Research Article

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IN VITRO EVALUATION OF SECONDARY METABOLITES AND HYDROXYL RADICAL SCAVENGING EFFICACY OF DIFFERENT EXTRACTS OF *CYPERUS ROTUNDUS* L. AND *RUBIA CORDIFOLIA* L.: PROTECTION AGAINST PHOTODAMAGES

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ABSTRACT

An antioxidant is simply a molecule that prevents another molecule from oxidizing. Overexposure to UV rays can lead to a significant reduction in the antioxidant supply, thus increasing oxidative stress. Herbal plants and their secondary metabolites have a long history to use in modern western medicine and in certain systems of traditional medicine and are the sources of important drugs. The antioxidant compounds, which are present in herbal plants in the form of secondary metabolites like phenolic acids, poly phenols and flavonoids scavenge free radicals such as peroxide and hydroperoxide. This study is aimed to investigate the secondary metabolites and antioxidant potential of ethanolic and acetone extracts of *Cyperus rotundus* L. and petroleum ether and 80 % ethanolic extracts of *Rubia cordifolia* L. using *in vitro* models in the reference of protection against photo damages. Different extracts of *Cyperus rotundus* L. and *Rubia cordifolia* L. were evaluated for their organoleptic properties, physicochemical properties, preliminary secondary metabolites analysis and hydroxyl radical scavenging potential with the help of standard procedures. Both extracts of *Cyperus rotundus* L. and *Rubia cordifolia* L. were found to be positive for many secondary metabolites like; flavonoids, alkaloids, saponins, tannins etc. The IC_{50} values of ethanolic and acetonic extracts of *Cyperus rotundus* were observed at 87 µg/ml and 98.2 µg/ml respectively and the IC_{50} values of pet ether and 80 % ethanolic extracts of *Rubia cordifolia* were observed at 100 µg/ml and 50 µg/ml respectively. Based on this, it may be concluded that, the herbal plants *Cyperus rotundus* L. and *Rubia cordifolia* L. are the rich source of secondary metabolites and have hydroxyl/free radical scavenging potential which shows antioxidant nature of the extracts against UV induced oxidative stress mediated photo damages.

Keywords: Antioxidants, *Cyperus rotundus,* Oxidative stress, Photo damages, *Rubia cordifolia,* Secondary metabolites

INTRODUCTION

An imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage, is termed 'oxidative stress'. Oxidants are formed as a normal product of aerobic metabolism but can be produced at elevated rates under patho-physiological conditions. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Further, some reactive oxidative species act as cellular messengers in redox signaling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling. A free radical is an atom, molecule, or compound that is highly unstable because of its atomic or molecular structure (i.e., the distribution of electrons within the molecule). As a result, free radicals are very reactive as they attempt to pair up with other molecules, atoms, or even individual electrons to create a stable compound. To achieve a more stable state, free radicals can "steal" a hydrogen atom from another molecule, bind to another molecule, or interact in various ways with other free radicals. The main characteristic of an antioxidant is its ability to trap free radicals. Antioxidants fill those electron needs without becoming free radicals themselves. The characteristic changes to skin induced by chronic UVA and UVB exposure is called Photo-aging or Photo-damages. The skin contains several antioxidants, including vitamin E, coenzyme Q10, ascorbate, carotenoids, superoxide dismutase, catalase and glutathione peroxidase. These antioxidants provide protection from reactive oxygen species produced during normal cellular metabolism. However, over exposure to UV rays can lead to a significant reduction in the antioxidant supply, thus increasing oxidative stress. Hence, these antioxidants are essential in the skin's defense mechanism against UV radiation and photo carcinogenesis.

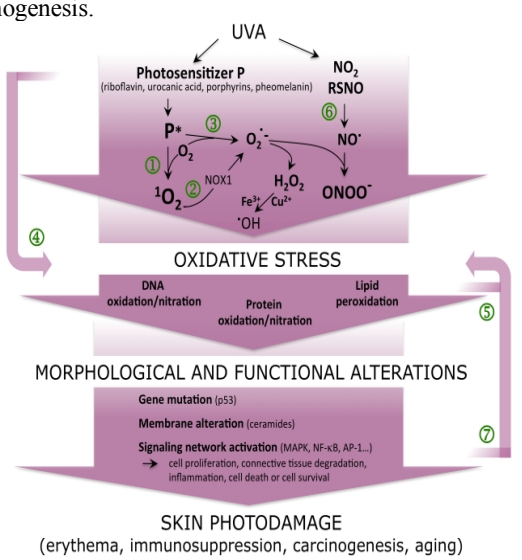


Figure 1: Effect of UV induced oxidative stress in causing and leading skin damages

Since origin of human's life, plants continue to play a curative and therapeutic role in preserving human's health against disease. The medicinal property of any herbal plant lies in some chemical substances known as secondary metabolites which produce a definite physiological action on human body. Phytochemicals or secondary metabolites are biologically active nonnutritive chemical compounds that occur naturally in plants. Some of these are alkaloids flavonoids, terpenoids, saponins, tannins, phenols etc[1]. Traditionally a lot of medicinal plants are used for treatment of ailments in human beings. These metabolites possess antipyretic, analgesic, antioxidant, anti-cancerous, anti-diabetic and many other important properties. For example flavonoids are a class of secondary plant metabolites that are thought to exert beneficial health effects through their antioxidant and chelating properties being the major contributor to the antioxidant capacity of vegetables[2,3]. Vitamin C has an antioxidant activity when it reduces oxidizing substances such as hydrogen peroxide[4], however, it will also reduce metal ions that generate free radicals. Thus, consuming a diet rich in antioxidant fruits, vegetables, herbs etc. will provide health-protective effects. *Cyperus rotundus* Linn belong to the family Cyperaceae. It is the world worst weed native to India and commonly known as 'Nagarmotha'. It grows in small clump up to 100 cm high. The extensive distribution of the nut-grass is due to its ability to adapt to a wide range of soil types, altitudes, temperatures, soil pH and moisture levels. It has wide range of medicinal and pharmacological applications. According to the Ayurveda, *C. rotundus* rhizomes are considered astringent, diaphoretic, diuretic, analgesic, antispasmodic, aromatic, carminative, anti tussive, emmenagogue, litholytic, sedative, stimulant, stomachic, vermifuge, tonic and antibacterial[5]. *Rubia cordifolia* Linn is a climbing or scrambling herb, with red rhizomatous base and roots belongs to the family Rubiaceae and commonly known as 'Manjistha'. *R. cordifolia* is considered as tonic, anti tussive and useful in chronic low fevers. It is used to cure tuberculosis and intestinal ulcer. Manjistha is highly recommended in skin diseases like hyper pigmentation, scabies, acne and allergies, edema and oozing. The wound and ulcers dressed with Manjistha ghrita heal promptly and get dried up and well cleansed. Especially the chronic non-healing and cozying wounds respond very well. The burns and scalds heal up magically without scar formation, when treated with Manjistha ghrita. Manjistha is valuable in a vast range of diseases[6].

MATERIAL AND METHOD

Collection of Plant material

Fresh plant parts were collected from Bhopal and forests of the Hoshangabad (M.P.), India having the rich diversity of medicinal plants by the permission of forest authorities. Plant material was washed thoroughly and shade dried at room temperature. The material was crushed using mortar- pestle and grinding machine. Powders were stored at room temperature in airtight containers.

Identification & Authentication of Plants

The root part of *Rubia cordifolia* and *Cyperus rotundus* were authenticated by Botanist, Dr. Zia Ul Hasan, Prof. & Head, Dept. of Botany, Safia Science College, Bhopal, Madhya Pradesh (India). The Voucher specimen No. for *Rubia cordifolia* root is 513/Bot/Safia/2015 and for *Cyperus rotundus* root is 514/Bot/Safia/2015.

Chemicals

All the Chemicals and Reagents used for the study were reagent grade and purchased from CDH, Renchem and Hi-Media Ltd., India.

Extraction

Cyperus rotundus (Nagarmotha) powder was extracted by hot extraction at 80^0C using 80 % Ethanol. The powder was also macerated with acetone at room temperature. The collected residues were kept at 45^0C in water bath to concentrate it and finally transfer into the hot air oven to dry it. Both the extracts were collected, dried, weighed and tagged as Ngm1 and Ngm2 respectively. *Rubia cordifolia* (Manjistha) powder (100 g) was extracted with petroleum ether at 70^0C using soxhlet; another 100 g was extracted using soxhlet at 80^0C by 80 % Ethanol. The collected residues were kept at 45^0C in water bath to concentrate it and finally transfer into the hot air oven to dry it. Extract was collected, dried, weighed and tagged as M1 and M2 respectively.

Organoleptic properties

The macroscopic characters like color, odor and consistency of the different parts of different plants were observed as per the reported method[7].

Physico-Chemical analysis

Various physico-chemical parameters such as ash value, acid insoluble ash and extractive values were examined for the samples according to reported methods[8].

Preliminary Phytochemical/Secondary metabolites analysis

Phytochemical technique mainly applies to the quality control of herbal medicine of various chemical components, such as saponins, alkaloids, flavonoids phenolic compounds etc. Different extracts were screened for presence or absence of secondary metabolites such as alkaloids, tannins, phenols, flavonoids, saponins, etc. using standard procedures to identify the constituents as described by Harborne[9].

Test for Carbohydrates

Dissolved small quantities of extracts in double distilled water and filtered. The filtrate was subjected to Molisch's test to detect the presence of carbohydrates.

Test for Alkaloids

3 ml of each extract was stirred with 3 ml of 1 % HCl on steam bath. After that the extracts were cooled at room temperature then Mayer's and Wagner's reagents were added to mixture. Turbidity of the resulting precipitate was taken as an evidence for the presence of alkaloid.

Test for Tannins

About 2 ml of the each extract was stirred with 2 ml of double distilled water and few drops of 1 % $FeCl_3$ Solution were added. Formation of blue, green or brownish green color indicated the presence of tannins.

Test for Saponins

A small amount of extracts were shaken separately with 4 ml of double distilled water in a test tube and warmed. The formation of stable foam was taken as an evidence for the presence of saponins.

Test for Flavonoids

About 3 ml of test sample of each extract was mixed with 1 ml of 10 % lead acetate solution. The formation of a yellow precipitate was taken as a positive test for flavonoids.

Test for Terpenoids

2 ml of the extracts were dissolved in 2 ml of chloroform separately and evaporated to dryness. Then 2 ml of concentrated sulphuric acid was added and heated for about 2 minutes. Development of a greyish color indicates the presence of terpenoids.

Tests for Glycosides

- **Sodium hydroxide reagent**
  Dissolved a small amount of extracts in 1 ml of double distilled water and added sodium hydroxide solution. Development of yellow color indicates the presence of glycosides.

- **Keller-Killani's test**
  Dissolved few amount of extracts in double distilled water followed by glacial acetic acid. Than one drop of 5 % $FeCl_3$ and conc. H_2SO_4 was added. Formation of reddish brown color at the junction of two liquid layers indicates the presence of glycosides.

Test for Phenols

The dried plant extracts about 100 mg was dissolve separately in double distilled water; few crystals of ferric sulfate were added. Formation of dark violet color indicates the presence of phenolic compound.

***In vitro* hydroxyl radical scavenging analysis**

Direct antioxidant activity and free/hydroxyl radical scavenging potential indirectly through stimulation of cellular antioxidant was tested for ethanolic and acetone extracts of *Cyperus rotundus* L. and petroleum ether and 80 % ethanolic extracts of *Rubia cordifolia* L. *In vitro* antioxidant activity of these different plant extracts were determined according to the De-oxyribose method of Halliwell *et al.,* (1987)[10]. The measurement of TBARS thus gives an index of free radical scavenging activity. The absorbance was measured at 532 nm. Ascorbic acid was used as positive control. The percentage inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = (\text{Control} - \text{Test})/ \text{Control} \times 100$$

RESULTS

Organoleptic properties

The extracts were evaluated for their organoleptic properties. The color, odor and consistency of the extracts were observed. Results are cited in Table 1.

Physicochemical analysis

The extractive Values of different extracts were calculated for all samples in which the acetone extract of *Cyperus rotundus* showed minimum extractive value whereas, the ethanolic showed maximum. In the case of *Rubia cordifolia* the ethanolic extract showed higher extraction value in the comparison of petroleum ether extract. The results are cited in Table 2a and 2b.

Preliminary phytochemical analysis

The medicinal value of plants lies in some chemical substances known as secondary metabolites that have a definite physiological action on human body to fight with different ailments. The ethanolic extract of *Cyperus rotundus* was found to be positive for the presence of carbohydrates, alkaloids, phenols, tannins, glycosides, flavonoids, saponins and acetone extract, for the presence of alkaloids. Petroleum ether extract of *Rubia cordifolia* was observed positive for the presence of carbohydrates, tannins phenols and the 80 % ethanolic extract, was positive for the carbohydrates, tannins, phenols, flavonoids and alkaloids. The results are presented in Table 3.

***In vitro* hydroxyl radical scavenging potential**

The ability of ethanolic and acetonic extracts of *Cyperus rotundus,* petroleum ether and 80 % ethanolic extracts of *Rubia cordifolia* to scavenge OH ' radical was assessed using the Fenton reaction assay. The Hydroxyl radical attacked to de-oxyribose and initiated a series of reaction that eventually resulted in the formation of Thiobarbituric acid reaction substances (TBARs). Extent of hydroxyl radical scavenged was determined by decreased intensity of pink colored chromospheres in the form of IC_{50} values. Lower IC_{50} values represent higher antioxidant activity.

The dose dependent inhibitions of TBARS formation at the different concentrations of these plants extracts ranging from 10 µg/ml to100 µg/ml. These values were compared to Ascorbic acid, which was used as positive Control. The graphs, % of Inhibition versus different concentrations of ethanolic and acetonic extract of *Cyperus rotundus* were plotted against ascorbic acid. In which IC_{50} values of ascorbic acid was found to be 46.4 µg/ml and ethanolic and acetone extract of *Cyperus rotundus* was observed at 87 µg/ml and 98.2 µg/ml respectively.

Table 1: Organoleptic properties of different extracts

S. No.	Plant Samples	Extracts	Color	Odour	Consistency
1.	*Cyperus rotundus* (Root)	Ngm1 (Ethanolic)	Chocolate	Pleasant	Powder
		Ngm2 (Acetone)	Dark brown	Pungent	Waxy
2.	*Rubia cordifolia* (Root)	M1 (Petroleum ether)	Dark yellow	Pleasant	Sticky powder
		M2 (80 % ethanolic)	Dark maroon	Pleasant	Viscous

Table 2a: Ash values of different plant powders

S. No.	Plant Sample	Total Ash (in %)	Acid Insoluble Ash (in %)
1.	*Cyperus rotundus* (Root)	11.95	4.85
2.	*Rubia cordifolia* (Root)	7.12	5.28

Table 2b: Percentage extraction values of all the plants with reference to different solvents

S. No.	Plant samples	Extracts	Wt. of Sample (g)	Wt. of Extract (g)	% Extraction (yield)
1.	*Cyperus rotundus* (Root)	Ngm1(Ethanolic)	100	3.27	3.27 %
		Ngm2 (Acetone)	100	0.86	0.86 %
2.	*Rubia cordifolia* (Root)	M1 (Petroleum ether)	100	0.56	0.56 %
		M2 (80 % ethanolic)	100	16.34	16.34 %

Table 3: Preliminary phytochemical screening of secondary metabolites in different plant extracts

S. No.	Plant samples	Extracts	Secondary Metabolites							
			C	A	G	T	P	Te	F	S
1.	*Cyprus rotundus* (Root)	Ngm1 (Ethanolic)	+	++	+	+	+	-	++	+
		Ngm2 (Acetone)	-	+	-	-	-	-	-	-
2.	*Rubia cordifolia* (Root)	M1 (Pet ether)	+++	-	-	+	+	-	-	-
		M2 (80 % ethanolic)	+	++	-	++	++	-	+	-

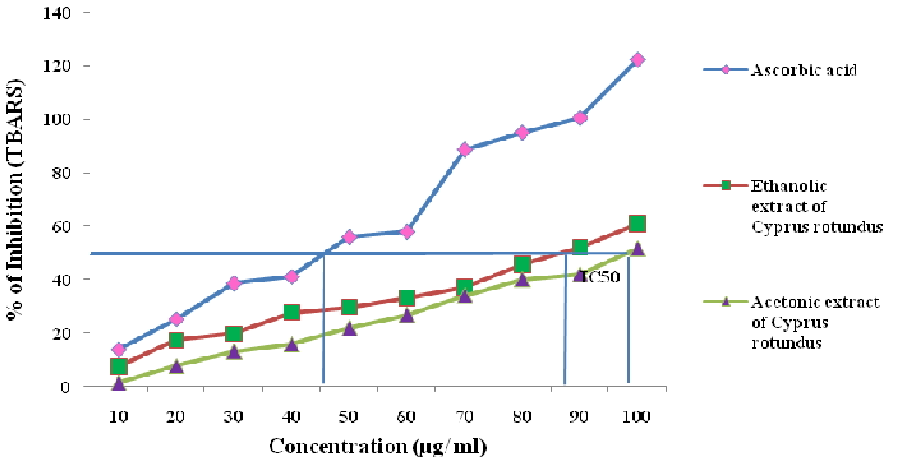
C – Carbohydrate, A – Alkaloid, G – Glycosides, T – Tannin, P – Phenol, Te – Terpenoid, F – Flavonoid, S – Saponin, '+++' Present in good amount; '++' Moderate Amount; '+' Less but present; '-' Absent

Table 4: *In vitro* Anti-oxidant potential of ethanolic and acetonic extracts of *Cyperus rotundus* in the comparison of Standard Anti-oxidant

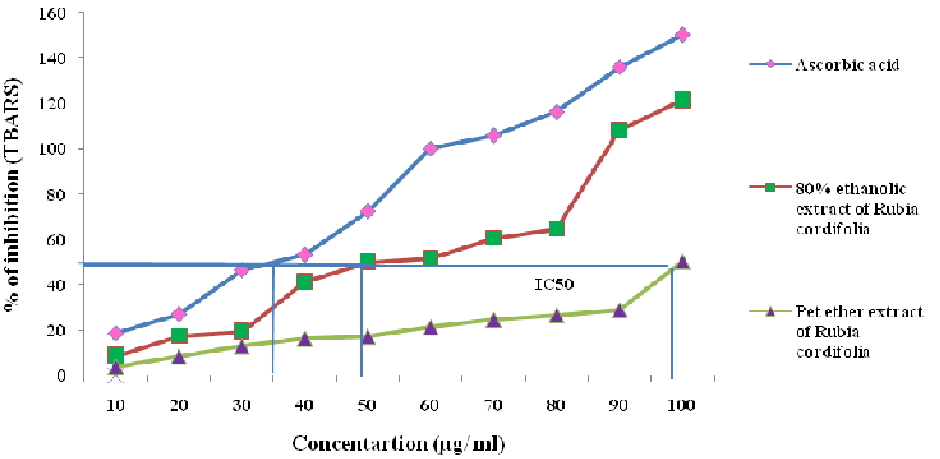
S. No.	Concentration (In µg/ml)	% Inhibition of Ascorbic Acid (In TBARS)	% Inhibition of ethanolic extract of Ngm1 (In TBARS)	% Inhibition of acetone extract of Ngm2 (In TBARS)
1	10	13.86	7.42	1.52
2	20	25.24	17.32	7.98
3	30	38.61	19.80	13.30
4	40	41.08	27.72	15.96
5	50	55.94	29.70	22.05
6	60	57.92	33.16	26.99
7	70	88.61	37.12	34.22
8	80	95.04	45.54	40.30
9	90	100.49	51.98	42.20
10	100	122.27	60.89	51.76

Table 5: *In vitro* Anti-oxidant potential of petroleum ether and 80 % ethanolic extracts of *Rubia cordifolia* in the comparison of standard anti-oxidant

S. No.	Concentration (In µg/ml)	% Inhibition of Ascorbic Acid (In TBARS)	% Inhibition of pet ether extract of M1 (In TBARS)	% Inhibition of 80 % ethanolic extract of M2 (In TBARS)
1	10	18.77	3.88	8.73
2	20	27.18	8.41	17.47
3	30	46.60	12.94	19.41
4	40	53.39	16.18	41.42
5	50	72.49	17.15	50.16
6	60	100.00	21.35	51.77
7	70	105.82	24.59	60.51
8	80	116.18	26.53	64.72
9	90	135.92	28.80	108.41
10	100	150.16	50.16	121.68



Graph 1: Antioxidant activity of ethanolic and acetone extracts of *Cyperus rotundus* Linn



Graph 2: Antioxidant activity petroleum ether and 80 % ethanolic extracts of *Rubia cordifolia* Linn

The graphs, % of Inhibition versus different concentrations of petroleum ether and 80 % ethanolic extracts of *Rubia cordifolia* were plotted against ascorbic acid. In which IC_{50} values of ascorbic acid was found to be 35 µg/ml and petroleum ether and 80 % ethanolic extracts of *Rubia cordifolia* were observed at 100 µg/ml and 50 µg/ml respectively. Results are summarized in Table 4 and 5 and also represented in graphs 1 and 2.

DISCUSSION

Medicinal plants are of great importance to the health of individuals and communities. As all the plants are able to synthesize a multitude of organic molecules/ phytochemicals, they are referred to as "secondary metabolites"[11]. The medicinal plants are rich source of secondary metabolites like alkaloids, glycosides, steroids and flavonoids, which are potential source of drugs. Nearly one third of the pharmaceuticals are plant origin[12]. Ash value is a criterion to judge the identity and purity of crude drugs. Extractive value is used for evaluating a crude drug as it gives idea about the nature of chemical constituent, soluble in that particular solvent which is used for extraction[1]. Prolonged human exposure to solar UV radiation may result in acute and chronic health effects on the skin, eye and immune system. Sunburn is the best-known acute effect of excessive UV radiation exposure. Over the longer term, UV radiation induces degenerative changes in cells of the skin, fibrous tissue and blood vessels leading to premature skin aging, photo-dermatoses and actinic keratoses[13]. Many natural and synthetic chemicals have been investigated in the recent past for their efficacy to protect against radiation-induced damage in biological systems. Natural antioxidants present in herbs and spices are responsible for inhibiting the severe consequences of oxidative stress. Phytochemicals especially plant phenolics constitute a major group of compounds that act as primary

antioxidants[14]. Herbs and spices contain certain polyphenols, flavonoids and phenolics which possess radical scavenging activity[13]. Present study explore the presence of many secondary metabolites in root extracts of *Cyperus rotundus* and *Rubia cordifolia* specially flavonoids, tannins, phenols, alkaloids which may be responsible for their hydroxyl radical scavenging potential. Many Ayurvedic natural products have properties to rejuvenate and protect the skin from environmental pollution, chemicals, atmospheric temperature fluctuation, UVA and UVB radiation, wrinkling, hyper-pigmentation (excessive tanning) and inflammation due to presence of secondary metabolites and their antioxidant nature. Naturally occurring herbal compounds such as phenolic acid, flavonoids and high molecular weight poly phenols are very useful for prevention of adverse effects of UV-R on the skin and also these herbal compounds which having its ability to stimulate the circulation of blood in skin and remove dead skin cells to giving fresher and younger appearance to the skin[15]. This study indicate that the herbal extracts of *Cyperus rotundus* and *Rubia cordifolia* root extracts may be include in the formation of good quality sunscreen. There are lot of herbal plants such as *Glycyrrhiza glabra*[16-18], *Tinospora cordifolia*[19], *Berberis aristata*[20], *Acacia catechu*[21] etc. evaluated for their phytochemical, antioxidant, anti mutagenic, hepatoprotective, etc activities.

CONCLUSION

This study may indicate the relationship between phytochemicals, antioxidants and UV-R induced skin disorders. The antioxidant activity of the herbal plants was evaluated by studying the UV absorption of the plants which show maximum antioxidant activity and develop sunscreen creams using them. Based on this it may be concluded that, the herbal plants *Cyperus rotundus* L. and *Rubia cordifolia* L. are the rich source of secondary metabolites and have hydroxyl/free radical scavenging potential which shows antioxidant nature of the extracts against UV induced oxidative stress mediated photo-damages.

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