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Stigmasterol expedites ester production in *Trigonella foenum-graecum* L: *In vivo* and *in vitro* propagates

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ABSTRACT

Fenugreek (*Trigonella foenum-graecum* L.), a popular spice, is explored in the literature for its chemical constituents and pharmacological potential. In the current investigation, a comparative analysis was conducted to evaluate the role of stigmasterol in the biochemical and therapeutic properties of fenugreek grown under controlled and natural environmental conditions. Fenugreek treated with stigmasterol significantly ($p < 0.001$) increased the production of alkaloids, flavonoids, phenolics, saponins, and tannins, as well as antioxidants, in field grown plants compared to *in vitro* calli. *In vivo*-grown plants also showed stronger antitumor ($IC_{50} = 0.001$ µg/mL) and antidiabetic activities ($IC_{50} < 1.0$ µg/mL) upon treatment with stigmasterol. GC-MS analysis depicted an augmentation of ester acids along with phytol and 2,4-di-tert-butylphenol production in stigmasterol-treated samples. This suggests that stigmasterol effectively boosts the medicinal properties of fenugreek and could be explored for enhancing the bioactive properties of other medicinal plants.

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1. Introduction

Fenugreek (*Trigonella foenum-graecum*) is an annual herb belonging to the family Leguminoseae. Seeds and leaves of fenugreek are commonly used in the dishes of the Indian subcontinent as culinary ingredients. The good adaptability of fenugreek to diverse atmospheric conditions, soils, and temperatures facilitated its cultivation in more than 20 countries in Asia, Europe, America, Africa, and some areas of Australia (Chaudhary et al., 2018). Various parts of fenugreek (leaves, roots, and seeds) have been evaluated in the literature for their chemical constituents. These chemical constituents are plant based bioactive compounds with significant therapeutic potential including, antimicrobial, antiallergic, antioxidant, antihelmintic, antispasmodic and antidiarrheal (Kumar et al., 2023). A number of phyto-constituents had been extracted from the seeds of fenugreek, including galactomannan, diosgenin, volatile oil, polysaccharide, yamogenin, saponin, mucilage, and alkaloids (Visuvanathan et al., 2022). Fenugreek has been explored for various treatments because of its presence in the Ayurvedic system of medicines for ages and its diverse phytoconstituents. The chemopreventive and antineoplastic effects of *T. foenum graecum* have been studied *in vivo* and *in vitro* (Iranmanesh et al., 2018). Beside this, other therapeutic activities like anticancer, anti-diabetic (Rampogu et al., 2018), hepatoprotective activity (Abd-Elrahman, 2019), hypolipidemic (Sharma and Choudhary, 2017), and anti-angiogenic effects have also been studied (Thakur and Ahirwar, 2019).

The phyto-profile of this plant fascinated many researchers, who employed different methods and strategies for enhancing the bioactive attributes of the plant. Under such a scenario, the general approach

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being adopted is the utilization of various chemicals and conditions to enhance the yield of plant secondary metabolites (Jurić et al., 2020). Elicitation could be an effective technique to boost the production of these valuable secondary metabolites. Elicitors are compounds that mimic the action of biotic and abiotic stresses, enhancing the plant defense system and resulting in enhanced production of secondary metabolites. Although elicitation can be carried out by various physical factors, the main methodology being adopted by biotechnological cell cultures is the addition of elicitors (Ruiz et al., 2018). Secondary metabolites in fenugreek have been enhanced by using certain elicitors like ethyl methane sulfonate, chitosan, and arginine (Badi et al., 2018; Kwon et al., 2019; Parchin et al., 2021). Silver nanomaterials have also been reported to elicit the bioactive properties of fenugreek (Jasim et al., 2017).

A considerable amount of work has been done to evaluate the medicinal properties of *Trigonella foenum-graecum* L., but the comparative functional performances of *in vitro* calli and field-grown plants under the effect of a selected (stigmasterol) elicitor have not been disclosed yet. Calli are an unorganized or undifferentiated masses of cells formed over a wounded or cut plant surface under various biotic and abiotic conditions. These cells are totipotent, meaning they are able to regenerate the whole plant body. Calli are not only a robust way of increasing the amount of secondary metabolites in plant species but also a commercial source of producing diverse chemical classes without harming parent plants (Fazili et al., 2022).

This study has been premeditated to quantify the difference between various bioactive constituents of fenugreek propagated *in vitro* through callogenesis and those grown in the field. Moreover, this study was also aimed at investigating the possible augmentation of secondary metabolites in fenugreek and their effect on the therapeutic properties upon treatment with selected elicitors.

2. Experimental

2.1. *In vivo* growth

Seeds of *Trigonella foenum-graecum* L. local variety (Kasuri methi) were soaked in water overnight and then sown in potted soil containing the mixture of soil and farmyard manure (4:1) at 25 ± 2 °C. The plants were watered twice a day and weeded as required. The whole plant was harvested after germination at a regular time period of fifteen days, *i.e.*, 45th, 60th, and 75th days for experimental analysis.

2.2. *In vitro* culture of Fenugreek

For *in vitro* culture, half-strength MS (Murashige and Skoog) media containing agar (0.8%) and sucrose (3%) was used. The seeds were sterilized with mercuric chloride (0.01%) for 10 seconds, followed by rinsing thrice with autoclaved distilled water. Seeds inoculated on prepared media were kept in dark for 48 h, followed by 16 h of photoperiod, illumination of 45 µE m^2s^{-1} and 60% relative humidity in a growth chamber at 25 ± 2 °C. Twenty explants (2 mm in size) from cotyledon, hypocotyls, and roots of germinated seedlings were cultured on full-strength MS media for callogenesis with optimized concentrations of auxins 1-naphthalene acetic acid, NAA) and cytokinins (6-benzylaminopurine, BAP), *i.e.*, NAA (0.5 mg/L) and BAP (1.5 mg/L).

2.3. Elicitation treatment

Three different concentration of stigmasterol (SIGMA: S2424) were tested, *i.e.*, 100, 50, and 10 nM for elicitation experiments. About 15 mL of each selected elicitor was added to the soil of one-month-old seedlings of *in vivo* plants (n = 5 for each treatment) for consecutive 15 days with a two-day regular interval. Plants were then harvested on 45th, 60th, and 75th days to inquire about the effect of elicitation at different growth stages. For *in vitro* culture, selected concentrations of elicitor were added to the optimized callogenesis media. Calli (n = 5 for each treatment) were harvested and analyzed after 45 days of treatment.

2.4. Bioactive phytoprofiling

Methanolic extracts (1.0 mg/mL) of *in vitro* calli and *in vivo*-grown plants were prepared according to the standard method reported by Ain et al. (2017), followed by the tests mentioned below.

2.4.1. Antioxidant assays

The DPPH inhibition assay was carried out according to the protocol reported by Chaves et al. (2020), where absorbance was measured at 517 nm by a UV-Vis spectrophotometer and percentage inhibition was calculated. Ferric reducing power assay (RPA) and phosphomolybdate assay (TAC) were carried out according to the protocol reported by Sadaf et al. (2021). Absorbance was measured at 700 nm and 695 nm, respectively. Ascorbic acid (100 µg/mL) was used as a positive control. Percentage antioxidant activity was calculated using Eqn. 1.

Antioxidant activity (%) = (Absorbance of sample/ Absorbance of positive control) × 100 (Eqn. 1)

2.4.2. Quantitative estimation

Total alkaloids, saponins, phenolics, tannins, and flavonoids were quantified by emulating reported protocols (Shanaida et al., 2018; Adusei et al., 2019; Bajad et al., 2019; Perveen et al., 2022). Total alkaloid and saponins content were measured in terms of weight of residue (µg) per weight of dry sample taken (mg). Gallic acid and quercetin were used as standards for the estimation of total phenolic and tannin content, respectively. Values were expressed as µg gallic acid (GAE) and quercetin (QE) equitant per milligram of the dry sample.

2.4.3. Potato disc antitumor assay

A potato disc assay was carried out to assess the antitumor potential of *in vivo* plants and *in vitro* calli (Rehmat et al., 2018). Samples of selected treatments were chosen based on their potent antioxidant value (10 nM stigmasterol). Controls (samples without any treatment) were used for comparison. About 10,000 µg/mL, 1,000 µg/mL, and 100 µg/mL concentrations of samples were prepared in dimethyl sulfoxide (DMSO). Vincristine (100 µg/mL) was used as a positive control. About 5 mm-thick potato discs, excised with the help of a borer and sterilized with mercuric chloride (0.1%), were placed on solidified agar. Inoculum (1000 µL) was prepared by adding 500 µL of distilled water, 400 µL of bacterial culture (*Agrobacterium tumefaciens* AT10), and 100 µL of the plant sample. Then, 50 µL of this prepared inoculum was poured on each disc. Plates were then incubated at 25 °C ± 2 for 21 days. Lugol's solution {KI (10.0%) + I_2 (5.0%)} was applied on each disc and observed under 3X magnification. Percentage tumor inhibition was calculated using Eqn. 2:

Percentage inhibition = 100 - (Average no. of tumors by test sample/Average no. of tumors by negative control) × 100 (Eqn. 2)

2.4.4. Anti-diabetic evaluation

The α-amylase inhibitory assay was conducted by mixing 250 µL of the plant sample with a concentration of 1.0 µg/mL, 50 µg/mL, and 100 µg/mL with 250 µL of sodium phosphate buffer (0.02 M) containing α-amylase solution (0.5 mg/mL). The pH of the phosphate buffer was maintained at 6.9. The solution was then incubated at 25 °C for 10 min, followed by addition of 250 µL of starch solution (1.0%). After 10 minutes, the reaction was terminated by the addition of 500 µL dinitrosalicylic acid reagent with further incubation of 5 mint at 100 °C. The mixture was cooled, and absorbance was measured at 540 nm.

For the α-glucosidase inhibitory assay, 100 µL of α-glucosidase (1.0 unit/mL) was pre-incubated with 50 µL of extracts (1 µg/mL, 50 µg/mL, and 100 µg/mL). After 10 min, 50 µL of *p*-nitrophenylglucopyranoside (3.0 mM) was added to start the reaction, followed by incubation at 37 °C for 20 min, and the reaction was stopped by adding 2 mL of Na_2CO_3 (0.1 M). Absorbance was measured at 405 nm (Chelladurai and Chinnachamy, 2018). The percentage of α-amylase and α-glucosidase inhibitory activity was calculated by Eqn. 3.

Percentage inhibition = (Absorbance of negative control - Absorbance of the sample)/Absorbance of negative control × 100 (Eqn. 3)

2.4.5. Gas chromatography-mass spectrometry (GC-MS)

GC-MS was carried out by the method adopted by Olivia et al. (2021) in order to identify the comparative bioactive constituents' percentage. The samples were first syringe-filtered using a Sterile Syringe Filter Millex-GP (0.22 µm x 25 mm) (Millipore, Boston, MA, USA) to get rid of any contamination and were then subjected to a DB-5 MS column. Operational parameters of spectrometry include: helium as the carrier gas with a 1 mL/min flow rate (split: 10:1); column temperature was programmed from 50 to 280 °C; inlet line temperature of MS program and source were fix at 200 °C; mass scan and electron energy were set to 40-600 and 70 eV, respectively. The initial temperature was maintained at 110 °C for 2 min and then rose to 280 °C at 5 °C increase per minute. Samples were injected at 250 °C and at a pressure of 60 kPa. The total retention time for each sample was about 20-30 min. The analysis was performed using GC-MS with a dimethylpolysiloxane column. Each sample was run thrice and compounds present were identified by comparing mass spectra from the inbuilt database of NIST-27 libraries.

2.4.6. Statistical analysis

For each assay, five plant samples were taken. All assays were performed in three biological replicates. Results are indicated as mean with standard deviations. Regression analysis was carried out to calculate the IC_{50} and EC_{50} values of selected assays. Tukey Pairwise Comparison and t-test were conducted for comparative analysis of biochemical production and bioactivity of *in vivo* plants and *in vitro* propagated calli.

3. Results and Discussion

3.1. Periodic trends in *in vivo* and *in vitro* grown control plants and calli

Stigmasterol is a natural plant molecule with an unsaturated structure. It belongs to a class of chemicals called tetracyclic triterpenes. Other natural sources include fungi and algae. Stigmasterol has a unique structure with two double bonds: One between the fifth and sixth carbons and the other between the 22nd and 23rd carbons and a hydroxyl group bonded to the third carbon position. The interactions that stigmasterol has with other molecules are influenced by these functional groups (Bakrim et al., 2022). Stigmasterol is the main ingredient of several plant extracts (Chen et al., 2016). This compound is known for its assorted therapeutics properties including antiviral, antiglycemic, antiosteoarthritis, antioxidant, antiproliferative, anti-inflammatory, antiparasitic, immunomodulatotory, antibacterial, neuroprotective and free radical scavenging (Bakrim et al., 2022). Therefore, we designed a study to investigate the effect of stigmasterol on plant growth (*in vivo*) and callus formation and proliferation (*in vitro*), as well as the production of valuable pharmacological compounds. *In vivo*-grown plants of *T. foenum* were harvested periodically at the 45th, 60th, and 75th days after germination. All physical parameters increased from 45 to 75 days; however, moisture content declined. Total length escalated from 17 cm ± 1 to 41.4 cm ± 3. Fresh and dry weight increased from 0.35 g ± 0.1 and 0.07 g ± 0.1 to 4.42 g ± 0.06 and 0.74 g ± 0.2 respectively (Table 1). The concentration of stigmasterol (10 nM) showed an increased in length and moisture

Table 1
Physiological parameters of *in vivo* grown *T. foenum-graecum* plants treated with elicitors.

Treatment	Total plant length after days of germination (cm)			Fresh weight after days of germination (g)			Dry weight after days of germination (g)			Moisture content after days of germination (%)		
	45	60	75	45	60	75	45	60	75	45	60	75
Stigmasterol (100 nM)	17 ± 1	33 ± 1	34 ± 2	0.35 ± 0.1	0.7 ± 0.1	1.2 ± 0.2	0.07 ± 0.1	0.17 ± 0.1	0.22 ± 0.2	78.86 ± 1.5	76.14 ± 4.5	82.02 ± 1.9
Stigmasterol (50 nM)	20 ± 4	30 ± 4	36 ± 4	0.72 ± 0.2	0.8 ± 0.2	1.4 ± 0.2	0.12 ± 0.2	0.16 ± 0.2	0.27 ±0.2	82.78 ± 0.1	67.00 ± 2.5	80.71 ± 0.2
Stigmasterol (10 nM)	23 ± 4	38 ± 2	39 ± 2	0.75 ± 0.3	0.9 ± 0.2	1.5 ± 0.1	0.15 ± 0.2	0.26 ± 0.1	0.46 ± 0.2	81.54 ± 1.2	80.38 ± 1.5	80.54 ± 1.4
Control	22.6 ± 1	28.6± 2	41.4 ± 3	1.06 ± 0.5	1.72 ± 0.2	4.42 ± 0.06	0.15 ± 0.2	0.31 ± 0.5	0.74 ± 0.2	85.85± 1.7	81.98± 6.01	83.26 ± 2.5

Data represent the mean of 05 replicates with standard deviation ± SD. Bold values represent best growth parameters. Control plants are healthy plants without any treatment. Moisture content in percentage was calculated using formula MC = Wet weight-dryweight/dry weight × 100. MC in the formula represents moisture contents.

content of *in-vivo* grown plants, which was selected for *in vitro* studies. The best callus induction frequency (100% from cotyledon and hypocotyls) was recorded in stigmasterol (10 nM)-treated and control samples, as mentioned in Table 2. Control plant samples were treated only with growth regulators, *i.e.*, BAP (1.5 mg/L) and NAA (0.5 mg/L).

Table 2
Physiological characteristics of calli grown on MS media containing selected elicitors.

Elicitation treatment	Callus source	Callus induction frequency (%)	Callus size (cm)	Fresh weight (g)
Stigmasterol (10 nM)	Cotyledon	100	1.5 ± 0.2	0.2 ± 0.11
	Hypocotyl	100	1.5 ± 1.1	0.1 ± 0.08
Control (No treatment)	Cotyledon	100	1.4 ± 0.3	0.1 ± 0.02
	Hypocotyl	100	0.9 ± 0.5	0.08 ± 0.05

Control is the condition where simple callogenesis took place at optimized hormone concentration of BAP and NAA (1.5:0.5 mg/L) in MS media without selected elicitors. Data represent the average of 05 replicates produced after thirty days of callogenesis with standard deviation (±SD). Bold value represents the highest physical parameter. Moisture content in percentage was calculated using following formula MC = Wet weight-dry weight/dry weight × 100. MC represents moisture contents.

3.2. Effect of elicitor on physical characters of *in vivo* plant and *in vitro* calli

The production of selected phytochemicals was evaluated in control *in vivo* plants over a period of 45 to 75 days. A steady decline in the production of selected phytoentities was observed after 45 days. In the case of elicitor treatment, the data was recorded on 45th, 60th, and 75th day-old seedlings. The physical characteristics of *in vivo* plants showed a steady change in plant length from the period of 45 to 75 days. No significant difference was recorded in the total length of plant in response to stigmasterol (10 nM), *i.e.*, 39 cm ± 2 compared to control 41.4 cm ± 3 after 75 days (Table 1 and Fig. 1a). In the case of calli, elicitor treatment confers a slight reduction in calli induction frequency. Stigmasterol slightly increased the size of calli compared to the control, *e.g.*, 1.5 cm ± 0.2 and 1.4 cm ± 0.3, respectively. But that difference was not significant (Table 2 and Fig. 1b).

3.3. Effect of elicitor on biochemical properties of *in vivo* plant and *in vitro* calli

Stigmasterol (10 nM) plays a principal role in escalating the bioactive potencies as well as the antioxidant potential of both *in vivo*-grown plants and *in vitro*-propagated calli compared to their control counterparts (Supplementary Material Table A). Calli propagated from cotyledon and hypocotyl was selected for the assessment of biochemical and antioxidant properties under stigmasterol treatment. Comparative analysis between cotyledon and hypocotyl calli revealed a significant increase ($p < 0.001$) in alkaloid, flavonoid, and tannin content in former. However, phenolics, and saponin contents were significantly increased ($p < 0.05$) in hypocotyl calli under the same treatment (Supplementary Material Fig. A (a)). The antioxidant potential of cotyledon calli was more increased under stigmasterol than control and hypocotyl calli, as shown in supplementary material Fig. A (b). In hypocotyl calli, stigmasterol only significantly increased ($p < 0.001$) phosphomolybdate reduction ability in comparison to control.

Calli propagated from cotyledon showed increased biochemical and antioxidant properties in comparison to those propagated from hypocotyl. That's why they were selected for further comparison with *in vivo*-generated plants under the effect of stigmasterol treatment. Comparative analysis revealed a significantly

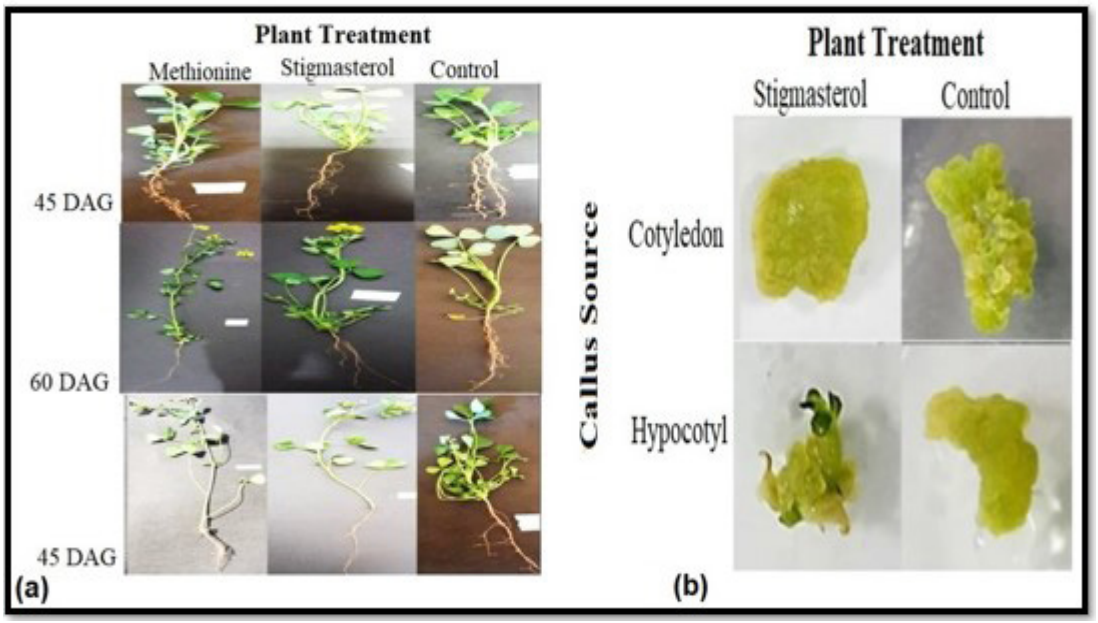

Fig. 1. Effect of elicitors on the growth performance of *in vivo* plants (a) and *in vitro* calli (b). DAG in Fig. 1a represents day after germination.

highest production of phytochemicals in *in vivo*-treated and control plants compared to *in vitro*. All selected phytoconstituents were significantly $p < 0.05$ higher in stigmasterol-treated *in vivo* plants than their control counterparts and *in vitro* propagates. Whereas, stigmasterol-treated *in vitro* calli only showed a significant increase in alkaloids ($p = 0.001$), flavonoids ($p = 0.006$), and tannins ($p = 0.002$) contents in comparison to their respective control counterparts, as shown in Fig. 2a. In contrast, stigmasterol treatment ensued a significant decline in phenolic content in priory mentioned samples. Our results are in harmony with those of Khorasani et al. (2015). They also have reported an elevated quantity of phenolics, and flavonoids with radiated antioxidant activity in the methanolic extract of *in vivo*-generated red clover plant, then there *in vitro*-propagated callus tissue.

DPPH inhibition, ferric, and phosphomolybdate reduction tests were performed to assess the antioxidant potential of *in vivo* and *in vitro*-treated and control germinates. All mentioned test revealed a significant $p = 0.0001$ increase in the antioxidant potential of *in vivo* plants compared to *in vitro* propagated calli. In stigmasterol-treated *in vivo* plants, a significant $p = 0.0002$ increased in antioxidant potential, followed by their untreated counterpart in comparison to *in vitro* control and treated calli (Fig. 2b). This trend of elevated quantities of secondary metabolites and antioxidant properties was found only untill the florescence stage of the *in vivo*-grown plant. As the plant reaches the florescence stage, both of the above-mentioned characters start to decline. Omezzine et al. (2014) and Ciura et al. (2015) also reported the highest levels of allele chemicals and diosgenin in *T. foenum-graecum,* respectively, during its vegetative stages, which started to decrease as the plant enters the flowering stage. Many factors contributed to this significant difference among the bioactive attributes of *in vivo*-grown plant and *in vitro*-propagated calli. One possibility might be the difference in the growth conditions. *In vitro* propagated calli only face elicitor stress, whereas *in vivo*-generated plants face both environmental (light, temperature, humidity, and soil microflora) and chemical (elicitor) stress. Vujčić et al. (2017) also reported that unfavorable *in vivo* growth conditions favor the production of a heterogeneous mixture of secondary metabolites. This can also be more robust in structure.

3.4. Antitumor and antidiabetic potencies

Stigmasterol (10 nM) also elicited the antitumor and antidiabetic potential of *Trigonella* as compared to the control. *In vivo* plants treated with stigmasterol which showed optimal antioxidant potential, were selected for antitumor and antidiabetic evaluations. The antitumor potential of stigmasterol-treated plants increases many folds, *i.e.,* having IC_{50} (0.001 µg/mL) for tumor inhibition. Among *in vitro* samples, cotyledon calli showed augmented antitumor potential, with an IC_{50} of 37.27 µg/mL under stigmasterol (10 nM) treatment.

α-amylase and α-glucosidase inhibitory assays revealed the high antidiabetic potential of fenugreek ($IC_{50} = 1.0$ µg/mL) without elicitation. However, stigmasterol (10 nM) treatment enhanced the antidiabetic potential with $IC_{50} < 1.0$ µg/mL of both *in vivo* and *in vitro* propagated samples (Table 3). Our results are in agreement with reported antidiabetic potential of fenugreek (Khorshidian et al., 2016). In another study, Keskes et al. (2018) also reported the highest α-amylase inhibitory activity of a methanolic extract of fenugreek seed.

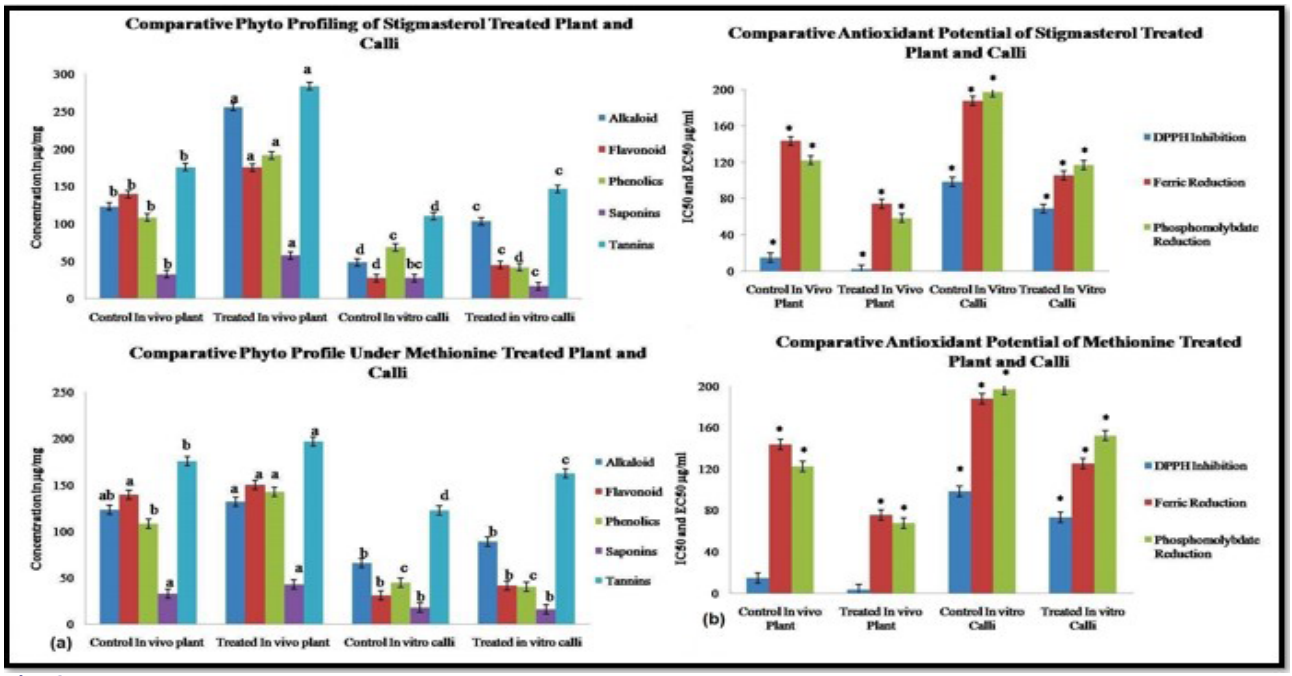

Fig. 2. Phytochemicals (a) and antioxidant potential (b) of *in vivo* grown plants and *in vitro* propagates of *Trigonella foenum graecum* from cotyledon explants under the effect of stigmasterol (10 nM) after 45 days.

Table 3
Antitumor and antidiabetic potential (IC_{50}) of *T. foenum graecum* under selected elicitor.

Plant treatment	Tumour inhibition (µg/mL)		α-Amylase inhibition (µg/mL)	
	In vivo	***In vitro* (cotyledon calli)**	***In vivo***	***In vitro* (cotyledon calli)**
Stigmasterol (10 nM)	0.001	37.27	< 1.0	< 1.0
Untreated Control	482.65	1668	1	1

The bold value represents IC_{50} concentration showing the most effective antitumor source and antidiabetic source in *in vivo* plant and *in vitro* calli harvested after 45 days. Vincristine (100 µg/mL) and acarbose (100 µg/mL) was used as positive control for antitumor and antidiabetic assay respectively having IC_{50} < 1.0 µg/mL.

Khlifi et al. (2016) improved the blood glucose level and decreased the serum α-amylase activity of diabetic patients by feeding them seeds of *T. foenum-graecum* seeds for thirty days regularly. Though the antitumor activity of fenugreek against various cancer cell lines has been assessed in the literature (Thakur and Ahirwar, 2019; Hamdani et al., 2020), but the effect of elicitors on the antitumor potential of fenugreek is missing in the literature. This study elucidates the information where stigmasterol is reported for the first time to enhance the antitumor potential of *in vitro* calli and *in vivo* plants.

3.5. Gas chromatography-mass spectrometry

In the current study, stigmasterol proved to be most effective and notable for enhancing the pharmacological properties (antioxidant, antitumor, and antidiabetic) of *Trigonella*. An increasing trend in these bioactive properties was in accordance with the increase in phytoconstituents. About seven medically important phyto-compounds were identified in *in vivo*-generated plants and *in vitro*-propagated calli of *T. foenum graecum* by GC-MS. Among the identified compounds, the highest percentage of butyl acetate was recorded *i.e.,* 40.48% and 24.79% among *in vivo* plants and *in vitro* calli, respectively, compared to the control ones (17.64% and 3.70%, respectively). The antifungal, antitumor, and antimicrobial properties of this phytoconstituent were already reported by Lens et al. (2016). Beside this, elevated concentrations of 1,1-dimethoxycyclohexane (14.51%), and 2-hexadecanoic acid methyl ester (12.03%) in treated *in vitro* calli then *in vivo* plants were recorded compared to their control counterparts. Stigmasterol not only increased the quantity of certain compounds in treated samples against to control, but it also caused the production of new one. Like phytol production, it was recorded only in treated *in vitro* calli and *in vivo* plants compared to their control. In the same way, among *in vitro* calli and *in vivo* germinated plants, 3-eicosene was only found missing in the case of the treated *in vivo* plant compared to its counter-control part. However, in *in vitro* calli its concentration was slightly higher than control. In the same way, 2,4-di-tert-butyl phenol was only found in small percentage in *in vivo*-treated plants as recorded in Table 4.
As 2,4-di-tert-butyl phenol (2,4-DTBP), detected only

Table 4
Identification of compounds by GC-MS among *in vivo* and *in vitro* grown fenugreek.

Retention time (min.)	Compound name	Percent peak value				Reported bioactivity
		In vivo plant		*In vitro* calli		
		Stigmasterol (10 nM)	Control	Stigmasterol (10 nM)	Control	
3.108	Butyl acetate	40.48%	17.64%	24.79%	3.70%	Antifungal, antitumor, antimicrobial activity (Lens et al., 2016)
4.635	1,1-Dimethoxycyclohexane	1.85%	19.78%	14.51%	1.39%	Antibacterial, antitumor, Antiviral, antiallergic, antimalarial, antitubercular, antidiabetic, immunomodulating (Valvi et al., 2014)
18.55	2-Hexadecanoic acid, methyl ester	3.43%	0.43%	12.03%	0.53%	Antibacterial and anticandidal properties (Tyagiaand Argawak, 2017)
12.495	2,4-di-tert-Butylphenol	1.78%	-	-	-	Anti-inflammatory, antimicrobial, antifungal, antioxidant, antitumor (Tyagiaand Argawak, 2017)
13.775	3-Eicosene	-	0.32%	1.86%	1.33%	Antioxidant and antimicrobial, antitumor, antifungal, cytotoxic (Tyagiaand Argawak, 2017)
21.908	Phytol	0.21%	-	0.93%	-	Antimicrobial,antioxidant action, induction of apoptosis and protective autophagy, anxiolytic and anticonvulsant effects, immune-modulating, antinociceptive and anti-inflammatory (Islam et al., 2018)
25.594	2-Chloropropionic acid, decyl ester	1.04%	1.23%	3.74%	9.62%	Antibacterial, antitumor activity (Keskes et al., 2018)

in *in vivo*-treated plants, was an auto-toxic lipophilic phenol. The anti-inflammatory, anti-microbial, antioxidant, anti-fungal, and anti-tumor activities of this compound are well known. This particular type of phenol was reported in diversified species. About 169 species were identified with this particular phenol. Among them are some species of fungi, liverwort, bacteria, pteridiphyta, diatoms, monocots, diacots, animals, and gymnosperms included. Besides this, several analogs of this phenol have also been identified in algae, bacteria, plants, insects, and fungi. The famous analogy included 2,6-DTBP, 2,5-DTBP, BHT etc. It demonstrated broad toxicity in all tested organisms, including producers. However, the available information failed to explain why an organism produces such a toxic substance. It was hypothesized in the endocide theory that an organism is more sensitive to its own endogenous metabolites than external molecules. So endocidal compounds commonly occurring in different species have a broad spectrum of toxicity, and 2,4-DTBP fits this example. This compound is a major component of volatile oils in many organisms, but its biosynthesis site is still uncovered. In a study, it was found that healthy rice plants had the same level of 2,4-DTBP as found in the plants of same species experiencing insect and viral infection. So there is a need to uncover this fact whether the production of this phenol can be induced under stress (Zhao et al., 2020).

The second important phytoconstituent is "phytol," which was detected in both *in vivo* and *in vitro* samples. This compound is derived mainly from chlorophyll, known as diterpene alcohol, and can be used as common food additive and dietary compound for cancer prevention. A little evidence was justifying their medical importance. As natural ligands of the peroxisome proliferator, it activated α, γ, and the retinoid X receptor. Some evidence in cell culture studies and limited evidence in animal models of anti-carcinogenic, anti-inflammatory, and anti-metabolic syndrome properties at physiological concentrations was found. It causes *in vitro* cytotoxicity in non-cancer cells and can cause morbidity and mortality in animal models. In human studies, evidence for the role of phytol and its metabolites in cancer prevention is currently limited and inconclusive (Bobe et al., 2020). A study found it effective against schistosomiasis. Schistosomiasis is a parasitic disease that is only cured by praziquantel (the only available drug). So phytol was found effective as an anti-schistosomalin *in vitro* studies against adult *Schistosoma mansoni* and *Schistosoma haematobium* juvenile (Eraky et al., 2016). In another study, it was also reported that it induces root knot nematode resistance in *Arabidopsis* by the ethylene signaling pathway (Fujimoto et al., 2021). In the same way, in another

study, phytol kinase and tocopherol were found to be important in handling the combined stress of high light and temperature in tomato model (Spicher et al., 2017). These results speculate on the role of stigmasterol, which is analogous to ergosterol (a component of the fungal cell wall), in triggering the defense mechanism of plants (Nasir and Besson, 2012). Though stigmasterol is produced naturally in plant for their immunity but its exogenous application might cause the expression of genes responsible for plant resistance against various biotic and abiotic stresses. The plant defense genes that could be activated against pathogens included *VST1* (*Synaptic Vesicle Transporter 1*), and *PR1* (*Pathogenesis Related Protein 1*). They are suspected to initiate the stilbene synthase pathway, the phenylalanine ammonia lyase pathway, the sesquiterpene cyclase pathway, the salicylic acid pathway, and the jasmonic acid pathway, leading to the production of important secondary metabolites and thus imparting significant biological properties to plants (Fig. 3). Our study finds stigmasterol eliciting the production of esters and phytol in *Trigonella* plant. One possible mechanism for stigmasterol-assisted augmentation in the said plant might be the effect of phytols in modulating the transcription of *VTE5* (*vitamin E pathway gene 5*) gene, which is well known for the biosynthesis of tocopherol (Pandey et al., 2017), thus imparting essential antioxidant capacity to plants.

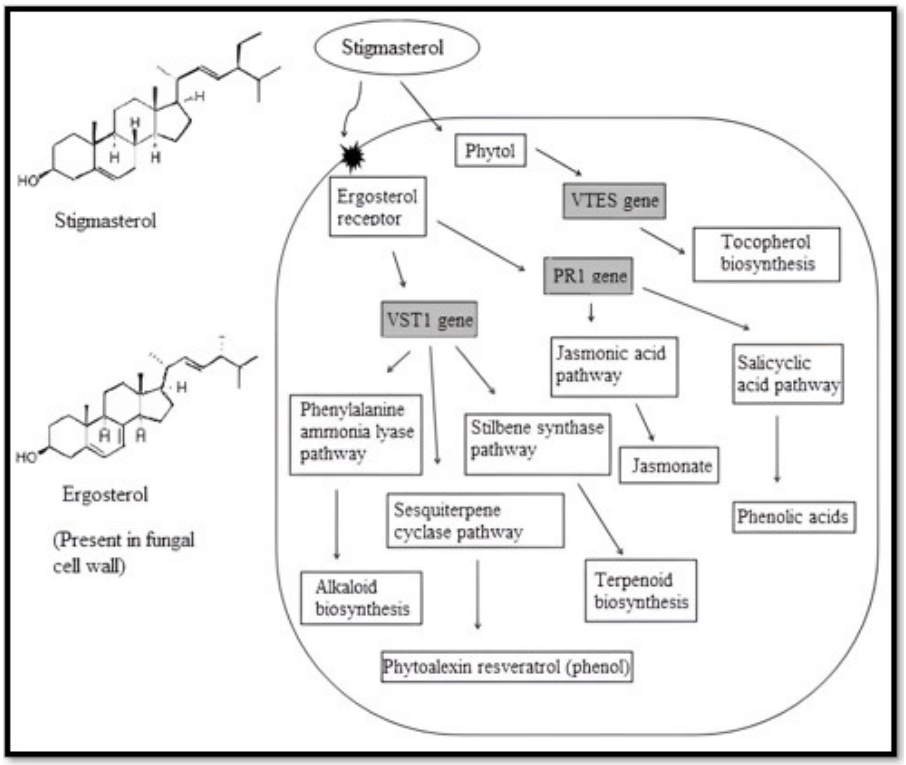

Fig. 3. Proposed mechanism for the effect of stigmasterol on *Trigonella Foenum graecum*.

4. Concluding remarks

This study elucidated the comparative analysis of physiochemical, biochemical and therapeutic activities of *in vivo* grown *Trigonella* plants against *in vitro* calli under the effect of stigmasterol. Stigmasterol proved good elicitor of choice for higher bioactive and pharmacological properties for *in vivo* grown plants compared to *in vitro* calli. Production of novel phytoconstituents like phytol and 2,4-di-tert-butylphenol under the effect of stigmasterol suggests improvement in fenugreek's therapeutic qualities. This study underscores the potential to enhance the bioactive characteristics of other therapeutic plants. Investigation of the interrelationship between elicitation and bioactive properties of fenugreek would explore the utilization and testing of other elicitors in fortifying the bioactive components of various medicinal and aromatic herbs for their effectual utilization in food and pharmaceutical applications.

Author contribution statement

Conceptualization, project administration, supervision, original draft was prepared by Naila Safdar. Original draft was written by Shaghufta Perveen. Design and experimental work was performed by Saman Fatima. Statistical analysis and Proof Reading was carried out by

Gul-e-Saba Chaudhry. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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