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Determination of antioxidant activity of rubia cordifolia

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ABSTRACT

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 vivo* do indeed translate *in vivo*.

Keywords: antioxidant, rubia cordifolia

INTRODUCTION

Antioxidants are compounds capable to either delay or inhibit the oxidation processes which occur under the influence of atmospheric oxygen or reactive oxygen species. They are used for the stabilization of polymeric products, of petrochemicals, foodstuffs, cosmetics and pharmaceuticals.

Antioxidants are involved in the defense mechanism of the organism against the pathologies associated to the attack of free radicals.

Endogenous antioxidants are enzymes, like superoxide dismutase, catalase, glutathione peroxidase or nonenzymatic compounds, such as uric acid, bilirubin, albumin, metallothioneins. When endogenous factors cannot ensure a rigurous control and a complete protection of the organism against the reactive oxygen species, the need for exogenous antioxidants arises, as nutritional supplements or pharmaceutical products, which contain as active principle an antioxidant compound. Amongst the most important exogenous antioxidants, vitamin E, vitamin C, β-carotene, vitamin E, flavonoids, mineral Se are well known, but also vitamin D and vitamin K3.

Exogenous antioxidants can derive from natural sources (vitamins, flavonoids, anthocyanins, some mineral compounds), but can also be synthetic compounds, like butylhydroxyanisole, butylhydroxytoluene, gallates, etc [1,2]

There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body, as well as the deterioration of fats and other constituents of foodstuffs [3].

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 [4]. 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 [5]. 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 [6,7].

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 [8].

Health Benefits of Antioxidants

Recently, antioxidants have attracted considerable attention in relation to radicals and oxidative stress, cancer prophylaxis and therapy, and longevity. Phenols and polyphenols are the target analytes in many such cases; they may be detected by enzymes like tyrosinase or other phenol oxidases, or even by plant tissues containing these enzymes [9].

The recommendations based on epidemiological studies are such, that fruits, vegetables and less processed staple foods ensure the best protection against the development of diseases caused by oxidative stress, such as cancer, coronary heart disease, obesity, type 2 diabetes, hypertension and cataract. The explanation consists in the beneficial health effect, due to antioxidants present in fruit and vegetables [10]. There are numerous antioxidants in dietary plants: carotenoids, phenolic compounds, benzoic acid derivatives, flavonoids, proanthocyanidins, stilbenes, coumarins, lignans, and lignins. Of the 50 analysed food products with high antioxidant content , 13 were spices, 8 were fruits and vegetables, 5 were berries, 5 were chocolatebased, 5 were breakfast cereals, and 4 were nuts or seeds. Considering the typical serving sizes, blackberries, walnuts, strawberries, artichokes, cranberries, brewed coffee, raspberries, pecans, blueberries, ground cloves, grape juice and unsweetened baking chocolate were at the top of the classification.

Fruit juices, beverages and hot drinks contain high amounts of antioxidants, like polyphenols, vitamin C, vitamin E, Maillard reaction products, β-carotene, and lycopene [22]. The consumption of fruit juices, beverages and hot drinks was found to reduce the morbidity and mortality caused by degenerative diseases [11,12]. Antioxidants are known to play a key role in the protective influence exerted by plant foods. Epidemiologic studies that analyse the health implications of dietary components rely on the estimation of intakes of sample populations, which are found in databases that provide the compounds found in commonly consumed foods. Thus, the availability of appropriate and complete food composition data is vital. Due to the diversity of chemical compounds with antioxidant activity present in foodstuffs, complete databases of antioxidant contents are not yet available. In addition, levels of single antioxidants in foodstuffs do not necessarily reflect their total antioxidant potential (TAP) [13]; the total antioxidant potential depends on the synergic and redox interaction among the different molecules present in food [14]. Geographical differences in food composition should also be considered when regional surveys are performed.

The total antioxidant potential is a relevant tool for investigating the relationship between dietary antioxidants and pathologies induced by the oxidative stress. This was confirmed by the data obtained from a recent population-based control study, proving that diet TAP resulted in reduced risk of both cardiac and distal gastric cancer[15]. Several analytical methods were recently developed for measuring the total antioxidant capacity of food and beverages: these assays differ in the mechanism of generation of different radical species and/or target molecules and in the way end-products are measured [16].

The consumption of fruits and vegetables, as well as of grains and nuts, has been associated with reduced risk of chronic diseases. Among food components fighting against chronic diseases, great attention has been paid to phytochemicals, plant-derived molecules endowed with steady antioxidant power. The cummulative and synergistic activities of the bioactive molecules present in plant food are responsible for their enhanced antioxidant properties. Hence, an appropriate investigation of the role of dietary antioxidants in disease prevention, should be based on a complete database of antioxidant-rich foodstuffs [17,18].

The evaluation of the total antioxidant capacity (TAC) may be an appropriate tool to determine the additive antioxidant properties of plant foods . The importance of TAC as a novel instrument to estimate the relationship between diet and oxidative stress-induced diseases, is presented in recent studies showing a negative association between dietary TAC and the incidence of gastric cancer or the levels of C-reactive protein. In order to assess the overall intake of TAC in population studies, the TAC of 34 vegetables, 30 fruits, 34 beverages and 6 vegetable oils, of varieties most often consumed in Italy, has been analysed using three different assays [19]. Among fruits, the highest antioxidant activities were found in berries, among beverages, coffee had the greatest TAC, followed by citrus juices, which exhibited the highest value among soft beverages . The TAC of spices, dried fruits, sweets, cereals, pulses, and nuts was determined with the aim to complete the Italian TAC database. In fiber-rich foods where phenolics are present in free or bound forms, such as cereals, legumes, and nuts, the contribution of bound antioxidant compounds to the TAC value was evaluated.

Various berries and fruit types of less common fruit species are known to contain antioxidants. The intake of high amounts of flavonoids, compounds endowed with antioxidant, antiproliferative and anti-inflammatory activity, may have a positive impact on human health, especially in the prevention of cancer and inflammatory diseases [20].

The Mechanism of Action of Antioxidants

LMWAs (low molecular weight antioxidants) are small molecules that frequently infiltrate cells, accumulate (at high concentrations) in specific compartments associated with oxidative damage, and then are regenerated by the the cell. In human tissues, cellular LMWAs are obtained from various sources. Glutathione (GSH), nicotinamide adenine dinucleotide (reduced form), and carnosine are synthesized by the cells; uric acid (UA) and bilirubin are waste products

of cellular metabolism; ascorbic acid (AA) , tocopherols and polyphenols are antioxidants obtained from the diet.[21]

Among these LMWAs, a considerable attention was focused on ascorbic acid (AA), known for its reductive properties and for its use on a wide scale as an antioxidant agent in foods and drinks it is also important for therapeutic purposes and biological metabolism.[22]

Ascorbic acid is an antioxidant with therapeutic properties, which plays an important role in activating the immune response, in wound healing, in osteogenesis, in detoxifying the organism, in iron absorption, in collagen biosynthesis, in preventing the clotting of blood vessels, and in many other metabolic processes [23].

Vitamin C can be easily oxidized, its degradation being accelerated by heat, light and the presence of heavy metal cations. Thus, due to its content variation, vitamin C represents an important quality indicator of foodstuffs and contributes to the antioxidant properties of food [24,25]. Special attention has been dedicated to the study of antioxidant action mechanism.

The excess free radicals circulating in the body oxidize the low density lipoproteins (LDL), making them potentially lethal; the excess free radicals can also accelerate aging processes and have been linked to other very serious pathologies, such as brain stroke, diabetes mellitus, rheumatoid arthritis, Parkinson's disease, Alzheimer's disease and cancer. Physiologically, the oxygenated free radicals are among the most important radical species. Reactive oxygen species (ROS) comprise species with a strong oxidizing tendency, both of a radical nature (the superoxide radical, the hydroxyl radical) and a non-radical nature (ozone, hydrogen peroxide) [26].

A number of chemical and physical phenomena can initiate oxidation, which proceeds continuously in the presence of (a) suitable substrate(s), until a blocking defence mechanism occurs. Target substances include oxygen, polyunsaturated fatty acids, phospholipids, cholesterol and DNA [27].

The essential features of oxidation via a free radical-mediated chain reaction are initiation, propagation, branching and termination steps. The process may be initiated by the action of external agents such as heat, light or ionizing radiation or by chemical initiation involving metal ions or metalloproteins [28].

MATERIALS AND METHODS

Reagents

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)

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, WhatmanInternational Ltd, Maidstone, England).

Plant Material Collection

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.

Preparation of plant extracts

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 50-60^{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.

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.

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.

RESULTS

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

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

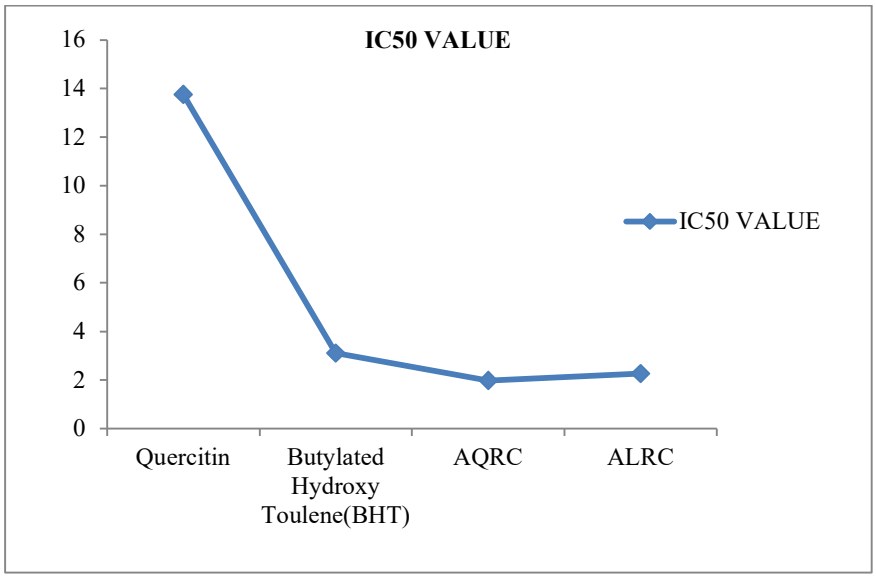

Fig 2: Reducing power of *Rubia cordifolia* Quercetin and BHT were used as reference antioxidant Values are means ± SD (n = 3).

Metal chelating activity of Rubia cordifolia

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

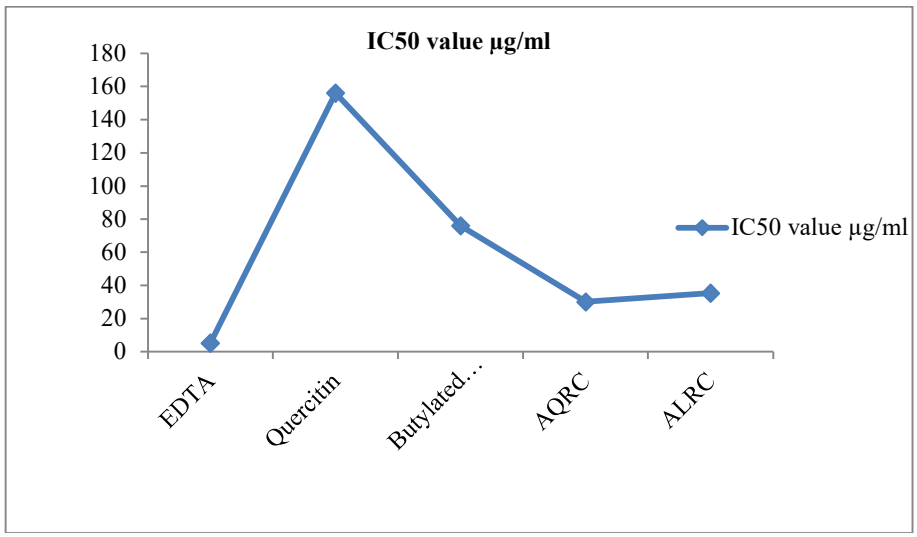

Fig 3: Metal chelating activity of *Rubia cordifolia* EDTA was used as positive control. Quercetin and BHT were used as reference antioxidants. Values are means ± SD (n = 3).

DPPH radical scavenging activity of Rubia cordifolia

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

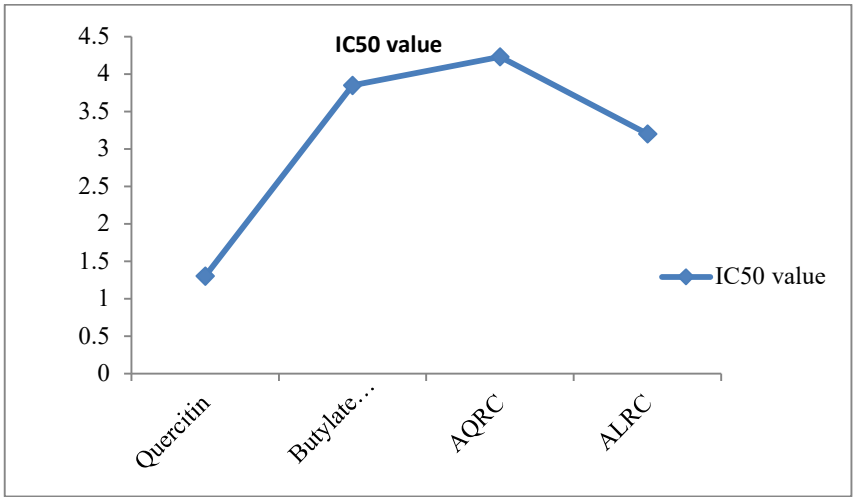

Fig 4: DPPH radical scavenging activity of *Rubia Cordifolia*. Quercetin and BHT were used as reference antioxidant.Values are means ± SD (n = 3).

Superoxide radical scavenging activity of Rubia Cordifolia

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

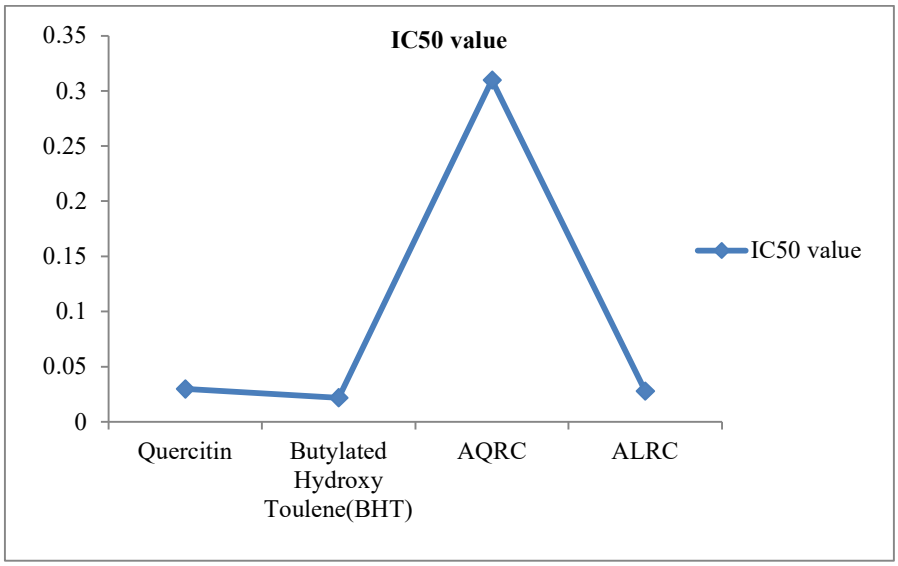

Fig 5: Superoxide radical scavenging activity of *Rubia Cordifolia* Quercetin and BHT were used as reference antioxidant. Values are means ± SD (n = 3).

Hydrogen peroxide scavenging activity of Rubia Cordifolia

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

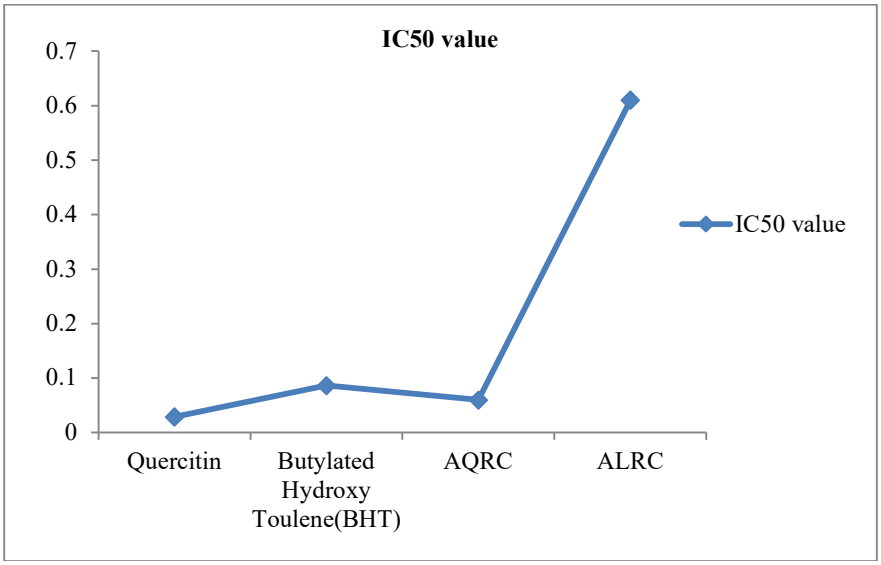

Fig 6: Hydrogen peroxide scavenging activity of *Rubia Cordifolia*. Quercetin and BHT were used as reference antioxidant. Values are means ± SD (n = 3).

SUMMARY

Phytochemistry has been making a rapid progress and plant products have become increasingly popular in various traditional, complementary and alternative systems as they are pharmacologically potent and have low or no side effects. Food derived products cannot be perceived as "medicine "and are highly interesting for development as preventive and protective agents that may find widespread, long-term use in populations at normal/high risk.

Rubia Cordifolia is a unique plant containing a rich and rare combination of phytochemicals. It is unparalleled in curing multitude of disorders and has aroused great interest for its potential role in helping in maintaining human health. The results obtained in this study led to the conclusion that,

- Leaves of *Rubia Cordifolia* possess substantial biological activities.
- Leaves have high level of polyphenolics and show significant antioxidant activity. *Rubia Cordifolia* could be regarded as a promising source of natural antioxidants and has a potential to be developed as an ingredient in health and functional foods.
- *Rubia Cordifolia* (alcoholic extracts of leaves) shows negligible cytotoxicity and genotoxicity to normal lymphocytes and exhibits potent protective effect against cell death and DNA damage in cells induced by H_2O_2 under ex vivo conditions.
- These could be related to the presence of polyphenolics in *Rubia Cordifolia* extracts as they possess significant capacity to remove reactive species by virtue of their ability to induce antioxidant enzyme system in the cells.
- *Rubia Cordifolia* (alcoholic extract of leaves) significantly inhibited the proliferation of several human cancer cells through induction of apoptosis.

Our findings suggest the use of *Rubia Cordifolia* extracts in functional foods and food supplements designed for the 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*.

CONCLUSION

The result of the present study showed that the aqueous and alcoholic extract of *Rubia Cordifolia* plant, which contains phenolic and flavonoidal compounds, exhibited the great antioxidant activity. The high scavenging property of methanolic extract of *Rubia Cordifolia* 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 *Rubia Cordifolia* 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 *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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