The underexploited biotechnology of overexploited *Origanum* species: Status, knowledge gaps, prospects and potential

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**ABSTRACT**

The genus *Origanum* is a group of phytochemically diverse, aromatic species. Distributed mainly in Eurasia and Mediterranean, they are used in traditional medicine, cosmetics and for culinary purposes. They possess antiproliferative, antioxidant, antiviral, anti-septic, anti-inflammatory, anti-hypertensive properties. The major constituents of *Origanum* spp. are carvacrol and/or thymol together with γ-terpinene, p-cymene, linalool, terpinene-4-ol and sabine hydrate. Several flavonoids and glycosides are also found. Although the *Origanum* spp. can either be cultivated or sourced from nature, high demand has necessitated increased production. Overexploitation from natural habitat has threatened these species. Also, due to its poor viability and small-sized seeds, cross-pollination abilities, less productive vegetative propagation, climate-dependent conventional propagation, its genetic improvement has been limited and thus scientific management of available germplasms through biotechnological approach is necessary. For *in-vitro* propagation, the literature review showed significant differences in culture protocols, genotypes and their success rates. Studies reported cell culture-based production of secondary metabolites or isolation of active compounds in different species of *Origanum*, which show antiproliferative activity in cancerous cell lines. However, significant knowledge gaps exist. The urgent need is to use advance technologies in enhancing either plant propagation thus the production of source material for active constituents or for genetic improvement of *Origanum* germplasms for contents, as well as to validate the therapeutic potential of *Origanum* constituents. This review critically appraises the status of mostly underexploited biotechnological know-how and research on highly valued medicinal herbs, *Origanum* and throws light on prospects and potential.

**Introduction**

The term “traditional medicine” is used interchangeably with complementary or alternative medicine (CAM) which broadly includes herbal medicine and natural medicine (1). Worldwide, 60 - 80% of people depend on traditional herbal medicine for their healthcare, body care and food products, leading to an increased demand for medicinal plant material (2, 3). Many of the medicinal plants containing aromatic essential oils are used not only as traditional medicines but also in food, flavour and fragrance industry (2).

The genus *Origanum* L., belonging to family Lamiaceae, tribe Mentheae comprises of forty-nine taxa including 38 species, 6 subspecies, 3 varieties and remaining hybrids and is characterized by vast taxonomic, morphological, anatomical and chemical diversity. The *Origanum* species are annual or perennial, shrubby herbs grown and naturally found across many geographical regions. In spite of the multi-purpose medicinal, culinary and cosmetic value of the *Origanum* species, only less than 50% species have been subjected to chemical profiling, characterization and biological studies (4).

**Distribution**

*Origanum* populations are primarily distributed in Eurasia and African regions. They have the highest recorded diversity in the Mediterranean, Euro-Siberian and Irano-Siberian regions (5). *Origanum* species are found on stony slopes and in rocky...
mountain areas at a wide range of altitudes (0–400 m) (6). With reference to biotechnological interventions, two Origanum species are, however, worth special mention. Origanum vulgare L., commonly known as Oregano, wild marjoram, Himalayan marjoram, or as Ban tulsi or Jangli Marwa in India, is widely distributed in Mediterranean areas and Northern Africa and in India, found in the temperate Himalayas from Kashmir to Sikkim at an altitude of 1500–3600 m (4, 5). Origanum majorana L., commonly known as Marjoram, Sweet Marjoram, Knotted Marjoram, Sampsishia or as Marwa in India, is although endemic to Cyprus, cultivated all over Asia, Arabian Peninsula and Africa (4) and is found in Europe as well (7). Both Oregano and Marjoram are one of the most important and largest selling culinary herbs (8).

Uses
Origanum spp. have been used as herbal medicine, in aromatherapy and as a culinary herb, spice and flavouring agent in food, across many nations and cultures (9). In traditional Indian medicine, medicinal properties of O. majorana were known (10). Various medicinal, culinary, cosmetic and other uses (11–34, 45, 46) of Origanum spp. have been summarized in Fig. 1. Whether used as an essential oil, leaves extract, powder, fresh or dried leaves, marjoram has numerous health benefits (35, 36).

Phytoconstituents
The Origanum biotypes vary in respect of content and/or composition of essential oil in them (36). Essential oil ‘rich’ species with an essential oil content of >2%, is mainly characterized either by the dominant presence of carvacrol and/or thymol (together with γ-terpinene and p-cymene) or by linalool, terpinene-4-ol and sabine hydrate as principal components (37–39). Besides the presence of essential oil, different qualitative phytochemical tests exhibited the presence of flavonoids, tannins, glycosides, cardiac glycosides, sterols, terpenoids in ethanol extract of leaves, root and stem and saponins and carbohydrates in aqueous extracts (40). Considerable amounts of phenolic compounds are generally available in water, methanol, acetone and ethylacetate/water extract of Origanum (11). The phytochemical constituents of O. majorana were extensively reviewed (36). Some phenolic glycosides found in marjoram essential oil are Arbutin, Methyl arbutin and Vitexin. Among flavonoids, Hesperetin has been identified in ethyl acetate extract; Catechin, Quercetin, Kaempferol, Naringenine and Eriodictyol in the hydroalcoholic extract and flavonoids like Diosmetin, Luteolin and Apigenin are found in marjoram essential oil (41–43). Rutin, a flavonoid glycoside was identified in marjoram hydroalcoholic extract (44). Besides having Rutin, Quercetin and Eugenol in aerial parts, which give it an advantage as a flavouring agent, O. majorana particularly has nutritional benefits as well. Nutritive analysis of dried leaves yields: energy, carbohydrates, fat, dietary fibre; Vitamins- folate, niacin, pantothenic acid, pyridoxine, riboflavin, thiamin, vitamin-A, vitamin-C, vitamin-E, vitamin-K; Electrolytes- sodium, potassium; Minerals- calcium, copper, iron, magnesium, manganese, zinc; and Phytonutrients, β-carotene, β-cryptoxanthin, lutein-zeaxanthin (45).

Propagation Means
Increasing demand for Origanum spp. in the pharma, perfumery and cosmetic industry has necessitated their large scale production. Increases in consumption in recent years may be due in part to the popularity of low-fat, low-salt diets (46). Whether vegetative parts (both green and dry), extracts or essential oil are used (47, 48), it necessarily requires better propagation means to make larger quantities of source material available.

Origanum species are either sourced from its natural occurrence in the wild or cultivated. Though they are half-hardy perennial sub-shrubs, they are treated as annual herb under cultivation. In nature, it is found growing on hill-sides and also on limestone and calcareous rocks. When cultivated, propagation is either via seeds or vegetative means (49). Vegetatively, Origanum species can be propagated by cuttings, division or by layering. Under cultivation, they generally require dry, warm, well-drained fertile garden loamy soil and frequent watering. However, it grows well under wide pH ranges from acidic, neutral to basic soils (7). Marjorams are generally cold-sensitive frost-tender herbs and can be grown outdoors as annuals and replanted in the spring. In plains, seeds are sown in October and in the hills from March to June (50). In contrast, at the higher elevation, sometimes propagation is done by cutting. Seeds can be sown in pots initially and then transplanted in the field. The crop is harvested in 3–4 months. The tops are cut at the time of flowering and dried in the shade. The volatile oil content of the leaves is maximum when the plant is harvested before seed formation. The flowers are hermaphrodite and are pollinated by bees (7).

Though the Origanum sp. can be cultivated and material can be sourced from its natural occurrence also, every cultivation and sourcing method has its problems. For example, propagation via seeds has a considerable disadvantage- as a cross-pollinating plant, the offspring of seeds represent populations differing in habit, colour, content and odour of the essential oil (51). Secondly, growing Oregano from seed is not always reliable as seeds have a long dormancy and they germinate poorly if proper temperature and moisture conditions following dormancy are not met (49). In its vegetatively propagated taxa, the poor rooting ability of the stem cuttings and thus non-significant survival efficiency (52) and lack of selected clones, restrain industrial exploitations (53).

On the other hand, sourcing large quantities of the plants material for the preparation, processing and testing of the product and thus overexploitation from natural resources are acting negatively on the populations of the plant and such activities have brought the species at the verge of being endangered. There is a strong need for the application of biotechnological techniques for multiplication and conservation of Origanum species (54) as well as to promote and popularize this wealth among the
farmers, entrepreneurs and pharmaceutical industry (53).

**Biotechnological Interventions**

Tissue culture efforts in *Origanum vulgare* started as early as 1970. A report, while studying the formation of volatile substances in plant tissue cultures, achieved callus induction from stem segments of field-grown *O. vulgare*, on MS media (55) supplemented with different auxins -IAA, NAA or 2,4-D (56). Tissue differentiation leading to regeneration was achieved; however, much later – in *O. vulgare* (57) and *O. majorana* (58). The biotechnological efforts put in the species of *Origanum* has been discussed here under these sections: Plant regeneration in *Origanum* spp.; Callus and cell suspension culture for production of secondary metabolites; Genetic transformation; and Antiproliferative properties: effects of extracts/preparations from *Origanum* species on cytotoxicity of cancer cells.

**Plant Regeneration in Origanum spp.**

*In-vitro* plant regeneration has been achieved in many species of *Origanum*. If not all, most of the studies resorted to getting regeneration from pre-existing meristems (PEMs) and very few reports claim to have morphogenesis in dedifferentiated cells. A comparative and summarized view of explants, basal culture media and plant growth regulators combination tested for *in-vitro* regeneration in different species of *Origanum* reported till date is presented in Table 1.

**Plant Materials:**

Explants from various plant parts- either young or mature seedlings or mature plant body, mostly containing PEMs, have been utilized to achieve *in-vitro* plant regeneration in many *Origanum* species (51–54, 57–71, 74).

**Surface Sterilization:**

In *Origanum* tissue culture, wherever seeds or seedlings parts have been used as explants, seeds have been surface-sterilized, subjected to germination and then explants from axenically grown seedlings have been harvested. In *O. vulgare*, *O. majorana*, *O. sipyleum* L. and *O. syriacum* L., mostly the first step is washing the mature seeds either with 50–70% ethanol or with 0.01–0.2% fungicide, followed by treatment with sodium hypochlorite, or mercuric chloride or other disinfectants with varying concentration and time and then washing with sterile water to remove traces of disinfectants (54, 57, 58, 62, 65, 70, 74). The surface-sterilized seeds were germinated on either 0.8% agar or half or full-strength MS medium with 2% or 3% sucrose. In *O. minutiflorum* Schwarz & Davis, *O. acutidens* (Hand.-Mazz.)Ietsw. and *O. onites* L., however, seeds were only treated with 5–15% NaOCl followed by 3-5 washes with water, before putting them for germination in MS or Gamborg B5 medium in the dark or in the presence of light (67, 68, 71).

Wherever *Origanum* explants were taken from mature or naturally grown plants, plant parts were...
first washed with either a detergent or with 50%, 70% or 95% ethanol followed by treatment with 1.5 – 2.0% or 5–10 % NaOCl or 5% Ca(ClO)$_2$, or sometimes with 0.1 % HgCl$_2$ solution for few minutes to disinfect the tissues followed by several washes with water and before preparing the explants for inoculation [O. majorana (59–61); O. vulgare (64, 72); O. sipyleum (66); O. syriacum and O. ehrenbergii Boiss. (69)]. In one of the reports, however, only a fungicide was used as a surface sterilant after washing with detergent for O. majorana stem nodal segments (52).

**Basal Media and Media Addenda:**
Tissue culture efforts on _Origanum spp._ for _in-vitro_ plant regeneration mostly used MS medium as basal medium and supplemented this medium with different plant growth regulators (PGRs) or other addenda. Other media formulations like Gamborg-B5 medium (75), Nitsch and Nitsch medium (76), Phillips

### Table 1. Studies on _In-vitro_ culture/Plant Regeneration and Callus/Cell Suspension Culture in _Origanum_ spp.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Explant</th>
<th>Basal media</th>
<th>PGR combinations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>O. majorana</strong></td>
<td></td>
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<tr>
<td>1</td>
<td>Shoot tips and nodal segment from 30-60 d old seedlings</td>
<td>MS</td>
<td>0.5 mg/L Kin.</td>
<td>(58)</td>
</tr>
<tr>
<td>2</td>
<td>Stem nodal segments</td>
<td>MS</td>
<td>0.1, 10.0 mg/L BAP; 0.1 mg/L IBA</td>
<td>(59)</td>
</tr>
<tr>
<td>3</td>
<td>Stem nodal segments and Leaf</td>
<td>MS with Sucrose/Maltose/Glucose</td>
<td>0.1, 0.2 mg/L 2,4-D; 2, 3 mg/L BAP; 0.2 mg/L IBA</td>
<td>(60)</td>
</tr>
<tr>
<td>4</td>
<td>Axillary buds</td>
<td>MS</td>
<td>2 mg/L Kin., 2 mg/L BAP</td>
<td>(61)</td>
</tr>
<tr>
<td>5</td>
<td>Stem nodal segments</td>
<td>MS / P&amp;C L2</td>
<td>9.3 uM Kin.</td>
<td>(52)</td>
</tr>
<tr>
<td>6</td>
<td>Hypocotyl and seed explants</td>
<td>MS</td>
<td>2 mg/L 2,4-D and 0.5 mg/L Kin.; 4 mg/L NAA and 0.4 mg/L BAP</td>
<td>(74)</td>
</tr>
<tr>
<td>7</td>
<td>Cotyledonary node from seedlings</td>
<td>MS</td>
<td>1 mg/L BAP</td>
<td>(62)</td>
</tr>
<tr>
<td><strong>O. vulgare</strong></td>
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<tr>
<td>1</td>
<td>Cotyledon, hypocotyl and root from 15 d old axenic seedlings</td>
<td>BS</td>
<td>2,4-D at 10$^{-7}$ M; NAA with BAP at 10$^{-4}$ M</td>
<td>(57)</td>
</tr>
<tr>
<td>2</td>
<td>Shoot tips and nodal segments from 30-60 d old seedlings</td>
<td>MS</td>
<td>1.0 mg/L BAP and 0.5 mg/L IBA</td>
<td>(58)</td>
</tr>
<tr>
<td>3</td>
<td>Young shoot tips from field-grown plants</td>
<td>MS</td>
<td>0.53 uM NAA and 0.28 uM BAP</td>
<td>(64)</td>
</tr>
<tr>
<td>4</td>
<td>Apical buds from <em>in-vitro</em> grown plants</td>
<td>MS</td>
<td>8.88 uM BAP and 2.26 uM 2,4-D</td>
<td>(53)</td>
</tr>
<tr>
<td>5</td>
<td>Hairy root segments</td>
<td>MS</td>
<td>0.25 mg/L 2,4-D; 0.25 mg/L BAP</td>
<td>(65)</td>
</tr>
<tr>
<td><strong>O. vulgare ssp. hirtum</strong></td>
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<tr>
<td>1</td>
<td>Nodal segments</td>
<td>BS</td>
<td>0.01 mg/L NAA</td>
<td>(63)</td>
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<tr>
<td>2</td>
<td>Stem tips from 1 wk. old seedlings</td>
<td>Lepoivre nutritional Environment L4 and L11</td>
<td>0.15 mg/L IAA</td>
<td>(51)</td>
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<tr>
<td><strong>O. sipyleum</strong></td>
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<tr>
<td>1</td>
<td>Shoot tips from 17d old seedlings</td>
<td>Modified MS with 550 mg/L CaCl$_2$</td>
<td>1 mg/L BAP; 0.5 mg/L BAP</td>
<td>(54)</td>
</tr>
<tr>
<td>2</td>
<td>Nodal segments from native plants</td>
<td>MS</td>
<td>0.5 mg/L BA; 0.2 mg/L GA$_3$; 1.5 mg/L IBA</td>
<td>(66)</td>
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<tr>
<td><strong>O. syriacum</strong></td>
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<tr>
<td>1</td>
<td>Stem nodal segments</td>
<td>MS</td>
<td>2 mg/L BAP; 0.5 mg/L IBA</td>
<td>(69)</td>
</tr>
<tr>
<td>2</td>
<td>Shoot tips and 1st nodes from 25 d old seedlings</td>
<td>MS</td>
<td>0.05 mg/L NAA and 0.5 mg/L Kin.; 1.5 mg/L IBA</td>
<td>(70)</td>
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<tr>
<td><strong>O. minutiflorum</strong></td>
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<tr>
<td>1</td>
<td>Nodal segments and shoot tips from 30-40 d old plants</td>
<td>MS / BS</td>
<td>2 mg/L BAP and 0.1 mg/L NAA</td>
<td>(67)</td>
</tr>
<tr>
<td><strong>O. acutidens</strong></td>
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<tr>
<td>1</td>
<td>Node explants from 1 wk. old seedlings</td>
<td>BS</td>
<td>1.8 mg/L BAP and 0.2 mg/L NAA</td>
<td>(68)</td>
</tr>
<tr>
<td><strong>O. onites</strong></td>
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<tr>
<td>1</td>
<td>Hypocotyl, epicotyl, cotyledons, primary leaves and apical meristem from 4 wk. old seedlings</td>
<td>MS</td>
<td>1.5 mg/L Kin.</td>
<td>(71)</td>
</tr>
<tr>
<td><strong>O. ehrenbergii</strong></td>
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<tr>
<td>1</td>
<td>Stem nodal segments</td>
<td>MS</td>
<td>2 mg/L BAP; 0.5 mg/L IBA</td>
<td>(69)</td>
</tr>
</tbody>
</table>

**Callus and cell suspension culture for production of secondary metabolites**

| **O. vulgare & O. syriacum** | | | | |
| 1 | Leaf | MS | 0.1-0.5 mg/L 2,4-D; 1-1.5 mg/L TDZ | (72) |
| **O. vulgare, O. vulgare ssp. hirtum & O. syriacum** | | | | |
| 2 | Leaf | MS / LS / BS | 0.5 mg/L 2,4-D and 5 mg/L Kin.; 0.5 mg/L NAA and 3 mg/L BAP | (73) |

Abbreviations: d: days; BM: Basal Media; MS: Murashige and Skoog Medium; P&C L2: Philips and Collins L2 medium; BS: Gamborg-B5 medium; LS: Linsmaier and Skoog medium; N&N: Nitsch and Nitsch medium; Kin.: Kinetin; BAP: Benzyl Amino Purine; IBA= Indole-3-Butyric Acid.; IAA: Indole-3-Acetic Acid; NAA: α-Naphthalene Acetic Acid; 2,4-D: 2,4- dichloro-Phenoxy Acetic Acid; GA$_3$: Gibberellic Acid; TDZ: Thidiazuron.
and Collins (L2) medium (77) were also used to test the explants’ response for regeneration.

Various PGRs have been used in tissue culture of species of Origanum. For shoot organogenesis, mostly BAP either alone or in combination with other cytokinins like Kinetin or auxins (IBA, IAA, NAA or 2,4-D) have been tested. However, other cytokinins like ZIF, Zeanin, Adenine sulphate and TDZ have also been used. For rooting, IBA has been the most preferred choice, although other auxins like IAA or NAA have also been used. Interestingly, GA₃ was also tested for shoot organogenesis in case of O. majorana by one work (52) and in O. sipyleum by another (66). Use of other media supplements like amino acids, casein hydrolysate, citric acid, ascorbic acid or activated charcoal was largely not found in the literature for Origanum spp. tissue culture. In our study on O. majorana in-vitro regeneration (unpublished data), it was found that the use of activated charcoal reduces polyphenolics and controls browning of the medium, thereby aiding shoot organogenesis. In one work (70), thiamine, glutamine and asparagine were tested as media additive for O. syriacum cultures, and it was found that only thiamine supported better shoot organogenesis.

**Carbon Source:**
Sucrose has been used as a carbon source by most of the researchers to accomplish plant regeneration in different species of Origanum. While 3% concentration of sucrose has been shown to be apt by most, 2% sucrose was used by one (59). Three different carbon sources were tested for O. majorana (60), and it was found that 3% maltose was best for supporting shoot organogenesis.

**Gelling agents:**
In all the reports on tissue culture of various Origanum species, agar has been used invariably as a gelling agent. Whereas a concentration of 0.8% agar has been most common, some have used different concentrations as well; 0.6% agar for O. minutiflorum (67), 0.62% agar for O. acutidens cultures (68), 0.7% agar for culturing O. syriacum explants (70) and 0.75% agar for achieving shoot regeneration in O. sipyleum (66). Use of Gelrite or Phytagel as a gelling agent was not found in the published reports on Origanum. This is interesting as the production of phenolic acids was demonstrated in O. majorana cultures (62). As Gelrite is known for its ability to leach out polyphenolics exuded by plant tissues in culture and thus reducing harmful effects on plant regeneration, it must be tested as a gelling agent for Origanum tissue culture with an imperative to obtain a better morphogenic response.

**Culture Conditions:**
Origanum spp. tissue cultures are incubated at a temperature of 25±1 °C to O. vulgare ssp. hirtum (Link) letsw. cultures. A temperature of 28±1 °C to O. vulgare ssp. hirtum (Link) letsw. cultures.

**Approaches in In-vitro regeneration of Origanum:**
In almost all in-vitro regeneration reports on any species of Origanum, organogenesis has been the regeneration mode. The shoot organogenesis has been mostly achieved from PEMs- either from shoot apex or from axillary buds. The reports where indirect organogenesis from dedifferentiated cells has been achieved are scanty [in O. majorana (52, 60); in O. vulgare (57, 65)]. Recently, it was reported that in O. onites L., all explants except shoot apical meristem (SAM) developed into non-morphogenic calli and did not show any regeneration (71). Organogenesis is a function of a group of cells having their division products set in fashion to differentiate in a particular organ, which in turn is preceded by meristem formation. Meristem organization is, therefore, very tightly regulated in Origanum and it neither disorganizes easily nor reforms (from dedifferentiated cells) easily. Regeneration via somatic embryogenesis is not reported, till date, in any species of Origanum. This also opens up new opportunities for researchers to understand plant regeneration in this important medicinal herb.

Although in-vitro plant regeneration has been demonstrated in many species of Origanum, two types of efforts may be categorized: (a) those where explants are from young seedlings-like cotyledon segments, hypocotyl pieces, cotyledonary node, root segments or shoot tips possessing or devoid of an organized or mature meristem, (b) those where explants are from mature plant parts possessing PEM-like shoot apical meristem, stem node, axillary buds or leaves.

**From seedling explants:**
In the pioneering study on O. vulgare (57), explants were subjected to Gamborg-B5 medium with 2,4-D. NAA and BAP individually and in various combinations of 0, 0.1, 1 and 10 μM. It was found that whereas callus proliferation was best at 0.1 μM 2,4-D, cotyledon explants was the best source of nodular callus. These calli, when transferred to NAA in combination with 1μM BAP, developed the highest number of shoots. The best rooting of shoots was achieved on half-strength B5 medium having 1% sucrose and NAA or IBA at 1μM concentrations. Shoot apices of O. sipyleum were subjected to MS medium with 550 mg/L CaCl₂ and 1 mg/L BAP by one study (54). Multiple shoots were obtained, which were subsequently rooted in MS containing 0.5 mg/L IBA. Node explants of O. acutidens were cultured on MS medium supplemented with 0.2 mg/L NAA in combinations with various BAP concentrations (0.6–2.4 mg/L) (68). Better shoot organogenesis was observed in this work on a combination of 0.2 mg/L NAA and 1.8 mg/L BAP. In another work (62), shoot part including buds from O. majorana seedlings were subjected to MS with 3% sucrose and various concentrations of BAP (0–1 mg/L). The maximum number of shoots was obtained with 1 mg/L BAP. They found that in O. majorana, the inclusion of BAP in medium enhanced the number of shoots. In contrast, its omission from medium led to an increase in the length of the regenerated shoots.
A detailed study of the in-vitro response of *O. syriacum* explants was done (70). Explants were cultured on MS, Nitsch and Nitsch- and Gamborg-B5-media with 3% sucrose for establishment. After establishment, explants were subjected to MS with 1 mg/L of Kinetin, 2iP and BAP and further on various levels (0–2 mg/L) of Kinetin. It was found that shoot tip was best responsive explant and MS was the best basal medium for culturing *O. syriacum* explants. Strikingly, Kinetin was best-suited cytokinin and shoots cultured on MS with 0.05 mg/L NAA and 0.5 mg/L Kinetin produced the highest amount of shoots.

The frequency of rooting in regenerated shoots was obtained on MS medium with 1.5 mg/L IBA. A similar observation was recorded by another study recently (71), which subjected *O. onites* explants to MS medium with varying 0.5–1.5 mg/L Kinetin. They found that SAM explants gave the highest shoot organogenesis in 1.5 mg/L Kinetin.

From mature plant parts:

Both *O. majorana* and *O. vulgare* were studied (58) for in-vitro regeneration response on MS medium with various concentrations of auxins and cytokinins, wherein best shoot organogenesis was reported on 0.5 mg/L Kinetin in *O. majorana* explants and on a combination of 1 mg/L BAP and 0.5 mg/L IBA in *O. vulgare*. Stem nodal segments of *O. majorana* were subjected (59) to MS having 2% sucrose and supplemented with 0.1–10.0 mg/L BAP for axillary shoot induction. It was found that there is a dose-dependent relationship between the number of shoots and BAP concentration, as the maximum number of shoot induction was obtained at 10 mg/L BAP. However, the length of shoots decreased with increasing the concentration of BAP and maximum shoot length was obtained at 0.1 mg/L BAP. The regenerated shoots rooted best on MS supplemented with 0.1 mg/L IBA. Both stem nodal segments and leaf explants from *O. majorana* were, however, tested on MS medium with various concentrations of sucrose, maltose and glucose and with 2 mg/L BAP for regeneration in another study (60). It was found that the maximum number of regenerated shoots per node was obtained on 3% maltose with 2 mg/L BAP. The calli obtained from stem and leaf explants on MS with 3% maltose augmented with various levels of 2,4-D (0.01–1.0 mg/L) were subjected to MS with BAP, either alone or in combination with IBA, IAA and Kinetin (0.1–10.0 mg/L). Whereas calli obtained from leaf explants which had not regenerate, maximum shoot organogenesis was observed in stem calli on MS with 3% maltose and a combination of 3 mg/L BAP and 0.2 mg/L IBA. Highest shoot induction of roots in the regenerated shoots was observed in 0.2 mg/L IBA. In another study on *O. majorana* (52), stem nodal explants were subjected to MS and Philips and Collins L2 medium supplemented with various concentrations of auxins (IAA, IBA, NAA) or cytokinins (BAP, Kinetin, Adenine sulphate, 2-i-P, TDZ) or GA3 either alone or in combinations (IAA and BAP, IAA and Kinetin and IBA and Kinetin). In general, the P&c L2 medium was better than MS for shoot organogenesis and both direct and indirect organogenesis was observed. The presence of Adenine Sulphate in medium promoted direct proliferation of shoots from the nodal region, while other cytokinins favoured indirect regeneration of shoots from the callus. The concentrations of 9.3 uM Kinetin (38 shoots) and 13.3uM BAP (33.3 shoots) showed a higher number of shoots from calli. Interestingly, this report demonstrated that even auxins or GA3 alone in the medium could promote shoot organogenesis from nodes, although in low numbers. In *O. minutiflorum*, nodal segments and shoot tips were tested on MS and Gamborg-B5 medium with combinations of BAP (0, 1, 2 or 3 mg/L) and NAA (0, 0.1 or 0.5 mg/L) (67). MS with 2 mg/L BAP and 0.1 mg/L NAA was found best for shoot organogenesis from nodal segments. In a tissue culture report (69) on *O. syriacum* and *O. ehrenbergii*, nodal explants were cultured on MS with 1, 1.5 or 2 mg/L BAP and it was found that 2 mg/L BAP was most suitable for shoot multiplication in these two species. Nodes from native plants of *O. sipyleum* were tested on combinations of BAP (0.1, 0.5, 1.0 mg/L) and GA3 (0.1, 0.2 mg/L) in MS-based medium and 85% shoot organogenesis response was obtained on a combination of 0.5 mg/L BA and 0.2 mg/L GA3 (66). 62.5% of regenerated shoots rooted in MS medium supplemented with 1.5 mg/L IBA.

Young shoot tips from field-grown *O. vulgare* x *applii* hybrid were cultured on MS with combinations of NAA (0.53, 5.3, and 5.83 uM) and BA (0.28, 2.58, and 3.86 uM) in order to get regeneration response (64). Highest shoot induction was observed by them on NAA (0.53 uM) and BAP (0.28 uM). Shoots with axillary buds of *O. vulgare* ssp. hirtum were subjected to Gamborg-B5 medium with 0.01 mg/L BAP (63) and shoot, and root induction was obtained on the same medium. Apical buds from *in-vitro* grown plants of *O. vulgare* were reported to be cultured (53) on MS medium with various concentrations of BAP (2.22 – 8.88 uM) in combination with either 2,4-D (2.26 – 11.3 uM) or NAA (2.68 – 13.42 uM) and best shoot induction was observed in MS with 8.88 uM BAP and 2.26 uM 2,4-D. In a report (61) on *O. majorana*, axillary buds were subjected to MS with Kinetin (1 – 3 mg/L) or BAP (1 – 3 mg/L). Highest (85%) shoot organogenesis response was noticed by this report on MS with 2 mg/L Kin (30 shoots), followed by MS with 2 mg/L BAP (62% response, 22 shoots).

All the *in-vitro* regeneration studies reported so far in any species of *Origanum* thus point to some of the generalizations: Most of the reports utilized organogenesis as chief mode; Both direct organogenesis from PEMs and indirect organogenesis from dedifferentiated cells are reported, but regeneration from PEMs are more common; direct or indirect somatic embryogenesis has not been achieved for any species of *Origanum*. Also, protoplast isolation and culture or regeneration from protoplasts for any species of *Origanum* is not reported. Similar is the case of *in-vitro* regeneration from inflorescence or any flowering part like sepals or petals. Anther culture, pollen culture, unfertilized ovary culture or production of haploids and regeneration has also not been reported in the published literature.

**Vitrification in *Origanum* tissue culture:**

Vitrification or hyperhydricity is a physiological malformation that is commonly associated with
tissue culture of many plant species and affects regeneration frequency. Vitrification in tissue culture of *O. vulgare* and strategy to rectify this problem with the help of bacteria isolated from oregano rhizosphere (*Pseudomonas* spp.) was reported (79). A plant-microbe (*O. vulgare*-*Pseudomonas* spp.) interaction that prevents physiological malformations associated with vitrification was suggested (79). Morphological and physiological variations such as water content, chlorophyll content, and total phenolics were compared between vitrified and unvitrified clones. Vitrified shoots were found to have lower water content and higher chlorophyll and total phenolics compared with vitrified controls. In a further study (80), it was identified that out of several different isolated strains of *Pseudomonas* spp., mucoidal strains A and F prevented hyperhydricity in *O. vulgare* cultures in a better way. Shoots inoculated with the non-mucoid strain-NMF showed hyperhydricity and had very similar levels of chlorophyll, solids and phenolics as uninoculated control. Later, hyperhydricity prevention in Oregano by several nonspecific polysaccharides producing rhizosphere bacteria, including *Pseudomonas mucidolens* and another *Pseudomonas* sp. was also shown (81). The endophytes and rhizospheric bacteria of *O. vulgare* were shown to possess high anti-oxidant and plant growth-promoting activities and to produce several hydrolytic enzymes (82). However, for commercial micro-propagation of *Origanum* sp., vitrification in organogenesis-based in-vitro regeneration methods remains a problem. Deploying bacteria to prevent this hyperhydricity is still far from any practical utility.

**Callus and cell suspension culture for production of secondary metabolites**

Two studies reported the development of callus and/or cell suspension culture in a few *Origanum* sp. (Table 1) and compared the presence and amounts of active constituents from their mature plant counterparts (72, 73). Leaf explants from *O. vulgare* and *O. syriacum* were cultured on MS medium supplemented with various levels of 2,4-D (0–2 mg/L) to develop callus cultures (72). Callus maintenance was tested on different levels of BAP or TDZ (0.5–2.5 mg/L) with or without 0.5 mg/L 2,4-D. Callus from 3rd generation was used to develop cell- suspension culture in MS liquid medium with 1 mg/L TDZ. Whereas better callus production and their fresh weight were observed on the lower range of 2,4-D (0.1–0.5 mg/L), best callus maintenance was observed on 1–1.5 mg/L TDZ without 2,4-D. Gas chromatography (GC) was performed and oil content and thymol contents were analyzed in greenhouse and in-vitro grown plants and calli and cell suspension culture extracts. *In-vitro* grown plants of *O. vulgare* had the highest oil content in comparison to its callus and cell culture, but no thymol was present. In *O. syriacum*, greenhouse-grown plants showed highest thymol percentage, and thymol was not detected in calli or cell suspension culture.

Another report (73), also utilized leaf explants from *O. vulgare, O. vulgare* var. *hirtum* and *O. syriacum* to develop callus cultures and compared the extracts of farm-grown plants and callus. Explants were subjected to MS, LS or B5 media along with various concentrations and combinations of 2,4-D, Kinetin, NAA and BAP. Out of the three media they tested for callus production and growth, MS was the best. A combination of 0.5 mg/L 2,4-D -5 mg/L Kinetin and 0.5 mg/L NAA- 3 mg/L BAP in MS produced highest amount of callus. GC and GC -MS of extracts showed the presence of 21 compounds in *O. vulgare* calli, 24 compounds in *O. vulgare* var. *hirtum* and 26 compounds in *O. syriacum* calli. Carvacrol and α-pinene were detected in all three species. Thymol was detected only in *O. vulgare*. Carene-8’-2’ was found only in *O. syriacum* and *O. vulgare*. The results obtained by later study (73) were, however, in contrast with earlier (72), who has not detected any thymol in *O. vulgare*.

Above two studies provided the basis that *Origanum* callus or cell-suspension culture may be utilized for obtaining active constituents. Nevertheless, significant scope exists in establishing the *Origanum* cell culture for enhanced production of the desired metabolite in a controlled environment. The clones with higher content(s) and genetic engineering for improved accumulation will be relevant to the needs of pharmaceutical industries. Moreover, it will reduce the overexploitation of *Origanum* sp. from nature for sourcing the plant material.

**Genetic transformation of Origanum spp.**

Since the advent of genetic engineering of plants for incorporating useful traits not available in their germplasms or from any of their wild relatives, a large number of plant species have been genetically transformed with many genes to bring in those useful traits in the transgenic plant lines, utilizing different direct or indirect genetic transformation methods. In spite of the much-acclaimed value of *Origanum* medicinal herbs, unfortunately, no report exists that detail the standardization of genetic transformation in any *Origanum* sp.; nor the development of a transgenic plant with a useful gene is reported in the published literature. Although a study (65) reported genetic transformation of *O. vulgare* with *Agrobacterium rhizogenes* and regeneration from hairy root-derived callus, their purpose was neither development of transgenic Oregano nor enhancing/tapping the secondary metabolite content in *O. vulgare* hairy roots. The leaf explants from 20-25 days old oregano plants were infected with two strains of *A. rhizogenes*. Bacterial cells were harvested at OD_{590} nm of 0.8, and the infection medium contained MS salts with 50 mg/L sucrose. Six co-cultivation media were tested, each containing MS medium with 100 uM acetosyringone but lacking one or more major or minor salt. After co-cultivation for two days, explants were cultured in hormone-free MS medium with 3% sucrose and 200 mg/L cefotaxime. Both strains were found effective in inducing hairy roots in oregano explants after two weeks. Highest induction (91.3%) was noted on co-cultivation medium-5 (lacking KH2PO4, NH4NO3, KNO3, and CaCl2). For transgenic plant development, usually, a high-frequency genetic transformation protocol is coupled to a reproducible in-vitro plant regeneration system. Preferably, compatible *A. tumefaciens* strain-binary vector system harbouring gene of interest is required. Infected explants are cultured on selection
medium to select transformed cells which can eventually be subjected to regenerate into complete plant body. Most of the regeneration protocol in *Origanum* species reported so far utilizes organogenesis from pre-existing meristems, and generally, meristem based regeneration of transformed cells lead to transgenic plants with the chimeric organization. Thus it is necessary to develop a compatible transformation-regeneration system in *Origanum* spp. for homogeneous, stable and faithful expression of the transgene in all the cells of transformed regenerants. Further, the optimizations of various factors affecting *A. tumefaciens* -mediated transformation are needed.

**Antiproliferative properties: Effects of extracts/ preparations from *Origanum* spp. on cytotoxicity of cancer cells**

Scientific investigation into anticancerous properties of *Origanum* phytoconstituents started with a study in 1987 (17), where hydroquinone isolated from *O. majorana* was demonstrated to have potent cytotoxicity on cultured rat hepatoma cells (HTC). Whereas this initial study indicated that marjoram constituents have antiproliferative potential, a detailed study and action mechanisms of these phytoconstituents were presented by a 2010 report (83). The antiproliferative activity of ethanolic extracts from marjoram leaves was tested on human lymphoblastic leukaemia cell line-Jurkat. At non-cytotoxic concentrations, the viability of cells decreased with the increase of plant extract concentration. The antiproliferative effect was also found to be dose-dependent. Marjoram extracts were shown to stimulate apoptosis, which in turn was induced by an up-regulation of p53 protein levels and down-regulation of Bcl-2a. The conclusions from this study suggested that phenolic content-rich marjoram extracts exhibit strong antioxidant and scavenging activities and high antiproliferative effect.

*O. majorana* and *O. vulgare* essential oils and their constituents were compared for cytotoxicity against different cancer cell lines (84). In the MTT assay, *O. majorana* essential oil (OmEO) was more cytotoxic than *O. vulgare* essential oil (OvEO) against different cancer cell types, such as MCF-7, LNCaP and NIH-3T3. Another study (85) reported the cytotoxic activity of ethyl acetate and ethanol extracts from leaves of *O. compactum* in human breast cancer cell line – MCF7.

The anti-metastatic and anti-tumour growth effects of marjoram ethanolic extracts on highly metastatic human breast cancer cell line- MDA-MB-231 was also presented (86), where it was shown that *O. majorana* promotes inhibition of tumour growth and metastasis *in-vivo*. Ethanol extract from *O. majorana* (87) was found to show significant cytotoxicity (P<0.001) to fibrosarcoma and least toxicity to normal human lymphocytes when compared to the controls. Ethanolic extract of *O. syriacum* was shown to inhibit human leukaemia THP-1 cells (18). In this work, ethanolic extracts from aerial parts of *O. syriacum* and *Thymus vulgaris* L. were investigated against the THP-1 leukaemia cell line and freshly isolated peripheral blood mononuclear cells (PBMCs). Both extracts exhibited a concentration-dependent reduction in viability of the THP-1 cells. However, *O. syriacum* was more potent against the PBMCs, while *T. vulgaris* was moderately selective. In *O. syriacum* the reduction in cells viability was caused by cytotoxic effect against leukemic cells of THP-1.

A study (88) identified 71 compounds in the essential oil obtained by hydrodistillation of aerial parts of *O. vulgare* and tested both crude essential oil (EO) extract as well as single substances for cytotoxicity to carcinoma and non-tumour cell line. The crude EO extract has significantly reduced the cell viability of the hepatocarcinoma cell line (HepG2) in dose-dependent mode, as compared to the vehicle control. Interestingly, results showed that oregano EO has lower cytotoxic activity against non-tumour cell line HEK293. While testing of single substances for cytotoxicity against HepG2 cells, they noted that carvacrol and citral showed the highest significant reduction of cell viability of hepatocarcinoma cells. Thymol and limonene showed a viability reduction of HepG2 cells.

Aqueous, petroleum ether, dichloromethane, ethylacetate and methanol extracts were prepared from aerial parts of marjoram, and these extracts were tested (89) for cytotoxicity against breast cell line (MDA-MB-231) and colon cancer cell line (HT-29 cells). All extracts of *O. majorana* tested against both types of cancer cells showed a more pronounced cytotoxic effect against MDA-MB-231 than HT-29 cells. *In-vitro* cytotoxic tests showed that extracts inhibit the proliferation of human cancer cell lines in a dose-dependent manner. Recently, the essential oil obtained from hydrodistillation of aerial parts of *O. onites* (OoEO) was examined for its antiproliferative activity against melanoma cells (A375), breast cancer cells (MCF-7), hepatocellular carcinoma cells (HepG2) and colon cancer cells (HT-29) (90). Interestingly, OoEO exhibited a dose-dependent antiproliferative activity against all human cancer cell lines tested. The most potent antiproliferative effect was observed in the HT-29 colon cancer cell line followed by A375 skin melanoma, MCF-7 breast carcinoma, and HepG2 hepatocellular carcinoma cells. OoEO attenuated migration and induced apoptosis-related morphological changes in both human (HT-29) and murine (CT26) colon cancer cell lines. The anticancerous activity of ethanolic extracts prepared from dried marjoram leaves was also recently studied on two human colon cancer lines (HT-29 and Caco-2) and the findings provide strong evidence that *O. majorana* extract (OmE) possesses strong anti-colon cancer potential, at least, through induction of autophagy and apoptosis (19). They showed that OmE exhibited this antiproliferative activity in a concentration- and time-dependent manner. OmE inhibited cell viability, colony growth and induced mitotic arrest of HT-29 cells. Also, OmE induced DNA damage, triggered abortive autophagy and activated a caspase 3 and 7-dependent extrinsic apoptotic pathway, most likely through the activation of the TNF-α pathway. The time-course analysis revealed that DNA damage occurred concomitantly with abortive autophagy 4 hours post-OmE treatment, while apoptosis was activated only 24 hrs later. Blockade of autophagy initiation (by 3-methyladenine) partially rescued OmE-induced cell death.

All the works mentioned above in this section lead to some generalizations: (i) Antiproliferative
activity of *Origanum* is concentration-dependent (in the range which is non-cytotoxic to normal cells), (ii) Although *Origanum* phytoconstituents have strong antioxidant and radical scavenging activity, the antiproliferative activity was shown to be mediated through induction of autophagy and apoptosis. (iii) Many different cancer cell lines—HTC, HepG2, Jurkat, THP-1, MCF-7, MDA-MB-231, HT-29, Caco-2, A375, LNCaP and NIH-3T3 have been tested to demonstrate the anticancerous properties of phytoconstituents of *Origanum* species, but still far from real practical use, (iv) Mostly, crude extracts have been tested, but a few have shown the efficacy of isolated constituents like Carvacrol, Citral and Hydroquinone for cytotoxicity of cancerous cells and (v) *O. marjorana* phytoconstituents have been mostly used to demonstrate this cancer cells cytotoxicity, followed by *O. vulgare*; however, phytoconstituents of *O. syriacum*, *O. onites* and *O. compactum* have also been reported.

**Conclusion**

Scientific investigations into medicinal plants nowadays are emphasizing on selecting and identifying useful compounds, validating their individual or in combination efficacy as well as to increase the production and availability of source material or plants. *Origanum*, with its many species and multi-purpose medicinal value, has been found to be an excellent source of such compounds. The present review point to the fact that significant opportunities exist for research in exploring high-frequency in-vitro regeneration and genetic transformation protocols of different species of *Origanum*. It will be prudent to develop somatic embryogenesis based mass propagation methods and high-frequency *A. tumefaciens*-mediated transformation protocols for *Origanum* spp. Model genotypes could be identified which respond to a wider range of regeneration protocols, or regeneration protocol could be developed which perform relatively better over a wider range of *Origanum* genotypes. The lines with higher contents of desired constituents, if subjected to mass propagation, will boost industries interested in *Origanum* compounds. *Origanum* cell culture systems need to be developed, and metabolic engineering should be attempted to enhance the bioactive secondary metabolites. In this context, biotechnological efforts on these species are overly underexploited, keeping in view ‘since time immemorial’ exploitation of this culinarily and medicinally valuable plant species.

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**Authors’ contributions**

All authors contributed equally.

**Conflict of interests**

Authors declare no conflict of interest.

**References**


