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Expanding the TLC studies on the unexplored medicinal plant *Reinwartdtia indica*

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ABSTRACT

The current study is an extension of our previously published work focused on TLC profiling of various extracts if *Reinwardtia indica*. In the previous report, we have shown that *R. indica* contains a vast spectrum of compounds that can find their role in novel drug development. Further, here we present quantitative reports of these important phytochemical compounds and their TLC profiling. The plant samples of *R. indica* were collected from Bugani village, Srinagar Garhwal, and crushed to powder. The plant powder was used as it is for quantitative analysis while extraction was carried out in four different solvents for TLC profiling. Results of the quantitative analysis showed a considerable amount of phenolics, saponins, flavonoids, alkaloids, and steroids. TLC profiling suggests the presence of more than single metabolites among all the groups that can be further exploited for novel drug formulations. Overall, the present study adds further scientific shreds of evidence to the acclaimed medicinal potential of *R. indica*. Therefore, the baseline data generated so far can be the basis of advanced research on this unexplored and underutilized plant.

Keywords: *Reinwardtia indica*, Phytochemicals, Quantitative analysis, TLC.

INTRODUCTION

Plants play an important role in our day-to-day life, from getting oxygen, to use as food, fodder, and furniture they are important in one way or the other. A more important aspect of plants is their use as a source of medicine; the phytochemicals found in the plants are the reason for their characteristic medicinal properties. The use of herbal drugs is increasing day by day due to their efficacy and no side effects.

India is a country with great diversity and huge natural wealth, from the Himalayas in the north, the Eastern Ghats in the east and the Western Ghats in West it possesses enormous biodiversity. The diverse plant species found all over the subcontinent consist of a large number of plants having medicinal properties and some of them found to be used by the native people in the treatment of various ailments.

The sector of Western Himalaya which stretches from 29° to 31°N latitude and 78° to 80°E longitude, comprising five districts of Uttrakhand, Pauri, Chamoli, Tehri, Uttarkashi, and Dehradun is popularly known as the Garhwal Himalaya. Garhwal Himalaya is a veritable emporium of medicinal plants. Indian Himalayan Region harbors 8644 plant species. Of which, about 2,500 plant species are being utilized in different Indian systems of medicines; more than 1,750 herbal species are native to the Indian Himalayan region, in which western Himalaya has a share of about 1,000 species which are still in use. At least 5,942 genera and 17,381 taxa of plants are represented by the Western Himalayan Region in which there are 8000 taxa of Angiosperms, 44 of Gymnosperms, 600 of pteridophytes, 1737 of bryophytes and 1159 of lichen [1].

There are more than 10, 000 plants that have been described in “Charak Samhita” the grand old book of natural medicines, as the sources of drugs against various ailments. The Unani and Siddha medicine systems also depend upon the natural products of plants for the treatment of diseases. The oldest Hindu scripture i.e. Rigveda also contains the description of many such plants and said treated as the earlier sources of ancient medicine system of Ayurveda. In his writings regarding the values, utilities, and identity description of such plants Charaka has described Himalaya as best suited for the growth of most of these medicinal plants. The plants and plant products that are generally used by the local healers and traditional medical practitioners are usually gathered from wild. Most of such plants and their parts are used directly and shows no side effects [2]. About 65% of the Indian population is dependent upon such traditional systems of medicine.

The knowledge of traditional medicines, skills, and practices based on the theories beliefs and experiences indigenous to the different culture, whether explicable or not, used in the maintenance of

health as well in the prevention, diagnosis, improvement or treatment of physical and mental illness. Present ethnobotanical studies have revealed the use of plants for various disease and ailments ranging from dysentery, cough, headache, sore throat, body ache, wound healing, mouth ulcers, bronchitis, delivery, inflammation, constipation, asthma, diarrhea, malaria, jaundice, mental disorder, menstrual disorders, and skin diseases, etc [3].

One of these plants is *Reinwardtia indica*. It is commonly called as Yellow flax or Pyoli. It is locally called as Feuli or Basanti. It is a species of Linaceae in the major group of Angiosperm found in the Himalaya. There are only two species of *Reinwarditia* found native to the southern and southeast Asia namely *Reinwardtia indica* and *Reinwardtia sinensis.* The medicinal properties of *Reinwardtia indica* make it a plant of choice for its phytochemical analysis. Also, there are meager scientific studies are available regarding this plant, previously we have reported the antioxidant activity, qualitative phytochemical analysis [4] and TLC fingerprinting and quantitative analysis of some metabolites of *R. indica* [5]. Further, the present study is focused upon quantifying some important secondary metabolites and TLC analysis of these metabolites.

METHODOLOGY

Sample Collection and Extract Preparation

The leaves of *Reinwardtia indica* were collected from Bugani village, Srinagar Garhwal. The leaves were shade dried and crushed in mortar-pestle using liquid nitrogen. The dried leaf powder directly used for the quantitative determination of various metabolites while extracts prepared for TLC analysis.

Quantitative Tests for Phytochemicals

Total Alkaloid Content [6]

5gm of the sample was weighed into a 250ml beaker and 200ml of 10% acetic acids in ethanol were added, covered, and allowed to stand for 4 hours. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and filtered. The residue was the alkaloid which was dried and weighed.

Total Carbohydrate [7]

100mg of the sample was weighed into a boiling tube and was hydrolyzed by keeping it in the boiling water bath for 3hrs with 5ml of 2.5N HCl and then cooled to room temperature. It was then neutralized with solid sodium carbonate until the effervescence ceases and then the volume was made up to 100ml. it was further, centrifuged at 3,000 rpm for 10 min. The supernatant was collected and 0.2 ml was taken in a test tube for the analysis. The prepared working standard was taken in the test tube in volume o.2ml 0.4ml 0.6ml, 0.8ml, and 1ml. Each test tube was made up of 1ml volume with distilled water and 4ml of Anthrone reagent added in each test tube. 1ml of distilled water with 4ml of Anthrone reagent is served as a blank. All test tubes were heated in the water bath for 8min and then cooled to room temperature. The dark green color was read at 630nm. The concentration of glucose in the sample was calculated by the standard graph.

Total flavonoid content [8]

10g of the sample was extracted with 100ml of 80% Methanol at room temperature for 24hr. The whole solution was filtered through Whatman filter paper no. 41. The filtrate was allowed to be evaporated to dryness over a water bath and weighed to a constant weight.

Total lipid Estimation [9]

1gm of the sample was macerated in a mortar and pestle with liquid nitrogen. 1ml chloroform: methanol (1:2) was added and homogenized well. The homogenate was transferred to a centrifuge tube. Another 2ml of chloroform: methanol (1:2) was added to the mortar to wash. The wash was transferred to the same tube. Then the tube was centrifuged at 3,000rpm for 15 minutes. The supernatant was transferred to another centrifuge tube. 3ml of chloroform: methanol (1:2) and 0.8ml of 1% KCl was added to the pellet obtained after centrifugation. Then the tube was vortexed well and centrifuged at 3,000rpm for 5 minutes. The supernatant was transferred to the previously prepared tube. 2ml of chloroform and 1.2ml of 1% KCl was added to the collected supernatant and vortexed well and then centrifuged again at 3,000rpm for 5min. The lower layer (lipid extract) was transferred to another tube which was weighed before use. Then it was dried under the liquid nitrogen stream and the tube with the lipid was weighed and total lipid content was calculated.

Total Saponins [10]

10gm of the sample was taken in a conical flask and 50ml of 20% methanol was added to the sample. The sample was heated over a hot water bath for 4hr with continuous stirring at about 55°C the mixture was then filtered and the residue re-extracted with another 100 ml of 20% ethanol. The combined extracts were reduced to 20ml over a water bath at about 90°C. The concentrated solution was then transferred into a 250ml separating funnel and 10ml of diethyl ether was added to the extract and vigorously shaken. The aqueous layer recovered while diethyl ether was discarded and it was repeated 3-4 times. Butanol (60ml) was added and this mixture was washed twice with 5ml of 5% NaCl. The remaining solution was then heated in a water bath and after evaporation, the samples were dried in the oven to a constant weight.

Total Steroid [11]

The standard working solution of cholesterol was prepared of 100µg/ml and taken in 5 test tubes with volume 0.2, 0.4, 0.6, 0.8, and 1ml. Methanolic extract of plant sample was prepared and taken in a test tube with volume 0.2ml. The volume of all test tubes was made up to 5ml using ferric chloride diluting reagent. A blank was prepared simultaneously by taking 5ml diluting reagent. 4ml of concentrated H_2SO_4 was then added to each test tube. After 30min of incubation intensity of the color developed was read at 540nm. The concentration of cholesterol was calculated by using the standard curve.

Thin Layer Chromatography analysis

To study the type of secondary metabolites present in different extracts of *Reinwardtia indica* leaves, thin layer chromatographic analysis was performed to get the basic details about the number and type of therapeutically important secondary metabolites present in this plant.

Extract preparation

Leaf extracts were prepared in different solvents of increasing polarity (Petroleum ether>Chloroform>Acetone>Methanol) using the soxhlet extractor. The extracts were further concentrated in a distillation unit and the sticky residue obtained finally was dried in a lyophilizer. The finally obtained product was semisolid, sticky residue which was subjected to TLC analysis.

TLC Plate Preparation

30gm of silica G was weighed and mixed well with 60ml of distilled water for a few minutes. Then it is poured evenly on the glass plates of 4x10cm dimension and placed under hot air oven for 30min at 110°C and then stored in a dry atmosphere and used whenever required.

Sample application

Before TLC, samples were prepared by diluting the crude extracts of petroleum ether, chloroform, acetone, methanol, and water with suitable solvent respectively, and then applied usually in 1-10µl volumes to the origins of a TLC plate 1cm above its bottom with the help of capillary tubes.

Chromatogram Development

The TLC glass chambers were filled with the mobile phase solvent and covered with the glass lid and left for 10 minutes for the saturation of the solvent. After the application of the sample on the adsorbent, the TLC plate was kept in the solvent in the TLC glass chamber and allowed the mobile phase to move through the adsorbent phase up to 3/4th of the plate. The separation took place and the color spots were obtained. The following table represents the phytochemical to be screened and the solvent system used for TLC.

Table 1: Chromatogram development of Phytochemicals

Phytochemicals	Solvent system	Confirmatory Test
Flavonoids	Chloroform: Methanol 24:1	3% boric acid + 10% oxalic acid spray
Steroids	P.E: E.A: Methanol 17:2:1	Phosphoric acid (85%) with water (1/1) v/v
Tannins	Chloroform: Methanol: Water 6.5:3.5:1	$FeCl_3$ spray
Coumarins	Butanol: Acetone: Water 4:1:5	1% solution of KOH in ethanol
Peptides and Amino Acids	Butanol: Glacial Acetic Acid: Water 6:1.5:3	Ethanolic ninhydrin spray
Phenol	Methanol: Water 6:3	$FeCl_3$ spray

RESULTS

Quantitative analysis

The quantitative analysis of the plant extract showed that the plant possesses a high amount of saponins that are 17.3gm per 100gm of plant extract as compared to the other phytoconstituents. Quantification of other phytochemicals shows the varying amount of these compounds in the plant. Different phytochemicals are estimated using different methods. Estimation of total carbohydrate and steroid were estimated by the colorimetric method while rests of the bioactive phytochemicals were estimated by the dry weight method. Table 2 shows the quantity of each of the phytochemical estimated.

Table 2: The values of phytochemicals estimated

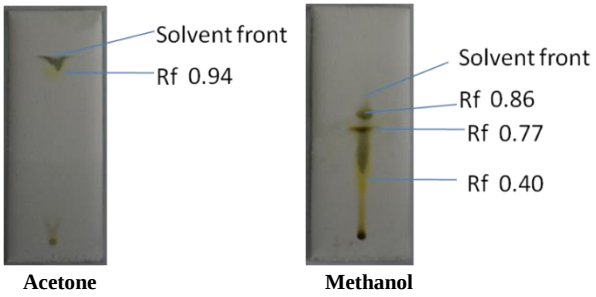
S. No	Phytochemical	Quantity (gm/100gm)
1	Saponins	17.30
2	Flavonoids	13.00
3	Alkaloids	2.04
5	Lipids	0.501
6	Carbohydrate	0.087
7	Phenolics	0.38
8	Protein	5.78
9	Steroids	0.30

TLC analysis

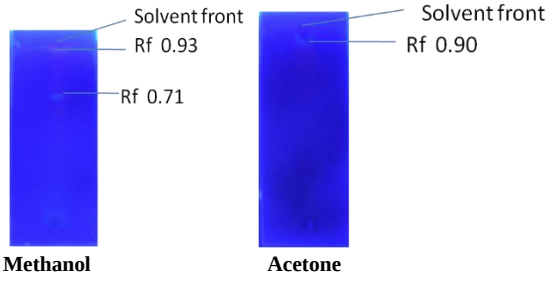
Table 3 shows the results obtained from the TLC profile of *R. indica* extracts. Each RF value represents a specific compound and hence gives an idea about the types of a particular phytochemical found in *R. indica* leaf extracts. A total of four alkaloids were observed, of which one was found in the acetone extract (0.94) while the other three were observed in the methanol extract (0.34, 0.75, 0.81). Three kinds of flavonoids were observed, two in methanol (0.72, 0.97) and the other in acetone (0.96). Three phenolic metabolites were observed, one each in acetone (0.85), methanol (0.59), and chloroform (0.85). Tannins were observed in acetone and methanol at 0.82 and 0.76 respectively. Two types of coumarins were observed, one each in acetone (0.78) and methanol (0.82). Three steroids were observed at 0.82, 0.84, and 0.84 in methanol, acetone, and chloroform respectively. A single spot at 0.17 shows the presence of amino acids.

Table 3: RF values of different phytochemical groups extracted from *R. indica*

S. No.	Phytochemical	Extract	RF Value
1	Alkaloid	Acetone	0.94
		Methanol	0.4,0.77,0.86
2	Flavanoid	Methanol	0.71,0.93
		Acetone	0.90
3	Phenol	Acetone Methanol Chloroform	0.88
			0.62, 0.27
			0.88
4	Tannins	Acetone	0.84
		Methanol	0.62
5	Coumarins	Methanol Acetone	0.82
			0.78
6	Steroids	Chloroform	0.80
		Acetone	0.71
		Petroleum ether	0.84
7	Amino acid and peptides	Acetone	0.34



Alkaloids



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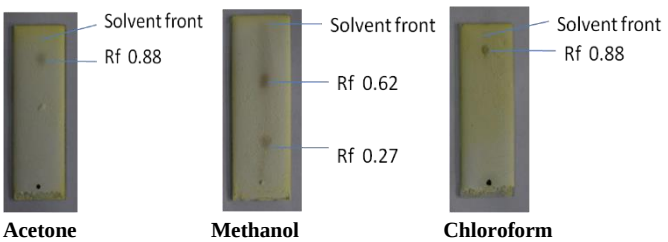
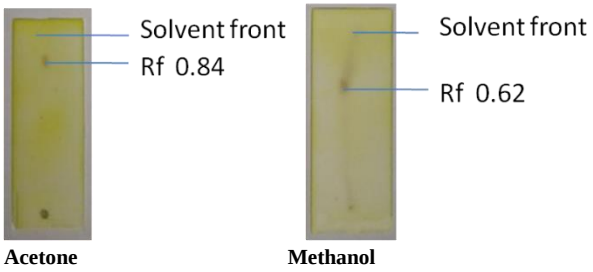


Phenols



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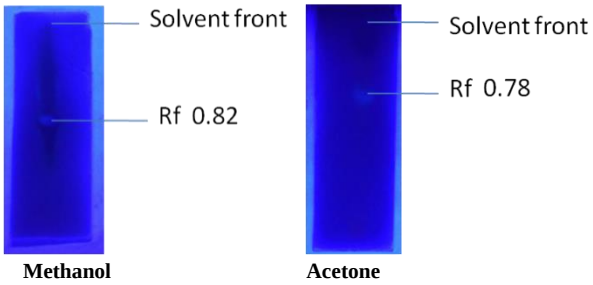
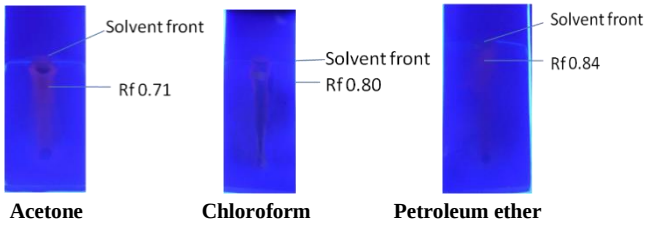
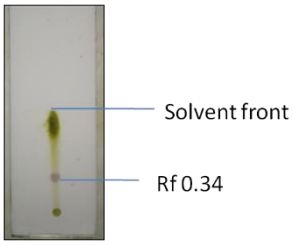


Coumarins



Steroids



Amino acids and peptides

DISCUSSION

Plants have been used since prehistoric times immemorial for their healing action and other medicinal properties shown by the variety of secondary metabolites or phytochemicals synthesized and accumulated in different parts of the plant. Screening of these compounds and the characterization of these bioactive molecules is the need of today as 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 use in disclosing new sources of economic compounds such as tannins. The most important of these bioactive compounds are alkaloids, flavonoids, tannins, and phenolic compounds [12].

The proximate study of *Reinwardtia indica* was shown in table 2 which revealed the different phytochemical constituents in varying amounts. The Saponins were found to be in the highest amount 17.3g/100g, followed by flavonoids (13g/100g) and alkaloids (2.04g/100g) while other bioactive phytochemicals were found in minute amounts. Saponin has been found therapeutically effective against cardiovascular diseases, lipid peroxide formation in kidney and liver, shown hypotensive activities [13]. Besides these saponins are also known for their analgesic, ant-nociceptive, antiviral and antifungal properties [14]. The flavonoid and terpenoids play a vital role in the defense against the free radicals [15]. These two phytochemicals show the various biological activities including anti-inflammatory, anti-viral, anti-bacterial, anti-fungal, hypoglycemic, and anti-cancerous properties [16].

The phenolic compounds are the most vital group of secondary metabolites and bioactive compounds which are a good source of natural antioxidants and are capable of free radicles scavenging activities, antidiabetic and anti-hypertension activity [17], prevent harmful effects of oxidative processes on macromolecules, such as proteins, carbohydrates, lipids and DNA and reduces the risk of cancer [18].

One of the major sources of phenolic compounds is tannins which are good radical scavengers and antibacterial agents that also show anticarcinogenic and antimutagenic potential [19]. A considerable amount of tannins were reported in *R. indica* leaves. The methanolic extract shows a significant amount of phenolic content this fact is also supported by earlier studies [5, 20]. Using standard graph total phenolic content is found to be 0.38g in 100g of the sample. These results show the potential medicinal value of *R. indica* and it can be used to derive the phenolics based phytochemicals.

Alkaloids are another group of phytochemicals that are known for antimalarial analgesic and stimulant properties [21]. A considerable amount of alkaloids were found in the plant.

Proteins, carbohydrates, and lipids are some important classes of biological macromolecules found in all organisms. These macromolecules are vital body nutrients present in every class of living beings. In the proximate analysis of *Reinwardtia indica* moderate amount of these macromolecules were found. Good fat content gives a major contribution towards energy requirements and can be used for feed and fodder of animals. Also, the nutritive value reflects the leaves as a good source of energy [22]. Quantitative analysis of steroid in done by Zak's method (1983) and about 0.3g of steroids was found in 100g of plant sample. The presence of Steroids helps the plant extracts to be used as antibacterial agents [23].

TLC Profiling of Plant Extracts in Different Solvent System Confirms the Presence of a Diverse Group of Phytochemicals. TLC profiling of all 4 extracts (Petroleum ether, Chloroform, Acetone, Methanol) reveals an impressive result, indicating the presence of different phytochemicals. Various phytochemicals give different Rf values in the different solvent systems. This variation in Rf values of the phytochemicals provides a vital clue in the understanding of their polarity and helps in the selection of an appropriate solvent system for the separation of pure compounds by Column Chromatography. Alkaloids were found in two extracts, acetone and methanol. The single compound was spotted in acetone extract at an Rf value 0.94 while 3 compounds were spotted in a methanolic extract with Rf 0.86, 0.77, and 0.40 respectively. Flavonoids were found in acetone with a single spot having Rf 0.90 and methanol with two spots having Rf 0.93 and 0.71. Phenols were spotted in 3 extracts viz. acetone methanol and chloroform. Two spots were observed in methanol with Rf 0.62 and 0.27 while a single spot was found in acetone with Rf 0.88. Tannins were spotted in two extracts acetone and methanol with Rf values 0.84 and 0.62 respectively. A single band of coumarins was spotted in acetone with Rf 0.78 and methanolic extract with Rf 0.82. Steroids were observed in 3 extracts, acetone with Rf value of 0.71, chloroform with Rf 0.80, and petroleum ether with 0.84 while amino acids and peptides are found in a single extract, chloroform with Rf value of 0.34. the TLC method stands out best among all known methods that are used for preliminary separation and gathering basic information on the type of compounds that are available in a certain plant. Different Rf values of the compounds indicate the type of compound [24]. This information will help in selecting an appropriate solvent system for further separation of the compounds from these plant extracts [25]. Similar work has been performed with six different medicinal plants by Solanki *et al.*, 2019 [24], they have also reported the presence of some important phytochemicals.

CONCLUSION

This study presents *Reinwardtia indica* as potential plant that can be looked upon for its medicinal properties. Though it was in use by the locals for some ailments but there was very meagre scientific records available. In regard this work can be considered and this less explored herb can be further utilized for therapeutic purposes.

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All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript.

Conflict of Interest

None declared.

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