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Research article

Physicochemical evaluation of microgreens and mature leaves of *Ocimum sanctum* using mid-infrared spectroscopy, scanning electron microscopy and energy dispersive x-ray spectroscopy

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ABSTRACT

Ocimum sanctum (holy basil) has been proved to cure multiple ailments since ancient times. It has multiple health benefits in each part of the plant. Since past few years, microgreens are gaining attention of each segment of society and health sector industries due to their highly concentrated nutritional profiles. This research was conducted on a comparative study between microgreens and mature basil involving scanning electron microscopy/ energy dispersive X-ray spectroscopy (SEM-EDS) and Fourier transform infrared spectroscopy (FT-IR) analysis. FT-IR analysis showed the occurrence of functional groups associated with several beneficial metabolites comparatively higher in concentrations in basil microgreens. SEM analysis showed the premature morphological structures in microgreen leaves with smaller oil glands, stomatal apertures and no trichomes as compared to the mature ones. Larger oil glands and stomatal apertures along with protective trichomes were observed in mature basil leaves. Elemental analysis using SEM-EDS revealed the higher percentage of elements like sodium, potassium, phosphorus, magnesium and sulphur in microgreens however, the amount of calcium were observed higher in mature basil.

1. Introduction

Tulsi (in Hindi), *Tulasi* (in Sanskrit), or holy basil is an aromatic herbaceous plant from the *Lamiaceae* family which is indigenous to the Indian subcontinent. *Ocimum sanctum* has been traditionally used for thousands of years in Ayurveda and Unani for its various medicinal properties and health care routines. The holy basil, 'Tulsi' is known for its religious and spiritual sacredness in India since ancient times. Charaka mentioned the medicinal properties of holy basil in ancient Ayurvedic text, the 'Charaka Samhita' (Pattanayak et al., 2010). Plethora of evidence suggest that *Ocimum sp.* are unique in addressing the physical, chemical, metabolic, biochemical stress through numerous combinations of pharmacological actions. There exist more than 150 species under the genus *Ocimum* and thus is known to be one of the biggest genera of *Lamiaceae* family (Pandey et al., 2014). Besides, holy basil has been used as nutraceuticals owing to a broad range of

therapeutic potentials including anti-cancerous, antibiotic, antifungal, anti-inflammatory, antidiabetic, cardio-protective antispasmodic, analgesic, diaphoretic antistress (Saxena et al., 2012; Sood et al., 2006). *Ocimum sanctum* is a species which possess higher content of phenolics and is profused with substantial antioxidant potentials which delay or inhibit of lipid oxidation by interfering with oxidative chain reaction thereby neutralizing the free radical formation (Javanmardi et al., 2003; Kwee & Niemeyer, 2011). *Ocimum sp.* contains a variety of essential oils, which are often used as perfumery rich in phytoactive compounds including the polyphenols such as anthocyanins and flavonoids. Additionally, the primary constituents of *Ocimum sp.* comprising ursolic acid, eugenol, rosmarinic acid and linalool have been known to induce anti-inflammatory and anti-oxidative responses (Kelm et al., 2000). One of the recent studies has revealed the biological importance of *Ocimum* in combating skin ageing by imparting

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the remarkable potentials including the anti-collagenase, anti-elastase and anti-hyaluronidase activities (Chaiyana et al., 2019).

Despite the fact that *Ocimum sp.* are regarded as “Queen of Herbs” owing to their brilliant phytochemical profiling, several studies also claim that the microgreens of basil contains yet higher concentrations of phytochemicals as compared to their mature basil (Chaiyana et al., 2019). Microgreens are young delicate plants of vegetables, herbs, spices and grains having one or two pairs of cotyledonous leaves and small tender stem which are known to endorse human health. The idea of microgreens has become popular as superfoods from past few years and its consumption is increasing significantly, as people are getting aware about their health (Lenzi et al., 2019). As compared to their mature counterparts, the microgreens possess higher nutritional profiles and can be highly endorsed for human health as per the records of the USDA, National Nutrient Database (www.nifa.usda.gov/microgreens-go-trendy-vegetables-functional-food). The basil microgreens are reported to contain the higher phenolic profiling as compared to their mature counterparts (Xiao et al., 2015). More recently, microgreens are being used as an exotic garnishing and flavoring agents in fine dines and high-end restaurants due to its vivid color range, flavors and delicate crunchy textures. Furthermore, the advancing research on microgreens unraveled its great nutritional profiling owing to which they are categorized as emerging superfoods having various health benefits and nutraceutical properties (Rizvi et al., 2022). This study would be the first report on the comparative fingerprinting of the functional groups corresponding to the phytochemicals present in the microgreens of holy basil (*Ocimum sanctum*) with the mature basil leaves. Fourier Transform infrared spectroscopy (FT-IR) was performed to acquire the functional groups of phytochemicals present in each. Additionally, the aim of the study is to observe the morphological and elemental details of both microgreens and mature basil for which scanning electron microscopic (SEM) imaging and energy dispersive X-ray spectroscopy (EDS) analysis, respectively, were performed.

2. Materials and methods

2.1 Cultivation of basil microgreens

The basil microgreens were cultivated in bottom of perforated pots filled with vermiculite and cocopeat based media. The seeds were allowed to germinate in a darker environment by keeping the pots in dark for initial 2-3 days. The germination took place after three days of sowing and then the pots were kept near windowsill for proper lighting and aeration. A gentle spray with clean water was given twice a day to maintain the relative humidity throughout the germination phase. The basil microgreens were harvested at the 15th day of sowing using a pair of sharp scissors to cut the delicate stem carrying a pair of cotyledonous leaves, closest to the growing substrate (Fig. 1). Harvested microgreens were washed properly to ensure the removal of seed coat and leftover cocopeat media and then were kept on the paper tower for proper removal of water. The fresh harvest of microgreens was used for the sample preparation protocols used for SEM and EDS analysis. The samples were then shade-dried at room temperature until

completely dried and stored for further use. On the other hand, the mature leaves of holy basil (*Ocimum sanctum*) were plucked, washed and kept at room temperature for shade-drying process.

2.2 Preparation of extracts

Both the mature and microgreens dried samples were gently pulverized in mortar and pestle to make fine powder. 10 grams of each powdered samples were taken in 50 mL of 80% methanol solvent was added and kept in incubator shaker in dark for about 60 hours to ensure proper extraction of metabolites. Afterwards, the extracted solution was filtered through Whatman filter paper to collect the filtrates which were further subjected to rotary vacuum evaporator (Model: UTS: 1.53, Thermo-Scientific) to obtain the dried extracts of mature and microgreens of holy basil.

2.3 Fourier transform infrared spectroscopy (FT-IR)

The mid-infrared spectroscopy was performed to determine the functional groups of phyto-active compounds and secondary metabolites present in the extract of holy basil microgreens and mature forms. The powdered extracts of each sample were thoroughly mixed to potassium bromide (KBr) (IR Grade with 99% purity) in a ratio of 1:30 and the mixture was macerated in hydraulic press operated under vacuum to get the thin pallets. The pallets were subjected to Fourier transform infrared spectrophotometer (Model: Nicolet TM 6700, Thermo Fisher Scientific, USA) to record the mid infrared absorbance spectrum between 4000-450 cm^{-1} (Kustiati et al., 2022). Wavelength of light absorbed is the characteristic of the chemical bond present in the extract. The spectra obtained and the chemical bonds were determined by interpreting the infrared absorption spectrum from the previous literature.

2.4 Scanning electron microscopy (SEM) and Energy dispersive X-ray spectroscopy (EDS)

The freshly harvested microgreens and mature greens of basil leaves were gently washed with autoclaved distilled water twice to prepare the samples for SEM-EDS analysis. Each sample leaf was cut gently to prepare the small discs and then were dipped 2.5% glutaraldehyde for initial fixation for at least 5 hours at 4°C. After the primary fixation, each sample was washed three times for 15 minutes each with 0.1 M phosphate buffer. Then 1% osmium tetrachloride treatment was given for post-fixation for 2 hours. Again, washing was done thrice with phosphate buffer (each wash for 15 minutes). The dehydration step was performed for 30 minutes by using 30, 50, 70, 90 and 95% acetone respectively. Finally, the samples were kept in 100% acetone solution before mounting on the aluminum stubs using double sided tape (Srividhya and Jeyakumar, 2022). Following the mounting, the stubs were kept in platinum sputter-coater (Auto Fine Coater, JFC 1600, JEOL, Japan) to make the leaf samples conductive. The samples were subjected to Scanning electron microscope (JEOL JSM 6490 LV, Tokyo, Japan) for SEM-EDS analysis at various magnifications (500X and 2000X) and accelerating voltage. The morphological and elemental details were obtained to study the comparison between basil microgreens and mature greens.

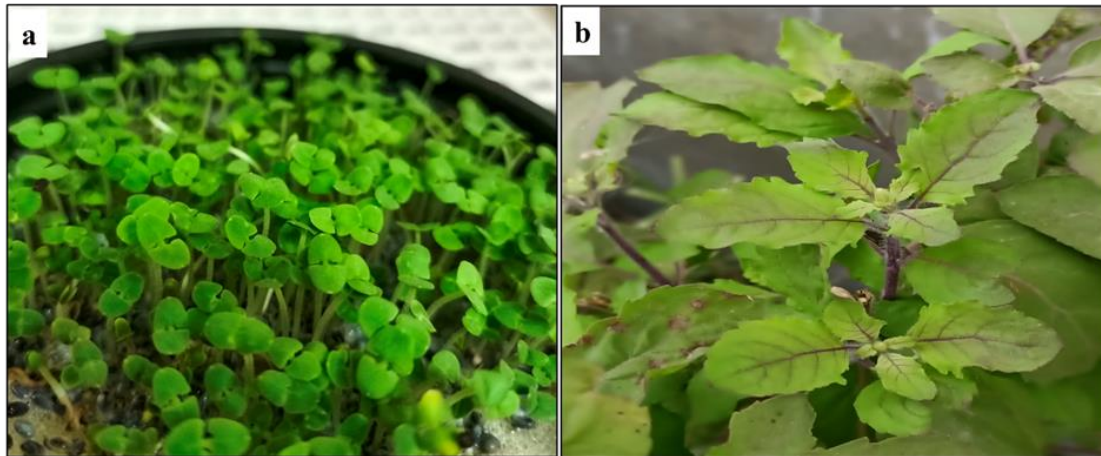


Fig. 1 (a) *Ocimum sanctum* microgreens grown on cocopeat and vermiculite based media (b) Mature basil

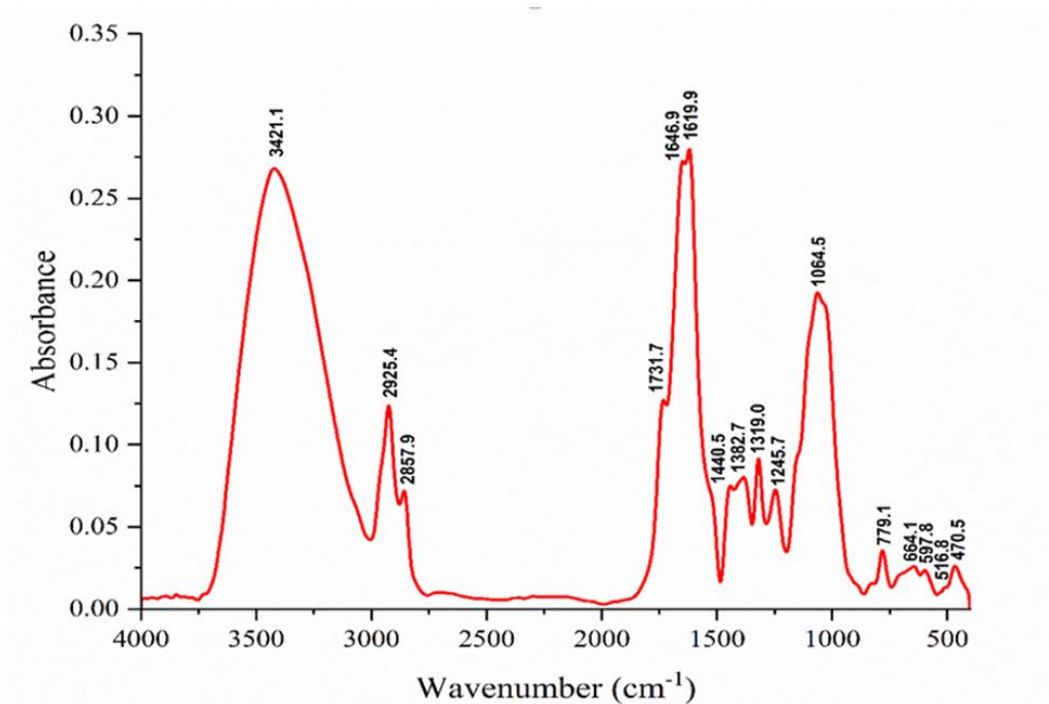


Fig. 2 FT-IR spectral peaks of *Ocimum sanctum* microgreens

3. Results and discussion

3.1 FT-IR spectral characterization and analysis

The mid-infrared spectral data obtained from extract of both basil microgreens and mature greens revealed the multiple peaks representing the various functional groups present in them indicated by wavenumber (cm^{-1}) and absorbance percentage. The absorbance peaks of spectrum of each extract were determined to interpret the specific functional groups as

per (Kustiati et al., 2022). The spectral data confirmed the occurrence of functional groups which are bioactive in nature including $-\text{OH}$, $-\text{CHO}$, $-\text{COOH}$, $-\text{SC} \equiv \text{N}$ and $-\text{COOR}$, etc. The important absorption frequencies recorded from the extract of basil microgreens and mature greens are tabulated (Table 1) along with the FT-IR spectrum which are recorded in the respective extracts are given in Fig. 2 and 3, respectively.

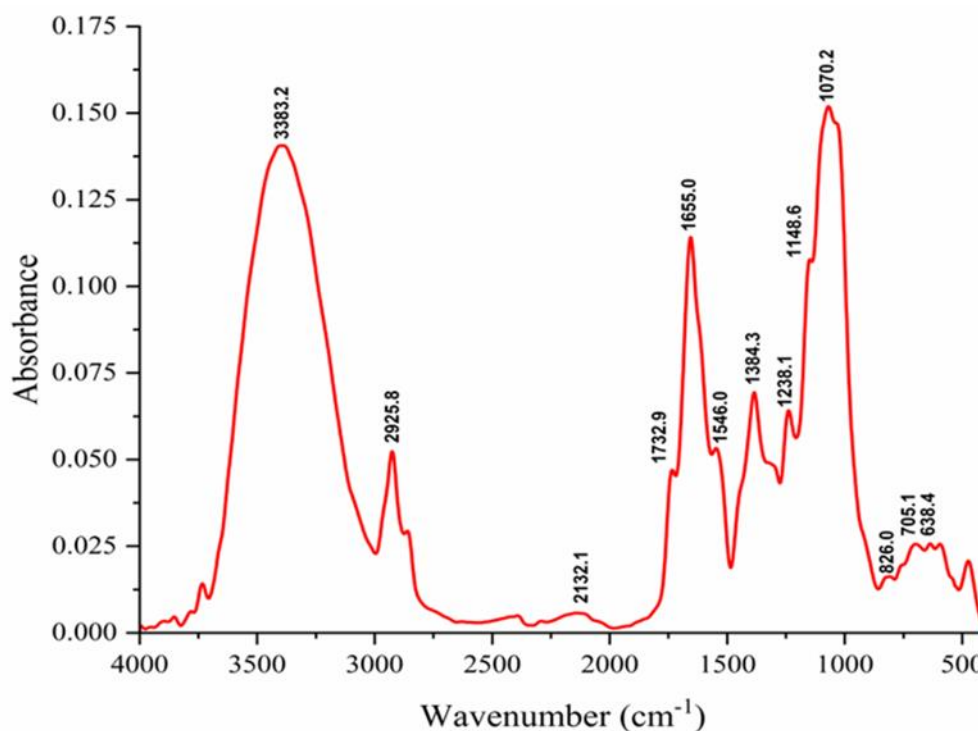


Fig. 3 FT-IR spectral peaks of *Ocimum sanctum* mature leaves

The peaks representing the O–H stretch and –NH stretching of alcohol, phenols and aromatic amines are commonly present in both the extracts. The –CH and C–N stretching is reported to be found common in both the extracts corresponding to the alkanes and primary aliphatic amines respectively. In case of basil microgreens, the spectral peaks obtained at wavenumber 3421.1 cm^{-1} clearly indicated the occurrence of O–H stretch corresponding to the presence of phenols and is showing higher absorbance than the one observed in mature basil at 3732.2 cm^{-1} which clearly indicated the higher phenolic concentrations in microgreens as compared to mature basil. There were certain absorbance peaks recorded in both basil microgreens and mature leaves at 1646.9 and 1655.0 cm^{-1} , respectively, which attributed to C = O stretching corresponding to the presence of secondary metabolites with carboxylic acids, primary/ tertiary amides, benzophenones, β -ketone esters and C–N stretch corresponding oximes and imines. Oximes are identified for their detoxifying activities against neurotoxic agents like organophosphates by reactivating acetylcholine esterase activity (Dhuguru et al., 2022). These chemical bonding and functional groups are characteristic features of phyto-active compounds which were interestingly detected in higher concentrations in microgreens. S–C \equiv N stretching was observed in both the samples representing the presence of thiocyanates which is a potent phyto-compound produced in plants as a bioactive product from glucosinolates, sulphur containing class of secondary metabolites (Bhat, 2022). The derivatives of thiocyanates are associated with excellent antimicrobial, antiparasitic, anti-inflammatory and anti-cancerous properties (Chen et al., 2022). Besides, thiocyanates also act as therapeutic component in inducing host defense mechanism for being a great antioxidant (Chandler and Day, 2012). In basil microgreens, the spectral absorbance obtained at

1319.0 cm^{-1} depicted the occurrence of N =N– O and CF_3 stretch representing the azoxy compounds and alkyl halide attached to benzene ring. Basil microgreens were also detected with S=O groups representing metabolites containing sulphoxides which are well known owing to their antimicrobial, anti-inflammatory and neuroprotective antioxidant attributes (Sanmartín-Suárez et al., 2011). The mid-infrared spectroscopic analysis therefore, detected the functional groups which certainly belong to the metabolic phyto-compounds including flavonoids, alkaloids, glucosinolates and other polyphenols and their derivatives, etc.

3.2 Morphological and elemental analysis using SEM-EDS

SEM analysis was performed to assess the morphological differences in leaf surfaces of microgreen and mature basil leaves. At 200X magnification, oil glands were seen in microgreen leaves and in mature leaves oil glands as well as trichomes both were observed. The average number of oil glands observed were approx. 22 and 52 per mm^2 in microgreen and mature basil leaves, respectively. The average diameter of oil glands observed were observed 33.89 and 50.49 μm in microgreen and mature basil leaves, respectively. The average length of trichomes present in mature basil leaves were found to be of 172.76 μm . The number of trichomes observed in mature basil leaves was approximately 39 per mm^2 . Further, the surfaces of the leaves were also analyzed at 500X magnification and the observations showed the occurrence of stomata as well as arrangement of subsidiary cells around the stomata. The number of stomata observed were approx. 207 and 385 per mm^2 in microgreen and mature basil leaves, respectively. At 2000X magnification, stomatal apertures were analysed and observed that the average length of stomatal pores were found approximately 7 and 11 μm with an average width

of about 0.08 and 2.20 μm in microgreen and mature basil leaves, respectively as shown in Fig. 4(a-f). Fig. 5 show Elemental analysis (EDS) showed the presence of carbon, oxygen, sodium, potassium, magnesium, phosphorus and

sulphur in both microgreen as well as mature basil (Fig. 5). However, calcium was not detected in basil microgreens (Table 2).

Table 1. Major bands observed and functional group annotation to the FT-IR spectral peaks of microgreen and mature leaves of *Ocimum sanctum*

<i>Ocimum sanctum</i> (Holy basil)	Frequencies observed (cm^{-1}) (stretch / band)	Corresponding functional groups	Probable Phytocompounds
Microgreens	3421.1 (O–H stretching), 2925.4 (–CH– stretching) 2857.9 (–CH– stretching), 1731.7 (C = O stretching), 1646.9 (C = O stretching, NH ₂ deformation, C–N stretching) 1619.9 (C = O stretching) 1440.5 (OH bending) 1382.7 (CH ₃ deformation) 1319.0 (N =N– O, CF ₃ stretch) 1245.7 (–CH– vibrations) 1064.5 (SO ₃ , C–N, S = O stretch) 779.1 (C – Cl stretch) 644.1 (S–C \equiv N stretch) 597.8 (SO ₂ scissoring) 516.8 (C–H stretch) 470.5 (NO ₂ rocking)	Alcohol and phenol Aliphatic compounds Alkanes/ alkanes Aldehyde Carboxylic acid / primary, secondary and tertiary amides/ oximes and imines/ benzophenone/ β -ketone esters β -diketone esters Carboxylic acid Tertiary butyl group Azoxy compounds/ alkyl halide with benzene ring Tertiary butyl hydrocarbons Sulphonic acid/ aliphatic amines/ sulphoxides Chloro-compounds Thiocyanates Sulfones Alkyl group Nitro compounds	Alkaloids, flavonoids, glucosinolate, thiocyanates, polyphenols, carboxylic acid containing phytocompounds, etc.
Mature leaves	3732.2 (O–H stretching), 3382.2 (O – H or N – H stretch) 2925.8 (C–H stretching) 2132.1 (C \equiv C stretching) 1732.9 (C = O stretching) 1655.0 (C = O stretching or C–N stretching) 1546.0 (ring stretch/ NO ₂ stretching) 1384.3 (C = O stretching) 1238.1 (C–N stretching) 1148.6 (C = S stretching) 1070.2 (C–N stretching/ SO ₃ Stretch) 826.0 (C–Cl stretching) 705.1 (C–S stretch) 638.4 (S–C \equiv N stretching)	Alcohol and phenol Alcohols/ phenols/ aromatic amines Alkanes Alkynes Aldehydes Primary and tertiary amides /Oximes/ imines/ benzophenones/ β -ketone esters Triazine compounds/ aromatic nitro compounds Carboxylic acid Aromatic compounds Thiocarbonyl compounds Primary aliphatic amine/ sulphonic acid Chloro-compounds Sulphonyl chloride/ sulfides Thiocyanates	Alkaloid, flavonoid, polyphenols, glucosinolates, thiocyanates, carboxylic acid containing phytocompounds, etc.

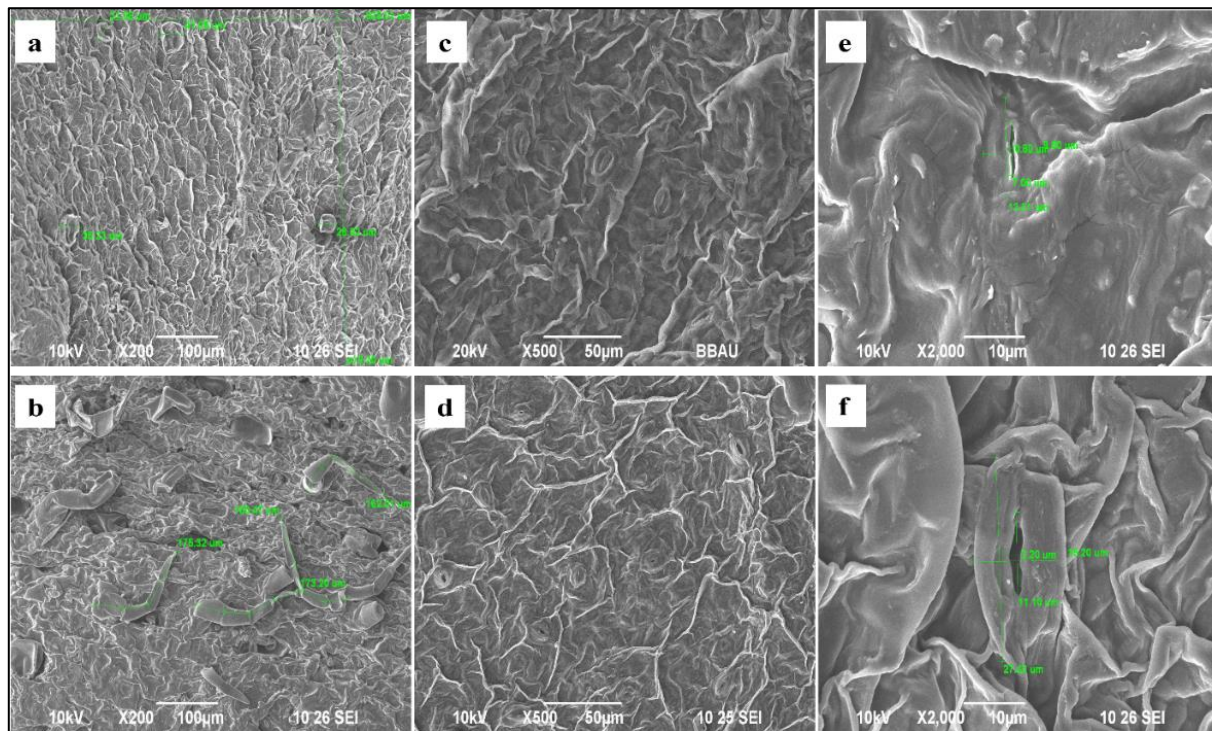


Fig. 4 SEM images showing various morphological features of abaxial surfaces of microgreen and mature leaves of *Ocimum sanctum* respectively; a and b showing oil glands and trichomes of microgreen and mature basil at 200X magnification, c & d showing distribution of stomata in microgreen and mature basil leaves at 500X magnification, e & f showing difference in the size of stomatal apertures of microgreen and mature basil leaves at 2000X magnification

Table 2 Major bands observed and functional group annotation to the FT-IR spectral peaks of microgreen and mature leaves of *Ocimum sanctum*

Elements	Weight percentages of elements in <i>Ocimum sanctum</i>	
	Microgreens	Mature leaves
C	34.71	41.25
O	51.43	49.72
Na	0.73	0.20
K	4.51	2.15
Mg	0.28	0.12
P	2.00	0.87
Ca	**	0.04
S	0.27	0.11

The number and diameter of oil glands observed were found lesser in microgreen leaves than the mature ones. The number of oil glands were found approximately 2.4 times higher and the diameter of oil glands were approx. 1.5 times larger in mature basil than that of the microgreen. The number of stomata found in microgreen leaves were observed approximately half the number of stomata found in mature basil leaves. The size of stomatal aperture in mature leaves were found about 1.5 – 3 times larger than that of microgreen. The higher number and larger size of stomata showed their important role in the process of photosynthesis in mature plants that is directly related to the higher amount of carbon detected in mature basil leaves during EDS analysis. The trichomes detected in larger size and higher in number shows their

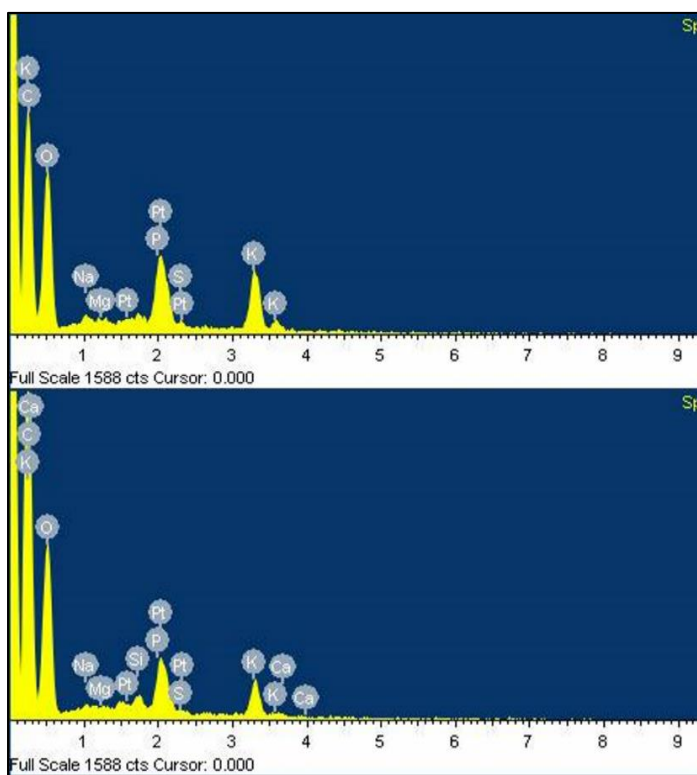


Fig. 5 Energy dispersive X-ray spectroscopic peaks of a microgreen and b mature leaves of *Ocimum santum*

importance in preventing water loss and minimizing loss incurred during transpiration. However, trichomes were not observed in basil microgreen leaves. The higher amount of carbon in mature basil might be due to the higher rate of photosynthesis. Mature leaves actively participate in photosynthesis so that they produce higher amount of sugars by carbon fixation and thus having more amount of carbon. Sodium, potassium, magnesium, phosphorus and sulphur were found up to 2-3 folds higher in concentration in basil microgreens. However, calcium was not detected in microgreen but was present in mature basil that plays an important role in membrane permeability and transport across the vacuoles.

4. Conclusion

Ocimum sanctum (holy basil) is renowned aromatic plant widely known for a plethora of medicinal values since ancient times. Although the curative and therapeutic importance of this genera is extensively studied, yet the detailed study about the metabolites involved in the respective mechanisms are to be studied further. Interestingly, the mid-infrared spectroscopic analysis revealed the functional groups and chemical bonds of secondary metabolites of basil microgreens to be more diverse and concentrated as compared to mature basil extract. SEM-EDS studies revealed the morphological differences along with element composition in both. The study found that the basil microgreens were having better element profile as far as the weight percentage is concerned. The weight percentages of sodium, potassium, magnesium and phosphorus were found as 0.73, 4.51, 0.28 and 2.00, respectively, which were observed about two to three times higher as compared to the mature basil leaves. The study paved the way towards the future research involving the analytical techniques for the structural annotation of the compounds that are directly or indirectly involved in remedial properties of the herb. The study concludes the importance of microgreens for being a potent candidate in therapeutics and nutraceuticals as they are having higher nutritional content including antioxidants and display better elemental profile.

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Author credit statement

Conceptualization, methodology, software, data curation, investigation, validation, formal analysis, writing- original draft preparation was equally done by Anamta Rizvi and Sailendra

Kumar. Conceptualization, supervision, resources, reviewing and editing was finalized and reviewed by Sangeeta Saxena. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that there are no competing interests to declare.

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