International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.5, No.4, pp 1670-1678, Oct-Dec 2013

Preliminary Phytochemical Screening, Antibacterial and Nitric Oxide Radical Scavenging Activities of Reinwardtia indica Leaves Extract

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Abstract: The study was designed to examine antibacterial and nitric oxide radical scavenging activities of water and carbinol extract of leaves of *Reinwardtia indica* (Family-Linaceae). The antibacterial activity of extracts was evaluated by agar disc diffusion method on *Staphylococcus aureus* and *Pseudomonas aeruginosa* at different concentrations. Highest activity was seen against *P.aeruginosa* with carbinol extract. They also showed good NO radical scavenging activity. Carbinol and Aqueous extract has IC_{50} equal to 725µg/ ml and 975µg/ml respectively. This was compared against standard (Ascorbic acid) which has IC_{50} equal to 825µg/ml. Phytochemical analysis of leaves extract revealed the presence of antibacterial active as well as NO radical scavenging agents such as alkaloids, glycosides, steroids, flavonoids, terpenoids, carbohydrates and saponins. All these experimental analysis established a good support to the use of this plant in herbal medicine and as a base for the development of new drugs and phytomedicine.
Keywords:*Reinwardtia indica*, phytochemical, nitric oxide radical, antibacterial.

Introduction

India is one of the leading countries in Asia in terms of the wealth of traditional knowledge system related to the use of plant species. India is also known to harbour a rich diversity of higher plant species (about 17000 species) of which 7500 are known as medicinal plants[1] and out of these species 1000 plant species have been used in the traditional system of medicines like Ayurveda, Unani, andSiddha.[2]There has been an increasing interest worldwide on therapeutic value of natural products.The nature provides the mankind, vast therapeutic flora with a wide variety of medicinal potential.[3]Although the popularity of herbal medicine recorded a sharp decline after the introduction of allopathic chemical drugs but herbal medicines again gaining interest because of cost effectiveness, eco-friendlyattributes and true relief from diseased condition.[4]

The medicinal power of these medicinal plants mainly depends on phytochemical constituents that have great pharmacological significance.[5] Knowledge of the chemical constituents of plants is desirable, not only for the discovery of therapeutic agents, but also because such information may be of valuein disclosing new sources of

such economic materials as tannins, oils, gums, precursor for the synthesis of chemical substances. The most important of these bioactive compounds of plant are alkaloids, flavonoids, tannins and phenolic compounds.[6]

In recent years there is an increasing interest in finding antioxidant phytochemicals because they inhibit the reactive oxygen species (ROS) generated from oxidation of cells.These ROS at low or moderate concentrations exert beneficialeffects on cellular responses and immune function but at high levels free radicals and oxidants generate oxidative stress that can damage cell structures including lipid, protein and DNA.[7] NO radical is also an important ROS as it has multiple cellular functions like regulation of cell growth differentiation and apoptosis and many physiological roles including modulation of blood pressure and synaptic plasticity. But high concentration of NO causes sepsis, hepatic failure coronary heart disorders and many other lethal diseases.[8] Apart from this, infections of different types have increased to a great extent and antibiotics resistance effect becomes an ever increasing therapeutic problem.Consequently the antimicrobial activities of plant extract have formed the basis of many applications including food preservative,pharmaceuticals, functional foods, nutraceuticals and alternative medicinal therapies.[9]To overcome these therapeutic problems one must rely on nature, as natural products of higher plants may possess a new source of chemical agent with possibly novel mechanism of action.[10]Antioxidants and antibacterial agents from plant resources are potent and safe due to their harmless nature and many herbs has been investigated for these properties.

*Reinwardtia indica*belongs to Linaceae family. Linaceae is a small family of about 6 genera and 220 species, distributed in tropical and subtropical regions also extending to north and south temperate region. This family is also known as flax family.[11]*R. indica* comes from foothills of Himalaya. Its common name is linum or yellow flax having a small evergreen shrub growing to about 1m tall. It forms bushy clump with erect stems sucking from the base. The oval shaped leaves are lime green in colour. In winters it producesmasses of five petteld butter yellow flower.[12] This plant is widely used by local communities for different medicinal purposes like fortongue wash,for increase in lactation period, in wound infection and against skin diseases etc.[13-16]

No experimental workhas been reported on this plant so the study was designed to screen the extracts of leaves of *R.indica*for preliminary phytochemical analysis, Nitric oxide (NO) scavenging activity and antibacterial activity.

Materials and method

Plant material

Leaves were collected in the month of March 2012 from Forest Research Institute (F.R.I) Dehradun and authenticated by Botanical Survey of India (B.S.I), Dehradun. A voucher specimen (Acc. no. 11094) was procured in the herbariumrecord of Botanical Survey of India, Dehradun.Fresh leaves were washed under running tap water, air dried at room temperature for 15 days, and then homogenised to fine powder and stored in air tight bottles for further extraction process and chemical testing.

Extract preparation

Extract were made by exhaustive soxhlet extraction technique. Fine powdered leaves of *Reinwardtia indica* (50 grams each) were extracted with water and carbinol for 10 hours and 17 hours respectively, until the solvent comes out of the extractor becomes pure and colourless.[17]Then the solvent was removed using a rotary vacuum evaporator at 40- 60°C togive the concentrated extract which was kept freeze dried until use.

Reagents

Reagents used are mainly carbinol (CDH), double distilled water, methanol (CDH), sodium nitroprusside (CDH), phosphate buffer, sulphanilic acid (Merck),1- naphthylaminehydrochloride (CDH), ascorbic acid (Merck), ciprofloxacin (CDRI, Lukhnow), Muller Hinton agar(High media) etc.

Phytochemical screening

Phytochemical screening of both the extracts was performed for different constituent like alkaloids, terpenoids, tannins, flavonoids, saponin, glycosides, steroids, carbohydrates. The following tests were performed for phytochemical analysis- [18, 19]

Alkaloids-

Extracts were dissolved in dilute hydrochloric acid and then filtered.

Mayer's test- Filtrates were treated with Mayer's reagent and occurrence of turbidity indicated the presence of alkaloids.

Wagner's test-Filtrates were treated with Wagner's reagent. Brownish redprecipitate indicated the presence of alkaloids.

Hager's test- Filtrates were treated with Hager's reagent and presence of yellow colour precipitate indicated the presence of alkaloids.

Flavonoids-

Alkaline reagent test- Extracts were treated with few drops of sodium hydroxide (NaOH) solution. Formation of intense yellow colour which became colourless on addition of dilute hydrochloric acid (HCl) indicates the presence of flavonoids.

Lead Acetate test- Extracts were treated with few drops of lead acetate solution. Yellow coloured precipitates give the presence of flavonoids.

Glycosides-

Keller-Killiani test- Extracts were mixed with chloroform and evaporated to dryness. 0.4 ml Glacial acetic acid (containing trace amount of ferric chloride) was added to dried extract. Transferred it to test tube and add carefully 0.5 ml of concentrated sulphuric acid by the side of test tube. Blue colour appeared in acetic acid layer showed the presence of glycosides (cardiac glycosides).

Legal test- Extracts were hydrolysed with dilute hydrochloric acid (HCl) and filtered. Filtrate was treated with sodium nitroprusside solution in pyridine and NaOH. Blood red colouration showed the presence of glycosides.

Carbohydrates-

Few quantity of extract were dissolved in 5ml of distilled water and filtered. The filtrates were used for the following tests-

Molisch test-Filtrates were treated with two drops of alcoholic α-naphthol solution in a test tube. Formation of violet ring at the junction indicated the presence of carbohydrates.

Benedict's test- Filtrates were treated with Benedict's reagent and heated gently on water bath. Occurrence of orange red precipitates showed the presence of carbohydrates.

Barfoed's test- To one ml of filtrate, one ml of Barfoed's reagent is added and heated on a boiling water bath for 2 minutes. Occurrence of red precipitates indicated the presence of sugar.

Tannin-

Ferric chloride test- 0.5gm of dried powdered sample was boiled with 20 ml of water and then filtered.1ml of ferric chloride solution was added to 1ml of filtrate. Brownish green precipitates were not formed which indicated the absence of tannins.

Terpenoids-

Salwoskii test- 1gm of extract was mixed with chloroform. 3ml of concentrated sulphuric acid was added from sides of test tube to form a layer. A reddish brown precipitates at the interface formed indicated the presence of terpenoids.

Saponin-

Foam test- The extract was diluted in 20ml distilled water and it was agitated in a graduated cylinder for 15 minutes. The formation of 1cm layer of foam showed the presence of saponin.

Steroids-

Test was performed by mixing crude extract with 2ml of chloroform. Then 2ml of each of concentrated sulphuric acid and acetic acid were poured into the mixture. The development of a greenish coloration indicated the presence of steroids.

Nitric oxide radical scavenging activity

Aqueous solution of Sodium nitroprusside (SNP) at physiological pH spontaneously released nitric oxide, which can be estimated by the use of Griess reaction.[20]The scavengers of nitric oxide reduce the production of nitric oxide. The reaction mixture (3 mL) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5ml) and the extract or the standard solution (0.5 ml) was incubated at 25 °C for 2.5 h. After incubation, 0.5 ml of the reaction mixture was pipette out and mixed with 1 ml of sulphanilic acid reagent (0.33 % in 20 % glacial acetic acid) and allowed to stand for 5 minutes for completion diazotization. 1 ml of 1-naphthylaimne hydrochloride (1 %) was added, mixed and allowed standing for 30 minutes, a pink coloured chromophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm.IC_{50} values were calculated which isdefined as the concentration of sample required to inhibit 50 % of the nitric oxide radical. The % activity was calculated by following equation[21]

% Scavenging activity =

$$\frac{\text{Absorbance before reaction- Absorbance after reaction}}{\text{Absorbance before reaction}} \times 100$$

Microbial Culture-

Present study was made on gram positive bacterial strain i.e. Staphylococcus aureus (MTCC no-3160) and other gram negative strain i.e. Pseudomonas aeruginosa (MTCC no-424).

Antibacterial assay-

The antibacterial activities of the extracts were determined by Kirby and Bauer agar disc diffusion method.[22]

Muller Hinton Agar (MHA) media was used for the antibacterial activity test. 15 ml of agar medium was dispensed into pre sterilized petridishes to yield a uniform depth of 4 mm and inoculated by both the bacterial strain separately. The sterile discs were impregnated with different concentration of leaves extract i.e.200mg/ml, 100mg/ml, 50mg/ml, 25 mg/ml diluted in ethanol with the help of micropipette. The discs were placed on agar surface with flamed forcep and gently

pressed down to ensure contact with agarsurface. Ciprofloxacin (0.01mg/ml) was used as standard for positive control and ethanol act as negative control which was used to made dilution of extract of different concentration. The discs were spaced enough to avoid reflections wave from the edges of the petridishes and overlapping rings of inhibition.

Finally the petridishes were incubated for 24 hours at 35 ±2°C for both the strains. The diameter of zone inhibition as indicated by clear area which was devoid of growth of bacterial colony was measured with the help of scale in millimetre (mm) and compares it with the zone of inhibition of standard drug.

Results and Discussion

Extractive yield-

Extractive yield of water extract was found to be more than that of carbinol extract. The result of extractive yield, appearance and consistency of both the extracts are given in Table.1.

Table.1- Yield details of R.indica leaves extract

Extracts	*Appearance*	*Consistency*	*Extractive yield*
Aqueous	dark brown	semi solid	37.4 %
Carbinol	dark green	waxy	28.2 %

Phytochemical constituents-

The phytochemical constituents investigated are summarised inTable-2. The results of phytochemical screening test revealed the presence of some active principles like alkaloids, glycosides, flavonoids, saponin, terpenoids, steroids and carbohydrates etc. The presence of these phytochemicals suggests that the plant might be of medicinal importance as out of these phytochemicals steroids are known to be important against cardio tonic activity and antimicrobial activities. [23]The presence of glycosides improves cardiac output and reduce heart diseases like congestive heart failure and cardiac arrhythmia etc.[24]Flavonoids are phenolic compounds that acts as primary antioxidant and possess antimicrobial, anti-inflammatory, antiallergic, anticancer activities etc.[25] Saponins acts as antibacterial agents and also used to treat hypercholesterolemia, hyperglycemia and obesity.[26] Alkaloids are the biggest class of phytochemical and exhibit many therapeutic effect like antimicrobial, sedatives, anti-inflammatory, antiallergic etc.[27]It is now established that phytochemicals are the source of the medicinal power of the plants.

Table 2- Preliminary Phytochemical analysis of extract leaves of Reinwardtia indica.

Phytochemicals	*Aqueous extract*	*Carbinol extract*
Alkaloids	+ve	+ve
Glycosides	+ve	+ve
Saponin	+ve	+ve
Tannin	-ve	-ve
Terpenoids	+ve	+ve
Steroids	+ve	+ve
Flavonoids	+ve	+ve
Carbohydrates	+ve	+ve

Key words- "+ve" = present, "-ve" = absent

Nitric oxide radical scavenging activity-

NO radical is a diffusible free radical that plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and anti-tumour activities.[28] Suppression of released NO may be partially attributed to direct NO scavenging as *R.indica* extracts decreased the amount of nitrite generated from the decomposition of SNP in vitro. The scavenging of NO by the extract was increased in concentration dependent manner. Figure. 1 illustrates a significant decrease in NO radical by both the extracts

and ascorbic acid which was used as reference drug. The IC_{50} values were found to be 725µg/ml, 975µg/ml and 825µg/ml for carbinol extract, aqueous extract and ascorbic acid respectively shown in Table.3. Carbinol extract shows better scavenging activity as IC_{50} of this extract is less than that of reference. The effective scavenge of NO radicals reveals the strong free radical scavenging activity of the plant and this is attributed to the presence of different phytoconstituents mainly as phenolic compounds such as flavonoids are considered to be responsible to the free radical scavenging properties and thus leading to antioxidant activity.[29]Saponins also act as antioxidant agents in many cases.[30]

Figure 1-NO radical scavenging ability of extracts and ascorbic acid at different concentrations

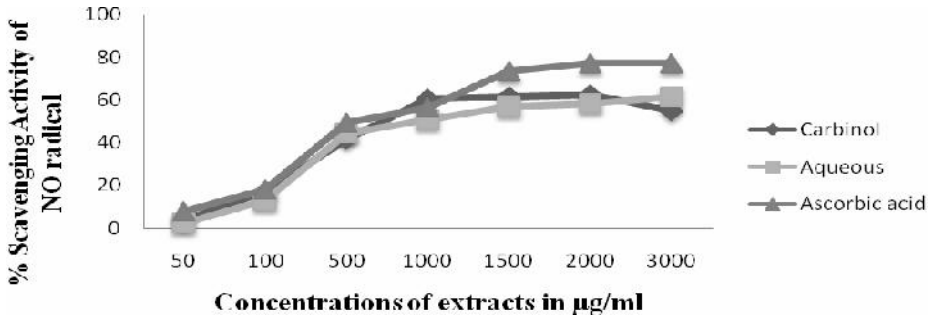
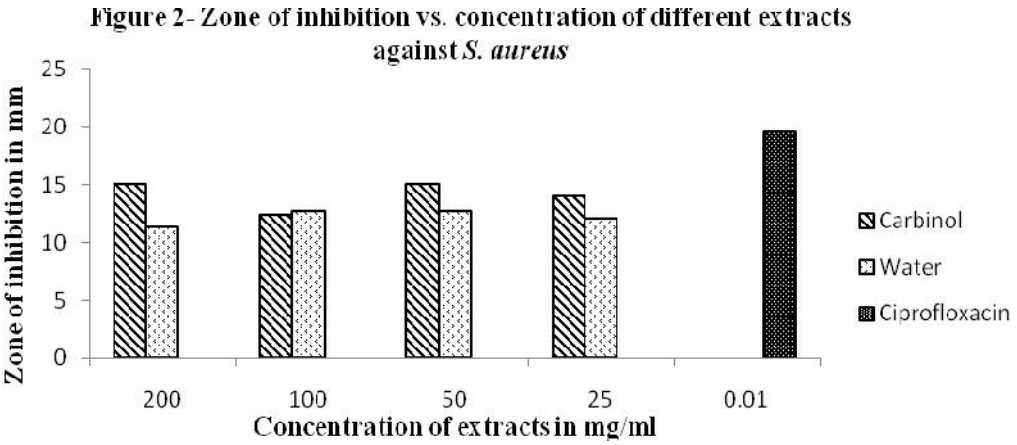


Table.3- Showing IC50 value of the extracts

Extract	*IC_{50} Value(µg/ml)*
Aqueous	725
Carbinol	975
Ascorbic acid	825

Antibacterial activity-

Results shows that both aqueous and carbinol extracts are active against both the bacterial strains. The zones of inhibition for *S. aureus* and *P. aeruginosa* were determined and results were shown in Table-4 and graphical representation is shown in Figure-2 and Figure-3.The gram negative *P.aeruginosa* is more sensitive than the gram positive *S.aureus*. Highest inhibitory activity was seen against *P.aeruginosa* (zone of inhibition 19.47mm) using the carbinol extract of 200mg/ml while the weakest activity was demonstrated against *S.aureus. R. indica* shows potent antibacterial activity and the factor responsible for this activity isvarious phytochemicals such as alkaloids, flavonoids, glycosides, saponin, terpenoids, steroids etc. as much literature has been cited for their involvement against antibacterial activity.[31]



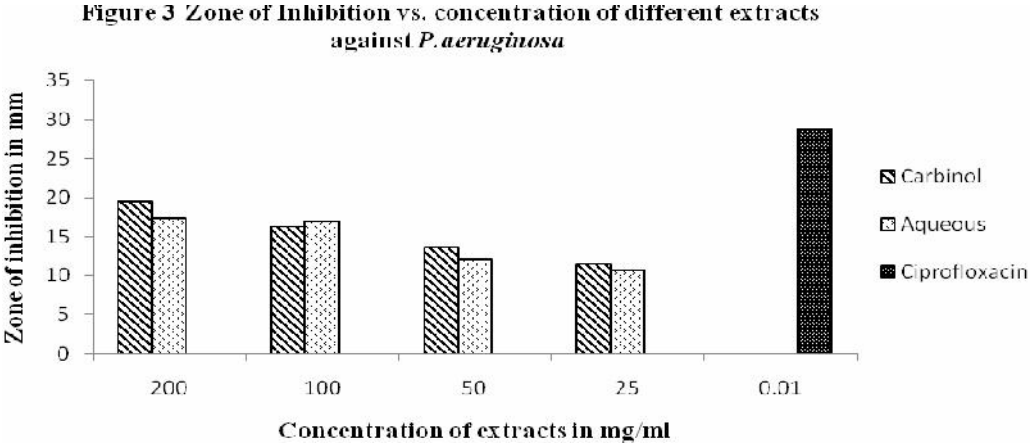


Table 4- Results of zone of inhibition of different extract of *R. indica*.

Extracts	***Concentrations in mg/ml***	***Zone of inhibition in mm***	
		S.aureus	***P.aeruginosa***
Aqueous	200	11.30	17.27
	100	12.71	16.96
	50	12.76	12.04
	25	12.08	10.70
Carbinol	200	15.09	19.47
	100	12.35	16.26
	50	15.11	13.63
	25	14.03	11.47
Ciprofloxacin	0.01	19.69	28.71

Conclusion

From above studies it is concluded that the susceptibility of both the bacterial strain to different concentration of extract of *R. indica* indicates that plant is a potential source for antibacterial compound and act as good scavenger of NO radical and can be useful for the treatment of many lethaldiseases. Both the activities of *R. indica* are reported for the first time. *R. indica* shows potent activities and so this plant can be used to discover bioactive natural products that may serve as leads for the development of new antibacterial and antioxidant drugs in the treatment of different diseases.

Acknowledgment

The authors are thankful to Department of Chemistry, Kanya Gurukula campus,Gurukula Kangri University, Haridwar for providing all the necessary facilities.

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