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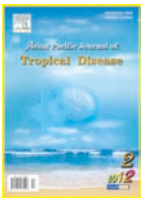

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Microscopic evaluation and physicochemical analysis of *Origanum majorana* Linn leaves

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ABSTRACT

Objective: To study the microscopic evaluation and physicochemical analysis of *Origanum majorana* Linn leaves. **Methods:** Fresh and dried powdered leaf samples was studies for its morphology, microscopy, organoleptic characters, fluorescence analysis and various other WHO recommended methods for standardisation. **Results:** Leaves are simple, petiolated, ovate to oblong-ovate, (0.5-1.5 cm) long, (0.2-0.8 cm) wide, with obtuse apex, entire margin, reticulate veination and symmetrical but tapering base. The microscopy revealed the dorsiventral nature of the leaf. Both the surfaces show presence of numerous covering trichomes, diacytic stomata and thin walled, wavy epidermal cells. The covering trichomes are multicellular, uniseriate, thin walled and pointed. In the midrib region, the epidermis is followed by collenchyma and vascular bundle (xylem and phloem). Whereas; the mesophyll exhibited only palisade cells and spongy parenchyma. **Conclusions:** It can be concluded that the pharmacognostic profile can serve as tool for developing standards for identification, quality and purity of *Origanum majorana* Linn leaves.

1. Introduction

Origanum majorana Syn. *Majorana hortensis* (M.) plant is an evergreen herbaceous plant belonging to the family Lamiaceae It is also known as Sweet marjoram[1,2]. The genus *Origanum* houses around 900 different species and many species are extensively used for the flavoring of alcoholic beverages, food products and in perfumery owing to their spicy fragrance[3]. In addition to their commercial importance, such plants have been traditionally used as condiments and spices for foods like salads, soups, sausages and meats. Recently antimicrobial[4], antimutagenic[5] antihyperglycemic, antilipidemic[1] and antiulcer[6] effect Their use for the management of diverse diseases was also in practice, being sudorific, stomachic, expectorant, emmenagogic, stimulant, antiseptic[7] hepatoprotective and nephroprotective[8,9].

Marjoram is a bushy tender perennial herb that grows up to 1 foot in height. It is native to Asia, but was naturalized in Europe where it was a favorite of the Greeks and Romans. The plant is well known as marwa in marathi and hindi. The branches have square stems and tiny, oval, gray-green leaves that may be fuzzy. The buds are knot-like and open to form clusters of white or pink flowers. The branches and leaves are steam distilled to produce an essential oil with a warm, woody, spicy, slightly peppery and nutty aroma that is calming and comforting. The scent (fresh & herbaceous; warm, sweet and slightly woody) and properties are milder than the closely related and potentially overwhelming Oregano.

Traditionally, the leaves are employed to cure diabetes, insomnia, catarrh, asthma and nervousness[10]. The leaf extracts have been scientifically proved to be effective as an antioxidant[11], hepatoprotecitve[12], antibacterial[13], antihypertensive[14] and antiplatelet aggregation[15] properties.

Both academic world and the food industry have been fascinated in the biological properties of *Origanum* extracts and essential oils due to their antimicrobial and antioxidant potential.

2. Material and methods

2.1. Collection and of authentication plant

The leaves of *Origanum majorana* Linn were collected in

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the month of November from the local markets of Pune and were authenticated by Botanical Survey of India, Western Circle, Pune with voucher specimen no. SWK-1.

2.2. Reagents and chemicals

All chemicals and solvents used in this project were procured from Merck (Germany), SD Fine chemical (India), Loba, Research Lab and Ranchem (India).

2.3. Morphology

Morphology of the leaves was determined by placing them over the stage of a simple microscope and observing through a 6 X lens.

2.4. Microscopic Studies

Light microscopy (LM): For bright field microscopy, specimens of fresh material were prepared. The fresh leaves were hand-sectioned transversely and paradermally with a sharp diamond edge blade. The sections were soaked in various reagents as explained in standard textual procedure. The following histochemical reactions were carried out: Toluidine Blue (TBO) at pH 5-6 as an indicative stain for polysaccharides Ruthenium Red for polysaccharides other than cellulose; Nadi reagent for essential oil detection Sudan Red 7B for lipids ferric chloride for polyphenols and Naphthol Blue Black to show up proteins and Berberine-Aniline Blue for suberin, lignin, and callose[16].

Photomicrographs of different magnifications (X 100 and X 400) were taken with Moticam 2300 camera and were analyzed by Motic Image-Plus 2.0 software. Magnifications are indicated below the figures. Histology and histochemistry of the entire drug was performed according to the methods described by Brain and Turner[17-19] the powder microscopy was studied according to the method described in the recent pharmacognosy practical books[20-22]. Terminology used for the anatomical features were as given in the standard books on plant anatomy[17,20,21,23].

2.5. Organoleptic and microscopic evaluation of powder

The leaves were dried in shade and crushed to yield coarse powder. The powder was stored in an airtight amber colored bottle throughout the study. The powder was sensed for its color, odor, texture and taste by placing in a Petri dish. The color was reported by placing the powder against a white background and observing in day light. The odor and taste was evaluated by sensing a dry powder as well as placing a pinch of powder in warm water.

2.6. Physicochemical analysis

Physicochemical parameters such as ash values, extractive values, foaming index, tannin content were determined according to the official method of the WHO guidelines on quality control methods for medical plants materials[24].

2.7. Preliminary phytochemical screening

Preliminary phytochemical screening was carried out with the help of standard procedure described by Kokate and Khandelwal[20,23].

2.8. Fluorescence analysis

Dried leaves were powdered and observed under visible light, short ultra violet light, long ultra violet light after treatment with different reagents like chloroform, ethyl acetate, methanol, petroleum ether (b.p. 600 - 800 C), 50% sulphuric acid, 50% nitric acid, 50% hydrochloric acid, 10 % sodium hydroxide, etc[25,26].

2.9. Essential oil extraction and analysis

The fresh *Origanum majorana* leaves were extracted in Clevenger type apparatus. An oily layer appeared over water in the receiver. The water in the receiver was shaken with diethyl ether to extract any dissolved phenols. The ether layer was evaporated and residual contents were mixed in volatile oil. The oil was collected and stored in an air tight container in a refrigerator[24].

3. Results

3.1. Morphology of Origanum majorana leaves

Leaves are smooth, simple, petiolated, ovate to oblong-ovate, (0.5-1.5 cm) long, (0.2-0.8 cm) wide, with obtuse apex, entire margin, symmetrical but tapering base and reticulate venation. The texture is extremely smooth due to presence of numerous hairs. Table I summarises the morphological characters of *Origanum majorana* leaves. Table 1 and Figure 1 shows the morphology of *Origanum majorana* leaves.

Figure 1 Morphology of *Origanum majorana* Linn leaves showing arrangement and size of leaves

Table 1

Morphological characteristic of *Origanum majorana* leaves

Parameters	Observations
Color	Dorsal surface –Grayish greenVentral surface – pale green
Odor	Aromatic and pleasant
Taste	Bland followed by sweet
Form	Simple
Shape	Ovate
Size	1–2 cm X 1 cm
Apex	Obtuse
Margin	Entire
Texture	Smooth
Venation	Reticulate
Base	Symmetrical and tapering
Arrangement of leaves	Opposite

3.2 Microscopic studies of Origanum majorana leaves

Paradermal study of the surface reveals the presence of diacytic stomata, wherein the stoma is covered with two guard cells followed by two subsidiary cells and epidermis layer. Epidermal cells are polygonal, thin and wavy walled. Surface analysis of the leaf also reveals the presence of veins, vein islet and vein terminations. Upper epidermis consists of polygonal cells and the outer wall which contains numerous covering trichomes and stomata. The covering trichomes are multicellular, uniseriate, thin walled and pointed. Lower epidermis is similar to upper epidermis (Figure 2).

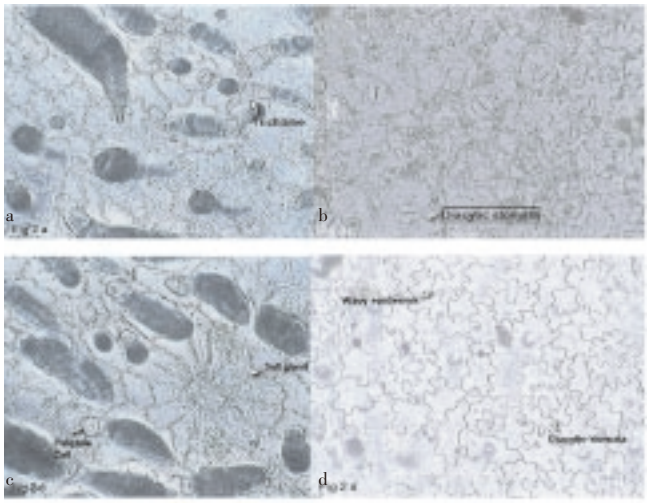

Figure 2 Paradermal characters of *Origanum* majorana leaves (X400) Leaf showing a – trichome and trichome bases, b – diacytic stomata of upper surface, c – Salt glands and palisade cells and e – wavy epidermis and diacytic stomata of lower surface

Transverse section of leaf shows the cuticularized epidermis followed by layers of compactly arranged chollenchyma followed by vascular bundles (xylem and phloem). Whereas; the mesophyll exhibited only palisade cells and spongy parenchyma. Collenchyma tissue consists of thick walled rounded parenchymatous cells. Xylem fibers are lignified whereas phloem fibers are non–lignified. Table 2, Table 3 and Figure 3 reveal the detailed microcopy of leaf.

Table 2

Microscopic characteristics of transverse and paradermal section of *Origanum majorana* leaves

Parameters	Observation
Type of leaf	Dorsiventral
Epidermis	Wavy, thin walled (upper & lower)
Trichomes	Covering, multicelular, uniseriate, bulbous base, pointed apex, non–warty, glandular trichomes absent
Stomata	Diacytic
Palisade cells	Columnar, radially elongated, loosely arranged
Parenchymatous cells	thin walled, spherical, loosely arranged
Collechyma	Thick walled, compactly arranged, few cells
Cortical Parenchyma	Loosely arranged cells, (cystolith and calcium oxalate crystals are absent)
Vascular bundle	Collateral arrangement, lignified xylem, non–lignified phloem
Stomatal index –lower surface	22.3±1.21
Stomatal index – upper surface	34.1±2.44

Values are expressed as mean±standard deviation. The leaf samples were analyzed in triplicate for determination of surface constants.

Table 3

Histochemical properties of transverse section of *Origanum majorana* leaves

Test	Color	Histological zone
Aniline sulphate + H_2SO_4	Yellow	Lignified xylem present
Phloroglucinol + HCl	Pink	Lignified xylem present
Conc. H_2SO_4	Green	Cellulose cell wall present
Weak Iodine solution	Pale blue	Starch present
Millons reagent	No red color	Protein absent
Dragendorffs reagent	No orange red	Alkaloids absent
H_2SO_4 (60% v/v)	No needle shaped crystals	Ca. oxalate absent
Antimony trichloride	Pink	Terpenes present

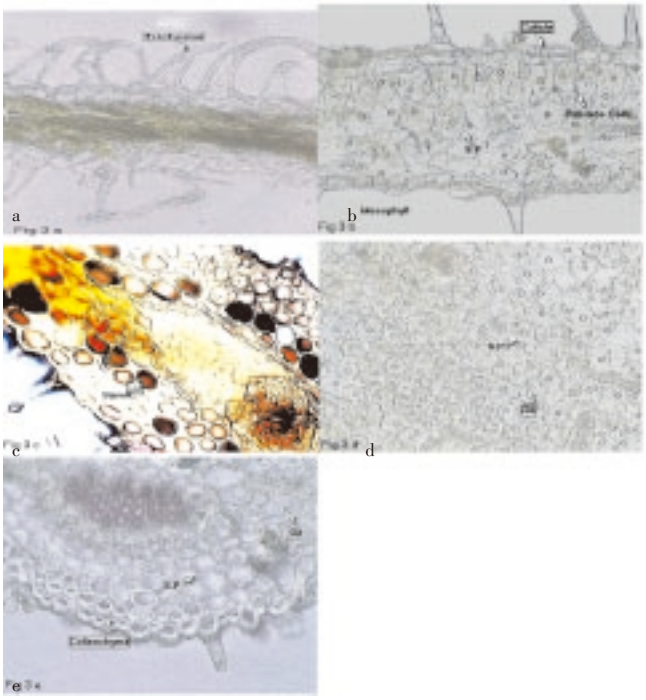

Figure 3 Microscopical characters of *Origanum* majorana leaves (X400)

Transverse section showing a – Covering trichomes, b – Mesolhyll with palisade cells, cuticularised epidermis and SP (spongy parenchyma), c – starch stores in SP (spongy parenchyma) d – SP (spongy parenchyma) with oil globules, e – compactly arranged collenchyma, SP (spongy parenchyma), oil globules, lignified xylem.

3.3. Organoleptic and microscopic evaluation of powder

The leaf powder when evaluated for organoleptic parameters, revealed the following characteristics. The leaf powder is grayish-green in color, with a characteristic and pleasant aromatic odour. The dry powder of leaves when tasted initially gives a bland taste followed by sweet sensation.

The leaf powder miocroscopy revealed different tissues such as stomata, trichomes, epidermal cells, palisade cells, starch granules, xylem and phloem fibres. The observed organoleptic and microscopic characters are reported in Table 4 and Figure 4.

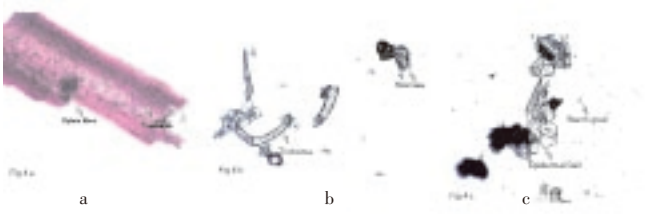

Figure 4 Microscopical characters of *Origanum* majorana powder (X 400)

Powder showing a – lignified xylem with tracheids, b– trichomes and stomata, c– starch grains and epidermal cells

Table 4

Organoleptic and microscopic characteristics of *Origanum majorana* leaf powder

Characters	Observations
Color	Greyish green
Odor	Spicy aromatic
Taste	Slighlty bitter
Texture	Rough
Xylem fibres	Scalariform tracheids, lignified
Phloem fibres	Nonlignified
Trichomes	Covering, multicelular, uniseriate
Stomata	Diacytic
Starch grains	Present
Oil cells	Present

3.4. Physicochemical analysis of the powder

Physicochemical analysis of the powder was performed and various parameters were evaluated. The foreign matter was 0.51%, LOD was found to be 10.05%, total ash 4.20%, water soluble ash 2.07%, acid insoluble ash 0.84%. The powder did not reveal the swelling and hemolytic index. The extractive values are primarily useful for the determination of the exhausted or adulterated drug. Extractive values recorded in petroleum ether, alcohol and water were 1.28, 3.71 and 4.88% w/w respectively. Table 5 summarizes the physicochemical analysis of leaf powder.

3.5. Preliminary phytochemical screening

The preliminary phytochemical screening basically revealed the presence of following phytoconstituents such as triterpenoids, phenols, tannins, carbohydrates etc. Table 6 reveals the details of phytochemical analysis of leaf powder.

Table 5

Physiochemical analysis of *Origanum majorana* leaf powder

Parameters	Value
Total ash	04.20±0.12 % w/w
Acid insoluble ash	00.84±0.03 % w/w
Water soluble ash	02.07±0.05 % w/w
Sulphated ash	00.43±0.06 % w/w
Foreign matter	00.51±0.04 % w/w
Loss on drying	10.05±0.24 % w/w
Haemolytic index	No hemolysis
Foaming index	< 100
Tannin content	01.11±0.06 % w/w
Swelling index	No swelling
Petroleum ether (bp:40 −60 0C) extractive	00.98±0.06 % w/w
Alcohol soluble extractive	03.71±0.08 % w/w
Water soluble extractive	04.88±0.20 % w/w

Values are expressed as mean±standard deviation. The powder samples were analyzed in triplicate.

3.6. Fluorescence analysis

Fluorescence analysis of the powder treated with different solvents and reagents is exhibited in table 7.

Table 6

Preliminary phytochemical analysis of different extracts of *Origanum majorana* leaf

Tests	Powder	Petroleum ether extract	Methanol extract	Aqueous extract	Hydrodistilled oil
Carbohydrates	+	−	−	+	−
Proteins	+	−	+	+	−
Amino acids	+	−	+	+	−
Fixed oils	−	−	−	−	−
Steroids	+	+	−	−	−
Volatile oils	+	−	+	−	+
Saponin glycosides	+	−	+	+	−
Flavonoids	+	−	+	+	+
Alkaloids	−	−	−	−	−
Phenols	+	−	+	+	+
Vitamin C	+	−	−	+	−
Tannins	+	−	+	+	−

Table 7

Fluorescence analysis of *Origanum majorana* leaf powder

Reagents	Visible	Short Ultra Violet	Long Ultra Violet
Pet Ether (bp: 40−600C)	Greenish−yellow	Green	Red
Ethyl acetate	Green	Green	Orange
Ethyl acetate: HCl (1:1)	Green	Green	Reddish−Orange
Methanol	Dark green	Pale Green	Red
Chloroform	Dark green	Green	Red
Acetone	Pale Green	Pale Green	Greenish−yellow
50 % H_2SO_4	Green	Dark green	Black
50 % HNO_3	Pale Green	Black	Dark Green
50 % HCl	Green	Black	Green
10 % NaOH	Green	Green	Green

3.7. Physicochemical analysis of essential oil

The essential oil extracted by hydrodistillation was analyzed for various physicochemical properties. The oil was pale yellow in color with characteristic aromatic odor and free flow. The yield was 1.72%; specific gravity and RI, when recorded at 200C were found to be 0.893 and 1.477 respectively. Oil was freely soluble in ethanol and ethyl acetate whereas insoluble in water. (Refer table 8 for detail physiochemical properties of oil)

Table 8

Physiochemical parameters of *Origanum majorana* Linn. oil

Parameters of Oil	Observations
Color	Pale yellow
Odor	Aromatic, fresh herbaceous
Appearance	Mobile liquid
Oil Yield (g 100g−1)	01.72±0.09
Refractive Index @ 20℃	1.477±0.02
Specific gravity @ 20 ℃	0.893±0.03
Boiling point @ 760 mm Hg	265.3±0.20 ℃
Solubility in ethyl acetate	soluble 1 in 2 parts
Solubility in ethanol (75 % v/v)	soluble 1 in 2 parts
Solubility in water	insoluble

Values are expressed as mean±standard deviation. The oil sample was analyzed in triplicate.

4. Discussion

Sensory evaluation plays a vital part in determining the suitability or denunciation of a crude drug in the market. Organoleptic testing of a crude drug is the technique for qualitative evaluation based on the observation of morphological and sensory profile[23]. In this report, various morphological, microscopical, physicochemical standards have been developed; that will help botanists for identification and standardization of *Origanum majorana* Linn.

The estimation of ash values and extractive values can help for recognition of adulteration[27]. Presence or absence of foreign inorganic matter such as metallic salts and/or silica can be easily determined by performing the total ash. Water soluble ash is the measure of physiological inorganic components of the crude drug. Rise in water soluble ash is normally due to supply of hard water excess mineralized soil for cultivation. Acid insoluble ash gives an idea about the non−physiological ash produced due to the adherence of inorganic dirt, dust to the crude drug. Increased acid insoluble ash means adulteration due to dirt, soil or sand[28]. Table 5 gives the details of ash values obtained for *Origanum majorana* leaves.

Extractive values are useful to estimate the chemical constituents present in the crude drug and are a measure

to determine the solubility of phytoconstituents from the crude drug in a given solvent[29]. Each crude drug looses specific amount and type of phytoconstituents when shaken with a particular solvent. Normally alcohol and water are used as solvents to determine extractive value as per the pharmacopoeias, but certain industries have their own standards. Table 5 highlights the extractive values of *Origanum majorana* leaf powder.

Loss on drying explains the amount of components that leaves the powder when heated at 1100C until constant weight is attained. High LOD value is undesirable as it leads to loss in weight and may attract microbial contamination. Swelling index gives an idea about the mucilage content of the crude drug[28]. No swelling was observed indicating absence of mucilage in the leaves. Foaming index determines the saponin content of the crude drug. Saponin glycosides are responsible for antiulcer and antidiabetic property of the drug. Excess amount of saponins in the crude drug may prove to be fatal as it causes hemolysis of the blood. *Origanum majorana* was found to contain fewer amounts of saponins hence; it is safe and can prove to be a better antidiabetic and antiulcer drug (refer table 5 for values).

Fluorescence is the phenomenon exhibited by numerous phytoconstituents present in the plant material. Many chemicals fluoresce in presence of certain reagents or solvents. The fluorescence color is specific for each compound. The fluorescence analysis of the leaf powder of *Origanum majorana* Linn exhibited different colors when treated with various chemical reagents (Table 3).

One of the simplest and cheapest methods to establish the accurate identity of plant material is microscopic analysis[30]. On observing the paradermal section, stomata were found to be diacytic as the longitudinal axes of two guard cells were exactly perpendicular to that of the subsidiary cell axes. The trichomes were covering type, multicellular (having 2-3 cells), uniseriate, with pointed apex and warts were absent[20]. The secretory glands were formed by fusion of 10-16 epidermal cells. The epidermis was lined with cuticle which was confirmed by staining with sudan red III reagent.

The upper and lower epidermal cells were found to be wavy with thin cell wall. The epidermal cells serve as a protective barrier for leaf both from the dorsal and ventral surface. The epidermal cells are transparent to facilitate the entry of sunlight towards the palisade cells for photosynthesis. Certain epidermal cells modifies into trichomes, stomata and oil secretory glands. Salt glands were found on the epidermis. Opening of the gland was found to be surrounded by 10-12 radially elongated cells. Recent reports suggest that the essential oil and fatty oil content and composition are affected by the salinity[31]. The oil is reported to have many beneficial reports such as antimicrobial, anticholiesterase, and antioxidant[32,33].

Study of transverse section of the leaf reveals its dorsiventral nature *i.e.* the palisade cells are found to be present only at the dorsal surface. The palisade cells have chlorophyll which aids in photosynthesis[20]. The mesophyll comprises of spongy parenchyma and a single layer of columnar, radially elongated, uniform palisade cells below the upper epidermis. The palisade cells manufacture monomer and transfer these monomers to the adjoining parenchyma, where they polymerize to form starch granules. The spongy parenchyma serves as storage reservoir for starch. Parenchymatous cells are loosely arranged with ample intercellular spaces.

The palisades cells are absent in the midrib region and are replaced by collenchyma. Collenchyma cells are compactly arranged without any intercellular spaces and have thick cell wall. Collenchyma provides mechanical strength to the neighboring vascular bundles[18]. The midrib appears like a sandwich of vascular bundle & spongy parenchyma between two collenchymatous layers.

Vascular bundle is restricted to the midrib region and comprises of collateral arrangement of xylem and phloem. Lignified xylem and non-lignified phloem fibers are arranged in stripes. The lignified xylem can be observed after staining the transverse section with phloroglucinol: HCl (1:1) proportion[19]. The intracellular components like starch can be observed by soaking the T.S. in dilute iodine solution. When the transverse section was treated with H2SO4 (60% v/v), no needle shape crystals of Calcium sulphate were observed indicating the absence of calcium oxalate crystals and cystolits ($CaCO_3$)[18]. Recent reports on *Origanum majorana* suggest its wide application in the field of skin care cosmetics[34], pharmaceuticals, agro-alimentary[35].

5. Conclusion

The present study was carried out with a vision to setup standards that could be beneficial for detecting the authenticity of this vital medicinal plant. Numerical standards reported in this work could be useful for the compilation of a suitable monograph of *Origanum majorana* Linn.

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Conflict of Interest

We declare that we have no conflict of interest.

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