



Original Research Article

Antimicrobial, Antioxidant Activities and Phytochemical analysis of Ethanolic extract of *Randia uliginosa* leaves

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ABSTRACT

Keywords

Disc diffusion, Resazurin, DPPH, superoxide, Hydroxyl radical, IC₅₀

Randia uliginosa is traditionally used to cure various ailments like detoxification, diabetes mellitus, gastrointestinal disorders helminthiasis etc, but very little chemical information is available for this species, hence in our preliminary studies an attempt was made to provide scientific evidences for this plant by selecting the Ethanolic extract of *Randia uliginosa* leaves. Phytochemical screening showed a wide variety of Phytoconstituents such as alkaloids, saponins phenols, flavonoids, tannins etc, antimicrobial activity evaluated against four clinical isolates *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillum niger* maximum activity was observed against *Staphylococcus aureus* (MIC 0.187mg/ml) followed by *Pseudomonas aeruginosa* and the fungal isolates (MIC 0.375mg/ml), and antioxidant potential was assessed by its ability to scavenge various free radicals like DPPH, Super oxide and the Hydroxyl radicals. IC₅₀ values of extract and the standard Ascorbic acid for stable free radical DPPH was found to be 95 and 30 µgs, for hydroxyl radical it was 90 and 40 µgs and that of super oxide radical was found to be 60 and 30 µg/ml. These results provide a scientific evidence for the traditional use of *Randia uliginosa* for the treatment of various diseases.

Introduction

Human beings are depending on botanicals directly or indirectly for the treatment of various ailments since ancient time, according to the World Health Organization, reports nearly 80%

psychological and physical health (WHO, 2002), mainly because of the two reasons one is they cannot afford the products of Western pharmaceutical industries and the second reason is due to

side effects of the synthetic drugs followed by lack of healthcare facilities. India has a rich culture of medicinal herbs and spices, with high potential abilities for Ayurvedic, Unani, Siddha traditional medicines but only very few have been investigated chemically and pharmacologically for their potential medicinal value. Rural masses of many developing countries still rely on traditional medicine for their primary health care needs and have found a place in day-to-day life. These medicines are relatively safer and cheaper than synthetic or modern medicine (Iwu *et al.*, 1999; Idu *et al.*, 2007; Mann *et al.*, 2008;).

Several reports have been published on antimicrobial activity of different herbal extracts (Balaji and Hriharan, 2007; Sampathkumar *et al.*, 2008) and also various studies have been carried out on 'oxidative stress' and its adverse effects on human health which has become a subject of considerable interest, it is well known that free radicals are related to several diseases such as arteriosclerosis, hypotension, diabetes mellitus cancer, inflammation, renal failure, liver disease, etc (Tiwari, 2004; Govindarajan *et al.*, 2005). Many efforts have been made to discover new antimicrobial and antioxidant compounds from various kinds of sources such as microorganisms, animals and plants (Tamokou *et al.*, 2008; Potchoo *et al.*, 2008; Asmah *et al.*, 2006; Rached *et al.*, 2010).

Our earlier studies on different solvent extracts of *Randia uliginosa* stem bark extracts has proved antibacterial activity against some of the selected Oral pathogens. (Khasim, *et al.*). Hence the present study is carried out on, Ethanolic extract of *Randia uliginosa* leaf to explore phytochemical and some of the pharmacological properties

Randia uliginosa is a small tree belongs to the family Rubiaceae having outer reddish brown scaly bark, thick, short, horizontal, numerous branches with 1-2 pairs of strong sharp thorns. Fruits are long, ovoid, smooth, yellowish brown, crowned with the persistent calyx and multi seeded. April – August is the Flowering seasons.

Ethnomedicinal uses

Randia uliginosa is widely used in Indian system of medicine Ayurveda, Siddha and Unani medicine. In Ayurveda it is used to get rid of phlegm, bile and internal toxic substance. In Unani it is used as aphrodisiac, haematinic & cardiac tonic and also used in biliousness, dysuria and strangury. The ashes of unripe fruit roasted in firewood are used to treat many gastrointestinal disorders like diarrhoea and dysentery, cholera, boils and gastric troubles. Fruit powder with honey has anthelmintic property. Fruit juice is used a hair tonic, regular use of this makes hair free from dandruff and lice. Boiled fruit with sugar before dawn is given to treat migraine. It is also used for fish poisoning. Unripe fruit is eaten as vegetable either alone or together with other vegetables in curries. The leaves are boiled and eaten. They are also used as fodder for deer and cattle. Flowers yield an essential oil similar to Gardenia oil. (Srivastava, R and Pandey V.N, 2013).

Materials and Methods

Plant Collection

Fresh healthy (disease free) branches of *Randia uliginosa* was collected from its natural habitat, from the forest region of Wayanad District, Kerala. The plant was identified and authenticated from the Taxonomist University of Mysore. From

the collected fresh plant material leaves are separated, washed in water, shade dried at room temperature and this dried material is mechanically powdered in a grinder to obtain coarse powder then passed through 40 mesh sieve. This powdered material is used for extraction.

Extraction of Plant Material

About 50gm of powdered leaves of *Randia uliginosa* was subjected to extraction by a hot percolation method with 100ml Ethanol in Soxhlet apparatus. Extraction step was carried out until the extractive becomes colorless, the extract was concentrated by using flash evaporator and the dried extracts were stored at 4°C for further study.

Tests For Phytoconstituents

Phytochemical screening

The primary metabolites like proteins, carbohydrates and fixed oils, fats, etc and the secondary metabolites like, alkaloids, flavonoids, saponins, phenolics, tannins volatile oils, terpenoids, glycosides, etc were assessed as per the standard procedures. (Gulnaz.A.R & Savitha.G. 2013)

Total Phenolic content

The total Phenolic contents were estimated as per the method of Taga, Miller & Pratt (1984). Briefly the, extract was mixed with two ml of 2% sodium carbonate and allowed to stand at room temperature for 2 min. Then, 100µl of 50% Folin Ciocalteu (F.C) reagent will be added and the reaction mixture was allowed to stand at room temperature for 30 min and readings were taken at 720nm. Gallic acid was used as standard for the calibration curve.

Total Flavonoid content

The amount of total Flavonoid was estimated as per the method of Decolour & Pratt (1984). Briefly the test extract was mixed with 5ml of Cinnamaldehyde and allowed to stand at room temperature for 30 min. The absorbance was measured against the blank at 640nm. The concentration of Total Flavonoid content was calculated from the standard calibration curve of Rutin.

Antimicrobial activity

Collection of Microbes

Human pathogenic organisms (bacteria and fungi) were isolated from clinical samples obtained from Department of Microbiology, Farooqia Dental College & Hospital Mysore, India. The organisms are identified and confirmed by clinical microbiologist. The organisms under study were *Staphylococcus aureus*, *Pseudomonas aeruginosa* *Candida albicans* and *Aspergillum niger*

Antimicrobial activity by Disc diffusion method

The disc diffusion assay was carried out as per the methods of Khasim, *et al.* with slight modifications Briefly, About 20 to 25ml of Muller Hinton agar for bacterial culture and Sabouraud dextrose agar for fungal culture (as a nutrient medium) was poured in the sterilized petridishes and allowed to solidify.

The inoculums of microorganisms were adjusted to 0.5 McFarland standards, and with the help of sterile swab test organisms are inoculated petridishes.

Application of disc on the Muller Hinton agar media

Discs of different concentration (50,100 and 200 µgs) were prepared using watmann filter paper. All the sample discs and standard disc (Ampicilin & Nystatin were used for bacteria and fungi respectively) were applied on microbes' inoculated plate with the help of flame sterilized forceps, followed by refrigeration of the plates for 30 min and incubation for 24hrs at 37⁰C for bacteria and at room temperature for fungi. After 24 hours Antimicrobial activities were evaluated by measuring inhibition zone (clear zone) diameters around the discs. Extracts with zones of inhibition greater or equal to 8-mm diameter were regarded as positive.

MIC by 96 well Resazurin based Microtiter Dilution

As per the standard procedures. (Gulnaz.A.R & Savitha.G. 2013), with little modification in brief under aseptic conditions, 96 well micro titre plates (Tarson) were used for Resazurin based Micro titre Dilution Assay. The first row of micro titer plate was filled with 50 µl of test materials in 10% (v/v) DMSO. All the wells of micro titer plates were filled with 50 µl of nutrient broth. Two fold serial dilution (throughout the column) was achieved by starting transferring 50 µl test material from first row to the subsequent wells in the next row of the same column and so that each well has 50 µl of test material in serially descending concentrations. 15 µl of Resazurin solution as indicator was added in each well, finally 15 µl of 0.5 McFarland standards microbial suspension is added to the wells. To avoid the dehydration plate were wrapped loosely with cling film. Each micro titre plate had a set of 3

controls: (a) a column with Ampicilin for Bacteria and Nystatin for fungal strains were used as positive control, (b) a column with DMSO blank control, and (c) a column with all solutions with the exception of the test extract/standard-negative control. The plates were incubated for 24 hrs at 37⁰ bacteria and at room temperature for fungal isolates. The color change in the well was observed visually, any color change observed from purple to pink or colorless was taken as positive. The concentration of leaf extract at which color change occurred was recorded as the MIC value.

Antioxidant Activity

DPPH scavenging activity

The antioxidant activity of the extract was measured based on the scavenging activity of the stable DPPH free radical. The activity was determined by the method described by Braca *et al* (2001). Briefly the plant extract/standard in different concentrations was added to 3ml of 0.004% DPPH solution. One ml of methanol in place of plant extract will be used for control. Absorbance was determined at 520nm after 30min and percent of inhibition will be calculated by formula

$$A_0 - A_t / A_0 \times 100$$

Where A₀= absorbance of control,

A_t= absorbance of extract

Superoxide scavenging activity

The scavenging activity of the extract for superoxide anion radicals was measured by the method of Liu & Chang (1997),briefly superoxide anions was generated in 3ml Tris HCl buffer containing 0.75 ml of NBT (300µM) solution,0.75 ml of NADH (936 µM) and different concentrations of the

extract/standard were added. Reaction was started by adding 0.75 ml of PMS solution (120 μ M). The mixtures were allowed to stand for 5 min at room temperature. The absorbance was read at 560 nm. The superoxide scavenging activity was calculated according to the following equation

$$\text{Scavenging rate} = [1 - (A_1 - A_2/A_0)] \times 100$$

Where; A_0 = absorbance of control A_1 = absorbance of extract A_2 = absorbance without PMS

Hydroxyl radical scavenging activity

The ability of the extract hydroxyl radical was carried out according to Halliwell *et. al.*(1987). The reaction mixture containing extract/standard of various concentrations, 500 μ l (5.6 mM) of deoxy ribose in KH_2PO_4 / NaOH Buffer (50mM, pH 7.5), 200 μ l of premixed 100mM FeCl_3 and 10 mM EDTA (1:1v/v) solution, 100 μ l of H_2O_2 (1.0mM) was taken in the test tubes. The tubes were vortexed and incubated at 50 $^\circ$ C for 30 min, thereafter 1ml of 2.8% TCA and 1ml of 1% TBA will be added and kept in a water bath for 30 min and cooled. The absorbance of the solution was taken at 540 nm. The extent of oxidation will be calculated by the formula

$A_c - A_s \times 100/A_c$, Where A_c = absorbance of control, A_s = absorbance of extract.

Statistical analysis: All the experiments were done in triplicate and data thus obtained was reported as mean \pm standard deviation (SD).

Results and Discussion

Phytoprofile of Ethanolic extract of *Randia uliginosa* leaf is presented in table -1, yield was found to be 5.8%. Phytochemical analysis revealed the presence of important phyto constituents

which is presented in table-2, the important Phyto constituents were primary metabolites like, Carbohydrates, Proteins and secondary metabolites were alkaloids, saponin tannins, flavonoids and, glycosides, and phenols but phytosterols and fixed oil were absent.

Alkaloids present in the extracts have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity, Saponins present in the extracts suggests that this plant may become one of the possible source in the treatment of cancer, The presence of tannins indicates that it is useful in the treatment of inflammatory conditions like ulcers and they have remarkable activity in cancer prevention. The Flavonoids present in extracts are of great importance, as these are involved in cell protection via their action on membrane permeability, and by inhibiting membrane-bound enzymes such as the ATPase and phospholipase A_2 . Cardiac glycosides are drugs used in the treatment of congestive heart failure and cardiac arrhythmia. Presence of phenols indicates that *Randia uliginosa* leaf has antioxidant potential. (Gulnaz.A.R & Savitha.G. 2013),

Total Phenolic and content Flavonoids content

Total Phenolic contents of the extract is expressed as Gallic acid equivalent, the total Phenolic content was found to be 85.5mg GAE/gram. Standard graph of Gallic acid is shown in figure-1, and that of Flavonoids content is expressed as Rutin equivalents, the total Flavonoids content was found to be 45 mg Rutin/gram. Standard graph of Rutin is shown in figure-2. In vitro antimicrobial activity Ethanolic extracts of *Randia uliginosa* leaves against selected bacterial

& fungal clinical isolates is presented in table-3. Antimicrobial potential of Ethanolic extracts of *Randia uliginosa* leaves was determined in terms of zone of inhibition in comparison with Ampicilin for bacterial isolates and Nystatin for fungal isolates at different concentrations (50-200µgms/disc), the zone of inhibition was found to be 13mm-30mm for bacteria, and 12 mm-13mm for fungi. Highest activity was against *Staphylococcus aureus* at 200µgms/disc, good anti bacterial activity was also seen at 100µgms/disc, but no antimicrobial activity was seen at the concentration of 50µgms/disc, for *Pseudomonas aeruginosa* and fungal isolates antimicrobial activity was observed only at 200µgms/disc.

MIC of Ethanolic extracts of *Randia uliginosa* leaves by Resazurin based Micro titer Dilution assay (RMDA)

MIC values of Ethanolic extracts of *Randia uliginosa* leaves against different pathogenic clinical isolates are presented in table-4, the results obtained were promising the extract showed maximum activity against *Staphylococcus aureus* (MIC 0.187mg/ml) followed by *Pseudomonas aeruginosa* and the fungal isolates (MIC 0.375mg/ml) Ampicilin was used as a positive control which showed the MIC value of 0.187mg/ml and that of Nystatin was 0.375mg/ml. Antimicrobial activity of medicinal plants is probably due to the presence of secondary metabolites like alkaloids and flavonoids (Moghadam *et al*).

Antioxidant activity Ethanolic extracts of *Randia uliginosa* leaves

Ethanolic extracts of *Randia uliginosa* leaves extracts was assessed for anti oxidant potential by its ability to

scavenge various free radicals like DPPH, Super oxide and the Hydroxyl radicals.

DPPH radical scavenging activity

Commercially available DPPH (2, 2-diphenyl-1-picryl hydroxyl) was used as source of stable free radical. The DPPH scavenging activity of plant extracts and the standard antioxidant Ascorbic acid is given in Figure-3. The scavenging activity was found to be directly proportional to the concentration of the extract. The DPPH scavenging activity of the extract and the standard was found to be in the range of 38- 68% and 49-89% respectively, at concentration of 25-200µg/ml, The concentration required for 50 percent (IC₅₀) inhibition of stable free radical DPPH was found to be 95 and 30µg respectively for the extract and the standard.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the extract and standard Ascorbic acid is shown in Figure-4. The scavenging activity of extracts on the hydroxyl radical was measured by studying the competition between deoxyribose and test compounds for hydroxyl radical generated from the Fe³⁺/Ascorbate /EDTA/ H₂O₂ system.

The scavenging activity of the extracts was concentration dependent, the hydroxyl radical scavenging activity of the extract and the standard was found to be in the range of 21-68% and 38-81% respectively at concentration of 25-300µg/ml, IC₅₀ for inhibition of hydroxyl radical was found to be 90 and 40µg respectively for the extract and the standard.

Table.1 Preliminary Phyto-profile of Ethanolic extract of *Randia uliginosa* leaf

S.no	Solvents used	Color	Consistency	% of yield
1.	Ethanol	Brownish black	Non sticky	5.8

Table.2 Phytochemical analysis of Ethanolic extract of *Randia uliginosa* leaf

S.no	Phyto component	Tests	
1	Alkaloids	a.Mayer's test	+
		b.Wager'test	+
		c.Dragondoff' test	+
		d.Hager's test	+
2	Phytosterols /triterpenoids	a. Liebermann Test	—
		b. Salkowski Test	—
3	Saponins	a. Froth test	+
		b. Foam test	+
4	Carbohydrate	a.Molisch's test	+
		b. Benedict's test	+
5	Tannins	Gelatin test	+
6	Flavonoids	a. Alk. reagent test	+
		b. Lead acetate test	+
7	Glycosides	Borntrager's test	+
8	Fixed oil/fat	Spot test	-
9	Phenol	Ferric chloride test	+
10	Proteins	a.Biurete test	-
		b.Lead acetate test	+
		c.Xanthoproteic tes	+
		d.Ninhydrin test	+

Table.3 In vitro antimicrobial activity Ethanolic extracts of *Randia uliginosa* leaves against selected bacterial & fungal clinical isolates

S.no	Test organisms	Zone of Inhibition in mm				
		Ethanolic extract Conc.in µg			Ampicilin	Nystatin
		50	100	200		
1	<i>Staphylococcus aureus,</i>	---	16	30	22	---
2	<i>Pseudomonas aeruginosa</i>	---	---	13	14	---
3	<i>Candida albicans</i>	---	---	12	---	12
4	<i>Aspergillum niger</i>	---	---	12	---	14

*values are mean +/- SD triplicates experiments (-) means no activity above 8mm.

Table.4 Minimum inhibitory concentration (MIC, mg/ml) of Ethanolic extracts of *Randia uliginosa* leaves against selected bacterial & fungal clinical isolates by Resazurin micro titre-plate assay

S.no	Test organisms	Ethanolic extracts	Ampicilin	Nystatin
01	<i>Staphylococcus aureus</i> ,	0.18 7	0.18 7	-----
02	<i>P. aeruginosa</i>	0.375	0.18 7	-----
03	<i>Candida albicans</i>	0.375	-----	0.375
04	<i>Aspergillum niger</i>	0.375	-----	0.375

Figure-1

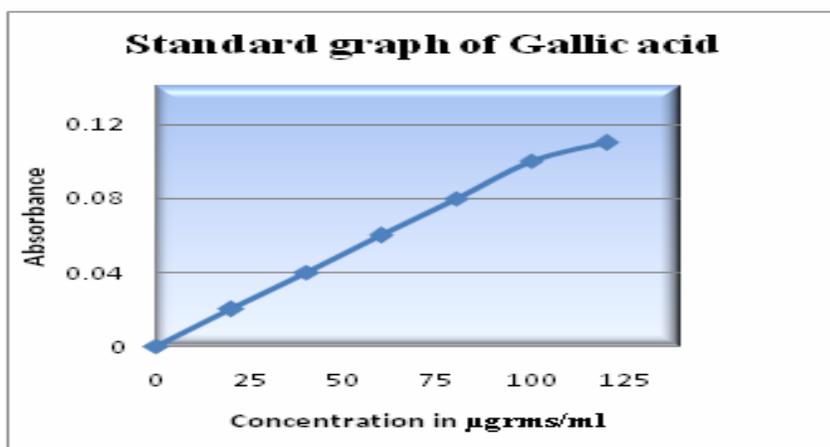


Figure-2

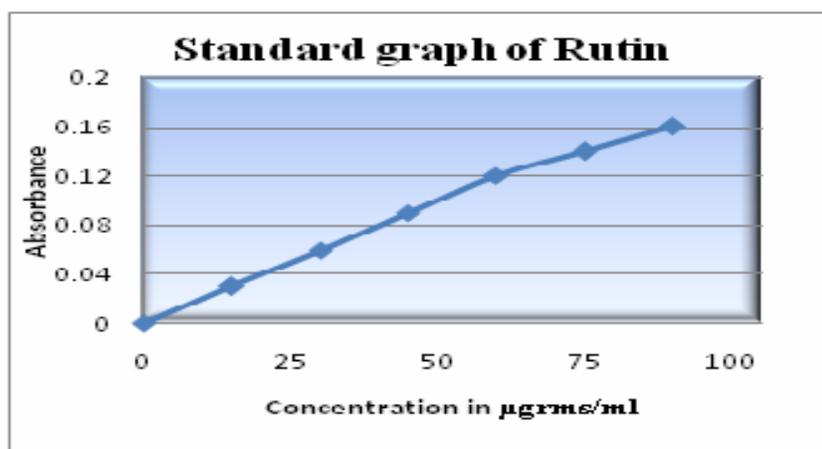


Figure-3

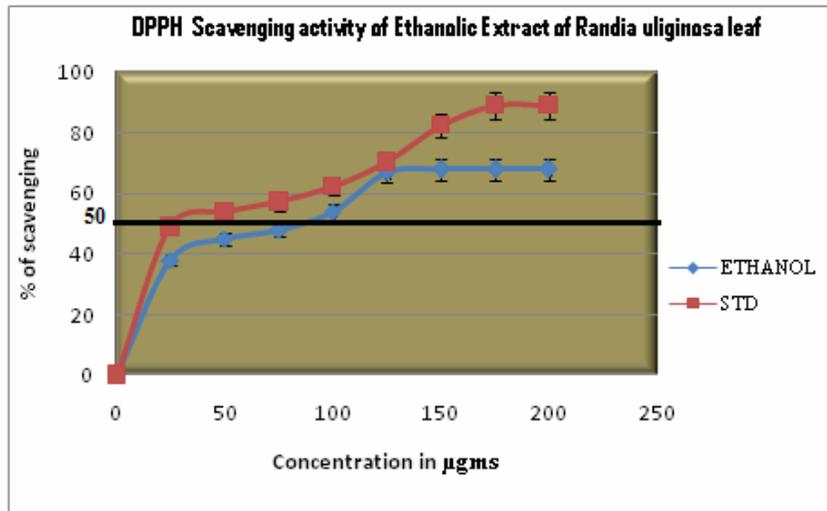


Figure-4

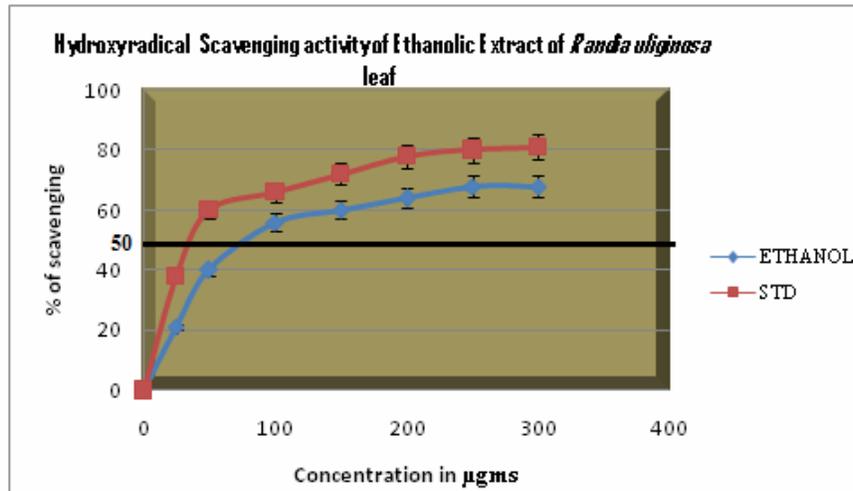
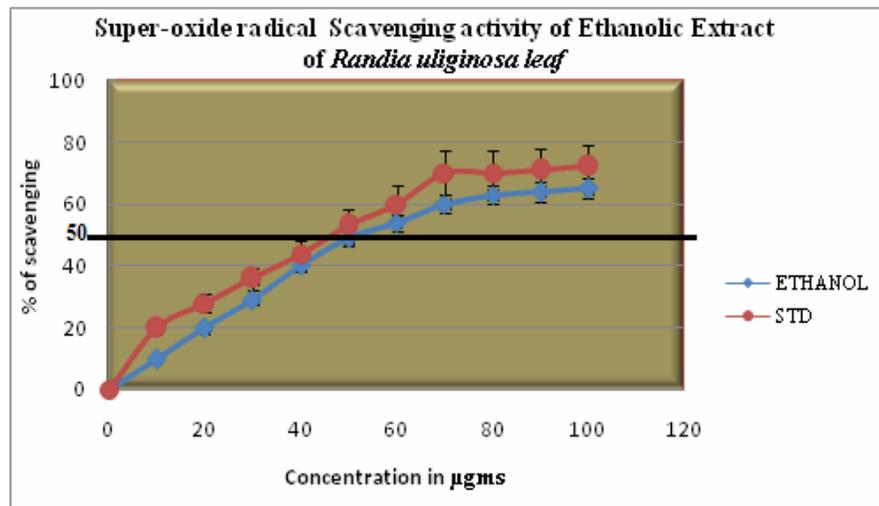


Figure-5



Super oxide scavenging

The scavenging activity of the extract and the standard for superoxide radicals generated in PMS-NADH is presented in figure-5. The scavenging activity was found to be increased with increase in concentration dependent. The superoxide radicals scavenging activity of the extract and the standard Ascorbic acid was found to be in the range of 10 to 65% and 20-72% respectively at concentration of 10-100µg/ml, IC₅₀ of extract and the standard was found to be 60 and 30 µg/ml.

The variation in scavenging different free radicals may be due to their different antioxidant mechanisms. A fair correlation between total Phenolic content and antioxidant activity was also observed. These observations clearly indicated a cross linkage between Phenolics and antioxidant activity. However a large number of Phyto-compound groups are implicated for antioxidant activity (Devasagayam TPA *et.al.* 2002) Many authors have also correlated antioxidant activity with their Polyphenolic or Phenolic contents, (Saleem *et.al.* 2001) (Kaur C & Kapoor H 2002).

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