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In vitro studies on antioxidant and anti-obesity properties of acetone extract from the leaves of *Rubia cordifolia*

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Abstract
The present study defines the screening of phytochemical screening of *Rubia cordifolia* based on their antioxidant and anti-obesity activities. The acetone extract of *Rubia cordifolia* demonstrated excellent antioxidant and anti-obesity potential with ABTS radical scavenging effect (68.34%) and pancreatic lipase inhibitory activity (EC_{50} value of 68.32 µg/mL). Hundred percent *Rubia cordifolia* acetone extract was found to be the most effective and showed highest antioxidant and anti-obesity activities. Moreover, metabolite profiling of acetone extract of *Rubia cordifolia* was also carried out using TLC. Results revealed *Rubia cordifolia* as promising medicinal plant for the development of new functional food with prodigious applications in obesity.

Keywords: antioxidant, anti-obesity, *Rubia cordifolia*, acetone extract

Introduction

Natural bioactive compounds especially from plant sources, including spices have been investigated for their characteristics and health effects. Plants are potential sources of natural bioactive compounds such as secondary metabolites and antioxidants. They absorb the sun light and produce high levels of oxygen and secondary metabolites by photosynthesis. Medicinal components produced are stored in plant leaves. Most of the secondary metabolites of herbs and spices are commercially important and find use in a number of pharmaceutical compounds. Flavonoids and phenolics acids are the most important groups of secondary metabolites and bioactive compounds in plants (Kim *et al*., 2010) [5]. They are also a kind of natural product and antioxidant substance capable of scavenging free superoxide radicals, anti-aging and reducing the risk of cancer. Secondary metabolites are chemicals produced by plants; and their functions in growth, photosynthesis, reproduction and other primary processes are not known yet. Secondary chemicals are important in plant use by widely used especially in Asia (Bodeker, 2000). It was found that flavonoids reduce blood-lipid and glucose of humans.

In the continuing search for novel anti-obesity agents, numerous plant derived phytochemicals have been screened for potential lipase inhibition activity. A recent extensive study examined 132 extracts from 106 plant species, used either as foods or medicinal herbs, screening them for pancreatic lipase inhibition activity.50 Surprisingly, the majority of extracts, 100 in total, exhibited some degree of inhibitory activity. All extracts from plants belonging to Brassicaceae, Ericaceae, Fabaceae, Rosaceae and Solanaceae showed inhibitory activity. Twenty-six extracts inhibited lipase activity by at least 40%, of which 10 exhibited over 70% inhibitory activity. All extracts from apples exhibited more than 70% inhibitory activity. The current review classifies these botanically derived inhibitors into the following chemical classes (Slanc *et al*., 2009) [11].

Rubia cordifolia is a perennial climbing herbaceous plant. It is also known as Indian madder, which belongs to Rubiaceae. The root of the plant is commonly known as Manjistha and is sweet, bitter, acrid. The extracts and phytochemicals of Rubia plants had drawn considerable attention due to their potent bioactivities. Leaves are arranged in four whorls whereas the stem is slender rough and woody at the base. Flowers are in cymes, greenish white. Fruits are smooth, shining, and purplish black when ripe. The roots, are cluster in the soil, are aubergine or orange-red. The elongating and rough stems slightly lignify at the base. The branches are four-edge shaped.

Materials and Methods

Phytochemical Screening

The aqueous extract of leaves of *Rubia cordifolia* were subjected to phytochemical screening to determine the presence of secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, glycosides, saponins and polyphenols using standard procedures (Harborne 1973) [3].

Thin Layer Chromatography

Thin layer chromatography of acetone extract of *Rubia cordifolia* was performed using standard procedures (Harborne 1973) [3]. The acetone extract of *Rubia cordifolia* was placed carefully in precoated aluminum silica gel 60 F, Merck F 254 using a microcapillary tube. The spots were allowed to dry for few minutes and the TLC plate was placed in the solvent mixture, Toluene, acetone and Formic

acid (6:6:1) or solvents of ethyl acetate-glacial acetic acid-formic acid-water (100:11:11:26 v/v/v/v). After drying, the TLC plates were observed under UV at 240nm and 360 nm in UV TLC viewer. The Rf value of the spots was calculated by using the standard formula,

$$\text{Retention factor Rf} \quad \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Pancreatic Lipase Inhibitory Activity

The lipase inhibition activity of plant extract was determined as per the method proposed by Kim *et al*. (2010) [5]. In this assay, the porcine pancreatic lipase activity was measured using p-nitrophenyl butyrate (NPB) as a substrate. Lipase solution (1 mg/mL) was prepared in a 0.1 mM potassium phosphate buffer (pH 6.0). To determine the lipase inhibitory activity, 1 ml of acetone extract of *Rubia cordifolia* were pre-incubated with 1 ml of lipase for 10 min at 37°C. The reaction was then started by adding 0.1 mL NPB substrate. After incubation at 37°C for 15 min, the amount of p-nitrophenol released in the reaction was measured at 405 nm using a UV-Visible spectrophotometer and the percentage of inhibitory activity was calculated.

ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical scavenging assay

ABTS radical scavenging activity of acetone extract of *Rubia cordifolia* was followed by Re *et al*. (1999) [9]. ABTS radical was newly prepared by addition 5 ml of 4.9 mM potassium persulfate solution to 5 ml of 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with distilled water to produce an absorbance of 0.70 at 734 nm and the same was used for the antioxidant activity. The final solution of standard group was made up to 1 ml with 950 µl of ABTS solution and 50 µl of Ascorbic acid. Correspondingly, in the experiment group, 1 ml reaction mixture encompassed 950 µl of ABTS solution and 50 µl of different concentration of each extracts. The reaction mixture was vortexed for 10 s and after 6 min, absorbance was recorded at 734 nm against distilled water by using a Deep Vision (1371) UV–Vis Spectrophotometer and compared with the control ABTS solution. Ascorbic acid was used as reference antioxidant compound.

ABTS Scavenging Effect (%) = $[(A_0 - A_1/A_0) \times 100]$ Where A_0 is the absorbance of the control reaction and A_1 is the absorbance of extract.

Inhibition of Lipid Peroxidation Activity

Lipid peroxidation induced by Fe^{2+}ascarbate system in egg yolk was assessed as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa *et al*. (1979) [6]. The experimental mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer (20 mM, pH 7.0); KCl (30 mM); $FeSO_4$ $(NH_4)_2SO_4.7H_2O$ (0.06 mM); and different concentrations of acetone extract of *Rubia cordifolia* in a final volume of 0.5 ml. The experimental mixture was incubated at 37°C for 1 h. After the incubation period, 0.4 ml was collected and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The final volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100 °C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The absorbance of butanol-pyridine layer was recorded at 532 nm in Deep Vision (1371) UV–Vis Spectrophotometer) to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard. Inhibition of lipid peroxidation (%) by the each extracts was calculated according to 1-(E/C) × 100, Where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample.

Superoxide Radical Scavenging Assay

This assay was based on the capacity of the acetone extract of *Rubia cordifolia* to inhibit the photochemical reduction of Nitroblue tetrazolium (NBT) in the presence of the riboflavin-light-NBT system (Tripathi and Pandey Ekta, 1999; Tripathi and Sharma, 1999). Each 3 ml reaction solution contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM Ethylene diamine tetra acetic acid (EDTA), NBT (75 µM) and different concentration of extracts. It was kept visible in fluorescent light and absorbance was taken after 6 min at 560 nm by using a Deep Vision (1371) UV–Vis Spectrophotometer. Identical tubes with reaction mixture were kept in the dark served as blanks. The percentage inhibition of superoxide radical activity was measured by comparing the absorbance of the control with test sample solution

$$\% \text{ Super oxide radical scavenging capacity} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 was the absorbance of control and A_1 was the absorbance of both plant extracts fraction.

Nitric Oxide Radical Scavenging Activity

Nitric oxide scavenging ability of acetone extract of *Rubia cordifolia* was measured according to the method described by Olabinri *et al*. (2010) [1]. 0.1 ml of sodium nitroprusside (10 mM) in phosphate buffer (0.2 M, pH 7.8) was mixed with different concentration of extracts and incubated at room temperature for 150 min. After treated period, 0.2 ml of Griess reagent (1% Sulfanilamide, 2% Phosphoric acid and 0.1% N-(1-Naphthyl) ethylene diamine dihydrochloride) was added. The absorbance of the experimental sample was read at 546 nm against blank. All readings were taken in triplicate and ascorbic acid was used as standard. The percentage of inhibition was calculated by following equation

$$\% \text{ Nitric oxide radical scavenging capacity} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid rich fraction.

Results and Discussion

Phytochemical Screening

The phytochemical screening of aqueous leaves extract of *R. cordifolia* studied presently showed the presence of alkaloids, flavonoids, polyphenol, terpenoids, and absence of glycosides and tannin (Table -1). *R. cordifolia* revealed the presence of anthraquinones, glycosides saponins, steroids, phenols, and flavonoids. Biologically active compounds are chemical in nature they have the potential to cure various diseases (Chang *et al*., 2000) [1].

Table 1: Phytochemical screening of aqueous leaves extract of *Rubia cordifolia*

S/No.	Constituents	Aqueous leaves extract of *Rubia cordifolia*
1.	Alkaloids-Dragendroffs reagent	+
	Alkaloids- Mayers Test	
2.	Flavonoids- Alkali reagents	+
3.	Tannin- $Fecl_3$ test	+
4.	Saponins- Frothing test	+
5.	Terpenoids - Nollers test	+
6.	Glycosides- Keller-Killiani Test	--
7.	Polyphenols-Ferrozine	+
8.	Anthocynin- Ammonia Test	+

+ indicate positive result; -- Indicate negative result

The partial characterization of acetone extract of *Rubia Cordifolia* by TLC

The acetone extract of *Rubia cordifolia* loaded on Pre-coated TLC plates (60F 254 Merck) and developed with a solvent system of hexane, ethyl acetate and acetic acid in the ratio of 10:5:0.5. The developed plate was viewed under UV 240nm and 360nm. The Rf value of compounds were shown in Fig-1.

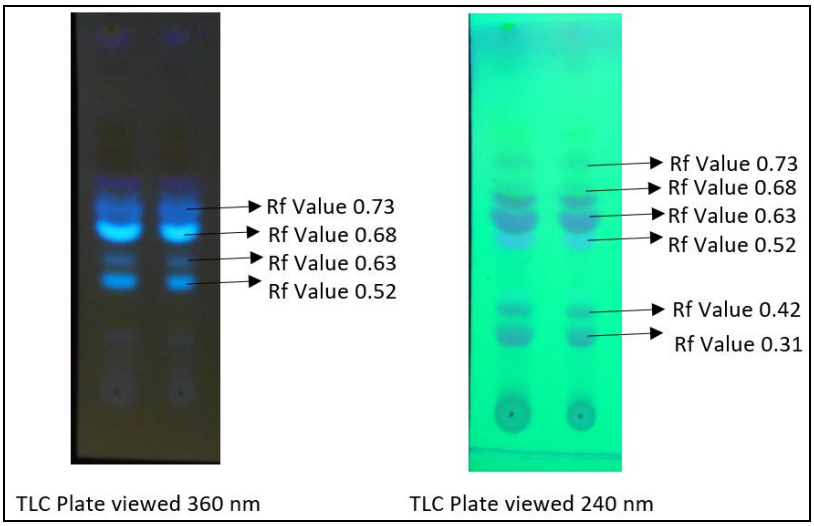

Fig 1: TLC profile of acetone extract of *Rubia cordifolia*

Free Radical-Scavenging Ability Using ABTS Assay

The radical scavenging ability was measured by ABTS assay as per given in table 4. The inhibition percentage of the ABTS radical activity was assessed on average and high free radical-scavenging values were found in acetone extract of *Rubia cordifolia.* In ABTS assay, inhibition percentage was high in acetone extract of *Rubia cordifolia* 68.34 % than standard vitamin-C 65.37% with EC_{50} value 76.32 µl/ml. The pure ascorbic acid was lower activity (Table-2). Nevertheless, in present study, it is showed that these activities were mainly due to anthocyanin and flavonoids compounds. It is known that vitamin C (ascorbic acid) and carotenoids are chief source of discrepancy of antioxidant/ antiradical activities in plant materials.

Table 2: Free radical-scavenging ability using ABTS assay

Different concentration of extract	% of ABTS radical activity	
	Acetone extract of *Rubia cordifolia*	ABTS radical activity Standard Vitamin-C
25 µl/ml	17.23±0.96	14.32±2.67
50 µl/ml	31.45±2.46	29.34±1.79
75 µl/ml	51.34±1.79	48.34±2.67
100 µl/ml	68.34±0.89	65.37±0.89
EC_{50} value	73.32	76.32

[a] Results are expressed as percentage inhibit of ABTS ability with respect to control. Each value represents the mean+SD of three experiments

Inhibition of Lipid Peroxidation Activity

Acetone extract of *Rubia cordifolia* also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates. Maximum inhibition was recorded in acetone extract of *Rubia cordifolia* 81.32% with EC_{50} value 61.23 µl/ml and lowest inhibition percentage ascorbic acid 73.64% with EC_{50} 68.34 (Table-3). As it is identified that lipid peroxidation is the net result of any free radical attack on membrane and other lipid components present in the system, the lipid peroxidation may be enzymatic (Fe/NADPH) or non-enzymatic (Fe/ascorbic acid). In the present study, egg yolk was used as substrate for free radical mediated lipid peroxidation, which is a non-enzymatic method. Generally, the mechanism of phenolic compounds for antioxidant activity includes neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals.

Table 3: Inhibition of lipid peroxidation activity of acetone extract of *Rubia cordifolia*

Different concentration of extract	Lipid peroxidation inhibition percentage	
	Acetone extract of *Rubia cordifolia*	Standard Vitamin-C
25 µl/ml	22.64±0.45	17.36±0.64
50 µl/ml	36.64±2.78	34.34±2.83
75 µl/ml	57.38±1.89	52.31±0.67
100 µl/ml	81.32±2.78	73.64±2.45
EC_{50} value	61.23	68.34

[a] Results are expressed as percentage inhibit of lipid peroxidation with respect to control. Each value represents the mean+SD of three experiments.

Superoxide Scavenging Assay Activity

Acetone extract of *Rubia cordifolia* exhibited powerful scavenging activity for superoxide radicals in a concentration dependent process than positive control. Acetone extract of *Rubia cordifolia* showed highest radical activity in the percentage of 79.34% with EC_{50} value 63.32 µl/ml when compared to positive control 74.67% with EC_{50} Value 69.64 µl/ml and (Table-4). One of the standard method to produce Superoxide radicals is through photochemical reduction of nitro blue tetrazolium (NBT) in the presence of a riboflavin-light-NBT system. These superoxide radicals are extremely toxic and may be produced either through xanthine activity or through mitochondrial reaction. Superoxide radicals are reasonably a weak oxidant may decompose to form stronger reactive oxidative species, such as singlet oxygen and hydroxyl radicals (Pandey and Rizvi, 2009) [8].

Table 4: Superoxide scavenging assay activity of acetone extract of *Rubia cordifolia*

Different concentration of extract	Percentage of Superoxide scavenging activity	
	Acetone extract of *Rubia cordifolia*	Standard Vitamin-C
25 µl/ml	23.24±1.36	18.34±1.39
50 µl/ml	37.65±1.78	34.56±0.78
75 µl/ml	58.34±1.63	56.34±2.34
100 µl/ml	79.34±1.58	74.67±0.89
EC_{50} value	63.32	69.64

[a] Results are expressed as percentage of Superoxide scavenging activity with respect to control. Each value represents the mean+SD of three

Nitric Oxide Radical Scavenging Assay

Nitric oxide radical quenching activity of acetone extract of *Rubia cordifolia* were identified and compared with the standard ascorbic acid. The acetone extract of *Rubia cordifolia* displayed the maximum inhibition of 65.32% at a concentration of 100 µg/ml, in a concentration-dependent process when compared to ascorbic acid with inhibition percentage 62.34% (Table-5).

In the current study, nitrite was produced by incubation of sodium nitroprusside in standard phosphate saline buffer at 25°C was reduced by acetone extract of *Rubia cordifolia* were identified and compared with the standard ascorbic acid. Significant scavenging activity may be due to the antioxidant property of flavonoid, which compete with oxygen to react with nitric oxide, leading to less production of nitric oxide. It is a diffusible free radical that plays many roles as an effector molecule in diverse biological systems, including neuronal communication, vasodilatation and antimicrobial and antitumor activities. Moreover, in pathological conditions, nitric oxide reacts with superoxide anion and forms potentially cytotoxic molecules, such as peroxynitrite (Shahidi *et al*., 2007).

Table 5: Nitric oxide radical scavenging assay of the acetone extract of *Rubia cordifolia*

Different concentration of extract	Percentage of Nitric oxide radical scavenging activity	
	Acetone extract of *Rubia cordifolia*	Standard Vitamin-C
25 µl/ml	15.10±2.34	14.35±2.37
50 µl/ml	30.21±1.63	27.34±1.78
75 µl/ml	48.34±1.45	45.37±1.67
100 µl/ml	65.32±1.12	62.34±0.45
EC_{50} value	72.34	76.32

[a] Results are expressed as percentage of Nitric oxide radical activity with respect to control. Each value represents the mean+SD of three experiments.

Inhibition of Pancreatic Lipase Activity

In the present study, acetone extract of *Rubia cordifolia* have been evaluated for lipid lowering activity through percentage inhibition of pancreatic lipase. Table-9 shows the results of pancreatic Lipase inhibition of the aqueous extract of selected medicinal plants at various concentrations. From the data of the results obtained, maximum percentage of lipase inhibition was shown by acetone extract of *Rubia cordifolia* (74.31 %). The standard orlistat has shown minimum activity (70.34 %) (Table-6). Pancreatic lipase inhibition is the most widely studied mechanism for the identification of potential anti-obesity agents. Only one blockbuster drug, Orlistat approved by FDA and available for the obesity treatment apart from the centrally acting anti-obesity drugs, is acting through the pancreatic lipase inhibition.

Table 6: Inhibition of pancreatic lipase activity of acetone extract of *Rubia cordifolia*

Different concentration of extract	Inhibition percentage of pancreatic lipase activity	
	Acetone extract of *Rubia cordifolia*	Standard Orlistat
25 µl/ml	20.31±1.13	15.34±2.78
50 µl/ml	39.64±2.14	34.65±1.36
75 µl/ml	55.34±1.18	51.78±1.45
100 µl/ml	74.31±1.19	70.34±2.36
EC_{50} value	68.32	71.34

[a] Results are expressed as percentage of Inhibition of pancreatic lipase with respect to control. Each value represents the mean+SD of three experiments.

Conclusion

Polyphenols are valuable plant constituents for the scavenging of free radicals because of their phenolic hydroxyl groups. This, together with the obtained results, suggests that as the amount of polyphenolic compounds increases, the antioxidant and anti-obesity, activity also increases. In conclusion, the present study demonstrates that the acetone extract of *Rubia cordifolia* can protect the body from oxidative stress from ROS, which may be due to the phytochemicals in the form of polyphenols that occur in the plant. Thus the results of this study indicate that the commonly used green leafy of, *Rubia cordifolia* significantly inhibit the activity of pancreatic lipase which can be attributed to the presence of saponins, phenols, flavonoids or alkaloids which is comparable to orlistat. These may be used in nutraceuticals and the food industry.

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