aqueous	Alcoholic
1.	Alkaloids	+	-
2.	Carbohydrates	-	+
3.	Glycosides	-	-
4.	Phytosterols	+	-
5.	Saponins	+	+
6.	Fixed oils & Fats	-	-
7.	Tannins & Phenolic compounds	+	+
8.	Protein & Free amino acids	+	+
9.	Gums & mucilage	-	-
10.	Flavonoids	+	-
11.	Lignin	+	+
12.	Volatile oil	-	-

Ferric reducing ability of *Rubia Cordifolia*

Antioxidant activity has been reported to be concomitant with reducing power of plant extract (Gordon, 1990). Significant ferric reducing ability of *Rubia Cordifolia* 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 *Rubia Cordifolia* have been shown to possess significant ferric reducing ability in their pure form, suggesting that ferric reducing ability of *Rubia Cordifolia* 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 etal, 2005).

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

Group	Drugs	IC_{50} value µg/ml
I	Quercitin	13.75±0.031
II	Butylated Hydroxy Toulene(BHT)	3.10 ±0.067
III	AQRC	1.98±0.084
IV	ALRC	2.26±0.056

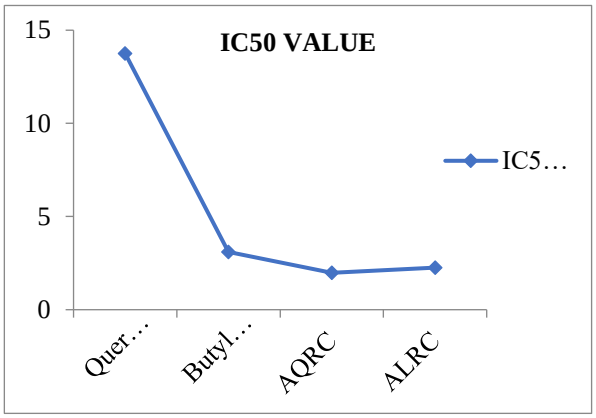

Fig 1: Reducing power of *Rubia cordifolia* Quercetin and BHT were used as reference antioxidant Values are means ± SD (n = 3).

Metal chelating activity of *Rubia cordifolia*

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, *Rubia Cordifolia* 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 *Rubia cordifolia*

Group	Drugs	IC_{50} value µg/ml
I	EDTA	5.22
II	Quercitin	156
III	Butylated Hydroxy Toulene(BHT)	76
IV	AQRC	30.11
V	ALRC	35.25

DPPH radical scavenging activity of *Rubia cordifolia*

Effective DPPH radical scavenging activity exhibited by *Rubia Cordifolia* 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 *Rubia cordifolia*. Rubia Cordifolia 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 *Rubia Cordifolia* leaves as natural antioxidants.

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

Group	Drugs	IC_{50} value µg/ml
I	Quercitin	1.3 ±0.003
II	Butylated Hydroxy Toulene(BHT)	3.85 ±0.061
III	AQRC	4.23 ±0.036
IV	ALRC	3.2 ±0.000

Superoxide radical scavenging activity of *Rubia Cordifolia*

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 *Rubia Cordifolia* extracts on superoxide radicals is shown in Figure 4.9. Extracts from different parts of *Rubia Cordifolia* 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 *Rubia Cordifolia* 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 *Rubia Cordifolia* and standard antioxidants on superoxide radical (O2•) as determined by their IC50, expressed as mg/ml.

Group	Drugs	IC_{50} value µg/ml
I	Quercitin	0.03 ±0.000
II	Butylated Hydroxy Toulene(BHT)	0.022 ±0.001
III	AQRC	0.310 ±0.005
IV	ALRC	0.028 ±0.004

Hydrogen peroxide scavenging activity of *Rubia Cordifolia*

Though hydrogen peroxide (H2O2) itself is not ver8y reactive, it can occasionally be toxic to cells, since it may give rise to potentially reactive hydroxyl radicals (Halliwell, 1991). The scavenging activity of *Rubia Cordifolia* extracts on H_2O_2 is shown in Figure 4.10 and compared with quercetin and BHT as standard antioxidants. *Rubia Cordifolia* 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 *Rubia Cordifolia* and standard antioxidants on hydrogen peroxide (H2O2) as determined by their IC50, expressed as mg/ml

Group	Drugs	IC_{50} value µg/ml
I	Quercitin	0.029±0.003
II	Butylated Hydroxy Toulene(BHT)	0.086±0.002
III	AQRC	0.060±0.001
IV	ALRC	0.610±0.043

CONCLUSION

The results obtained in this study (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 *Rubia Cordifolia* 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 *Rubia Cordifolia* act via its free radical scavenging to prevent lipidperoxi- dation. Therefore, natural antioxidants and phenol compounds in *Rubia Cordifolia* have the capability to be used medically and in food systems to preserve food quality.

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