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Research Article

Phytochemical Evaluation, Proximate Analysis and Biological Activity of *Reinwardtia indica* Dum. Leaves

Abha Shukla[1*], Swati Vats[1], Rishi Kumar Shukla[2], Deepak Painuly[2], Anirudh Porval[2], Ritu Tyagi[1]

[1]*Department of chemistry, Kanya Gurukula Campus, Gurukula Kangri Vishwavidyalaya, Haridwar, Uttarakhand-249404, India.*
[2]*Department of chemistry, Gurukula Kangri Vishwavidyalaya, Haridwar, Uttarakhand-249404, India*

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ABSTRACT
The leaves of *Reinwardtia indica* was analysed for preliminary phytochemical screening, proximate analysis and biological activity. Proximate analysis has been performed to evaluate content of ash (11.09±0.10%), moisture (12.62%), fat (11.44%), fibre (2.44%), protein (29.23%), carbohydrate (45.79%) and nutritive value (403.05 Kcal/100 grams of leaves). The results of phytochemical screening revealed the presence of various medicinal active phytoconstituents. Total phenolic content was highest for chloroform extract (41.66 mgGAE/g dry weights). The antioxidant potential of extracts was performed by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assay. In DPPH assay highest activities shown by aqueous extract with IC_{50} value of 0.45mg/ml and also have FRAP value 1.62. Antimicrobial activity performed by agar well diffusion method and activity towards the selected microbes was best shown by chloroform extract. Overall results revealed that *Reinwardtia indica* leaves exhibits excellent nutritive value along with potent antioxidant and antimicrobial activity. It can be used in medicine as antimicrobial as well as in food supplements to prevent their oxidation and rancidity of food.

Keywords: Phytochemical, proximate, nutritive value, total phenolic content, antioxidant activity, antibacterial activity, antifungal activity

INTRODUCTION

Nature has been a source of medicinal agents since time immemorial. The importance of herbs in the management of human ailments cannot be over emphasized. Plants can act as herbal drugs, neutraceuticals, food supplements, folk medicine, pharmaceuticals intermediates and new chemical entities for synthetic drugs, sweetness, fragrance and number of healthcare products. The medicinal power of plants mainly depends on phytochemical constituents that have great pharmacological significance[1]. With the recent years, infections have increased to greater extent and antibiotic resistant effects become an ever increasing therapeutic problem. A lot of work has been done in the field of medicines still infectious diseases caused by bacteria, fungi and parasites are a major threat to the human health[2]. Also there is an increasing interest in the measurement and use of plant antioxidant for scientific research as well as industrial purposes. The oxidation induced by free radicals in human body can results in cell membrane disintegration, membrane protein damage, DNA mutation which can further initiate or propagate the development of various diseases such as cancer, liver injury, cardiovascular diseases, arthritis, diabetes etc[3].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. Antioxidants and antibacterial agents from plant resources are potent and safe due totheir harmless nature and many herbs has been investigated for these properties. *Reinwardtia indica* comes from the foot hills of the Himalayas. It belongs to Linaceae family. Its common name is yellow flax. This plant is widely used by local communities for different medicinal purposes like for tongue wash[4], for increase in lactation period[5], in wound infection[6] and against skin diseases[7] etc. One scientific study has been made for antibacterial and NO radical scavenging activity[8]. Therefore it is great interest to carry out a screening of *R. indica* in order to validate their use in folk medicine to reveal the active principle. Present study focuses on proximate analysis, nutritive value and biological activity such as antimicrobial and antioxidant on leaves of *R. indica*.

MATERIALS AND METHODS

Reagent and chemicals

Ascorbic acid (Rankem), Muller Hinton Agar (Hi-Media), Muller Hinton broth(Hi-Media), Sabourd dextrose agar(Hi-Media), Sabourd dextrose broth(Hi-Media), 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ)(Sigma Aldrich), Ofloxacin (Welcure Drugs and Pharma Ltd.), fluconazole (Cipla), 1,1-diphenyl-2-picrylhydrazyl [DPPH]((Sigma

*Author for Correspondence

Table 1: Proximate composition of dried leaves of *Reinwardtia indica*

Parameter % dry weight basis	*Reinwardtia leaves* (% dry weight basis)
Moisture content	12.62±0.16
Ash content	11.09±0.10
Crude fibre	2.44±0.004
Crude fat	11.44±0.09
Total protein	29.23±0.39
Total carbohydrate	45.79
Nutritive value	403.05*

*nutritive value is calculated in Kcal.100 gm^{-1} dry weight of stem

Table 2: Extractive yield of *Reinwardtia indica* leaves extracts.

Extracts	Appearance	Consistency	% Yield (w/w)
Petroleum ether	Green	Waxy	4.89
Chloroform	Dark Green	Dried Wax	3.06
Ethanol	Green	Crystalline solid	7.54
Water	Brown	Crystalline solid	4.10

Aldrich), sodium carbonate (CDH), Gallic acid (Merk), Butylated hydroxyl toluene (BHT) (Rankem), sodium acetate (Fisher Scientifics), Folin Ciocalteau (Merk), Ferric chloride (Thomas Baker) were purchased. All the other solvents and chemical used were of HPLC or analytical grade and different microbial isolates was purchased from MTCC, Chandigarh, India.

Plant Materials

Fresh leaves of *R. indica* were collected from Dehradun district of state Uttarakhand, India. Get authenticated by Botanical Survey of India (B.S.I) Dehradun. A herbarium (accession no- 114094) was also procured in BSI Dehradun. A voucher specimen of both the plants has been deposited in Department of Chemistry, Kanya Gurukula Campus, Gurukula Kangri Vishwavidyalaya, Haridwar under the registry No. 1/1 for further reference. The fresh leaves were washed with water and then dried under the shade for about 15 days and then crushed in grinder into powder form then stored in air tight container for further extraction and various processes.

Proximate Analysis

Proximate analysis of the powdered leaves includes estimation of moisture content, ash content, crude fibre, crude fat, protein content[9], whereas total carbohydrate was calculated by the equation-

Total carbohydrate = 100 - (% Ash+ % Moisture + % Crude fibre+ % Crude protein)

Nutritive Value

Nutritive value of leaves was expressed in KCalories/ 100 gram of dry weight of leaves and calculated by using the formula[10]: Nutritive value = (4× % Protein) + (9× % Crude fat) + (4× % Total carbohydrate)

Extraction of Plant Material

150 gram of the dried powdered leaves of R. indica were weighed, loaded and extracted by soxhlet apparatus using 1.3 litres each of petroleum ether, chloroform, ethanol and water respectively in increasing order of polarity. Extraction was continued for about 72 hours or until the solvent coming out of the siphoning tube become colourless[8]. Extracts were concentrated under reduced pressure in vacuum evaporator and refrigerated for further use.

Phytochemical Screening

Phytochemical analysis for various phytoconstituents of the extracts was undertaken using standard qualitative methods[11-12]. The extracts were screened for the presence of biologically active compounds like alkaloids, flavonoids, tannins, glycosides, terpenoids, steroids, fat and oil, saponins, protein etc.

Total Phenolic Content

The total phenolic content of obtained extracts of leaves of R. indica was determined by using Folin ciocalteu reagent with some little modifications[13]. Phenolic content from the sample reduces the molybdenum metal and changes from yellow colour to prussian blue. The intensity of blue colour is directly proportional to the phenolic content. The extracts were diluted with methanol to form a concentration of 1000 µg/ml and also standard solution of gallic acid is made of concentration range of 25µg/ml to 300µg /ml for plotting the calibration curve. 1ml of extracts was added to 10ml of 10 % Folin Ciocalteu reagent. To this reaction mixture add 8 ml of 7.5 % sodium carbonate after 8 minutes. Further total volume is made up to 20 ml by adding distilled water. The complete reaction mixture was incubated for about 2 hours in dark and at room temperature of about 25°C±2. Same reaction mixture was set up with gallic acid standard dilutions and also with blank where methanol is taken in place of extract. After incubation, the absorbance was measured at 765nm with UV-VIS spectrophotometer (Systronic, Visible spectro-105).

Calculations were made using calibration curve of gallic acid. The total phenolic content of extracts was expressed as mg gallic acid equivalents (GAE)/ gram of dry mass by following equation[14]-

$T = C \times V/M$

Where, T= Total phenolic content mg/gm of plant extract in GAE,

C= Concentration of Gallic acid from the calibration curve,

V = Volume of the extract in ml,

M =Weight of the pure plant extracts.

Free Radical Scavenging Assay

The free radical scavenging power of all the obtained extracts of leaves of *R. indica* were evaluated by stable DPPH free radical scavenging assay with little modification[15]. DPPH is a free radical of violet colour. The antioxidants in the sample scavenge the free radical and turn it into yellow in colour. The change in colour from violet to yellow is proportional to radical scavenging activity of sample. A working solution of 0.004% DPPH was freshly prepared in methanol. 1ml of sample or standard dilutions of different concentrations (0.5 to 3.5 mg/ml) added to 3ml of working solution of DPPH. After

Table 3: Phytochemical constituent in *Reinwardtia indica* leaves extracts.

Phyto constituents	Test performed	Extracts			
		Petroleum ether	Chloroform	Ethanol	Water
Alkaloids	Wagner's test	-	-	-	-
	Hager's test	-	+	+	+
	Dragendroff's test	-	-	-	-
	Mayer's test	-	-	+	+
Flavonoids	Alkaline test	-	+	+	+
	Lead acetate test	-		+	+
Carbohydrates	Molisch's test	-	-	+	+
	Bendict's test	-	-	+	-
Tannins	Ferric chloride test	-	+	+	-
	Gelatin test	-	+	-	-
Glycosides	Legals test	-	+	+	-
	Keller Killiani test	_	+	+	-
Terpenoids	Liebermann burchard test	+	+	+	-
	Salwoski test	+	+	+	-
	Salwoski test (Triterpenes)		-	+	-
Steroids	Liebermann burchard test	+	+	+	-
Fat and Oil	Saponification test	+	+	-	-
	Filterpaper test	+	+	-	-
Saponin	Foam test	-	-	+	+
	Froth test	-	-	+	+
Protein	Ninhydrin	-	-	-	+
	Biuret	-	-	-	-

Table 4: Total phenolic content of *Reinwardtia indica* leaves extracts.

Extracts	Total phenolic content ($mgGAE.gm^{-1}dw$)
Petroleum ether	28.55 ±0.39
Chloroform	41.28±0.28
Ethanol	41.58±0.38
Water	40.37±0.70

Table 5: IC_{50} of *Reinwardtia indica* leaves extracts in DPPH assay.

Extracts/ Standard	IC_{50} Values in $mg.ml^{-1}$
BHT	0.050 ± 0.27
Petroleum ether	1.68 ± 0.09
Chloroform	0.75 ± 0.67
Ethanol	0.68 ± 0.18
Water	0.45 ± 0.07

30 minutes incubation in dark at room temperature 25°C±2. The absorbance of reaction mixture were taken at 517nm with UV-VIS Spectrophotometer (Systronic VIS-105) which was compared with the corresponding absorbance of same dilution range of standard 2,6-di-tert-butyl-4-hydroxytoluene (BHT). 1ml of methanol with 3ml of working DPPH solution serves as control. The % radical scavenging activity or % inhibition was calculated by-

% inhibition =

$$\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

IC_{50} of all the extracts and standard BHT were calculated by graphical method by plotting % inhibition versus concentrations. IC_{50} is defined as the amount of antioxidant material required to scavenge 50% of free radical in the assay system. Results were expressed in terms of IC_{50}.

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was performed on the given extracts by the method with the little modification[16]. The stock solution includes 300mM acetate buffer (pH-3.6), 10mM TPTZ solution (in 40mM HCl) and 20mM $FeCl_3.6H_2O$ solution. The working FRAP reagent was freshly prepared by mixing of above reagents in ratio of 10:1:1 (v/v) respectively and then warmed at 37°C±2 before using it. Antioxidant potential was determined by a reaction mixture which consists of 1ml of extract (1500µg/ml) and 10 ml of working FRAP reagent. This reaction mixture is kept for 30 minutes incubation in BOD incubator at 37°C±2. Absorbance was taken in U.V spectrophotometer at 593 nm. Ascorbic acid standard solution was tested in similar way. The standard curve was plotted between (100µM-600 µM) ascorbic acid. 1ml of methanol in 10ml of working FRAP reagent act as control and working FRAP reagent serves as blank. Calculations were made by calibration curve. Results were expressed in µM/ml and FRAP value of sample is calculated by[17]:

FRAP Value =

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100$$

Anti-Bacterial Activity

The inhibition of microbial growth under standardised conditions may be utilised for demonstrating the therapeutic efficacy of plant extracts. For this agar well diffusion method was used for the study[18]. The method depends on the diffusion of sample from a vertical cylinder through a solidified agar layer in a petridish or plate to an extent such that growth of the added micro-organisms were

Table 6: FRAP value *Reinwardtia indica* leaves extracts.

Extracts/ Standard	Ferric reducing antioxidant power (µM.ml^{-1})	FRAP value
Ascorbic Acid	489.75 ± 0.45	2.000
Petroleum ether	276.50 ± 0.12	1.13
Chloroform	393.75 ± 0.34	1.61
Ethanol	352.50 ± 0.09	1.44
Water	395.50 ± 0.24	1.62

prevented entirely in a zone around the cylinder containing a solution of the sample.

Bacteria strains

Bacterial strains viz. *Bacillus cereus* (MTCC-1688), *Salmonella enteric* (MTCC-98), *Pseudomonas aeruginosa* (MTCC-430), *Staphylococcus aureus* (MTCC-737) were purchased from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh.

Preparation of culture media

Culture media consists of nutrient which supports the growth of micro-organisms. It was prepared by dissolving known amount of media in water and sterilized by autoclaving at a pressure of 15lbs/inch at a temperature of 120°C for 15 minutes. Muller Hinton Agar (MHA) was used as culture media for all bacterial strains.

Preparation of inoculum

Bacterial cultures, freshly grown at 37°C in Muller Hinton Broth were appropriately diluted in sterile normal saline solution and the turbidity of the suspension was adjusted equivalent to a 0.5 McFarland standard by adding more organisms, so as to obtain the cell suspension between 10^5 to10^8 CFU/ml.

Antibacterial assay

Purified liquid medium was inoculated with the requisite quantity of suspension of bacterial strains. 0.1ml of diluted inoculum of test organisms was added in MHA media having temperature of 40-45°C. Immediately the medium was shaken slowly and 25 ml poured in sterilized petriplates. All the petriplates were rotated gently and allowed to get solidified. Ensure that the layer of medium is of uniform in thickness. The prepared plates were stored in laminar airflow condition. After solidification wells of 6mm diameter were punched with the help of borer in agar medium and filled with 40µl of plant extract and antibiotic drug standard (Ofloxacin). DMSO served as negative control. Each extract assayed in triplicate and the mean value is calculated. The plates were incubated ate 37°C for 18-24 hours. The antibacterial activity was interpreted from the size of diameter of zone of inhibition measured in millimetre. Clear zone around extract shows inhibitory nature of the sample. It was measured with the help of zone reader or vernier calliper.

Antifungal Activity

To analyse the antifungal activity of the plant extracts agar well diffusion method was used[18].The method depends on the diffusion of sample from a vertical cylinder through a solidified agar layer in a petridish or plate to an extent such that growth of the added micro-organisms were prevented entirely in a zone around the cylinder containing a solution of the sample.

Fungal strains

Aspergillus flavus (MTCC-277) and *Candida albicans* (MTCC-227) were purchased from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh.

Preparation of culture media

Culture media consists of nutrient which supports the growth of micro-organisms. It was prepared by dissolving known amount of media in water and sterilized by autoclaving at a pressure of 15lbs/inch at a temperature of 120°C for 15 minutes. Sabourd dextrose Agar (SDA) was used as culture media for all fungal strains.

Preparation of inoculum

Fungal cultures, of 48-72 hours old grown on Sabourd dextrose agar were used for preparing inoculums and appropriately diluted in sterilized water and the turbidity of the suspension was adjusted equivalent to a 0.5 McFarland standard by adding more organisms, so as to obtain the cell suspension between 10^5 to10^8 CFU/ml.

Antifungal assay

Purified liquid medium was inoculated with the requisite quantity of suspension of fungal colonies. 0.1ml of diluted inoculum of test organisms was added in SDA media having temperature of 40-45°C. Immediately the medium was shaken slowly and 25 ml poured in sterilized petriplates. All the petriplates were rotated gently and allowed to get solidified. Ensure that the layer of medium is of uniform in thickness. The prepared plates were stored in laminar airflow condition. After solidification wells of 6mm diameter were punched with the help of borer in agar medium and filled with 40µl of plant extract and antibiotic standard drug (fluconazole). DMSO served as negative control. Each extract assayed in triplicate and the mean value is calculated. The plates were incubated ate 37°C for 48-72 hours. The antifungal activity was interpreted from the size of diameter of zone of inhibition measured in millimetre. Clear zone around extract shows inhibitory nature of the sample. It was measured with the help of zone reader or vernier calliper.

Statistical Analysis

Results were presented as mean ± standard deviation of triplicate measurements and analyzed as one-way analysis of variance (sigmastat ver. 2.0). The significant differences between means were calculated by a one way analysis of variance (ANOVA) using dunnett multiple-range test at $P<0.05$.

RESULTS

The proximate analysis of *R. indica* leaves were shown in Table 1 which revealed moisture content of 12.62 %, ash content 11.09 %, crude fibre 2.44 %, fat content 11.44 %, protein content 29.23 %, total carbohydrate 45.79 % and nutritive value of leaves was 403.53 Kcal/100 grams. The extractive yield of *R. indica* leaves during the successive extraction with petroleum ether, chloroform, ethanol and water was shown in Table 2. The preliminary phytochemical screening revealed the presence of medicinally active phytoconstituents. The phytochemical

Table 7: Antibacterial and antifungal activity of *Reinwardtia indica* leaves extracts.

ZONE OF INHIBITION IN MILLIMETER (mm)						
	Strains	Petroleum Ether	Chloroform	Ethanol	Water	Standard Ofloxacin
Bacterial strains	*Staphylococcus aureus*(+)	11.9±0.15	9.1±0.27	9.2±0.91	11.3±0.30	44.1±0.10
	Bacillus cerus(+)	11.5±0.61	9.5±0.50	9.9±0.10	-	55.8±0.15
	Salmonella entrica (-)	-	15.3±0.81	9.9±0.50	-	45.2±0.27
	Pseudomonas aeruginosa(-)	-	-	-	-	49.7±0.35
						Standard Fluconazole
Fungal strains	*Aspergillus flavus*	14.5±0.55	12±0.40	12.4±0.40	14.1±0.15	41.8±0.20
	Candida albicans	9.6±0.92	10.1±0.15	11.3±0.27	10.3±0.35	42.1±0.31

of the leaves extracts of R. indica are summarised in Table 3. Phytochemicals found present were alkaloids, flavonoids, tannins, glycosides, fat, saponins, carbohydrates, terpenoids, steroids, protein and amino acids. The total phenolic content of different extracts was determined by Folin ciocalteu method. The TPC was expressed as milligram GAE / gram dry weight using the standard curve equation- $y= 0.0044x + 0.0187$, $R^2 = 0.9989$ Where y is the absorbance at 765nm and x is the total phenolic content in 1000µg/ml of extracts. TPC was found highest in chloroform (41.65), followed by ethanol (41.20), water (40.98) and in petroleum ether (28.93). The results of TPC are shown in Table 4. The free radical scavenging activity (DPPH assay) was maximum for water extract (IC_{50}= 0.45mg/ml), followed by ethanol (IC_{50}= 0.68mg/ml), chloroform (IC_{50}=0.75mg/ml), petroleum ether (1.68mg/ml). Figure 2 shows the % inhibition of DPPH has linear relationship with the concentrations of extracts. Results are expressed in terms of IC_{50} in Table.5. The antioxidant activity determined by FRAP assay was expressed as µM/ml using the standard curve equation: $y = 0.004x-0.137$, $R^2 = 0.998$, Where y is the absorbance at 593nm and x is the ferric reducing ability in 1500 µg/ml of extracts in µM/ml. the ferric reducing antioxidant power in (µM/ml) was highest in (395.5) followed by (393.75), ethanol (352.5) and lowest in petroleum ether (276.5). The FRAP value were demonstrated in Table 6. All the extracts showed activity against the tested micro-organisms. When compared with one another chloroform is active against all the strains except *P. aeruginosa*. Ethanol extract is active against the fungal strains, *S. aureus* and *S. entrica*. Petroleum ether showed best activity towards *A. flavus* and not active against P. aeruginosa and *S. entrica*. Water exhibits less activity. Detailed results in terms of zone of inhibition were expressed in Table 7.

DISCUSSION

The proximate results revealed that leaves contains good amount of ash which is the measure of inorganic mineral element present in the sample. Moisture content is in between the range of 5-15%[8]. Leaves are good source of carbohydrates and proteins which act as building blocks of life and can be used for feed and fodder of animals. Good fat content gives major contribution towards energy requirement. Also nutritive value reflects the leaves as good source of energy[19]. Phytochemical studies reveal the presence of alkaloids which are known as antimalarial, analgesics and stimulants[20]. Flavonoids are major contributors to the antioxidant activity of plants and also import anti-inflammatory and hypoglycaemic activities. Tannins are major source of phenolic compounds and are good radical scavengers and antibacterial agents[21]. Glycosides have hypoglycaemic activity and can also serve as defence mechanism against many micro-organisms, insects and herbivores[17]. DPPH is stable free radical with a characteristic absorption at 517nm, was used to study the radical scavenging effects of extracts. Antioxidants either donate protons to the radical or act by free radical quenching mechanism as a result of which absorption reduces[22]. The decrease in the absorption is taken as a measure of radical scavenging activity which is demonstrated in the form of IC_{50}, a parameter representing the extract concentration able to inhibit 50% of the DPPH. The antioxidant activity highest for water can be attributed to the high content of phenols (flavonoids and tannins) in the extract. FRAP assay measures the reducing potential with a Fe^{3+} changes to Fe^{2+}. The reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating the hydrogen atom[23]. The ethanol extracts shows good reducing power attributes to various phytochemicals presence in this extract. The FRAP activity of extracts may be due to the presence of phenolic hydroxyl or methoxy group, flavones hydroxyl, keto groups, free carboxyl group and other structural features like triterpenes and their derivative. Antimicrobial activity showed reduction in bacterial growth in terms of zone of inhibition, indicating that leaves exhibits excellent antimicrobial activity. The extracts showed good activity towards gram positive and gram negative bacteria as well as have great potency against both the fungal strain. This shows that the compounds present in the potent extracts are versatile in nature and can have wide range of antimicrobial spectrum. The antimicrobial activity may be attributed to presence of various phytochemical like tannins, alkaloids, phenolic content and terpenoids etc. which are known to be active against microbes.

CONCLUSION

In conclusion present study illustrated that *Reinwardtia indica* leaves can be used in energy drinks or formulations as have high nutritive value. It can be a good source of

antioxidant agent and can replace harmful synthetic antioxidants. It may become a part of natural antibiotics, cosmetics, and medicine as it provides potent antimicrobial activity towards wide range of microbes. Presence of various active secondary metabolites is the main cause of its different biological efficacy. How ever these active principles need to be isolated, identified and characterized, and the structure need to be elucidated.

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