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*Full Length Research Paper*

# Analysis of total flavonoids and phenolics in different fractions of bark and needle extracts of *Pinus roxburghii* and *Pinus wallichiana*

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**Total flavonoid and phenolic content was estimated quantitatively by using colorimetric method in various fractions of bark and needle extracts of *Pinus roxburghii* and *Pinus wallichiana*. Flavonoids may exist as free aglycone, but usually they are bound to sugar as glycosides and any one aglycone may occur in single plant in several combinations. For the measurement of total flavonoid content, original plant extract was hydrolysed and aglycone was measured before and after hydrolysis to calculate the presence of free aglycone content. Flavonoid contents (aglycone) of the extracts, in terms of quercetin equivalent (the standard curve equation: y = 0.0877× -0.0595, $r^2$ = 0.9981), were in the range of 60.1 ± 4.5 to 484 ± 2.5 before and after hydrolysis. As such, the values fell in the range of 76.9 ± 3.2 to 943 ± 2.6. The total phenol varied from 394 + 0.03 to 1331 + 0.24 measured in terms of gallic acid equivalent (with standard equation: y = 0.0197 × -0.0076, $r^2$ = 0.9997).**

**Key words:** Flavonoid, phenolics, aglycone, *Pinus wallichiana*, *Pinus roxburghii*.

## INTRODUCTION

Plant phenolics are secondary metabolites with diverse chemical nature and potential including: phenolic acids, flavonoids, tannins, coumarins, lignans, xanthones and stilbenes (Liu, 2004; Harborne, 1980). Flavonoids and phenolics detected and isolated from the bark and needles of *Pinus roxburghii* and *Pinus wallichiana* are listed in (Table 1). These extracts are reported to exhibit antioxidant activity (Maimoona et al., 2011).

Flavonoids play important role in imparting bright colours to flowers, fruits and berries that make the biosphere beautiful (Brouillard and Dangles, 1993). In addition to their biological, nutraceutical and clinical effects (Maimoona et al., 2011), flavonoids including proanthocyanidins are implicated in various plant defense mechanisms (Stafford, 1988). Flavonoids may act as Synder and Nicholson, 1990; Dixon, 1986). These bioactive defense compounds are also responsible for plant responses to environmental hazards, such as temperature fluctuations (Alonso et al., 2007), air pollution (Gietych and Karolewski, 1993) and UV radiation (Tegelberg et al., 2004; Kan et al., 1998). Phenolic compounds vary greatly after heat damage to stems and crown, proving their worth as bioindicators of thermal stress. Accidental fire or prescribed burning activates secondary metabolism to produce phenolic compounds (Alonso et al., 2002; Cannac et al., 2007).

The increasing numbers of automobiles, industries and thermal power plants have continuous additive effect on atmospheric pollutants. Plants, which are rightly spoken as the lungs of nature, act as the sinks for various pollutants by absorbing, accumulating and integrating them. Pollution can be detected at low levels and at an early stage by computing the chemical compounds as the physiological changes are faster than the morphological and anatomical parameters (Pasqualini et al., 2003). Moreover, a significant increase in total flavonoid and phenolic content has been related with the increased vehicular pollution (Qayoom et al., 2009). Total phenolic content of *Pinus halepensis* showed positive correlation

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**Table 1.** Flavonoids/phenolics detected and isolated from bark and needles of *Pinus roxburghii* and *Pinus wallichiana.*

| Flavonoids/Phenolics | *Pinus roxburghii* | | *Pinus wallichiana* | | Reference |
|---|---|---|---|---|---|
| | Bark | Needles | Bark | Needles | |
| Quercetin | ++ | ++ | ++ | ++ | Naeem et al. (2010) and Willfor et al. (2009) |
| Taxifolin | ++ | -- | -- | -- | Willfor et al. (2009) |
| Catechin | ++ | -- | ++ | -- | Willfor et al. (2009) |
| Quercetin derivative | ++ | -- | -- | -- | Willfor et al. (2009) |
| Taxifolin derivative | -- | -- | ++ | -- | Willfor et al. (2009) |
| Catechin and gallocatechin derivative | ++ | -- | ++ | -- | Willfor et al. (2009) |
| Kaempferol | ++ | -- | ++ | -- | Naeem et al. (2010) |
| Rhamnetin | ++ | -- | ++ | -- | Naeem et al. (2010) |
| Isorhamnetin | -- | -- | ++ | ++ | Naeem et al. (2010) |
| Myricetin | -- | ++ | ++ | -- | Naeem et al. (2010) |
| 3,4-dihydroxybenzoic acid | ++ | -- | ++ | -- | Willfor et al. (2009) |
| 3,4-dihydroxycinnamic acid | ++ | -- | -- | -- | Willfor et al. (2009) |
| Monomethyl Pinosylvin | -- | -- | ++ | -- | Willfor et al. (2009) |
| Dihydro-monomethyl Pinosylvin | ++ | -- | ++ | -- | Willfor et al. (2009) |
| Resveratrol glycoside | -- | -- | ++ | -- | Willfor et al. (2009) |
| Pinoresinol | ++ | -- | -- | -- | Willfor et al. (2009) |
| Secoisolariresinol | ++ | -- | ++ | -- | Willfor et al. (2009) |

with $SO_2$ and negative correlation with nitrogen oxide concentration, while ozone gives no significant results (Pasqualini et al., 2003). Flavonoids and phenolics have been proven to be UVB protectants in many studies in different plants (Kenneth et al., 2008; Ryan et al., 1998), including conifers (Fischbach et al., 1999). In addition, plant phenolics not only hinder the pest attack at larval stage (Clifford et al., 1997), but also provide defence against mammalian herbivory (Harborne, 1991) acting as antifeedant.

Conifers, which represent the largest group of present day gymnosperms and which constitute the major boreal forest, famous for excellent timber and other commercially important products, are reported for their strong allelopathy. These canopy plants determine the pattern of understorey vegetation through allelopathic interactions which are caused by leaching of phenolics largely from litter of tree needles which take longer time to decompose (Singh et al., 1999). The fact that herbicides used for weed control are costly and have negative impact on environment has provoked the need of searching and developing alternatives which would be cheaper and environment friendly (Albuquerque et al., 2010). Naturally occurring plant phenolics may be assorted on the basis of allelochemical structures and action mechanism for their potential to act as lead compounds for synthetic herbicides and pesticides (Li et al., 2010).

The proposed research is aimed at analyzing the extracts from barks and needles of two pine species for total flavonoid and phenolic contents so that utilization of pine forests may be extended in the light of the role of flavonoids and phenolics discussed previously. The research may have considerable advantage to agri-cultural and horticultural industry and authorities involved in environmental conservation.

## MATERIALS AND METHODS

Based on the significance of total flavonoids and total phenolics, experimental procedures were adapted to analyze their content in different fractions of crude extracts obtained from bark and needles of *P. roxburghii* and *P. wallichiana* using different extracting solvents. Flavonoids and phenolics were calculated as quercetin and gallic acid equivalent, respectively.

### Plant material

Fresh plant material was collected from different parts of Murree Hills, Pakistan in May 2009.

The plant had previously been identified and deposited in Prem Madan herbarium of Lahore College for Women University, Lahore, with voucher specimen PM≠0101 for *P. wallichiana* and PM≠ 0102 for *P. roxburghii.*

### Instruments

UV/Vis double beam spectrophotometer (Hitachi, U2800) and 1 cm quartz cells were used for all absorbance measurements.

### Reagents and solutions

The extracting solvents used were all of analytical grade, including methanol, *n* -hexane, dichloromethane, ethylacetate and butanol, and were obtained from Merk Germany. Other reagents and

**Table 2.** Extractions and fractionation: Percentage yield of different extractants from bark and needles of two pine species (dry weight bases).

| Material | MeOH ext. (%) | n-Hexane fraction (%) | Dichloromethane fraction (%) | EtOAc fraction (%) | Butanol fraction (%) | Aqueous fractions (%) |
|---|---|---|---|---|---|---|
| *Pinus roxburgii* bark | 18.64 | 0.001 | 0.039 | 1.15 | 2.35 | 0.109 |
| *Pinus roxburgii* needles | 12.86 | 0.84 | 0.69 | 0.26 | 1.10 | 8.96 |
| *Pinus wallichiana* bark | 6.22 | 0.001 | 0.002 | 0.21 | 1.03 | 0.31 |
| *Pinus wallichiana* needles | 10.07 | 0.44 | 0.61 | 0.24 | 1.03 | 4.70 |

standards: acetone, hydrochloric acid, hexamethylenetetramine, sodium nitrite, aluminium chloride, sodium hydroxide, sodium carbonate, folin-ciocalteu reagent, quercetin and gallic acid, were purchased from Sigma Aldrich available commercially.

#### Preparation of plant extracts

The crude methanolic extract of both bark and needles of the two plant species were obtained by dipping the air-dried material of each into 100% methanol for ten days. After evaporating the solvent at low pressure in rotary evaporator at temperature below 40°C, the extract was dissolved in deionized water and then fractionation was done using different solvents with polarity gradient. (Table 2) shows the details of extraction and fractionation procedure along with the weight of each fraction obtained.

#### Determination of total flavonoids

For the assessment of flavonoids, the colorimetric method introduced by Dewanto et al. (2002) was adapted. As this method is restricted to aglycone part, quercetin was used as standard and falvonoid contents were measured as quercetin equivalent. For this purpose, the calibration curve of quercetin was drawn. Many flavonoids exist in the form of glycosids. So, flavonoid content was calculated in plant samples before and after hydrolysis and in this way, the amount of flavonoids present in the form of free aglycone was also measured. To determine the amount of flavonoids by the aforementioned method, 1.50 ml of deionized water was added to 0.25 ml of the sample extract from stock solution (concentration of 1 mg/ml of methanol), followed by 90 µl of 5% sodium nitrite ($NaNO_2$). Six minutes later, after addition of 180 ul of 10% $AlCl_3$, the mixture was allowed to stand for another 5 min before addition of 0.6 ml of 1 M NaOH. By adding deionized water and mixing well, final volume was made up to 3 ml. Using blank, absorbance was measured at 510 nm.This procedure was performed for each sample extract before hydrolysis to know about the aglycone content. For the purpose of hydrolysis, 10 ml of acetone, 1 ml of 25%HCl and 0.5 ml of 0.5% hexamethylenetetramine was mixed with 15 ml of the extract and refluxed at 56°C for 30 min and finally tested for total flavonoids.

#### Determination of total phenolics

To analyze the total phenolic content (TPC), the method of Kim et al. (2003) was used to make the Folin Ciocalteu reagent. For 0.2 ml of the extract (prepared in methanol with a concentration of 1.0 mg/ml), 0.4 ml of Folin-Ciocalteu reagent was mixed and the solution was allowed to stand at 25°C for 5 to 8 min before adding 0.2 ml of 7.0% sodium carbonate solution. Using deionized water, the final volume was made to 10.0 ml. After two hours, absorbance was measured at 765 nm. Thus, the calibration curve was drawn using gallic acid as standard for total phenolics (TPC) which was measured as mg gallic acid equivalents (GAE) per gram of the sample (mg/g).

#### Statistical analysis

The results of the spectrophotometric analysis were expressed as Mean ± SD (SDOM) upon three independent analyses.

## RESULTS AND DISCUSSION

All fractions of the bark and needles of two pines, with the exception of aqueous fraction, contain flavonoids and phenolics to a varying extent. Flavonoids exist in plants in different combinations, as glycosides so dependent on the sugar moity, appear in different fractions. However, the maximum flavonoid content as aglycone of the two barks was present in ethylacetate and dichloromethane fractions of *P. roxburghii and P. wallichiana*, respectively. The total flavonoid content, including glycosides after hydrolysis, is at its maximum in dichloromethane fraction of the two barks, while the total phenolic content is at its maximum in *n*-hexane fraction of *P. roxburghii* and ethylacetate fraction of *P. wallichiana.* In the case of needles, with only an exception of the total phenolics of *P. wallichiana* which appear in dichloromethane fraction, all others are at their maximum in ethylacetate fractions of the needles.

Usually, most of the flavones and flavonols get extracted in dichloromethane and ethylacetate fractions due to their polar nature. The presence of flavonoids and simple phenolics like phenolic acid in different pine species has been reported variously (Ye-sil-Celiktas et al., 2009; Senthilmohan et al., 2003; Rohdewald et al., 2002), but as mentioned earlier, plant phenolics are quite variable, including stilbenes, coumarins, tannins, lignans and xanthones as well. Stilbenes, the plant phenolics with somewhat nonpolar nature, have been reported in barks of different plants (Oleszek et al., 2001;Ito et al., 2005; Muhtadi et al., 2006; Wieslaw et al., 2001; Cui et al., 2008). Resveratrol is the stilbene most commonly found in pine wood and may occur with phenolic hydroxyls, methylated or as glycosides (Norin et al., 1989); whereas pinosylvin is the stilbene found in *Pinus sylvestris* (Hovelstad et al., 2006). Coumarins, like umbelliferone

**Table 3.** Analysis of contents of total flavonoids and total phenolics of the two pine species.

| Plant species (Part used) | Fractions (Solvent) | Aglycone content mgQE/100 g (Dry mass) | Total flavonoids mgQE/100 g (Dry mass) | Total phenolics mgGAE/100 g (Dry mass) | Flavo/Phen ratio |
|---|---|---|---|---|---|
| *Pinus roxburghii* (Bark) | *n*- Hexane | 129±2.64 | 257±3.56 | 1331±0.24 | 0.193 |
| | Dichloromethane | 140±1.67 | 740±2.09 | 810±0.31 | 0.913 |
| | EtOAc | 223±2.31 | 334±1.91 | 891±0.03 | 0.374 |
| | Butanol | 151±1.11 | 276±6.53 | 893±0.15 | 0.309 |
| | Aqueous | -- | 97.4±5.12 | -- | -- |
| *Pinus roxburghii* (needles) | *n*-Hexane | -- | 108±3.12 | 947±0.05 | 0.1140 |
| | Dichloromethane | -- | 160±4.01 | 942±0.19 | 0.169 |
| | EtOAc | 262±6.23 | 428±2.17 | 1008±0.06 | 0.424 |
| | Butanol | 213±1.04 | 391±2.03 | 855±0.28 | 0.457 |
| | Aqueous | -- | 100.9±6.31 | 394±0.03 | 0.256 |
| *Pinus wallichiana* (Bark) | *n*-Hexane | -- | -- | -- | -- |
| | Dichloromethane | 484±2.5 | 943±2.6 | 515±3.03 | 1.83 |
| | EtOAc | 281±1.37 | 522±4.03 | 1261±1.25 | 0.413 |
| | Butanol | 110.0±1.14 | 253±2.73 | 591±2.31 | 0.428 |
| | Aqueous | 145±1.92 | 195.5±1.23 | 510±3.07 | 0.383 |
| *Pinus wallichiana* (needles) | *n*-Hexane | 60.1±4.5 | 76.9±3.2 | -- | - |
| | Dichloromethane | 113±3.02 | 417±1.17 | 510±1.81 | 0.817 |
| | EtOAc | 120±2.2 | 395±3.21 | 409±0.43 | 0.965 |
| | Butanol | 79±1.15 | 259±1.09 | 406±1.12 | 0.637 |
| | Aqueous | -- | -- | -- | -- |

and its derivative hernarin, have been reported from different plants (Iqbal et al., 2009). Marmin is the coumarin extracted from the bark of Bael tree and grapefruit skin. So, different phenolics with degree of polarity exist in different fractions of the pines' bark and needles extracts. The comparison of the results obtained from the methods discussed previously is made on gram bases, that is, the flavonoid content in the extracted solutions is calculated as mg quercetin equivalent per 100 g of the dried plant material, as presented in (Table 3).

## Conclusion

The two pine species are rich in flavonoid and phenolic content. Barks and needles contain various types of phenolics and flavonoids. The function of plant flavonoids and phenolics is thought to be that of providing resistance against fungi and insects. So, pine wood is the best in the wood industry and is rich in tannins. Pines also play a role as antipollutant, keeping the air fresh and this is probably attributable to their flavonoid and phenolic contents which act as sinks for different pollutants. Therefore, to control pollution in urban and industrial areas, plantation of pine trees should be promoted if possible. Conifers show allelopathy which is mostly due to phenolics of the needles litter on the forest floor. Nonetheless, utilization of this raw material as herbicides might be possible.

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