International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 14-02-2025, Accepted: 13-03-2025, Published: 29-03-2025
Volume 10, Issue 3, 2025, Page No. 79-82

Secondary metabolic profile of Olive leaves in different cultivars and sites at Bikaner (Rajasthan)

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Abstract
Olive tree is native to Mediterranean climate but the climate of Rajasthan in North-western India resembles it. Olive leaves are known for their potent antioxidant and anti-inflammatory properties. This study evaluated the quantitative profile of secondary metabolites in leaves of olive cultivars ('Arbequina', 'Barnea', 'Coratina' and 'Koroneiki') grown at two sites, *viz*., Lunkaransar and Napasar, located in Rajasthan state of India. The total flavonoid content was estimated using the Aluminium chloride colorimetric assay method. The Folin-ciocalteu assay method was used to determine the total phenol content. Phytosterol was solvent extracted and gravimetric quantified. Maximum total phenolic content was present in leaves of 'Coratina' cultivar of Napasar. Maximum total flavonoid content was found in 'Arbequina' cultivar of Napasar. Maximum phytosterol content was found in 'Arbequina' of Lunkaransar. Thus, it was possible to conclude that the cultivar and the site significantly affect the secondary metabolites

Keywords: Abiotic stress, cultivars, mediterranean, olive leaves, secondary metabolites

Introduction

The Mediterranean basin dominates global olive production, accounting for 95% of all olive orchards. Olive trees are distributed worldwide in different tropical, subtropical and temperate region and are best grown in tropical and temperate regions of Asia. *Olea europaea* L. is the most popular member of the genus *Olea* belonging to family *Oleaceae* (Zohary *et al*., 2012) [30]. Olive is an evergreen tree, has many twiggy branches with opposite branchlets (Hashmi *et al*., 2015) [10]. It has thousands of cultivars but only some are grown extensively. Olive trees are native to Mediterranean climate, have high tolerance to poor soils and adaptability to drought salinity so well suitable to arid and semi-arid regions. New olive orchards are being planted outside the Mediterranean region (Bouwmeester *et al*., 2019) [2] and expanding in the rest of the world (Wang *et al*., 2018) [27] (Porfirio *et al*., 2016) [18].

Olive tree leaves have been broadly used in traditionally medication. In recent years, health effects of various herbal teas have been arising great interest. Olive-leaf is one of the most common, traditional herbal teas used among Mediterranean people to cure certain disease. Thus, plants which can withstand harsh environment are important source of secondary phytochemicals and have medicinal importance. (Kurian, A. and Sankar, M.A., 2007) [13]. Olive leaves are known for their commanding antioxidant and anti-inflammatory properties and is affected by secondary metabolites (Haloui *et al*., 2010) [9] whose level may be influenced by cultivar and place of production. However, olive tree is native to Mediterranean climate but the climate of Rajasthan in North-western India resembles it. The phytochemical profile of plants through their secondary metabolism can be influenced by the environmental conditions of the regions combined with intrinsic genetic factors (Ruiz Rodriguez *et al*., 2014; Maieves *et al*., 2015) [16]. According to the evaluation of the available literature, secondary metabolites in olive leaves needs more insight, together with the role of site where the trees are grown. The climate of Bikaner district situated in Rajasthan state of North-western India is generally arid. For this reason, the aim of this research was to critically examine the influence of cultivar and site on the quantitative property of secondary metabolites of olive leaves. Diverse selected cultivars were compared for their secondary metabolites in the same site to assess the role played by their genetic origin and also the comparison was made between cultivars at two sites. Efforts with appropriate basics and applied research can result in profitable cultivation of olives in India (Subbiah, 1994) [23]. Thus, the present study emphasizes on better understanding about the quantitative nature of secondary metabolites found in response to prevailing environmental conditions in selected olive cultivars.

Material and Methods

The present study deals with the study of cultivars of *Olea europaea* L. *viz*., 'Arbequina', 'Barnea', and 'Coratina' and 'Koroneiki' grown at Lunkaransar and Napasar (Bikaner) in which quantitative aspects of secondary metabolites at two sites were studied**.** Fresh leaves of selected cultivars were collected, dried and pulverized for evaluation of different secondary metabolites.

Total Flavonoids Extraction

For the extraction of flavonoids, 10 gm of each of the eight shade-dried and powdered plant samples were Soxhlet extracted with 250 ml of 80 % ethanol for twenty- four hours on water bath separately. The ethanol solvable fractions were filtered and each of the extract was concentrated. Then the extract was re-extracted with petroleum ether (Fraction-1), diethyl ether (Fraction-2) and ethyl acetate (Fraction-3) in succession (Subramanian and Nagarajan, 1969) [24]. To ensure complete extraction each step was repeated three times. Fatty acids were highly present in Fraction 1, so it was discarded while Fraction-2 and fraction-3 were evaluated for free and bound flavonoids individually. Fraction-2 was concentrated and reserved for quantitative investigation. Fraction 3 was hydrolyzed by refluxing with 7% H_2SO_4 for 2 hours, filtered and filtrate extracted three times with ethyl acetate. The ethyl acetate layer (bound flavonoid) of each extract was neutralized with

distilled water and concentrated. Fraction 2 and 3 were re-dissolved in ethanol and used for quantification.

Quantification

The total flavonoid content was estimated using the Aluminium chloride colorimetric assay method (Kaviarasan *et al*., 2007) [12]. 1 ml of each sample extract and 4 ml of distilled water was taken in a volumetric flask of 10 ml. To the flask, 0.30 ml of 5 percent Sodium nitrite was added and after 5 minutes, 0.30 ml of 10 percent Aluminium Chloride was mixed. After 5 minutes, 2 ml of 1M Sodium hydroxide was added and diluted to 10 ml with distilled water. A blank was also prepared in which 1 ml of distilled water was only taken and instead of extract all the constituents were the same as that of extract samples.

Standard curve was prepared by using quercetin as standard flavonoid. Stock solution of quercetin was prepared. A set of reference standard solutions of quercetin (20, 40, 60, 80 and 100 µg/ml) were prepared in the identical manner as above. The absorbance for test and standard solutions were measured against at 510 nm with a spectrophotometer. The total flavonoid content was calculated by referring the optical density of the sample with the regression curve of the quercetin standard (Sahu, R., & Saxena, J., 2013) [22]. Three replicates of each sample were examined and their mean value were noted. The results were expressed in mg QE/g dry weight of the sample

Total phenol Extraction

For the extraction of total Phenol, soxhlet extraction method was used. 10 gm of all the eight dried olive leaves sample were placed in the thimble of soxhlet apparatus and extracted with 250 ml of 80 % ethanol in different test tubes. Thus Ethanol was used as a solvent for extraction at 60ºC. Extract was cooled at room temperature and filtered. The filtered extracts were then evaporated in rotary evaporator at room temperature under vacuum. The concentrated extract was stored in refrigerator at 8-10°C until used (Yateem *et al*., 2014) [28].

Quantification

The Folin-ciocalteu assay method was used to determine the total phenol content (Bray and Thorpe, 1954). 1 ml of the extract was treated with 1 ml of Folin-Ciocalteu phenol reagent and shaken well. After 5 minutes, 2 ml of 20 percent Sodium carbonate (Na_2CO_3) solution was treated to the mixture. Each test tube was then heated in a water bath for 1 minute and cooled the final volume was adjusted to 25 ml. A blank was also prepared in which 1 ml of H_2O was only taken and instead of extract all the constituents were the same as that of extract samples. A Stock solution of caffeic acid was prepared in 80 % alcohol. 0.1 to 0.9 ml of aliquots of the stock solution were transferred into separate test tubes and the volume in each test tube was adjusted to 1 ml with 80% ethanol, and the same procedure was followed for the standard solution. The absorbance of the test and standard solutions was measured against the blank at a wavelength of 750 nm using a spectrophotometer. Three similar replicates were taken and their mean value were noted. The total phenol content was determined by referring the optical density of the test sample with the regression curve of the standard caffeic acid. Total phenol was expressed as mg of caffeic acid equivalent per gm dry weight of extract (Ghasemzadeh *et al*., 2010 [8]; Rasool *et al*., 2011) [20].

Phytosterol Extraction

For the extraction of phytosterol, 10 gm dried powder of leaves sample was placed in the thimble of a soxhlet apparatus and extracted using 85 ml petroleum ether solvent at 70°C-80°C. When colorless solvent in the siphon tube was appeared, it indicated complete extraction and based on that further extraction was terminated. The solvent was recovered under pressure to obtain dark green mass. The petroleum ether extract of leaves of the plant was saponified using 36 ml 1 M alcoholic KOH to remove fatty material and then picked up in petroleum ether and Defatted materials were then dried at ambient temperature and hydrolyzed in 30% HCl for 4 hours. All hydrolyzed samples were washed with dH_2O to maintain pH at 7 and dried later. The dried preparative sample was again extracted with 500 ml benzene for twenty-four hours the solvent was evaporated to yield unsaponified extract. This fraction contains lesser number of components than the unsaponified extract (Kamboj *et al*., 2013) [11] (Sutar, *et al*. 2014) [25].

2.3.2 Quantification

The extract was then transferred into the previously weighed empty beaker and evaporated to a thick paste on the water at 50°C to obtain petroleum ether extract. The extract was then air dried thoroughly to remove all the traces of the solvent and the percentage yield was calculated and then stored in an air tight container below 10°C (Patil and Gaikwad, 2011) [17]. The crude extracts were dissolved in benzene before chromatographic investigation.

Results and discussion

The parameters analyzed were total flavonoid content, total phenolic content and phytosterol content. Maximum total phenolic content 4.71 ± 0.11 mg/g DW was present in leaves of 'Coratina' cultivar of Napasar. Second highest total phenolic content 4.46 ± 0.46 mg/g DW was found in 'Coratina' cultivar of Lunkaransar. Minimum phenolic content 3.57 ± 0.15 mg/g DW in leaves of 'Arbequina' of Lunkaransar. Thus 'Coratina' is comparatively better cultivar for total phenolic content in the entire estimation. The results obtained in our study are in complete coherence with the previous study where the researchers found maximum content of phenol in 'Coratina' variety in comparison to 16 other olive varieties including 'Arbequina' (Ghasemi *et al*. 2018) [7]. Similar results have been reported in previous studies, where too, the researchers found significant variability in the phenolic content of olive leaves from various cultivars (Ramirez *et al*., 2022 [19]; Edziri *et al*., 2019 [5]; Lama Munoz *et al*., 2020). Contrary to this, Maria E.M-N *et al*. (2023) [15] concluded that 'Arbequina' had highest phenol values among 'Picual', 'Koroneiki', and 'Kalamon'. A number of studies have shown that olive leaves harbour phenolic compounds that have the potential of anti-inflammatory agents (CAI *Et al*., 2004) [4] (Table 2) (Fig. 1).

Maximum total flavonoid content was found 1.88 ± 0.09 mg/g DW in 'Arbequina' cultivar of Napasar. Minimum total flavonoid content was found 1.22 ± 0.07 mg/g DW in cultivar 'Barnea' of Napasar. Thus, Napasar has both maximum and minimum total flavonoid content cultivar. This is due to inherent genetics of cultivar**.** A recent study also showed variability in flavonoid content of olive leaves belonging to different varieties and olive leaves were found

to be excellent source of flavonoids (Zhang *et al*. 2022) [29] (Table 2) (Fig. 1).
Maximum phytosterol content 23.49 ± 0.39 mg/g dw was found in 'Arbequina' of Lunkaransar. It can be inferred from the result that 'Barnea' cultivars of olive at both sites have the noticeable content of phytosterol in their leaves. The highest phytosterol content was present in the 'Meski' leaves from Southern region followed by Central and Northern region. This pattern shows spatial variation in the phytosterol content of olive leaves from the same species due to different climatic conditions (Ben Mansour-Gueddes *et al*. 2020) [1]. Other studies have shown the contribution of phytosterols in mitigating biotic and abiotic stress_tolerance in plants (Vriet *et al*., 2013 [26]; Ferrer *et al*., 2017) (Table 2) (Fig. 1).

Table 1: Sample names and Sample codes of Olive Cultivars according to Study Sites

Sample name	Sample code	Study site	Cultivar
S1	NA	Napasar	'Arbequina'
S2	NB	Napasar	'Barnea'
S3	NC	Napasar	'Coratina'
S4	NK	Napasar	'Koroneiki'
S5	LA	Lunkaransar	'Arbequina'
S6	LB	Lunkaransar	'Barnea'
S7	LC	Lunkaransar	'Coratina'
S8	LK	Lunkaransar	'Koroneiki'

Table 2: Secondary Metabolite content in leaves of Olive Cultivar

Sample name	Sample code	TFC (mg/g dw)	TPC (mg/g dw)	Phytosterols (mg/g dw)
S1	NA	1.88 ± 0.09	4.12 ± 0.09	21.42 ± 0.30
S2	NB	1.22 ±.07	3.71 ±0.09	23.48 ± 0.29
S3	NC	1.55 ± 0.09	4.71 ± 0.11	19.71 ± 0.27
S4	NK	1.37 ± 0.04	3.85 ± 0.06	18.43 ± 0.22
S5	LA	1.51 ± 0.09	3.57 ± 0.15	23.49 ± 0.39
S6	LB	1.53 ± 0.06	4.36 ± 0.15	22.54 ± 0.37
S7	LC	1.40 ± 0.09	4.46 ± 0.46	20.54 ± 0.41
S8	LK	1.38 ± 0.17	4.25 ± 0.10	21.41 ± 0.34

Mg/gdw = milligram per gram dry weight; TPC=Total Phenolic Content; TFC=Total Flavonoid Content Sample names and Sample codes of Cultivars as per Table 1 Values are expressed as means ± SD of three samples; SD: Standard deviation

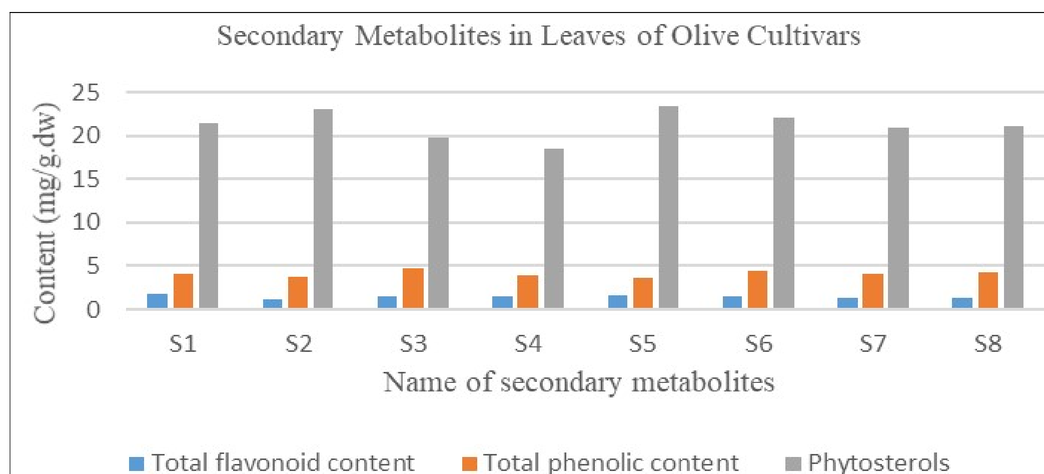

Fig 1: Secondary Metabolites in Leaves of Olive Cultivars Mg/gdw = milligram per gram dry weight; Sample names as per Table 1

Conclusion

The presented in this paper seem to indicate, in spite of limited number of number of cultivars analysed that secondary metabolites composition might represent a useful contribution to biochemical characterization of olive leaves. Moreover, the leaves of some cultivars could be interesting source of secondary metabolites. At Napasar, high amount of total flavonoid content and total phenol content was found in 'Arbequina' cultivar which shows its importance for secondary metabolites but maximum level of phytosterol in 'Barnea'. However, at Lunkaransar, maximum level of total flavonoid content was found in 'Barnea' cultivar, total phenol content in 'Coratina' and phytosterol in 'Arbequina' cultivar. These findings could be used for manage production process and correlating the cultivar type and study site to the quantity of secondary metabolite pattern.

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