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Studies on trigonelline from *Moringa oleifera* and its *in vitro* regulation by feeding precursor in cell cultures

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Abstract: Trigonelline (*N*-methylnicotinate) biosynthesized from nicotinate is one of the metabolically active pyridine alkaloid, widely distributed in plant kingdom. In the present study trigonelline has been isolated from various plant parts and callus cultures of *Moringa oleifera* Lam., Moringaceae, and was identified using TLC, GLC, GC-MS, which was comparable to that of the standard trigonelline. The trigonelline recovery was found to be maximum in the pods and minimum in flowers. In order to enhance the production of trigonelline *in vitro* grown cultures, different treatment doses of nicotinic acid (250, 500 and 750 mg L^{-1}) were supplemented in the medium as precursor. Maximum increase (up to 1.10 fold) was observed in the treatment dose of 500 mg L^{-1} of nicotinic acid.

Article

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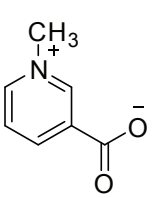
Introduction

Plants are regarded as molecular factory, as they have capacity to synthesize enormous diversity of metabolites termed as bioactive compounds. Alkaloids have gained attention of the biochemists because of their diversified physiological and pharmacological actions (Verpoorte et al., 1991). Trigonelline (*N*-methylnicotinate) (**1**), a pyridine alkaloid mostly reported from family Fabaceae, is biosynthesized from nicotinate (Joshi & Handler, 1960; Kogan et al., 1953; Taguchi et al., 1986). Trigonelline is metabolically active as hypocholesterolemic agent (Evans et al., 1979) and its nutritional benefits have also been well documented (Viani & Horman, 1974; Taguchi et al., 1986). It has been reported it also possess glucose lowering activity (Olthof et al., 2011).



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Biosynthetic pathway of any bioactives is regarded as the key point for industrial production of any pharmaceutical, which may lead to a drastic effect at a commercial level by use of precursor (Patel, 1998). Exogenous supply of a precursor to culture medium has been advocated to increase the yield of the desired product in cell cultures (Staba, 1985). Successful attempts have been made to increase the production of metabolites by supplying precursor or intermediate compounds *in vitro* (Moreno et al., 1993; Whitmer et al., 1998; Silvestrini et al., 2002; Namdeo et al., 2007).

Moringa oleifera Lam. commonly called as *drum stick* belongs to family *Moringaceae*, which bears fourteen species among which *M. oleifera* is most commonly found. The plant is native to northern India where it was noted as medicinal plant around 2000 BC. The pods and leaves are rich in protein and minerals and cooked as vegetable (Verma et al., 1976), posses certain bioactivities such as antiurolithiatic (Karadi et al., 2008), anti-inflammatory (Sulaiman et al., 2008), antiasthmatic (Agrawal & Mehta, 2008), antioxidant and hepatoprotective (Fakurazi et al., 2008) have been reported. In literature, the presence of trigonelline was not previously recorded in *M. oleifera.*

The present study deals with the isolation, identification and quantitative determination of trigonelline (**1**) in various plant parts and tissue cultures of *M. oleifera.* The regulation of its production by

nicotinic acid (precursor) feeding at different treatment doses was also attempted. The present investigation is the first report of trigonelline in *M. oleifera.*

Material and Methods

Plant material

Moringa oleifera Lam., Moringaceae, (flowers, leaves, stem, pods and roots) was collected from fields at Jaipur and authenticated by Dr. N J Sarna, Department of Botany, University of Rajasthan. Plant parts were cleaned and oven dried at 35 ºC for 30 min and then at 25 ^{o}C till constant weight was achieved and then powdered. The voucher (RUBL No. 20393, Rajasthan University Botanical Library) of experimental plant was deposited in Herbarium at Department of Botany, University of Rajasthan, Jaipur).

Reference compound

Standard trigonelline used were of analytical grade and obtained from Sigma- Aldrich (Germany)

Tissue culture

Surface sterilization and inoculation

Nodal segments were surface sterilized with mercuric chloride ($HgCl_2$) solution (0.1% w/v) for 2 min and subsequently rinsed several times with sterile distilled water and treated with antibiotic (ciprofloxacin, 250 mg L^{-1}) prior to inoculation in order to remove any kind of microbial interactions. Surface sterilization was done in a horizontal laminar flow hood fitted with ultraviolet light. Nodal segments were inoculated in the flasks containing culture medium aseptically having combination of BAP:IBA (3:3 mg L^{-1}), BAP: NAA (1:0.2 mg L^{-1})

Incubation

Cultured flasks were incubated in culture chamber at the temperature 25±1 °C and light of intensity (1200 lux) provided from fluorescent tubes and incandescent bulbs (40 watt) for 16 h. The cultures were observed and examined every week and at final morphogenetic data were recorded.

Growth index

The maintained calli were harvested regularly at the transfer age of 2, 4, 6, 8 weeks. Each of the callus samples was harvested and their growth indices (GI) calculated on fresh weight basis

$$GI = \frac{\textit{Final wt. of the tissue} - \textit{Initial wt. of the tissue}}{\textit{Initial wt. of the tissue}}$$

Five such replicates were examined in each case and average values calculated.

Trigonelline

Extraction

Powdered and weighed plant materials (flowers, leaves, stem, pods and roots) along with callus tissue were taken in 100 mL Erlenmeyer flasks added 50 mL distilled water for each g of the dried plant/tissue material (v/w) and 5 mL of 0.05 N sulphuric acid was added to it. Mixture was macerated for 3-4 h and boiled gently for 25 min. Heavy magnesium oxide (2.5 g/g) was added to the mixture and again boiled gently for 20 min. It was cooled at room temperature and 20 mL of volume was completed to 100 mL with distilled water 10 mL of alcohol was added to remove the mucus. Mixture was filtered through Whatman filter (having diameter-12.5 cm) paper 1 (Kogan et al., 1953). Filtrate was evaporated to dryness *in vacuo*, reconstituted in distilled water for further analysis.

Qualitative analysis

Thin layer chromatography

Extracted sample were dissolved in distilled water and applied on activated TLC plates, along with authentic samples of trigonelline. These plates were developed in presaturated chromatographic chamber containing solvent mixture of butanol:acetone:water (4:1:5) (Johnson & Lin, 1953) and isobutanol and water (85:15) of (Kogan et al., 1953). Plates were visualized under UV light and fluorescent spots were marked and then were sprayed with Dragendorff's reagent to develop coloured spot co comparable with authentic sample applied simultaneously.

PTLC (Preparative thin layer chromatography)

Preparative thin layer chromatography of isolated trigonelline samples was also carried out as above on thick Silica Gel G coated plates (500 µm thick) by spotting extract along with the trigonelline. The developed plates were compared with authentic samples under UV light. Spot coinciding with standard compound was marked and collected by scrapping silica gel G, dried, weighed (mg) and crystallized in methanol and water. Crystals were subjected to GLC and GC-MS analyses.

Gas Liquid Chromatography (GLC)

The extract and the standard samples were analyzed by GLC. A Perkin Elmer OV-17 gas chromatograph equipped with a flame ionization detector on gas Chrom Q. The operating temperature used for analysis was 260 °C. Nitrogen was used as carrier gas at a flow rate 0.6 mL/min, chart speed 0.28 cm/min.

Gas Chromatography and Mass Spectroscopy (GC-MS)

The extract and the standard samples were analyzed by GC-MS of Hewlett-Packard 6890/5973 operating at 1000 eV ionization energy, equipped with using Agilent 7890A/5975C GC HP-5. Capillary column (phenyl methyl siloxane, 25 m×0.25 mm *i.d*) with Helium (He) was used as the carrier gas with split ratio 1:5. Oven temperature was 100 °C (3 min) to 280 °C at 1 to 40 °C/min; detector temperature, 250 to 280°C; carrier gas, He (0.9 mL/min). Retention indices were determined by using retention times of samples that were injected under the same chromatographic conditions. The components of the standard and plant samples were identified by comparison of their mass spectra and retention time with those given in literature and by comparison with the mass spectra of the Wiley library or with the published mass spectra.

Effect of precursor feeding

Six months old maintained calli as suspension cultures were transferred in the fresh medium supplemented with various dose treatment of nicotinic acid (250, 500 and 750 mg L^{-1}, separately) as precursor. A control was run in parallel without supplementation of nicotinic acid. The cultures were harvested at different time intervals of 2, 4, 6 and 8 weeks of precursor treatment, cells cultures were harvested, filtered and kept at 100 °C for 20 min to inactivate the enzymes and then at 60 °C till constant weight was achieved and were analyzed for trigonelline content.

Data analysis

Data were subjected to one-way analysis of variance (ANOVA) using SPSS version 10. The significance level was determined at p=0.05. When there were significant differences, the means were separated using Duncan's multiple range test (DMRT). Differences are denoted in tables and figures by different letters. The standard error was also noted where appropriate.

Results

Tissue culture

Nodal segments were used for the tissue culture studies. The GI was found to be minimum in two weeks (0.75) and maximum in six weeks old tissue (3.06). Thereafter, the GI steadily declined thus showing a characteristic sigmoid curve of growth (Figure 1)

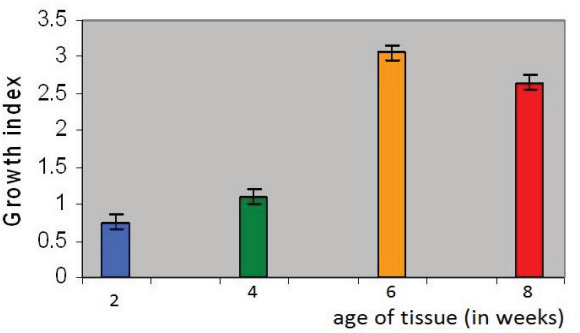

Figure 1. Growth Index (GI) of *Moringa oleifera* grown on modified MS medium.

Trigonelline

The qualitative and quantitative analysis of trigonelline from various plant parts and callus cultures of *M. oleifera* was carried out.

Qualitative analysis

Isolated alkaloid gave single fluorescent spot on thin layer chromatographic plates developed in solvent system of butanol:acetone:water (4:1:5) under UV lamp. On spraying with Dragendorff's reagent, brick red coloured spot was observed at the R_f 0.09 which coincided with standard trigonelline.

Quantitative

Among various plant parts maximum trigonelline content was observed in pods (3.55 mg/g) followed by leaves (2.60 mg/g), roots (2.15 mg/g), stem (1.90 mg/g) and minimum in flowers (1.60 mg/g). Callus cultures had lower (2.38 mg/g) content than pods and leaves but higher than other plant parts (Figure 2).

GC-MS

The GC-MS studied showed that the retention time and peaks of the isolated trigonelline was comparable with that of standard. The active principles with their retention time (RT) and concentration (%) in the standard and sample are presented. Compounds found in standard are methyl pyridine-3-carboxylate (retention time-7.457* and 69.20% area), 1*H*-pyrrole-3-carboxylic acid

(retention time-10.100 and 2.42% area), 1*H*-indole, 1,3-dimethyl (retention time-10.382 and 0.66%), 1*H*-pyrrole-3-carboxylic acid (retention time-10.758 and 21.90% area), hydroxy(4-nitrophenyl) acetic acid (retention time-12.034% and 5.82%) and 49 compounds are identified in alkaloid extract isolated from pods of *M. oleifera* along with their biological activity. Phytocompounds and its biological activities obtained through the GC-MS study are presented in Table 1 and 2.

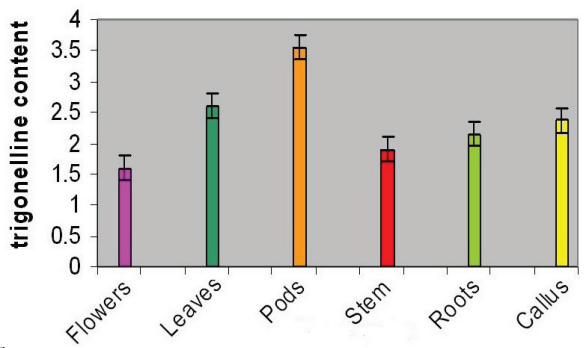

Figure 2. Trigonelline content (mg/g) in various plant parts and callus cultures of *Moringa oleifera*.

GLC

The GLC studied showed that the retention time and peaks of the isolated trigonelline was comparable with that of standard (Figure 3).

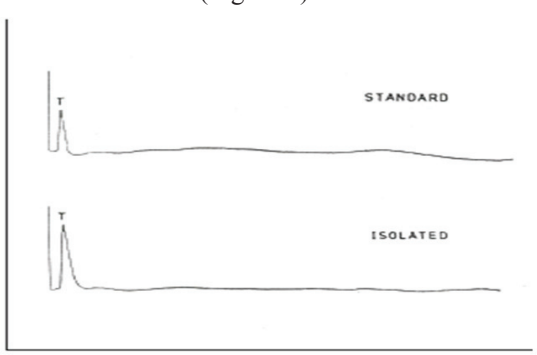

Figure 3. Gas liquid chromatographic analysis of standard trigonelline and isolated from pods of *Moringa oleifera.*

Effect of precursor feeding

The quantification data of trigonelline obtained from different treatment doses of nicotinic acid gave maximum content of 3.27 mg/g in callus obtained from 50 mg% nicotinic acid supplementation dose, which was significantly superior to all other treatments and minimum content of 2.12 mg/gdw (gram dry weight) was observed in callus of control, which was at Par with callus of 25 mg% nicotinic acid treatment dose (2.14 mg/g). The marginal mean content in relation to time showed maximum content in six weeks old callus (2.97 mg/g), which was significantly superior to other treatments and minimum (2.12 mg/g) was in two weeks old callus (Table 3).

Discussion

In the present investigation callus was raised from nodal segments on each of the combination hormonal doses, the best response was observed in combination doses of BAP: IBA (3 mgL^{-1}, each). The presence of auxins and cytokinin in the culture medium regulates various aspects of dedifferentiation and differentiation (Woodward & Bartel, 2005; Wang et al., 2008) at cellular levels in a particular dose.

There is no uniform and clear definition of growth of plant cell cultures and dry weight or fresh weight methods have been in use for determining GI, because of its preciseness, accuracy in observing variation (Grossmann, 1988). Several workers have observed the sigmoid growth pattern of the callus culture (Staba, 1980; Endress, 1994). In the present study a sigmoid pattern of growth curve was observed in *M. oleifera* with maximum GI at the 6^{th} week of subculture indicated the exponential growth phase and minimum at 2^{nd} week of subculture. An increase in GI after supplementation of various growth regulators finds support that the growth of tissue some time depends upon the culture medium and also controlled by other factors like pH, treatment dose and combinations of growth regulators used (Barz et al., 1977; Heble, 1985; Schripsema et al., 1990).

Table 1. Activity of phyto-components identified in the standard trigonelline by GC-MS.

Peak	Retention time	Area %	Name of compound	Biological activity
1	7.457*	69.20	methyl pyridine-3-carboxylate	Antineoplastic, cytotoxicity
2	10.100	2.42	1*H*-pyrrole-3-carboxylic acid	Antimicrobial, antioxidant
3	10.382	0.66	2-dihydro-3,3-dimethyl-2-oxo-3*H*-indole	Antiviral, ATP-competitive platelet derived growth factor
4	10.758	21.90	1*H*-pyrrole-3-carboxylic acid	Antimicrobial, antioxidant
5	12.034	5.82	hydroxy(4nitropheny)acetic acid	Immunostimulant, cancer preventive

*Identified as trigonelline

Table 2. Biological activity of various compounds identified in pods of *Moringa oleifera* by GC-MS.

Peak#	Retention Time	Area %	Name of compound identified	Biological activity
1	6.433	4.01	5-undecanone	Insectisidal, antioxidant
2	6.633	7.53	4-methyl-phenol	Antimicrobial, antioxidants
3	6.868	12.36	2,3-dihydro-5-hydroxy-6-methyl-4*H*-pyran-4-one	New compound
4	7.336*	4.76	methyl nicotinate	Antineoplastic, cytotoxicity
5	7.677	3.67	4-hydroxydihydro-2(3*H*)-furanone	Antifungal, cytotoxic and antioxidant
6	8.417	0.72	5-methoxy-2-pentyne	Antihypertensive, hepatoprotective
7	8.576	4.57	4-hepten-3-one-4-methyl	Antifungal
8	9.514	4.37	4hydroxy toluene	Antioxidant, antidiabtic
9	9.816	1.68	oxiranecarboxylic acid, 3-methyl-ethyl ester	Control of fatty acid metabolism
10	10.229	2.15	4-hydroxy methyl phenol	New compound
11	10.375	6.17	α-D-galactopyranoside	Antioxidant, antimicrobial, antidiabetic activity
12	10.460	4.42	4-hydroxybenzaldehyde	Diphenolase activity,
13	10.683	1.71	5-oxo-pyrrolidine-2-carboxylic acid methyl ester	Antiinflammatory, antiarthritis
14	11.118	1.08	3-chloro-1-phenyl-1-propanol	Antimicrobial,
15	11.605	0.85	2-butylphenol	Antioxidant, autocatalytic
16	11.977	3.16	benzeneacetonitrile,4-hydroxy	Cytotoxicity, antinephropathic
17	12.180	1.78	α-methylbenzylamine	Antimicrobial, antitumour, anthelmintic
18	12.758	0.22	dodecanoic acid	Antiinflammatory, Hypocholesterolemic, cancer preventive, hepatoprotective, nematicide, antiandrogenic, antiarthritic,
19	13.102	0.67	5-tetradecene	Anti-inflammatory, anti-spasmodic,
20	13.942	0.91	4,5-dihydropyrrole-5-one-2-propionamide	Antioxidant
21	14.362	1.04	1-cyclopentylacetonitrile	Cytotoxicity, antitumor
22	14.692	0.19	2,3,4,4α,5,8,8α-octahydro-4,8α-dimethylnaphthalen-4a-ol	Insecticidal
23	14.867	1.29	hydrazine, 1-(3 hydroxybenzyl)-	Vasorelaxant
24	15.151	0.35	9-eicosene	Antimicrobial, larvicidal
25	15.525	0.14	benzeneacetic acid	Trypsin inhibition
26	15.585	0.45	tyyrosine	Neuroprotective
27	15.950	0.70	phthalic acid, butyl tetradecyl ester	Hepatoprotective, antiasthmatic
28	16.161	1.08	*N,N*-dimethyl-1-nonadecanamine	New compound
29	16.584	2.92	hexadecanoic acid	Antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant, antiandrogenic, flavor, hemolytic
30	16.708	0.76	1,2-benzenedicarboxylic acid	Used in the formulation of drugs by both oral and transdermal delivery routes
31	16.791	0.39	1-octadecanol	Antioxidant, antidiabetic
32	17.043	0.27	heptadecanoic acid	Radioprotective
33	17.598	5.06	9-octadecenoic acid	Antimicrobial
34	17.738	2.25	octadecanoic acid	Antimicrobial
35	17.884	3.10	heptadecene	New compound
36	17.998	2.55	9-octadecenoic acid	Antifungal
37	18.155	1.25	14-methyl-8-hexadecyn-1-ol	New compound
38	19.745	0.51	9-tetradecenal	Neurotransmitter

39	20.134	0.25	eicosane	Antimicrobial, larvicidal
40	20.236	0.36	methyl dihydromalvalate	Antitumor
41	20.368	0.72	docosanoic acid	Antimicrobial
42	20.663	1.20	1,2-benzenedicarboxylic acid	Used in the formulation of drugs by both oral and transdermal delivery routes
43	20.849	0.36	eicosane	Antimicrobial, larvicidal
44	21.948	0.53	tetracosanoic acid	Antimicrobial
45	23.033	0.44	2,6,10,14,18,22-tetracosahexaene	Antitumor

Table 3. Two way interaction doses of nicotinic acid and time treatment (in weeks) on trigonelline content (mg/g) in *Moringa oleifera* static cultures.

Time Treatment (in weeks)	Trigonelline content (mg/g) Treatment dose of nicotinic acid in (mgL^{-1})			Control
	250	500	750	
2	2.14±0.32	2.32±0.41	2.28±0.34	2.12±0.29
4	2.43±0.35	2.52±0.81	2.45±0.75	2.38±0.31
6	3.12±0.54	3.27±0.53	3.18±0.46	2.97±0.46
8	2.62±0.47	2.73±0.55	2.64±0.43	2.60±0.38

Each value is the mean of three replicates±SE.

In many plant species alkaloids get accumulated in seeds in relatively high concentration as a chemical defense. During germination alkaloids get metabolized and their nitrogen is reused for seedlings metabolism (Wink & Witte, 1985). Preliminary reports on presence of alkaloids in *M. oleifera* have been shown (Ramchandran et al., 1980; Dangi et al., 2002; Patel et al., 2008).

Trigonelline, serves as a storage form of nicotinic acid (Blaim & Wanner, 1960) has been reported from other plants also. Trigonelline from seeds of *T. foenum-graecum* (0.38%) was reported by Kuhn & Gerhard (1943). Samola & Gerhard (1983) reported (0.8% to 1.1%) trigonelline from green coffee. Kaushik & Khanna (1990) reported 0.014% from *Dolichos lablab* seeds and 0.0068% of trigonelline from its callus cultures.

Present study is the first report of presence of trigonelline in *M. oleifera.* The maximum content was found in pods (3.55 mg/g). The content in callus cultures was lower than pods and leaves and higher than rest of the plant parts. In tissue culture trigonelline has been reported from *Trigonella foenum-graecum* (Antony et al., 1975; Willeke et al., 1970, Radwan & Kokate, 1980), *Pisum sativum* and *Glycine max* (Tramontano et al., 1985), *Trigonella polycerata* (Kamal et al., 1996), *Dolichos lablab* (Kaushik & Khanna, 1990).

Use of precursor in the culture medium enhance end product have been advocated (Staba, 1980). Nicotinic acid acts as precursor in the biosynthesis of trigonelline (Johnson & Lin, 1953). Khanna & Jain (1972) observed an increase in growth and trigonelline in tissue cultures of *Trigonella foenum-graecum* fed with nicotinic acid in 8 week old callus cultures. Later, Khanna et al. (1989) reported 2 fold increase in six weeks old callus obtained from 500 mg L^{-1} nicotinic fed medium in *Allium cepa* callus cultures. In the present studies, similar results were observed in *M. oleifera* callus cultures, where the trigonelline content increased (1.10 fold) in six weeks old tissue fed with nicotinic acid (Figure 2), whereas the growth of callus decreased remarkably, which may be due to enhanced trigonelline content which checks cell proliferation by cell arrest as suggested by Lynn et al., (1984).

Conclusion

The present investigation is the first report of trigonelline from *M. oleifera* confirmed by GLC and GC-MS analyses. The biosynthesis of trigonelline was enhanced in *M. oleifera* by feeding the precursor nicotinic acid in its cell cultures. Our results confirm that specific plant metabolites could be modulated by exogenous precursor supplemented according to their specific metabolite pathway.

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