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Anti-viral and in-vitro free radical scavenging activity of leaves of *Rubia cordifolia*

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Abstract

The aim of this research was to develop the pharmacognostical parameters and phytochemical screening along with histological studies and the leaf powder of *Rubia cordifolia*. The dried leaves of *Rubia cordifolia*. (Family: Rubiaceae) were subjected to standardization by following pharmacognostical and phytochemical screening methods. *Rubia cordifolia* was investigated for preliminary phytochemical analysis and characterization by various instrumental techniques. Methanolic extracts of *Rubia cordifolia* leaves was very good antibacterial activity and also minimum inhibitory concentrating of different virus using HEL cell cultures, HeLa cell cultures and Vero cell cultures but MIC of Herpes simplex - 1 and 2, vaccinia virus, vesicular stomatitis and Herpes simplex-1 (TK ACV^{I}) were observed very good antiviral activity of *Rubia cordifolia* leaves DMSO extracts has good minimum cytotoxic concentration activity and also screening for various pharmaceuticals activities. Such as anti oxidant and microbial activities.

Keywords: Antiviral and Microbial activity, Phytochemical and Pharmacognostical screening, DPPH, free radical scavenging activity, *Rubia cordifolia*.

Introduction

Free radicals are generated continuously in the body due to the metabolism and disease [1]. In order to protect themselves against free radicals, organisms are endowed with endogenous (catalase, superoxide dismutase, glutathione peroxidase/ reductase) and exogenous (C and E vitamins, β-carotene, uric acid) defences: yet these defense system are not sufficient in critical situation (oxidative stress, contamination, UVexposure,etc.) When the production of free radicals significantly increases [2]. An excess generation of free radicals and a deficient cellular antioxidant defense system may lead to a state of oxidative stress, which may contribute to the development of cancer [3]. Free radicals are known as the cause of qualitative decay of food in food industry [4].

Rubia cordifolia (Rubiaceae) also kown as 'manjistha' is an important medicinal plant, which is used for treatment of various ailments such as anti-tumor, anti-inflammatory [5], urinary disorders [6], antistress antimicrobial [7], hepatoprotective [8], radioprotactive [9] and anticancer [10].

The roots of *Rubia cordifolia* L. have been used as a traditional Korean medicine to treat cough,bladder and kidney stones, joint inflammation, uterine, hemorrhage and uteritis. Anthraquinone, anthraquinone glycoside, napthaquinone, napthaquinone glycoside, furomollngin, mollugin, alizarin,lucidine, pimeveroside, ruberythric acid, purpurin, xanthopurpurin, cyclohexapeptide, alkaloid and lignan have been reported from Rubaia species

[11-14]. Anti cancer constituent from the roots of *Rubia cordifolia* [15]

Rubia cordifolia Linn. is a flowering plant species. It is commonly known as Manjistha. Roots and stems are active part of plant. Plant has many pharmacological actions like blood purifier activity, anticancer, astringent, antidysentric, antiseptic, deobstruent properties and antirheumatic, hepatoprotective [16-17]. Hepatoprotective action is mainly shown by Rubiadin [18]. Plant contains various chemical constituents like Anthraquinones [19]. Iridoids [20] Hexapeptides, Rubiprasins, Quinones, and Triterpenoids [21]. Literature survey reveals that Quality control protocol was developed for stems of *Rubia cordifolia* Linn [22].

Material and Methods:

Collection of plants leaves and Material:

Plant leaves of *Rubia cordifolia* was collected from the Kerala, India. DPPH and Dragendroff's reagent, Wagner's reagent, Hager's reagent, Mayer's reagent from Hi-media chemical co. All the other chemicals used were of analytical grade.

Preparation of Extracts:

The leaves of *Rubia cordifolia* was collected in the month of October 2008, shade dried and powdered. 250gm of powder was subjected to successive soxhlet extraction by various solvent such as methanol, chloroform, hexane, acetone, ethyl acetoacetate, water and dimethyl sulphoxide. The solvent was then removed under reduced pressure the yield obtained with respect to which were used for phytochemical analysis, anti-viral activity, free radical scavenging activity and microbial activity.

Phytochemical screening

Phytochemical screening for extract were carried out and reported as Tst for Alkaloids, Flavanoids, Glycosides, Saponins, Tannins, Triterpenoids, Anthraquinone [23-28]. The phytochemical screening of the test compound are showed in table-1.

Table-1: Result of Phytochenical screening of leaves extracts of *Rubia cordifolia* leaves.

Constituent	Test	ME	HE	CE
Alkaloids	a) Mayer's reagent	+	-	-
	b) Dragendroff's reagent	+	-	-
	c) Hager's reagent	+	-	-
	d) Wagner's reagent	+	-	-
Glycosides	a) Keller-Killani test	+	-	-
	b) Borntrager's test	+	-	-
	c) Legal's test	-	-	-
	d) Baljet's test	+	-	-
Flavanoids	Shinoda test	+	+	+
Tannins	a) Ferric chloride solution	+	+	+
	b) Lead acetate test	+	+	+
	c) Gelatin solution test	+	+	+
Saponin		-	-	-
Anthraquinones		+	+	+
Triterpenoids	Libermann - Burchard test	+	+	+

ME: Methanol Extracts, HE: Haxene Extracts, CE: Chloroform Extracts.
(+ Positive, - Negative)

Physicochemical study

Physico-chemical parameters for extracts were determined as per WHO guidelines and reported as total ash, water soluble ash, acid insoluble ash, water soluble extractive, alcohol soluble extractive, and loss of drying [29]. The physiochemical study of the data are showed in table-2.

Assay methods for antioxidants

Several methods have been developed to evaluate the total antioxidant activity of fruits or other plants and animal tissues. Among them, trolox equivalent antioxidant capacity, total radical absorption potentials, oxygen radical absorption capacity assays and the ferric reducing ability of plasma (FRAP) assay are commonly used and are the representative methods frequently used in various investigations. [30-34]. One of the methods is the DPPH assay, which can accommodate a large number of samples in a short period of time and is sensitive enough to detect natural compounds at low concentrations so it was used in the present study for the primary screening of antioxidants.

DPPH method

This method was given by [35] and later modified by [36]. It is one of the most extensively used antioxidant assay for plant samples. Recently the assay has been used to determine antioxidant activity in Tanacetum [37], Moldavian balm [38], and Phyllanthus-amarus [39]. This method is based on scavenging of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) from the antioxidants, which produces a decrease in absorbance at 515 nm. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, the reduced form of the radical is generated accompanied by loss of colour. This delocalization is also responsible for the deep violet colour, characterized by an absorption band in ethanol solution at about 520 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity.

Tabel-2: Physico-chemical parameter of *Rubia cordifolia* leaves extracts.

	Evaluation parameter	Yield (%w/w)
Ash values	Total ash	4.25
	Water-soluble ash	0.81
	Acid insoluble ash	0.35
Extractive Values	Alcohol soluble extractive	92.32
	Water soluble extractive	18.23

Table-3: Antiradical activity of extracts *Rubia cordifolia* leaves .

Compound	Concentration µg/ml	% Inhibition	EC_{50} (µg/ml)
Standard Pyrogallol	1.0, 1.2, 1.4, 1.6, 1.8, 2.0	22, 30, 36, 44, 54, 62	1.7 µg/ml
Rubia cordifolia	10 20 40 60 80	16.24 29.70 50.07 68.72 88.03	33.42 µg/ml

Antiradical activity :-
Antiradical activity is measured by decrease in absorbance at 515 nm, of methanol solution of colored DPPH [40-41]. Decrease in absorbance in the presence of the test compound at different concentrations was measured after 15 minutes. Ec_{50} is the concentration of the test solution that can bring about 80% decrease in absorbance in this study pyrogallol was used as a reference standard. The anti radical activity of the test compound are showed in table-3.

Superoxide scavenging activity :-
Superoxide radical is known to be very harmful to the cellular components. For superoxide scavenging activity, the ability of the test solution to inhibit formation by scavenging the superoxide radicals generated in riboflavin light-NBT system was measured [42]. The reaction mixture contains 50 mM phosphate butter PH 7.6, 20µg riboflavin, 12 mM EDTA and NBT o.1 mg/3 ml added in that sequence. The absorbance was measured at 590 nm. Ascorbic acid was used as the standard anti oxidant. The anti radical activity of the test compound are showed in table-4.

Nitric oxide scavenging activity :-
Nitric oxide is implicated in inflammation, cancer and other pathological condition [35]. The procedure for nitric oxide scavenging activity is based on the principle the sodium nitroprusside in aqueous solution at physiological PH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using 0.5 ml Greiss reagent (1% N-(1-napthyl) ethylenediamine, dihydrochloride). The absorbance of the chormophore formed was read at 546 nm, while using curcumin, as positive control form the result [43]. One fact emerges our regarding the efficacy of *Rubia cordifolia* leaves have exhibited very significant activity in test system. The anti radical activity of the test compound are showed in table-5.

Table-4: Superoxide anion activity of extracts *Rubia cordifolia* leaves observed with riboflavin- light-NBT system.

Sample	Concentration µg/ml	% Inhibition	EC_{50} (µg/ml)
Standard Ascorbic acid	5, 10, 15 20, 25	24.06, 40.34, 58.04, 75.24, 92.55	12.5 µg/ml
Rubia cordifolia	5 10 15 20 25	31.40 40.44 53.30 61.11 78.12	11.03 µg/ml

Table-5: Nitric oxide activity of extracts *Rubia cordifolia* leaves observed with Griess reagent.

Sample	Concentration µg/ml	% Inhibition	EC_{50} (µg/ml)
Standard Curcumin	5, 10, 15 20, 25	37.56, 47.30, 58.33, 65.38, 77.30	11.2 µg/ml
Rubia cordifolia	20 25 30 35 40	37.23 44.35 52.22 56.11 61.19	26.11 µg/ml

Antiviral activity:

In antiviral activity live cell cultures were used. In this method cell was grown on solid media. After any cytopathic effect was checked with comparing uninoculated cell line. Various cell lines are use for the study of antiviral activity of drug e.g. HEL cell cultures, Hela cell cultures and Vero Cell cultures etc. The minimum concentration of extract which the viral inhibited or reduced virus induced cytopathogenicity by 50% is good for drug. There is a co-relationship between MIC (minimum inhibition concentration) and MCC (minimum cytotoxic concentration). Higher MCC and lower MIC indicate usefulness of drug. For this reason the study of viruses are not possible like bacteria. The viral activity is checked by Cytopathic effect in which morphological change in particular cell line is observed. Normally, the drugs, which are nucleotide analogue, protein inhibitor, or drugs, which prevent the assembly of virus, are used [44].

Sample preparing:

For antiviral activity of herbal plants of *Rubia cordifolia* leaves was extracts in DMSO solvent and also extracts in water and methanol [45].

Different viruses infect different cell lines:

For the result, minimum inhibitory concentration of different virus using HEL cell cultures. Here Brivudin, Ribavirin, Acyclivir and Ganciclovir are used as control. MIC of Herpes simplex-1 and 2, vaccinia virus, vesicular stomatitis and Herpes simplex-1 (TK ACV^{l}) were observed very good antiviral activity of *Rubia cordifolia* leaves DMSO extracts and good minimum cytotoxic concentration activity. *Rubia cordifolia* leaves have been good antiviral agent because their MIC for all five viruses are less than 10mmg/ ml and their MCC is less than 50 mg/ml. The respective data are given in Table no- 6.

Table – 6 Cytotoxicity and antiviral activity in HEL cell cultures.

Compounds	Minimum cytotoxic concentration[a] (µg/ml)	Minimum inhibitory concentration[b](µg/ml))				
		Herpes simplex virus-1 (KOS)	Herpes simplex virus-2 (G)	Vaccinia Virus	Vesicular stomatits Virus	Herpes simplex virus-1 TK KOS ACV^{r}
Rubia cordifolia	50	>10	>10	>10	>10	>10
Rubia cordifolia	>50	>50	>50	>50	>50	>50
Brivudin (µM)	>250	0.08	0.8	6	>250	250
Ribavirin(µM)	>250	250	250	50	150	250
Acyclovir(µM)	>250	0.4	0.16	>250	>250	150
Gancicolvir(µM)	>100	0.032	0.096	>100	>100	4

[a]Required to cause a microscopically detectable alteration of normal cell morphology.
[b]Required to reduce virus-induced cytopathologenicity by 50%

The result of *Rubia cordifolia* leaves extracts For antiviral activity viruses using HeLa cell cultures, and Vero cell cultures. Here Brivudin, Ribavirin, Acyclovir, Ganciclovir are used as control. Minimum inhibitory concentration (MIC) of vesicular, stomatitis, Coxsackie virus, Respiratory syncytial virus were observed very good antiviral activity of *Rubia cordifolia* leaves DMSO extracts and good minimum cytotoxic concentration (MCC) activity. *Rubia cordifolia* leaves have been good antiviral agent because their MIC for all three viruses are less than 10 µg/ml and their MCC is less than 50 mg/ml. The data are given in Table no-7 and 8.

Table - 7 Cytotoxicity and antiviral activity in HeLa cell cultures.

Compounds	Minimum cytotoxic concentration[a] (µg/ml)	Minimum inhibitory concentration[b](µg/ml))		
		Vesicular stomatitis virus	Coxsackie virus B4	Respiratory syncytial virus
Rubia cordifolia	50	10	>10	>10
Rubia cordifolia	>50	>50	>50	50
Brivudin (µM)	>250	250	>250	>250
(S)- DHPA(µM)	>250	150	>250	>250
Ribavirin(µM)	>250	30	150	10

[a]**Required to cause a microscopically detectable alteration of normal cell morphology.**
[b]**Required to reduce virus-induced cytopathologenicity by 50%**

Table – 8 Cytotoxicity and antiviral activity in Vero cell cultures

Compounds	Minimum cytotoxic concentration [a] (µg/ml)	Minimum inhibitory concentration[b](µg/ml))				
		Para Influenza-3 Virus	Reo virus-1	Sindbis virus	Coxsacke virusB4	PuntaTor o virus
Rubia cordifolia	>50	>50	>50	>50	>50	>50
Rubia cordifolia	>50	>50	>50	>50	>50	>50
Brivudin (µM)	>250	>250	>250	>250	>250	>250
(S)- DHPA(µM)	>250	>250	>250	>250	>250	>250
Ribavirin(µM)	>250	150	150	150	>250	50

[a]**Required to cause a microscopically detectable alteration of normal cell morphology.**
[b]**Required to reduce virus-induced cytopathologenicity by 50**

Antibacterial activity:

The antimicrobial drugs occupy a unique was the vehicle in the history of medicine. The realization that certain microorganisms are successfully resisting the "wonder drugs" not only impels a lease less search for new systemic antimicrobial agents but also forced for sober return to certain ancillary art of the medical and surgical treatment of infectious disease. The data are given in Table no- 9

Conclusion

The *Rubia cordifolia* plants were subjected to extractions in various solvent including methanol, chloroform, hexane and water. Highest antioxidant potential was *Rubia cordifolia* leaves in methanolic extract. From the present data methanol was the best solvent for extraction of *Rubia cordifolia* plants. From the experiment it was observed that these plants have certain important constituents which were responsible for Radical scavenging activity. Radical scavenging activity was observed when discoloration was occurred. When the difference was high between the DPPH solution and sample, the percent free radical activity is high or the sample was high potential to scavenge the free radical of DPPH. This study reveals that tested plant materials have significant free radical scavenging activity. The result of the present study suggests that these plants can be used as a source of antioxidants for different diseases. These plants are an effective potential source of natural antioxidants. This free radical scavenging protocol is efficient for *Rubia cordifolia* need to be biotechnology techniques for feasible and increase continuous production of biologically active compounds at a comparable rate to commercially available antioxidants. For the result, minimum inhibitory concentration of different virus using HEL cell cultures. Here Brivudin, Ribavirin, Acyclovir and Ganciclovir are used as control. MIC of Herpes simplex-1 and 2, vaccinia virus, vesicular stomatitis and Herpes simplex-1 (TK ACV^{I}) were observed very good antiviral activity of Acacia arabica seeds DMSO extracts and good minimum cytotoxic concentration activity. *Rubia cordifolia* leaves have been good antiviral agent because their MIC for all five viruses are less than 10mmg/ ml and their MCC is less than 50 mg/ml.

Table– 9: Antibacterial activity of *Rubia cordifolia* leaves extracts on different bacteria.

Treatment	Concentration µg/ml	Zone of inhibition (mm)			
		S.aureus	***S.epidermidis***	***P.aeruginosa***	***K.pneumoniae***
Methanolic extract	150	16	21	19	20
Chloroform extract	150	14	13	16	15
Hexane extract	150	16	17	15	16
Standard Ciprofloxacin	2	28	30	30	30

Table– 10: Antifungal activity of Rubia cordifolia leaves extracts on different bacteria.

Treatment	Concentration µg/ml	Zone of inhibition (mm)	
		C.albicans	***A.niger***
Methanolic extract	150	17	19
Hexane extract	150	15	16
Standard Greseofulvin	2	30	30

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