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# A comparative evaluation of phytochemicals in bark, leaves and seeds of *Putranjiva roxburghii* Wall. (Putranjivaceae)

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**Abstract**
The bark, leaves and seeds of *Putranjiva roxburghii* Wall. (Putranjivaceae) were compared for their phytoconstituents using a series of solvents with varying degree of polarity. A preliminary qualitative evaluation for secondary metabolites in bark, leaves and seeds was carried out in five different solvents – petroleum ether, chloroform, ethyl acetate, methanol and water. The methanolic extracts were evaluated to quantify the phytoconstituents using standard protocols for alkaloids, flavonoids, phenolic compounds, tannins and terpenoids. A comparison of extraction efficacy of solvents indicated that polar solvents such as methanol and water were highly promising than the non-polar systems. All the phytoconstituents were present in higher quantity in leaves than bark and seed. Even though the seed is widely used in traditional medicine and Ayurveda, the present study suggested that the quantity of secondary metabolites was maximum in leaves, followed by bark and the seeds contain comparatively lesser level of all major classes of therapeutically promising secondary metabolites.

**Keywords:** Phytochemicals, *Putranjiva roxburghii*, secondary metabolites, methanolic extract

## Introduction
*Putranjiva roxburghii* Wall. Is a moderate-sized, evergreen tree, growing up to 12 m in height? It has pendant branches and dark grey bark having horizontal lenticels. Leaves are simple, alternately arranged, dark green, elliptic-oblong, distantly serrated. Female flowers 1-3 in the axil of leaf, male flowers are with short stalks, in rounded axillary clusters, fruits ellipsoid or rounded drupes; seed normally one, stone pointed, rugose, very hard [1].
In traditional medicine, leaf, bark and seeds of *P. roxburghii* Wall. Are used as medicine. Fresh juice of Putranjivaka is given to treat elephantiasis and the paste of the leaf is applied over the affected leg. The paste of the leaf is applied over the area affected with burning sensation. Ayurvedic classics have highlighted the importance of Putranjivaka as helps in pregnancy. The powder of the seed is given in a dose of 1-3 g with milk to improve the sperm count in males and help in maintaining the foetus in pregnant women. Leaves and fruits are used as medicine for rheumatism [2]. The leaf extracts and bio-oil extracted from seeds are mostly utilized in Ayurveda, Herbal and Unani medications [3].
The present study aims to analyze the phytochemical constituents of the bark, leaves and seeds of P. *roxburghii* Wall. Through successive extraction of plant parts in solvents with increasing polarity. A comparison of secondary metabolites in methanolic extracts on quantitative basis is also attempted for bark, leaves and seeds.

## Materials and Methods
### Chemicals
For the phytochemical studies of *Putranjiva roxburghii* Wall following chemicals were used. Wagner's reagent, Dragendroff's reagent, NaOH, $NH_4OH$ solution, HCl, ethyl acetate, chloroform, methanol, petroleum ether, ferric chloride, KOH, ethanol, Fehling's A and B, Folin-Ciocalteu reagent, glacial acetic acid and sulphuric acid. These chemicals were analytical grade and procured from SRL, Sd fine and Himedia.

### Collection of Plant Material
The plant material *Putranjiva roxburghii* Wall. Was collected from Thiruvananthapuram district, Kerala. Leaf, bark, and seed were procured for a comparative phytochemical evaluation on a qualitative and quantitative level.

**Preparation of Successive Extracts**

All plant parts used in the study were washed thoroughly in tap water, dried and powdered into a fine powder. Five gram of dried powder of bark, leaf, and the seed was extracted successively with 25 ml each of petroleum ether, chloroform, ethyl acetate, methanol and water. These five samples were placed in a water bath at a temperature of $40^0$C for 10 hours and then placed in a gyratory shaker at 120 rpm for 48 hours. Each type of extract was filtered using Whatman No.1 filter paper, dried to attain consistent weight and the yield and quality of extract were recorded. The final residues were stored in a refrigerator at $4^0$Ctill further use.

**Preliminary Qualitative Analyses**

A 30 ml stock solution (mg/ml) of extract from each type of solvent was prepared using the mother solvent. These extracts along with blanks were analyzed qualitatively for the presence of various phytochemicals. Phytochemical examinations were carried out for all the extracts of leaf, stem, and seed of *P. roxburghii* Wall**.** as per the standard methods[4].

**1. Test for Alkaloids**

Approximately 50mg of the extract was dissolved in 5 ml of distilled water and 2ml of HCl was added until complete precipitation and filtered. The filtrate was tested for the presence of alkaloids as below.

**a) Dragendroff's test**: 1ml of Dragendroff's reagent was added to 2ml of the filtrate along the side of the test tube. Formation of a reddish brown precipitate indicated the presence of alkaloid.

**b) Wagner's test**: Two drops of Wagner's reagent was added to 1ml of the test solution along the side of the test tube. The formation of yellow or brown precipitate confirmed the presence of alkaloids

**2. Test for Flavonoids**

A small quantity of each extract was heated with 10ml of ethyl acetate in boiling water bath for 3 minutes. The mixture was filtered and filtrates are used for the following tests.

**a) Ammonium test**: the filtrate was shaken with dilute ammonia solution (1ml, 1%, v/v). The layers were allowed to separate. A yellow color observed at ammonia layer indicated the presence of flavonoid.

**b) Alkaline reagent test**: The extract (2ml) was treated with few drops of 20% (w/v) NaOH solution. Formation of intense yellow color, which turned colorless on the addition of dilute HCl indicated the presence of flavonoids.

**c) Shinoda test**: A few magnesium turnings and 5 drops of concentrated hydrochloric acid were added drop wise to 1ml of test solution. A crimson red color appeared after few minutes confirmed the presence of flavonoid.

**3. Test for Phytosterols/ Terpenoids**

**Liebermann-Burchard's test:** 2 mg of the extract was dissolved in 2ml of acetic acid anhydride, heated to boiling, cooled and then 1ml of concentrated sulphuric acid was added along the side test tube. A brown ring formation at the junction confirmed the test for the presence of phytosterols.

**4. Test for Tannin**

**Ferric chloride test:** A few drops of 5% (w/v) $FeCl_3$ solution was added to the test solution (2ml). Formation of bluish black color indicated the presence of hydrolysable tannin.

**5. Test for Cardiac Glycosides**

**Keller-Killiani test:** Added glacial acetic acid (.4 ml) and a few drops of 5% ferric chloride solution to a little of dry extract. Further concentrated $H_2SO_4$ (0.5ml) was added along with the side of the test tube carefully. The presence of blue color in acetic acid layer confirmed the test.

**6. Test for Phenol**

Extracts were treated with 3-4 drops of 10% (w/v) $FeCl_3$ solution. Formation of greenish black color indicates the presence of phenol.

**7. Test for Triterpenoids**

**Salkowski test:** Dry extracts (2mg) was shaken with chloroform (1ml) and a few drops of Conc. $H_2SO_4$ were added along the side of the test tube. A red-brown color formed at the interface indicated the presence of triterpenoids.

**8. Test for Anthraquinone Glycosides**

**Hyhydroxyanthraquinone test:** To extract (1ml), added a few drops of 10% (w/v) KOH solution. The formation of red color confirmed the test.

**9. Test for Proteins**

**Biuret test:** To 2ml of the test solution added 5 drops of 1% (w/v) $CuSO_4$ solution and 10% NaOH (2ml). Mix thoroughly, Formation of purple or violet color confirmed the presence of protein.

**10. Test for Fats and Fixed Oils**

To 5 drops of the sample was added 1ml of 1% CuSO4 solution and a few drops of 10% NaOH. Formation of clear blue solution confirmed the test.

**11. Test for Saponin**

**Foam test**: The test solution (5ml) taken in a test tube and shaken well for five minutes. Formation of stable foam confirmed the test.

**Quantitative Analyses of Secondary Metabolites**

**1. Detection of Alkaloids- (Harborne, 1984) Method [5]**

Powdered sample, 1 g was treated with 40 ml of 20% acetic acid and kept undisturbed for 40 h. The solution was filtered and the volume was reduced to one fourth in a water bath. To this sample, concentrated ammonium hydroxide was added drop wise for precipitation reaction. The whole solution was allowed to settle and the precipitate was collected by filtration and weighed [6]. The percentage of total alkaloid content was calculated as:

$$\text{Percentage of total alkaloids (\%)} = \frac{\text{weight of residue} \quad \text{x } 100}{\text{Weight of sample taken.}}$$

**2. Determination of Total Phenolics**

To 0.1ml of the extract add 3.9 ml of distilled water and 0.5ml of Folin Ciocalteau reagent. Incubate the solution at room temperature for 3 minutes and then add 2ml of 20% (w/v) sodium carbonate solution to the mixture. The solution is kept in a boiling water bath for 1 minute, cooled and the absorbance was recorded at 650 nm. Gallic acid was used as standard to express the total phenol content [7].

**3. Determination of Total Flavonoids**

The protocol by Zhishen *et al.* [8], employing aluminium chloride colorimetric assay was used to quantify the total flavonoids. 1ml of plant extracts were diluted with 200 µl of distilled water followed by the addition of 150 µl of sodium nitrite (5%) solution. This mixture was incubated for 5 minutes and then 150µl of aluminium chloride (10%) solution was added and allowed to stand for 6 minutes. Then 2ml of sodium hydroxide (4%) solution was added and made up to 5ml with distilled water. The mixture was shaken well and left it for 15 minutes at room temperature. The absorbance was measured at 510 nm. The appearance of a pink color showed the presence of flavonoid content.

**4. Determination of Total Tannin**

**a) Reagents**

**Coloring agent**: dissolve 1.6221gm of ferric chloride (0.1M), 0.9 ml of hydrochloric acid (0.1N) and 263.4 mg of potassium Ferro cyanide (0.008M) in 100 ml of water. Tannic acid was used as standard and the tannin content was recorded as tannic acid equivalent.

**Protocol**: Boil 1gm of the sample with 100 ml of water for 30 minutes, cool and filter through a Whatman no.1 filter paper and it makes up to 100ml with distilled water. Take 0.5ml of the coloring agent. Read the blue color developed at 760nm against reagent blank after 30 minutes at room temperature. Coloring agent was added to standard tannic acid samples with concentrations in the range –20-100µg and calculate the amount of tannic acid equivalent. The value was expressed as mg of tannic acid equivalent (TE) per gram of dried sample [9].

**Determination of Total Terpenoid**

To estimate total terpenoid content in the plant extract, 0.5 gm of each powder was dissolved in 5 ml methanol and 7.5 ml water. The mixture was shaken well and centrifuged at 3000-4000 rpm for 10minutes. The filtrate was taken. Take 1ml of extracts, add 2ml of chloroform followed by 3ml of concentrated sulphuric acid. Terpenoids estimated by reading the absorbance of reddish brown solution at 538nm. The blank used was 95% methanol.

**Results and Discussion**

**Yield of Extracts**

The successive extracts of seed leaf and bark of *Putranjiva roxburghii* Wall. Were compared for their quality and yield. The yield of crude extract was obtained by measuring its dry weight. The yield was found to be low in petroleum ether due to its low polarity and more in methanol and aqueous phases. The yield of extract in various solvent systems is shown in Table. 1.

**Phytochemical Analysis**

**Qualitative Analysis**

A preliminary screening of each extract was performed following the standard phytochemical analysis protocol l [10]. The results of the qualitative analysis of extracts of seed, leaf and bark of *P. roxburghii* Wall. Are illustrated in Tables 2. On basis of the intensity of the reaction product of qualitative tests, the data were graded as very high, (+++), high (++), moderate (+) and nil (-). The phytochemical tests employed indicated that methanolic and aqueous extracts contained most of the secondary metabolites. However, the extractions carried out with petroleum ether and ethyl acetate, chloroform indicated the little number of phytoconstituents. The major phytoconstituents detected were alkaloids, flavonoids, triterpenoid, phenol, phytosterols, saponins, fat and fixed oil.

Alkaloids were present in chloroform, ethyl acetate, methanol, aqueous extract of leaf, seed, bark; but it is absent in petroleum ether extract of leaf, seed, bark Alkaloids are comparatively higher in leaf and bark than seed. Methanolic and aqueous solvent better for the alkaloid extraction than chloroform and ethyl acetate.

Flavonoids were present in leaf, bark and seed of *P. roxburghii* Wall. From the Alkaline test and Shinoda test, it is found that leaves have higher flavonoid content than bark and seed. All the solvent except petroleum ether and ethyl acetate showed a positive result for flavonoid. Methanolic and aqueous extracts are most suitable for flavonoid extraction.

Triterpenoid was present in small amount in the methanolic and aqueous extract of leaf, bark and seed, and it's found higher in aqueous extract of the three plant parts. It is found absent in ethyl acetate, petroleum ether and chloroform extracts. Tannin is found in higher amounts (+++) in the methanolic and aqueous extract of leaf, bark has a moderate amount of tannin and seed has comparatively lesser amount of tannin. All other extracts don't show a positive result for Ferric chloride test.

Phenols are also found higher in leaf than bark and seed. Methanolic and aqueous are best for phenol extraction than others.

**Table 1:** The yield and nature of extracts of Bark, Leaf and Seed samples of *P. roxburghii* Wall. After successive extraction. (Duration of extraction = 60 h; Treatment temperature = $45^0$C)

| Name of part | Weight of powder (g) | Name of solvent | The yield of extract (g) | Yield/ 100g | Nature of extract |
|---|---|---|---|---|---|
| Leaf | 5 g | Petroleum ether | 0.099 | 1.98 | Dark green |
| | | Chloroform | 0.177 | 3.51 | Dark green |
| | | Ethyl acetate | 0.237 | 4.74 | Dark green |
| | | Methanol | 0.516 | 10.32 | Dark green |
| | | Water | 0.664 | 13.28 | Light brown |
| Bark | 5 g | Petroleum ether | 0.026 | 0.52 | Light green |
| | | Chloroform | 0.064 | 1.28 | Light green |
| | | Ethyl acetate | 0.062 | 1.24 | Light green |
| | | Methanol | 0.196 | 3.92 | Light green |
| | | Water | 0.209 | 4.18 | Light brown |
| Seed | 5g | Petroleum ether | 0.205 | 4.10 | Light yellow, Oily |
| | | Chloroform | 0.478 | 9.56 | Light yellow, Oily |
| | | Ethyl acetate | 0.482 | 9.64 | Light yellow, Oily |
| | | Methanol | 0.288 | 5.76 | Light yellow, Oily |
| | | Water | 0.367 | 7.34 | Light yellow, Oily |

Anthraquinone was found in a lesser amount (+) in aqueous extract of leaf, seed and bark, and also found in the methanolic extract of the leaf. In other solvent hydroxy anthraquinone test don't show a positive result.

Presence of cardiac glycosides is tested by Keller-Killiani test and found that in all solvents it is absent. Saponin is tested and found in the methanolic and aqueous extract of leaf, bark, and seed. Leaf (+++) and bark (+++) has comparatively higher amount of saponin than seed.

Proteins are found absent in all solvents of leaf, bark, and seed. The methanolic, chloroform, ethyl acetate, petroleum ether extract of seed indicated the presence of oil, aqueous extract doesn't show the presence of oil since it is a highly polar solvent. Phytosterols are found present in the methanolic and aqueous extract of leaf, bark, and seed methanolic extract of leaf showed (+++) higher amount of phytosterols. In other solvents, the Liebermann Burchard test yielded a negative result.

Significant differences in the quantity of phytoconstituents were observed in different solvents for bark, seed and leaf samples. In the present study, the polar solvents were detected as more effective in extracting the secondary metabolites from bark, seed and leaves of *P. roxburghii* Wall. The poor efficacy of non-polar solvents could be due to insolubility of secondary metabolites in non-polar solvents. It may also be due to poor permeation level of non-polar solvents and their inefficiency to act at intracellular regions to extract the phytochemicals Alcohol, being more polar. Can easily penetrate the cell wall and membranes to extract the intracellular ingredients. Earlier reports on screening of solvents for yield of phytoconstituents from various plant sources also suggested the superiority of polar solvent extracts [11-14]. Though traditional healers use primarily water to prepare herbal decoctions, extracts employing organic solvents have been found to give more consistent activity in comparison to aqueous extracts [15]. Another drawback of aqueous based preparations is that they are more likely to be easily contaminated by microbial growth.

**Table 2:** Phytochemicals present in the successive extracts of Leaf, Seed, and Bark of *P. roxburghii* Wall. (Duration of extraction = 50hours; Treatment temperature = $45^0$C)

| Name of test | Petroleum ether | Chloroform | Ethyl acetate | Methanol | Water | |
|---|---|---|---|---|---|---|
| Alkaloids Wagner's test Dragendroff's test | - | + | ++ | +++ | ++ | Bark |
| | - | + | ++ | +++ | +++ | Leaf |
| | - | + | + | ++ | ++ | Seed |
| | - | + | ++ | +++ | ++ | Bark |
| | - | + | ++ | +++ | +++ | Leaf |
| | - | + | + | ++ | ++ | Seed |
| Flavonoids Alkaline test Shinoda test | - | - | - | + | ++ | Bark |
| | - | + | - | ++ | +++ | Leaf |
| | - | - | - | + | + | Seed |
| Triterpenoids Salkowski test | - | - | + | + | + | Bark |
| | - | - | + | ++ | ++ | Leaf |
| | - | - | - | + | - | Seed |
| Tannin $Fecl_3$ | - | - | - | + | ++ | Bark |
| | - | - | - | +++ | +++ | Leaf |
| | - | - | - | - | - | Seed |
| Phenol | - | - | - | + | ++ | Bark |
| | - | - | - | +++ | +++ | Leaf |
| | - | - | - | + | + | Seed |
| Anthraquinone Hydroxy anthraquinone | - | - | - | - | + | Bark |
| | - | - | - | + | + | Leaf |
| | - | - | - | - | + | Seed |
| Cardiac glycosides Keller-Killiani test | - | - | - | - | - | Bark |
| | - | - | - | - | - | Leaf |
| | - | - | - | - | - | Seed |
| Saponins | - | - | - | +++ | +++ | Bark |
| | - | - | - | +++ | +++ | Leaf |
| | - | - | - | +++ | ++ | Seed |
| Protein | - | - | - | - | - | Bark |
| | - | - | - | - | - | Leaf |
| | - | - | - | - | - | Seed |
| Fats & fixed oil | - | - | - | - | - | Bark |
| | - | - | - | - | - | Leaf |
| | +++ | +++ | ++ | +++ | - | Seed |
| Phytosterol Libermann-Burchards test | - | - | - | + | + | Bark |
| | - | - | - | +++ | ++ | Leaf |
| | - | - | - | ++ | ++ | Seed |

(+++ High; ++Moderate; + Low; - Nil)

**Quantitative Analysis**

**Total alkaloid content**

The total alkaloid content was recorded as high in leaf and low in seed and the observations are depicted in Table 3. The results on the alkaloid level suggested that both leaf and bark are ideal sources of alkaloids.

**Total Flavonoid content**
As illustrated in Table 3 the total flavonoid was highest in the methanolic extract of leaf (18.437mg/g) than bark (9.875mg/g) and seed (2.250mg/g).

**Total Phenolic content**
The total phenolic content was found highest in methanolic extract of the leaf (196.54 mg/g), and bark (66.66 mg/g) than seed (21.27mg/g) as illustrated in Table 3.

**Total Terpenoid content**
In the comparative study of leaf, bark, seed, total terpenoid was found highest in leaf (7.264 mg/g). Bark has more terpenoid content (2.048 mg/g) than seed (1.036 mg/g). It is illustrated in Table 3.

**Total Tannin Content**
The concentration of tannin, estimated as tannic acid equivalent (TE mg/g) was recorded to be in the range of leaf (65.6 mg/g), bark (22.4 mg/g) and seed (3.36 mg/g) in methanolic extract (Table 3)

**Table 3:** Total phytochemical content (mg/g) in methanolic extracts of Bark, Leaf and Seed of *P. roxburghii* Wall.

| Phytochemicals (mg/g) | | | | | |
|---|---|---|---|---|---|
| **Extract** | **Alkaloid** | **Flavonoid** | **Phenol** | **Tannin** | **Terpenoid** |
| Bark | 0.045 | 9.875 | 66.66 | 22.4 | 2.048 |
| Leaf | 0.031 | 18.437 | 190.54 | 65.6 | 7.204 |
| Seed | 0.016 | 2.250 | 21.27 | 3.36 | 1.036 |

**Conclusions**
A comparative evaluation of phytochemical constituents in bark, leaf and seed suggested that leaf contained highest levels of all secondary metabolites. The phytoconstituents were best eluted in polar solvents and the major phytochemical in bark, leaf and seeds was phenolic compounds. In general, phenolic compounds have antioxidant and anti- inflammatory potential and therefore further studies are to be focused on the response of leaf extract.

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