Original Article

Antioxidant activities in stem bark, leaves, and fruits of *Olea ferruginea* Royle grow in Himachal Pradesh, in relation to altitudinal changes

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Abstract

Background: *Olea ferruginea* Royle is one of the important plant which is commonly used by local people for their health benefits as folk medicine. The secondary metabolites of plants vary due to different abiotic and biotic stresses. In the present study, variation in phenolics and flavonoids content and antioxidant activity due to altitude, variation has been studied.

Aim: The aim of the study was to investigate the phytochemical content and antioxidant activity of the stem bark, leaf, and fruits of *O. ferruginea* collected from five populations (Thalaut, Sapangi, Suind, Kolibeher, and Kais) of North-west Indian Himalayan.

Materials and Methods: Stem bark, leaf, and fruit extracts were prepared by the maceration process using 80% (v/v) methanol and phytochemicals (phenolics and flavonoids) contents as well as their antioxidant activities were analyzed using *in vitro* assays, namely DPPH (2, 2-Diphenyl-1-picrylhydrazyl) ABTS (2, 2'-Azino-bis 3-ethylbenzothiazoline- 6-sulfonic acid), and ferric-reducing antioxidant power (FRAP).

Results: The results revealed that phenolics (mg GAE/g fw) and flavonoids (mg QE/g fw) varied between 5.2–9, 5.1–9.4 and 4.6–8, and 0.8–1.8, 8.8–22.8 and 0.8–1.1, respectively, in stem bark, leaf, and fruits. Average DPPH, ABTS, and FRAP activities were found highest in the methanol extracts of stem bark, leaf, and fruits, respectively, of *O. ferruginea* plants. The biochemical attributes of the test plant's parts showed positive and significant correlations with altitudes ($R^2 = 0.86–0.99$; $P < 0.01$). Principal component analysis showed that Sapangi and Kais population is biochemically different from other populations (Thalaut, Kolibeher, and Suind).

Conclusion: The present study reveals that stem bark, leaf, and fruits of the *O. ferruginea* are a rich source of natural antioxidants and may be exploited for commercial and health benefits.

Keywords: Altitude, antioxidant activity, *Olea ferruginea* Royle, phenolics, population

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INTRODUCTION

Free radicals are generated during the aerobic metabolic process and are involved in various regulatory mechanisms such as cell proliferation, apoptosis, and gene expression but when generated in excess, they can hamper the defensive potential of the antioxidant system, damage the essential biomolecules in the cell due to oxidation of membrane lipids, cell proteins, carbohydrates, DNA, and enzymes.[1] Oxidation reactions of biomolecules are not only important to the food industries and inside the human system but also required to avoid the deterioration of products found in cosmetics, pharmaceuticals, and plastic industries.[2] Antioxidants are substances that have the ability to inhibit or delay the oxidation of biomolecules caused by free radicals. These antioxidant compounds have the capacity to neutralize the free radical.

Nowadays, several synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxyl toluene are commercially available and are frequently being used but because of their synthetic origin and adverse effect on the biological system, it is important to replace them by exploring the potential of biodiversity elements as a natural source of antioxidants. The demand for natural additives, including antioxidants, has grown worldwide in recent years.[3] It is well-known that plants are one of the major sources of food, fodder for humans and cattle, and also is being used for the therapeutic purpose since ancient times. Because of technological advancement, it is known that plants used for the therapeutic purposes are an excellent source of many phytochemicals such as phenolics, flavonoids, carotenoids, anthocyanin, and tocopherol.[3-5] Most of the phytochemicals, especially phenolics and flavonoids have antioxidant potential, are used in food products, cosmetics, and pharmaceutical industries.[6,7]

Olea ferruginea Royle (syn *O. cuspidate* Wall. Ex G. Don) is a small tree that grows widely in the Himalayas from Kashmir to Kumaun up to an altitude of 2500 m.[8,9] It belongs to the family *Oleaceae* and generally known as Indian olive, which is one of the six species of Olea found in India. This is one of the important plants which are commonly used by local people for their health benefits as folk medicine.[10] Stem bark, leaf, and fruits of *O. ferruginea* plant is used as antiperiodic in fever and debility, toothache, astringent medicines, mouth ulcer, sore throat, mild digestive aid, antiseptic, etc.[11,12] Therefore, this study aimed to evaluate Phytochemical (phenolics and flavonoids) contents in the methanol extracts of leaf, bark bark, and fruits of *O. ferruginea* plants collected from five different populations (Thalaut, Sapangi, Suind, Kolibeher, and Kais) of the Kullu valley of Himachal Pradesh, India, and to explore their antioxidant activities.

MATERIALS AND METHODS

The present study was carried out in the Kullu district of Himachal Pradesh, India. Five geographically different sites, namely Thalaut, Sapangi, Suind, Kolibeher, and Kais were selected for the sampling purpose [Table 1]. Plant samples (leaf, stem bark, and fruit) were collected from each site in triplicate. The samples were brought to the laboratory and washed thrice using tap water followed by double distilled water to remove the dust particles and chopped into small pieces.

Table 1: Geographical locations of different populations of *Olea ferruginea* in North-Western Indian Himalaya

Populations	Altitude (m amsl)	Latitude	Longitude
Thalaut	981	N 31° 42. 734’	E 077° 06. 739’
Suind	1151	N 31° 54. 418’	E 077° 09. 348’
Sapangi	1184	N 31° 45. 226’	E 077° 06. 739’
Kolibehar	1293	N 31° 54. 656’	E 077° 06. 739’
Kais	1460	N 31° 01. 207’	E 077° 08. 242’

Chemicals

Chemicals such as DPPH (2, 2-Diphenyl-1-picrylhydrazyl) ABTS (2, 2'-Azino-bis 3-ethylbenzothiazoline- 6-sulfonic acid, and 2,4,6-tripyridyl-S-triazine were purchased from Sigma-Aldrich Pvt. Ltd., India. Whereas, Folin Ciocalteu phenol reagent, gallic acid, quercetin, ascorbic acid, sodium carbonate, methanol, aluminum chloride, etc., were purchased from Merck, Pvt., Ltd., India. All the chemicals used were of analytical grade and stored at 4°C in the refrigerator.

Extract preparation

For the preparation of extract, one (1 ± 0.01) gram of each plant sample was weighed and crushed in 10 ml aqueous methanol (80% v/v) using mortar and pestle. The crushed samples were kept at 4°C in a refrigerator for 48 h and then centrifuged at 10,000 g. The supernatant of the centrifuged plant samples was collected as a plant extract and further stored at 4°C for further analysis.

Determination of phenolics

After slight modification, the Folin Ciocalteu Phenol Reagent method was used to quantify the total phenolics in methanol extracts.[13] In brief, 1 ml of the plant extract was mixed with the same volume of Folin Ciocalteu phenol reagent and 2 ml of 2% (w/v) sodium carbonate, and the total volume of the reaction mixture was maintained up to 10 ml using double distilled water. The reaction mixture was then heated in a water bath at 80°C for 30 min. After cooling, the absorbance of the blue-colour mixture was measured at 650 nm using a spectrophotometer (Ultraspec 2100 Pro, Healthcare Bioscience AB, Uppsala, Sweden). A standard curve was prepared by the different concentrations of gallic acid, and results were expressed in terms of mg GAE/g fw.

Determination of flavonoids

Total flavonoids in methanol extracts of *O. ferruginea* were quantified by the aluminum chloride colorimetric method.[14] In brief, 1 ml of the plant extract was thoroughly mixed with the same volume of 2% ethanolic $AlCl_3$ (w/v). The reaction mixture was then allowed to stand for 60 min at the room temperature, and absorbance was recorded at 420 nm using a spectrophotometer (Ultraspec 2100 Pro, Healthcare Bioscience AB, Uppsala, Sweden). Different concentrations of quercetin were used to prepare the standard curve. The results of total flavonoids in methanol extracts were expressed as mg QE/g fw.

In-vitro antioxidant measurements

DPPH radical assay

DPPH radical scavenging activity of the methanol extract

was measured by a method described by.[15] Briefly, 5 ml of DPPH (0.135 mM) prepared in methanol was mixed properly with 1 ml of the plant extract and the reaction mixture was incubated in dark for 30 min. The absorbance of the reaction mixture was recorded at 517 nm using a spectrophotometer (Ultraspec 2100 Pro, Healthcare Bioscience AB, Uppsala, Sweden). A blank was prepared using 1 ml methanol instead of plant extract. The results were expressed in terms of mM AAE/g fw.

ABTS radical assay

ABTS radical scavenging capacity of the methanol extract of *O. ferruginea* was measured by the method given by.[16] ABTS radicals ($ABTS^+$) were generated by allowing the reaction between ABTS solution and potassium in dark for 16 h. ABTS cation was then diluted by using 80% aqueous methanol and absorbance of the solution was maintained 0.700 ± 0.02 at 734 nm. One ml of the methanol extract was then mixed with 5 ml of $ABTS^+$ and shaken properly. The absorbance of the reaction mixture was measured at 734 nm using a spectrophotometer (Ultraspec 2100 Pro, Healthcare Bioscience AB, Uppsala, Sweden) and results were expressed in terms of mM AAE/g fw.

Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) of methanol extract of *O. ferruginea* was determined as per the method described by.[17] FRAP assay was initiated by adding 10 ml of 300 mM acetate buffer to 1 ml of 10 mM 2,4,6-tri-2pyridyl-1,3,5-triazine in 40 mM HCl and 1 ml of 20 mM ferric chloride. The mixture was then prewarmed at 35°C before use. 150 µl of plant extract was added to 3 ml of the above mixture and kept at the room temperature for 10 min. The absorbance was taken at 593 nm using a spectrophotometer (Ultraspec 2100 Pro, Healthcare Bioscience AB, Uppsala, Sweden). A standard curve was prepared using different concentrations of ferrous sulfate solution. The results were expressed in terms of mM AAE/g fw.

Statistical analysis

All experimental measurements were carried out in triplicates, and results were expressed as a mean of three analyses ± standard error. The statistical analyses were performed using the statistical software (SPSS Version 16.0, Chicago, USA). Significant differences between the means were achieved by Duncan's multiple range tests at $P \leq 0.05$. Principle component analysis was also performed to access the biochemical relationships among populations.

RESULTS AND DISCUSSION

Plants are the most important source of natural compounds which are important for the health benefits of humans and other animals. They are the major source of natural phenolic and flavonoid compounds which are responsible for boosting the health benefits value such as antioxidant activity in human beings.[18,19] Several authors have reported that olive fruits and leaves contain an immense quantity of polyphenols and antioxidant activities.[9,18,20-23] Sharma *et al*.[18] have studied the antioxidant activities in methanol extract from raw and ripe seeds of *O. ferruginea* and found a significant level of phenolics and antioxidant activity. Antioxidant activity in the methanol extract of *O. ferruginea* fruits was studied and concluded that this plant part can serve as a source of natural antioxidants for the local population and can also be exploited for the commercial purposes.[9] Al Juhaimi *et al*.,[22] investigated that in the olive plant drying process influencing the total phenolic contents and antioxidant activity, and gallic acid was found as a major phenolic compound. Mehmood and Murtaza[23] have studied the antimicrobial and antioxidant activities of *O. ferruginea* and suggested that leaves and bark can be used as a source of natural antioxidants.

In the present study, total phenolics and flavonoid content in methanol extracts of stem bark, leaf, and fruits of *O. ferruginea* plants are shown in Table 2. The results showed that total phenolics and flavonoid contents in all the tested parts of *O. ferruginea* were significantly increasing with increasing altitudes ($P < 0.05$). Total phenolics and flavonoid content (expressed as mg GAE/g fw and mg QE/g fw, respectively) ranged between 5.2–9 and 0.8–1.8 in stem bark, 5.5–9.4 and 8.8–22.8 in leaf, and 4.6–8 and 0.8–1.1 in fruits, respectively [Table 2]. The highest average content of total phenolics and flavonoids was found in leaf followed by stem bark and least in fruits [Table 2]. In the present study, the amounts of phenolics and flavonoids were found maximum in the leaf which may be due to the presence of a higher level of secondary metabolites, formed from primary metabolites due to more absorption of ultraviolet (UV)-radiation.

The variations in phenolic components in tested parts may also be ascribed to variations in altitudes, habitats, growth stages, harvesting stages, exposure times, surface areas, etc.[18] Sharma *et al*.[9] have reported that *Withania somnifera* collected from the roadside have more phenolic compounds than those found in the undisturbed area. It has been reported that total phenolics content in the methanol extract of fruits of *O. ferruginea* ranges between 3.4 and 2.3 mg

Table 2: Phenolics and flavonoids content in the methanol extracts of stem barks, leaf, and fruits of *Olea ferruginea* populations of North-Western Indian Himalayas

Populations	Phenolics (mg GAE/g fw)			Flavonoids (mg QE/g fw)		
	Stem bark	Leaf	Fruit	Stem bark	Leaf	Fruit
Thalaut	5.17^{d}	5.51^{c}	4.61^{e}	0.81^{d}	8.75^{e}	0.76^{e}
Suind	6.26^{c}	7.95^{b}	5.63^{d}	0.97^{c}	9.80^{d}	0.82^{d}
Sapangi	6.29^{c}	8.60^{b}	6.67^{c}	1.22^{b}	14.73^{c}	0.90^{c}
Kolibeher	6.98^{b}	8.57^{b}	7.34^{b}	1.35^{b}	20.15^{b}	1.00^{b}
Kais	8.99^{a}	9.43^{a}	7.95^{a}	1.78^{a}	22.78^{a}	1.11^{a}
Average	6.74	8.01	6.44	1.23	15.24	0.92
F	89.22***	83.33***	54.42***	69.83***	692.09***	67.90***

***Significant ($P<0.001$). Values are mean of three replicate determinations. Mean values in each column with different letters are significantly different ($P<0.05$). Standard errors values are not shown as the values are <10% of mean value

TAE/g fw which was found lower as compared to the methanol extracts of leaf and stem bark of *O. ferruginea* [Table 2].[9] Debib and Boukhatem[24] have reported the total phenolics, antioxidant, and antimicrobial activity of *Olea europea* leaf extract and found that total phenolics range from 3.64 to 21.47 mg GAE/g dw). Several studies have investigated that total phenolics content in plants is positively related to antioxidant activity.[25,26]

Flavonoids are a special group of polyphenols, which are exclusively present in all the parts of plants. Different organs or tissues of various plants, including the leaves, flowers, fruits, seeds, and roots have been reported to be rich in flavonoids.[27] Many researchers have shown that flavonoids have a wide range of pharmacological activities, including antioxidant, anticancer, enzyme inhibition, and anti-inflammatory properties; therefore, flavonoids have applications in human health, pharmaceutical, and industrial sectors.[28,29] Higher flavonoids content in the leaf extract maybe because of more conversion of primary metabolites into secondary metabolites as leaf contain more carbohydrates as compared to stem bark and fruits of the plants.[30] In the present study, total flavonoid contents in leaf extracts were found higher than those reported by Cheurfa *et al*., (2019)[31] in the leaf of *O. europea* (0.98–1.06 mg QE/g fw).

Free radicals are involved in many disorders such as neurodegenerative diseases, cardiovascular diseases, and cancer.[32] Antioxidants are the molecules/compounds which inhibit the oxidation caused by free radicals by donating the electrons. The free radical scavenging properties of bioactive compounds contribute to the protective effect against the free radicals. Free radicals can be neutralized by providing an electron to them. It is well documented that plant bioactive compounds such as phenolics, flavonoids, and other secondary metabolites group donate an electron to the free radicals generated under adverse conditions for mitigating the effects of free radicals.[33,34] Besides the antioxidative effects other protective effects such as anti-inflammatory, anticancer, and antimicrobial properties have also been reported which contribute to the health benefits.[35-38] For free radical scavenging activity for reactive species, DPPH has been widely used as an assay to evaluate the antioxidant activity of extract from the plant sources due to its less time-consuming in the analysis compared to other methods.[39]

The results of *in vitro* antioxidant assays such as DPPH, ABTS, and FRAP are shown in Table 3. The results showed that DPPH, ABTS, and FRAP activities expressed as mM AAE/g fw in different populations ranged between 19.5–20.7, 2.2–4.5, and 29.2–37.5 in stem bark, 18.5–19.3, 3.9–4.4, and 29.7–32.6 in leaf, and 19.1–20, 0.9–4.3, and 38.9–46.8 in fruits extracts, respectively [Table 3]. The results showed that antioxidant activities in tested plant parts also increase significantly with increasing the altitudes ($P < 0.05$) which may be due to increasing phenolics and flavonoid contents. The average DPPH, ABTS, and FRAP antioxidant capacity was found the highest in stem bark, leaf, and fruits, respectively [Table 3]. Variations in antioxidant activities in the methanol extract obtained from stem bark, leaf, and fruits may be due to variations in altitudes, habitats, soil properties, the intensity of UV radiations, exposure time, vehicular, and other emissions.[9,40] Kabbash *et al*.[41] reported that DPPH inhibition was found to be varied between 86.56%–90.09% in O. europaea leaf extract and also varied with season. Sharma *et al*. found that DPPH (2, 2-Diphenyl-1-picrylhydrazyl) ABTS (2, 2'-Azino-bis 3-ethylbenzothiazoline- 6-sulfonic acid), and FRAP activity in terms of mM AAE/g fw varied between 0.15–0.24, 0.0019–0.013, 28.02–31.43, respectively, in the fruit of *O. ferruginea* collected from different populations. Bouarroudj *et al*.[42] further reported that DPPH and ABTS antioxidant activities varied between 59%–85% and 31%–75%, respectively, in oil obtained from *O. europaea*.

The tested biochemical attributes of stem bark, leaf, and fruits of *O. ferruginea* showed a strong relationship (cc values: 0.863–0.994) with altitudes [Table 4]. FRAP activity in stem bark had the strongest correlation with altitude where phenolic content in leaf showed a poor relationship with altitudes. Principal component analysis (PCA) was performed to investigate the relationship among five populations based on the biochemical attributes of stem bark, leaf, and fruit extracts of *O. ferruginea*. PCA is represented by PC1 (95.87%) and PC2 (2.61%) and showed that Sapangi and Kais populations are biochemically different from other populations, i.e., Thalaut, Suind, and Kohibeher [Figure 1]. The variations among the population may be ascribed to the phytochemical composition of plant extracts which may be induced or inhibited by different environmental factors such as altitudes, UV radiation, leaf size, soil properties, and plant-microbe interaction.[5]

CONCLUSION

Plants are rich in many bioactive compounds as utilized in traditional medicine for a long time. Many plants are even not explored for their utilization in various industries and human health benefits. In the present study, different parts of *O. ferruginea* (stem bark, leaf, and fruit) were investigated for phenolics and flavonoids as well

Table 3: Antioxidant activities in methanol extracts of stem barks, leaf, and fruits of *Olea ferruginea* from North-Western Indian Himalayas

Antioxidant capacity (mM AAE/kg fw)	Populations	*Olea ferruginea* fractions		
		Stem bark	Leaf	Fruits
DPPH assay	Thalaut	19.53^{d}	18.48^{c}	19.11^{c}
	Suind	19.98^{c}	18.79^{b}	19.30^{c}
	Sapangi	20.11^{c}	18.84^{b}	19.33^{c}
	Kolibeher	20.31^{b}	19.11^{a}	19.69^{b}
	Kais	20.72^{a}	19.27^{a}	19.99^{a}
	Average	20.13	18.90	19.48
	F	52.27***	8.59**	22.31**
ABTS assay	Thalaut	2.20^{d}	3.86^{c}	0.92^{e}
	Suind	3.50^{c}	4.03^{b}	3.49^{d}
	Sapangi	4.11^{b}	4.14^{b}	3.77^{c}
	Kolibeher	4.30^{a}	4.26^{a}	4.09^{b}
	Kais	4.48^{a}	4.42^{a}	4.29^{a}
	Average	3.72	4.14	3.11
	F	188.15***	10.31**	575.64***
FRAP assay	Thalaut	29.19^{e}	29.72^{b}	38.88^{d}
	Suind	30.92^{d}	30.37^{b}	43.43^{c}
	Sapangi	33.26^{c}	30.86^{b}	44.39^{b}
	Kolibeher	35.16^{b}	31.08^{b}	44.50^{b}
	Kais	37.52^{a}	32.62^{a}	46.79^{a}
	Average	33.21	30.93	43.60
	F	243.32***	100.13***	496.46***

***Significant ($P<0.001$), **Significant ($P<0.01$). Values are the mean of three replicate determinations. Values in each column of respective assay with different letters are significantly different ($P<0.05$). Standard errors values are not shown as the values are <10% of mean value. DPPH: 2, 2-Diphenyl-1-picrylhydrazyl, FRAP: Ferric-reducing antioxidant power, ABTS: 2, 2′-Azino-bis 3-ethylbenzothiazoline-6-sulphonic acid

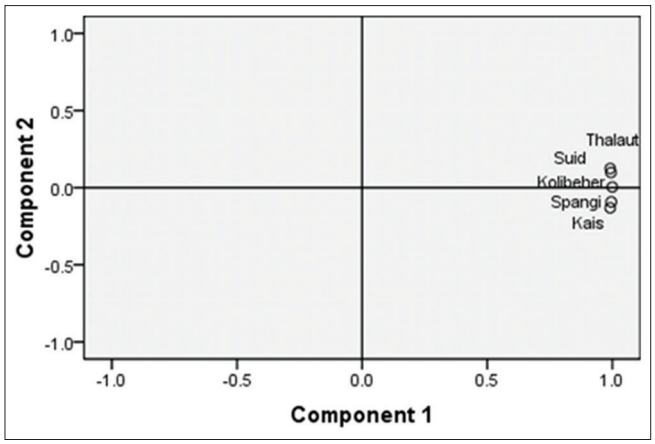

Figure 1: Variation among population of *Olea ferruginea* based on its biochemical attributes analysed using principal component analysis tool

Table 4: Correlation coefficients between altitudes and various biochemical properties of *Olea ferruginea*

Altitudes	CC values	Altitudes	CC values
Phenolics-stem bark	0.921**	ABTS-stem bark	0.911**
Phenolics-leaf	0.863**	ABTS-leaf	0.895**
Phenolics-leaf	0.970**	ABTS-fruits	0.845**
Flavonoids-stem bark	0.963**	FRAP-stem bark	0.994**
Flavonoids-leaf	0.980**	FRAP-leaf	0.933**
Flavonoids-fruits	0.976**	FRAP-fruits	0.906**
DPPH-stem bark	0.958**		
DPPH-leaf	0.865**		
DPPH-fruits	0.916**		

**Significant ($P<0.01$). DPPH (2, 2-Diphenyl-1-picrylhydrazyl) ABTS (2, 2'-Azino-bis 3-ethylbenzothiazoline- 6-sulfonic acid), CC: Correlation coefficients

as antioxidant activities. The results revealed that the tested parts/plants contain the significant amounts of phenolics and flavonoids as well as possesses antioxidant activities Thus, the present study suggests that different parts of *O. ferruginea* could be considered as an effective alternative for natural antioxidant and a potential source of natural antioxidants for pharmaceutical and food industries and could further be promoted for human and animal consumption.

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Conflicts of interest

There are no conflicts of interest.

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