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Bioactive Compounds and Health Benefits of *Trigonella Foenum-Graecum Linn*.: A Study on Seed And Callus

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A B S T R A C T

An effective method was developed to induce callus formation using various fenugreek explants. The study compared the phenolic, antioxidant, and antimicrobial properties of seeds and calli from different fenugreek explants. Fenugreek, renowned for its medicinal properties in pharmaceuticals and nutraceuticals, was evaluated. Three explants—hypocotyl, root, and cotyledons—were used for callus induction. These explants were cultured on MS medium supplemented with varying concentrations (0.5-6 mg/L) of 2, 4-D, NAA, and TDZ. Antioxidant activity was assessed using DPPH and Folin-ciocalteu assays, while antimicrobial activity of calli and seed extracts was also evaluated. Friable callus was successfully induced from all explants using all plant growth regulators (PGRs) except for 2, 4-D, which failed to stimulate callus formation in root explants. Among the explants, root segments showed superior callus induction, achieving a maximum fresh weight of 5.29 g with TDZ at 2 mg/L, and the highest callus index of 4.3 with TDZ at 0.5 mg/L. Hypocotyl-derived callus exhibited the highest phenolic content (246.9 mg GAE/g DW), followed by root callus (243.5 mg GAE/g DW), seed (176.2 mg GAE/g DW), and cotyledon callus (64.9 mg GAE/g DW). Seed extracts demonstrated the highest antioxidant activity at 44.3%, compared to 34.7%, 24.3%, and 16.7% recorded for cotyledon, hypocotyl, and root calli, respectively. In terms of antimicrobial activity, hypocotyl-derived callus exhibited the largest zone of inhibition (19 mm) against E. coli. Overall, fenugreek calli demonstrated promising biological activities compared to seed extracts.

Keywords: Fenugreek, Callus, Tdz, Diameter Zone Of Inhibition, Explants

Introduction

Fenugreek (Trigonella foenum-graecum Linn, Fabaceae) is a longstanding and widely used traditional medicinal and culinary plant in northern and eastern Africa, the Middle East, China, and India[1]. Traditionally, fenugreek extracts have been utilized to treat various ailments such as colic, flatulence, dysentery, diarrhea, dyspepsia with loss of appetite, gastric troubles, and joint pains, particularly in older individuals[1,2]. Additionally, fenugreek seeds extracts have exhibited diverse biological activities including carminative, tonic, aphrodisiac, and anticancer properties[3]. Metabolites found in fenugreek seeds extracts include polysaccharides, galactomannan, various saponins such as diosgenin and yamogenin, mucilage, lipids, flavonoids (apigenin luteolin,

quercetin), and alkaloids such as choline and trigonelline[4]. Similarly, fenugreek leaves extracts, like seeds, have been studied for their nutritional and therapeutic properties. Phytochemical analysis has revealed that fenugreek leaves contain saponins, ascorbic acid, and β-carotene. However, phenolics and flavonoids are the predominant compounds identified in various parts of fenugreek, contributing to its antioxidant capabilities[2].

Plant tissue culture techniques have been developed to facilitate rapid and large-scale production of cells and their secondary compounds. This approach ensures a continuous and dependable supply of secondary metabolites at higher concentrations compared to whole plants[5]. Callus induction represents the initial phase in establishing a cell suspension system, influenced by factors such as the type of explant, plant growth regulators, and genotype. Aasim et al.[6] conducted a review of plant tissue culture research on fenugreek, highlighting that much of the work has focused on callus and cell cultures for the production of important metabolites. Various significant phytochemicals have been identified in in vitro cultures of fenugreek, including diosgenin found in both callus[7,8] and cell suspensions[9], isoflavonoids in cell suspensions[10], 4-hydroxyisoleucine in callus [11], and alkaloids such as choline, trigonelline, and carpaine in callus[12]. Various authors have reported the establishment of callus cultures for fenugreek using different types of explants. However, the most responsive explants for callus induction in fenugreek have varied, including leaf[9], shoot apex[13,14], cotyledons[15], hypocotyl[16], root[7,17], and embryonic axis[11]. Different plant growth regulators have been identified as suitable for achieving maximum callus induction, with some authors favoring 2, 4-D while others prefer NAA or a combination with cytokinin, as documented by Hassan and Jassim[12]. The variability in callus formation potential among fenugreek explants is likely influenced by genetic factors. Screening of 21 Iranian landraces of fenugreek for callus induction revealed significant differences in callus formation capacity among the studied genotypes[11].

The objective of this study was to establish an efficient system for callus induction using various fenugreek explants, followed by a comparison of the phenolic, antioxidant, and antimicrobial potentials between seeds and calli derived from different fenugreek explants.

Material and Methods

Callus Induction Procedure

Mature seeds of Trigonella foenum-graecum (fenugreek) were procured from a local market in Khartoum, Sudan. The seeds underwent surface sterilization in a 10% solution of Clorox® (0.5% free chlorine) for 15 minutes, followed by four rinses with sterile distilled water. Subsequently, the seeds were cultured on Murashige and Skoog's (MS) basal medium[18], supplemented with 3% sucrose and 0.7% agar, adjusted to pH 5.8. The cultures were kept in the dark for 10 days.

After 10 days, in vitro germinated seedlings of fenugreek, aged 10 days, were used to obtain three types of explants: hypocotyls (5-10 mm), roots (3-5 mm), and cotyledons (see Fig. 1). These explants were inoculated on MS medium supplemented with either 2, 4-dichloro-phenoxyacetic acid (2, 4-D), naphthalene acetic acid (NAA), or thidiazuron (TDZ) at varying concentrations (0.0, 0.5, 1.0, 2.0, 4.0, and 6.0 mg/L). The cultures were then incubated at 25±2 ºC under a 16-hour photoperiod using cool white fluorescent lights (1000 lux).

After 6 weeks of culture, the performance of callus induction was qualitatively assessed based on parameters such as percentage of induction, callus color, and size index, rated from 1 to 5: 1 indicating callus formation at one end of the explant, and 5 indicating callus formation covering both ends, sides, and surfaces of the explant. Subsequently, after an additional week, calli were harvested from the cultures, and the fresh weight of callus per explant was determined.

Plant Material and Extracts Preparation

Calli (7 weeks old) obtained from hypocotyl, cotyledon, and root explants were freeze-dried and ground into homogeneous particles. Fenugreek seeds were washed with distilled water to remove foreign matter and air-dried at room temperature. The dried seeds were then ground using a blender and sifted through a mesh sieve to obtain a fine powder.

Ten grams of each callus and seed material were macerated in 200 mL of 80% methanol with intermittent shaking at room temperature for 48 hours. The extracts were filtered through Whatman filter paper No. 1. The residues were re-extracted twice under the same conditions to ensure complete extraction.

The resulting extracts were concentrated at 45°C under reduced pressure using a rotary evaporator, and their dry masses were recorded. The dried extracts were stored at 4°C until further experimental use.

Determination of Total Polyphenol Content

The concentration of phenolics in fenugreek seed and callus (roots, cotyledons, and hypocotyls) extracts was determined using the Folin-Ciocalteu method [19]. Methanolic solutions of the fenugreek samples at a concentration of 1 mg/mL were used for the analysis

For the analysis, the reaction mixture was prepared by combining 0.5 mL of the sample solution with 2.5 mL of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 mL of 7.5% NaHCO3. A blank solution was prepared simultaneously using 0.5 mL of methanol, 2.5 mL of 10% Folin-Ciocalteu's reagent dissolved in water, and 2.5 mL of 7.5% NaHCO3.

The reaction mixtures were then incubated in a thermostat at 30°C for 90 minutes. After incubation, the absorbance was measured using a spectrophotometer at λmax = 765 nm. Each sample was prepared in triplicate for analysis, and the mean absorbance value was calculated.

A standard solution of gallic acid was similarly analyzed to construct a calibration curve. Based on the measured absorbance values, the concentration of phenolics in the extracts was determined from the calibration curve and expressed as milligrams of gallic acid equivalent per gram of dry weight of extract (mg of GA/g DW).

Free Radical Scavenging Assay

The antioxidant activity of the four fenugreek extracts was assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) inhibition method, adapted from Shyur et al.[20] with some modifications. Various concentrations (1.56–100 µg/mL) were prepared from stock solutions (1 mg/mL in 98% ethanol) of the fenugreek extracts.

For each concentration of the extracts, 0.9 mL of tris-HCl and 1 mL of DPPH solution (0.1 mM) were added to test tubes. In control samples, the same volumes of tris-HCl and DPPH were added to 0.1 mL of ethanol, while in blank samples, 0.9 mL of tris-HCl was added to 1.1 mL of ethanol. The tubes were then incubated at room temperature in the dark for 30 minutes.

Following incubation, the absorbance of each mixture was measured at 517 nm using a spectrophotometer. The scavenging ability of the plant extract against DPPH radicals was calculated using the formula:

$$\text{Scavenging activity (\%)} = \frac{A\ control - A\ sample}{A\ control} \times 100$$

The total antioxidant activity was expressed as ascorbic acid equivalent/g dry weight extract.

Antimicrobial Activity

The antibacterial and antifungal activities of fenugreek seed and callus extracts were evaluated in vitro against various pathogenic organisms. Extracts were prepared at a concentration of 100 mg/mL and tested against the following microorganisms using the agar diffusion method[21], with slight modifications:

1. *Pseudomonas aeruginosa (ATCC 27853)*
2. *Escherichia coli (ATCC 25922)*
3. *Salmonella typhi (ATCC 0650)*
4. *Staphylococcus aureus (ATCC 25923)*
5. *Candida albicans (ATCC 7596)*

Data Collection and Statistical Analysis

Result were observed at regular intervals and data were collected from three independent experiment and analyzed by using analysis of variance procedure (ANOVA) on Microsoft Excel program. Means were separated by Duncan's multiple range test (DMRT) and presented as average ± standard error.

Results and Discussion

Callus Induction

All cultured fenugreek explants successfully developed friable callus when cultured on media supplemented with both TDZ and NAA. However, root explants cultured on media augmented with 2, 4-D did not produce any callus. The quality and quantity of callus induced varied significantly depending on the type of explant and the plant growth regulators (PGRs) used (refer to Tables 1 and 2 for details)

PGRs; plant growth regulators, H; hypocotyl, C; cotyledon, R; root, callus size index; 1: one end; 2: two ends; 3: two ends+ one side; 4: two ends+ two sides; 5: two ends+ two sides+ surface of explant.

The highest fresh weight of callus obtained was 5.29±0.4 g from hypocotyl explants cultured on medium supplemented with 2 mg/L TDZ (Table 2). However, this value did not show a significant difference ($P<0.05$) compared to the fresh weight of callus from root explants (4.77±0.5 g) cultured on medium containing 0.5 mg/L TDZ, or cotyledon explants cultured on medium with 2 mg/L TDZ. The significantly ($P<0.05$) lowest fresh weight of callus, 0.30±0.1 g, was recorded from cotyledon explants cultured on medium supplemented with 0.5 mg/L NAA (Table 2). Oncina et al[7] reported that stem segments producedthe maximum calluS fresh and dry weights compared to leaf and root explants of fenugreek. In contrast, Prabakaran and Ravimycin[15], Elaleem et al.[23] and Vaezi et al.[24] found that cotyledon explants were more effective in callus production than hypocotyl explants in fenugreek. This evidence suggests that callus induction in fenugreek is influenced not only by the concentration of plant growth regulators and the type of explant, but also by genotype.[14]

Values are means ± standard error. Means value followed by different letter are significantly different at 0.05% probability level using Duncan multiple range test. PGR; plant growth regulator.

Total phenolic contents and antioxidant activity of fenugreek extracts

The content of phenolic compounds varied considerably depending on the part of fenugreek that was extracted (refer to Table 3). The highest phenolic content recorded was 246.9 mg GAE/g DW in hypocotyl callus extract, followed closely by 243.5 mg GAE/g DW in root callus extract. This indicated that calli derived from hypocotyl and root exhibited higher phenolic content compared to seed extract, which measured 176.2 mg GAE/g DW. Cotyledon callus showed the lowest phenolic content at 64.9 mg GAE/g DW (Table 3).

In contrast, Ali and El Nour[25] reported that cotyledon callus exhibited higher phenolic content (412.1 mg/L) compared to hypocotyl callus (211.2 mg/L). Several studies on the quantification of polyphenols in fenugreek seed extracts using the Folin-Ciocalteu method with gallic acid as a standard have reported varying total phenolic content values. Seasotiya et al.[4] reported high phenolic contents up to 186 mg GAE/g DW in methanolic extracts of fenugreek seeds, while other studies reported lower values ranging from 9.7 mg GAE/g [3] to 45.4 mg GAE/g [26] and 78.1 mg GAE/g.[27]

Numerous factors can influence the secondary metabolite content in plant species, such as genetics, environmental conditions, seed maturity, and extraction methods. Rahmani et al.[28] observed significant variations in total phenolic content among four varieties of fenugreek from Algeria.

Table 1.Qualitative measurements of callus induction on different explants of fenugreek after 6 weeks of culture

PGRs (mg/L)		Callusing (%)			Callus size index			Color
		H	C	R	H	C	R	H, C, R
Control		0	0	0	0	0	0	-
TDZ	0.5	100	100	100	3.3	3.5	4.3	Light green
	1	100	100	100	3.2	3.3	4.0	
	2	100	100	100	3.3	3.5	2.7	
	4	100	100	100	4.2	3.0	4.2	
	6	100	100	100	3.3	3.0	4.0	
NAA	0.5	94	50	100	2.3	2.2	2.5	Green
	1	81	75	100	2.2	2.0	2.3	
	2	75	68	75	2.2	2.5	2.2	
	4	75	60	56	2.5	2.5	2.2	
	6	69	50	31	2.2	2.0	2.0	
2,4-D	0.5	100	58	0	2.2	2.5	0	Yellowish green
	1	100	66	0	2.3	2.5	0	
	2	25	66	0	2.5	2.2	0	
	4	16	33	0	2.0	2.2	0	
	6	16	16	0	2.0	2.0	0	

Table 2.Callus fresh weight induced on different explants of fenugreek using different concentrations (0.5-6 mg/L) of TDZ, NAA and 2, 4-D after 7 weeks of culture

PGR (mg/L)	Callus fresh weight (g)	Hypocotyl	Root	Cotyledon
Control	-	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}
TDZ	0.5	4.25 ± 0.5^{a}	4.77 ± 0.5^{a}	3.24 ± 0.4^{ab}
	1	4.70 ± 0.9^{a}	4.19 ± 0.6^{a}	3.83 ± 0.3^{ab}
	2	5.29 ± 0.4^{a}	3.95 ± 0.4^{ab}	4.98 ± 0.8^{a}
	4	5.28 ± 0.6^{a}	3.98 ± 0.5^{ab}	2.63 ± 0.4^{b}
	6	4.66 ± 0.5^{a}	3.92 ± 0.6^{ab}	2.53 ± 0.4^{b}
NAA	0.5	0.31 ± 0.1^{c}	0.42 ± 0.1^{c}	0.30 ± 0.1^{c}
	1	0.41 ± 0.0^{c}	0.42 ± 0.0^{c}	0.31 ± 0.1^{c}
	2	0.51 ± 0.0^{bc}	0.45 ± 0.0^{c}	0.42 ± 0.1^{c}
	4	0.57 ± 0.1^{bc}	0.46 ± 0.1^{bc}	0.43 ± 0.1^{c}
	6	0.31 ± 0.0^{c}	1.25 ± 0.0^{bc}	0.35 ± 0.6^{c}
2, 4-D	0.5	2.37 ± 0.3^{b}	0.00 ± 0.0^{d}	2.22 ± 0.3^{b}
	1	2.00 ± 0.3^{b}	0.00 ± 0.0^{d}	2.04 ± 0.3^{b}
	2	1.97 ± 0.3^{bc}	0.00 ± 0.0^{d}	1.33 ± 0.2^{bc}
	4	1.76 ± 0.3^{bc}	0.00 ± 0.0^{d}	0.95 ± 0.2^{bc}
	6	1.69 ± 0.2^{bc}	0.00 ± 0.0^{d}	0.94 ± 0.2^{bc}

Table 3. Phenolic contents and free radical scavenging potential DPPH activity of fenugreek

Extract source	TPCs mg GAE/g	Activity % ± SD
Hypocotyl callus	246.9	24.3 ± 0.06
Root callus	243.5	16.7 ± 0.1
Cotyledon callus	64.9	34.7 ± 0.04
Seed	176.2	44.3 ± 0.04

The results of the in vitro investigation into the antioxidant activities of fenugreek extracts using DPPH radical scavenging technique are summarized in Table 3. Fenugreek seed extracts exhibited the highest antioxidant activity (44.3 ± 0.036%), followed by cotyledon callus extract (34.7 ± 0.04%), hypocotyl callus (24.3 ± 0.064%), and root callus extract (16.7 ± 0.1%). These findings indicate that the different extracts of fenugreek demonstrated moderate to low radical scavenging activity.

The antioxidant activity of fenugreek seed extract has been previously reported with varying values. For instance, Haliem and Al-Huqail[29] studied the antioxidant potential of fenugreek seed among seven wild accessions and found scavenging activities ranging from 42.92% to 54.23%. Conversely, higher values of antioxidant activity in fenugreek seed extract have also been documented, such as 67.9%[4], 80.5%[25], and 89.7%.[3]

Additionally, antioxidant activity of fenugreek callus extracts has been reported, with cotyledon and hypocotyl callus showing scavenging activities of 91.5 ± 0.16% and 85.46 ± 0.29%, respectively[25]. The genotype of fenugreek has been identified as a significant factor influencing chemical constituents and antioxidant properties of its seeds.[1, 30]

Antimicrobial activity of extracts

The extracts obtained from fenugreek seeds and calli derived from roots, cotyledons, and hypocotyls at a concentration of 100 mg/mL were evaluated for their antimicrobial activities against four standard bacteria and one fungus using the in vitro agar well diffusion method (refer to Table 4). Hypocotyl callus exhibited the highest zone of inhibition recorded (19 mm) against E. coli. Methanolic extracts at 250 mg/mL from hypocotyl callus showed the maximum inhibition zone of 11 mm against S. aureus.[16]

In contrast, the lowest zone of inhibition recorded (10 mm) was against E. coli by root callus in the present study (Table 4). Among seed extracts, the highest activity recorded was 16 mm against P. aeruginosa. El Nour et al.[16] previously reported a maximum inhibition zone of fenugreek seeds (petroleum ether extract at 250 mg/mL) as 17 mm against E. coli, while showing no activity against P. aeruginosa. However, Sharma et al.[2] reported that a methanol extract of fenugreek seed at 100 µL exhibited no activity against E. coli and Staphylococcus spp

Table 4. Antimicrobial activities of the fenugreek extracts

Extract type (100 mg/mL)	Zones of inhibition diameter (mm)					
	Ec	Pa	Sa	Bs	Ca	Extract mean
Seed	13	16	12	15	-	14
Hypocotyl callus	19	16	12	-	-	16
Cotyledon callus	11	-	-	13	12	12
Root callus	10	14	-	13	12	12
Microbial mean	13	15	12	14	12	-

Bs: Bacillus subtitles, Sa: Staphylococcus aureus, Ec: Escherichia coli, Pa: Pseudomonas aeruginosa, Ca: Candida albicans. MIZD (mm): > 18 mm: sensitive; 14–18 mm: intermediate; < 14 mm: sesistance.

In general, hypocotyl callus demonstrated higher average antimicrobial activity (16 mm) against the studied bacteria, followed by seed extract (14 mm). However, both extracts showed no activity against the studied fungus C. albicans, consistent with results reported by El Nour et al.[16] On the other hand, root and cotyledon callus extracts exhibited activity against C. albicans with the same inhibition value (12 mm).

Regarding the susceptibility of different fenugreek extracts, P. aeruginosa appeared moderately sensitive to most extract types, with an average inhibition zone of 15 mm. The other microbes studied generally showed complete resistance, with average inhibition zones ranging from 21 to 14 mm (Table 4).

Several studies have investigated the antimicrobial activity of fenugreek seed extracts against similar microbial strains using different solvents, yielding variable results.

Conclusion

A straightforward and effective method for producing friable callus from in vitro seedling explants of Trigonella foenum-graecum was successfully developed. Root segments proved to be the most effective explants for callus induction, particularly with TDZ at 2 mg/L yielding the highest fresh weight of green callus. Cotyledon callus followed seed extract in antioxidant activity, while hypocotyl callus extract exhibited a higher phenolic content. The antimicrobial activity observed in hypocotyl callus extract may be attributed to its high phenolic content. These findings suggest that fenugreek callus represents a promising source of biologically active compounds. Further phytochemical investigations are necessary to identify the specific bioactive compounds responsible for the activity of fenugreek callus extract.

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