

Determination of volatile glucosinolate degradation products in seed coat, stem and *in vitro* cultures of *Moringa peregrina* (Forssk.) Fiori.

S. Dehshahri^{1,*}, S. Afsharypuor¹, G. Asghari¹ and A. Mohagheghzadeh²

¹Department of Pharmacognosy and Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R.Iran.

²Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, Shiraz University of Medical Sciences, Shiraz, I.R.Iran.

Abstract

Moringaceae, a monogeneric family in Capparales (glucosinolate-containing species), includes 14 species. One of them is *Moringa peregrina* (Forssk.) Fiori., a small tree, which grows in south east of Iran. Volatile constituents of seed coat and stem of *M. peregrina* were determined by GC and GC/MS. Moreover, extracts of seed and different cultured cells were analyzed by TLC and GC. Three volatile isothiocyanates including isopropyl isothiocyanate (4.2%), sec-butyl isothiocyanate (< 0.1%) and isobutyl isothiocyanate (92.9%) were found in the volatile oil of the stem, while only two volatile isothiocyanates namely isopropyl isothiocyanate (7.0%) and isobutyl isothiocyanate (51.5%) were determined in the seed coat of the tree. For the first time, the callus and suspension cultures of *M. peregrina* were initiated and established successfully on Murashige and Skoog medium, containing plant growth hormones. Different precursors and elicitors were fed to the cultures to induce glucosinolates production. This is the first report of *in vitro* culture production of *M. peregrina*. There was no production of volatile isothiocyanates in *M. peregrina* callus and suspension cultures with different treatments.

Keywords: Moringaceae; *Moringa peregrina*; Isothiocyanates; plant cell culture

INTRODUCTION

Moringa peregrina (Forssk.) Fiori is a desert tree of the monogeneric family, Moringaceae, distributed from tropical Africa to east India (1-3). Moringa tree is growing in Sistan and Baluchestan, the south east province of Iran. The tree has often numerous stems with raised grey branches. Its fruit is 20-40 cm pod which contains 8-15 unwinged seeds (1).

M. peregrina is cultivated as a source of gums, oil and pungent principals (4). Occurrence of several isothiocyanates in the volatile oil of the seed kernel and leaves of *M. peregrina* has been reported previously (5).

Isothiocyanates (hydrolyzed products of glucosinolates) have various biological activities including anti oxidative, anti bacterial, anti cancer and chemoprotective properties (6-7). In several studies, it has been shown that there is an inverse correlation between dietary intake of isothiocyanates and cancer risk of several

organs. These findings have generated more interest about isothiocyanates as potential cancer-preventive agents in humans (8). In addition, it was reported that some isothiocyanate containing spices and vegetables (such as mustard and horseradish) showed gastro-protective effects (6).

The medicinal importance of isothiocyanates justifies attempts first for finding new sources for isothiocyanates and second producing them through plant cell cultures. To the best of our knowledge, there is no report on volatile glucosinolate production in plant cell culture of *M. peregrina*. However there are several reports on producing some glucosinolates in other genera of Cruciferae and Capparidaceae (9-10). It is of interest to find out whether glucosinolate production biosynthetic pathway in plant cell cultures of *M. peregrina* is active. If so, the mass production of glucosinolates can be achieved by biotechnological processes via plant cell culture.

*Corresponding author: Shadab Dehshahri, this paper is extracted from the Ph. D thesis No. 387053
Tel. 0098 9173031679, Fax. 0098 311 6680011
Email: Dehshahri@pharm.mui.ac.ir

In the current study, volatile constituents of seed coat, stem and cultured cells of *M. peregrina* were investigated. In addition, various strategies were used to produce glucosinolates in cell culture conditions.

MATERIALS AND METHODS

Plant material

Seeds and stems of *M. peregrina* were obtained from the department of Natural Resources of Chabahar, Sistan and Baluchestan province, Iran, and identified by the same department. Voucher specimens of the seeds, leaves and stems (no. 2025) were deposited in the herbarium of Pharmacognosy Department, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences (Iran).

Autolysis and collection of the volatile constituents

The powdered seed coat and air dried stem (40 g of each) were mixed with distilled water (400 ml), separately. The mixtures were left for autolysis at 25°C for 17 h in a container. Autolysis which is hydrolytic breakdown of glucosinolates led to the formation of volatile isothiocyanates by separating the glucose moiety from glucosinolate. To each mixture, 6 ml of cyclohexane were added and agitated well for 10 min. Then volatile constituents were collected by 3 h distillation. The organic layer of each distilled sample was concentrated and subjected to GC/ GC-MS analysis (11).

Plant cell culture

Callus cultures were obtained from the sterilized young leaves of *M. peregrina* on Murashige and Skoog medium (12) supplemented with coconut water (15% v/v), sugar (3% w/v), kinetine (0.5 mg/L), 2, 4-dichlorophenoxyacetic acid (0.5 mg/L), α -naphthalene acetic acid (1 mg/L) as plant growth regulators and agar (0.8% w/v) for solidifying the medium. The pH of the medium was adjusted to 5.7 ± 0.1 before sterilization by autoclaving. The callus cultures were kept at $25 \pm 2^\circ\text{C}$ under constant light and were subcultured every 4 weeks.

In order to establish *M. peregrina* suspension culture, after 3 subcultures, 5 g of

callus was inoculated in to the liquid medium (40 ml) of the same media ingredients used for callus, in 200 ml conical flask maintained at $25 \pm 2^\circ\text{C}$ on a rotatory shaker operated at 120 rpm under constant light exposure. The cell suspension cultures were transferred in to the fresh media every 10 days from the old media.

Different treatments for production of isothiocyanates in callus and cell suspension cultures were applied. Methionine (2 mM), cysteine (2 mM) and phenylalanine (1 mM) as amino acid precursors and salicylic acid (10 μM) as elicitor were added to the solid and liquid Murashige and Skoog media separately. Callus and suspension cultures were harvested from all treatments after 3 subcultures on each medium. Cell cultures were freeze dried just before extraction.

Extraction of cultured cells

The freeze dried cells (callus and suspension cultures) as well as seeds were powdered separately and 0.25 g of each was mixed with distilled water (2.5 ml) and left for 17 h at 25°C for autolysis. Each mixture was shaken with dichloromethane (DCM) for 10 min, then organic phase was separated and concentrated to analyze immediately by GC and TLC. Seed volatile oil was used as a control for isothiocyanates (11).

TLC analysis

The DCM extracts were chromatographed on TLC, silica gel 60 F₂₅₄ plates (Merck, Germany) after thiourea derivatization. In order to prepare thiourea, 0.5 ml of ethanol and 0.5 ml of ammonia (25%) were added to the concentrated DCM extracts. The solutions were kept in refrigerator for 12 h. The thiourea derivatives were applied on TLC (silica gel G60 F254) with the lower phase of solvent system of ethyl acetate:chloroform:H₂O (40:30:30) (11,13). After developing and drying, the plates were sprayed with Grote's reagent which is adequate for identification of thiourea derivatives by blue color spots (13).

GC analysis

A Perkin Elmer 8500 instrument using BP1 capillary column (30 m \times 0.25 mm; film thickness: 0.25 μm) was used for analysis of volatile constituents and DCM extracts. The

carrier gas was nitrogen with a flow rate of 2 ml/min. The oven temperature was programmed from 60°C to 275°C at 4°C/min. Injector and detector temperatures were set at 275°C and 280°C, respectively (5).

GC/MS analysis

Determination of volatile constituents was performed on an Agilent 7890 A with Mass detector under the following conditions: injection volume, 0.1 µl, HP-5 MS capillary column (30 m× 0.25 mm; film thickness: 0.25 µm); carrier gas, He; flow rate, 2 ml/min; injector temperature, 250°C; temperature program, 60-275°C at 4°C/min; mass spectra: electronic impact, ionization potential 70 eV, ion source temperature 250°C, ionization current 1000 µA, resolution 1000 and mass range 30-300. Identification of the constituents was performed by computer matching against the library spectra (library database Wiley 275), their retention indices with reference to an n-alkane series in a temperature programmed run, interpreting their fragmentation pattern and comparison of the mass spectra with the literature data (14).

RESULTS

The identified volatile constituents and their

relative percentages in the seed coat and stem of *M. peregrina* are listed in Table 1. As it is clear, isobutyl isothiocyanate with 93% and 51.6% was the main volatile isothiocyanate of the stem and seed coat, respectively. Mass spectra of isopropyl isothiocyanate (Fig. 1), isobutyl isothiocyanate (Fig. 2) and *sec*-butyl isothiocyanate (Fig. 3) have been shown.

M. peregrina callus culture was produced successfully from young leaves on Murashige and Skoog medium with Hormones. Three subculture of the callus to the same medium resulted in yellow color, slow growing and hard calli. Transferring the callus formed on Murashige and Skoog medium with hormones to different treatment media resulted in different morphological characteristics (Table 2).

The GC analysis of the seed extract showed two main peaks with retention times of 5.2 and 8.7 min, respectively while such peaks related to isothiocyanates were not found in callus and cell suspension cultures intervened with mentioned treatments.

Analysis of the extracts of seed and cultured cells after thiourea derivatization on TLC plate showed only two blue spots for seed extract. Their R_f values were 0.6 and 0.4, respectively. As it was predictable by GC analysis, no isothiocyanates from cultured cells was detected on TLC.

Table 1. Volatile components of the seed coat and stem of *M. peregrina* (Forssk.) Fiori, Agricolto.

Components	RI (Retention indices)	Seed coat (%)	Stem (%)	Identification method
n- Octane	800.0	20.2	-	RI
Isopropylisothiocyanate	835.4	7.0	4.2	RI+MS
<i>Sec</i> butyl isothiocyanate	937.0	-	t ^a	RI+MS
Benzaldehyde	963.0	t	-	RI
Isobutyl isothiocyanate	974.0	51.5	92.9	RI+MS
p- cymene	1026.2	t	-	RI
n-nonanol	1170.8	t	-	RI
Isobornyl acetate	1286.0	-	2.8	RI
Hexadecanoic acid	1973.0	21.0	-	RI+MS
Total identified constituents		99.7	99.9	

a: t=trace (< 0.1%)

Table 2. Morphological characteristics of *M. peregrina* callus cultures, grown on different treated culture media.

Treatments	Calli morphological characteristics
Methionine	Yellow to brown- almost fast growing- fresh
Cysteine	Yellow to brown- almost fast growing- fresh some parts hard
Phenylalanine	Yellow to green- loose-fresh- fast growing
Salicylic acid	Yellow to brown- inside parts hard- slow growing

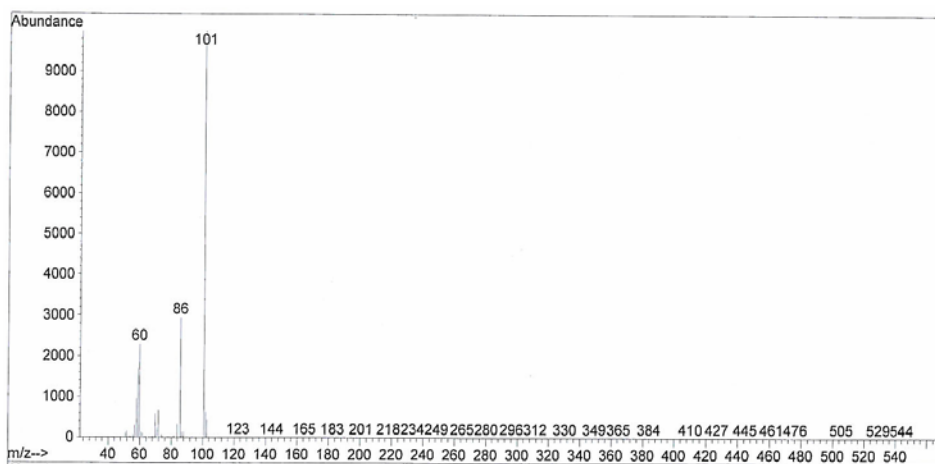


Fig. 1. Mass spectrum of 2-isothiocyanato propane (isopropyl isothiocyanate) on HP-5 MS capillary column; RI=835

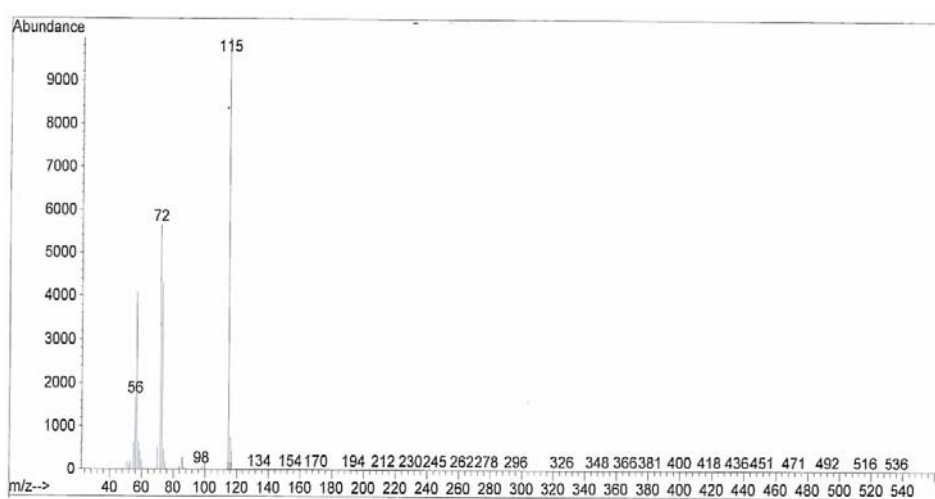


Fig. 2. Mass spectrum of 1-isothiocyanato 2-methylpropane (isobutyl isothiocyanate) on HP-5 MS capillary column; RI=974

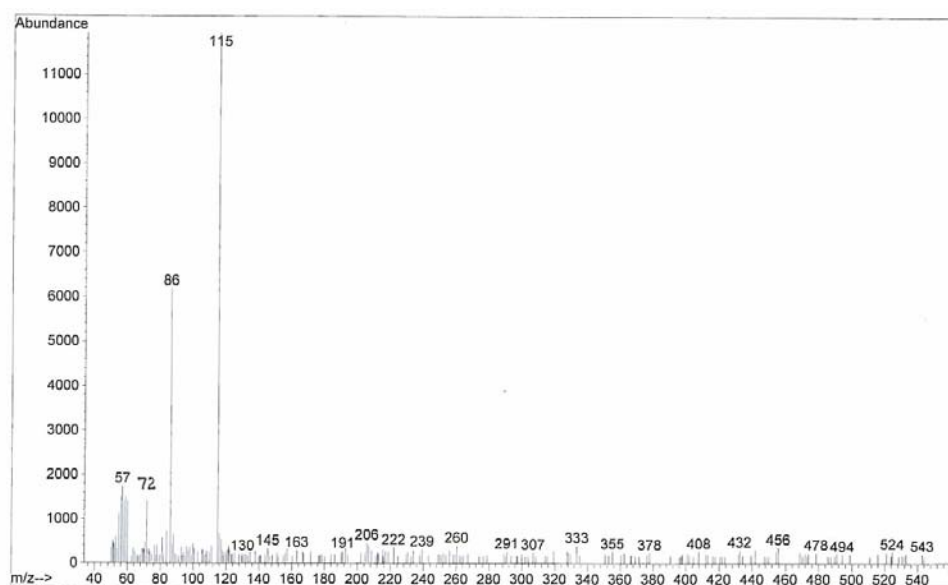


Fig. 3. Mass spectrum of 2-isothiocyanato butane (*sec*-Butyl isothiocyanate) on HP-5 MS capillary column; RI=937

DISCUSSION

The main isothiocyanates of seed coat were only isobutyl isothiocyanate (51.5%) and isopropyl isothiocyanate (7.0%). However the authors who previously analyzed the seed kernel oil of the same plant reported the occurrence of isobutyl isothiocyanate (94.0%), isopropyl isothiocyanate (4.9%), sec-butyl isothiocyanate (0.5%), n-butyl isothiocyanate (0.5%) and benzyl isothiocyanate (< 0.1%) (5).

The volatile oil of the stem contained isopropyl isothiocyanate (4.2%), isobutyl isothiocyanate (92.9%), isobornyl acetate (2.8%) and sec-butyl isothiocyanate (< 0.1%) while isothiocyanate content of the leaf of this plant was isobutyl isothiocyanate (88.5%), isopropyl isothiocyanate (10.2%) n-butyl (0.4%) and sec-butyl isothiocyanate (< 0.1%) (5). A literature survey revealed that reports on the volatile components of stem, seed coat and cultured cells of *M. peregrina* are very scarce. Several researchers have also identified glucosinolates (non-hydrolyzed isothiocyanates) in the seed of some *Moringa* species. These glucosinolates included of isopropyl, 2-methylpropyl, and traces of isobutyl and 4'-o-acetyl-4-(R-L-rhamnopyranosyloxy)-benzyl in *M. pterygosperma*, and 4-(R-L-rhamnopyranosyloxy)-benzyl in *M. oleifera* and *M. stenopetala* (15).

Although the formation of callus has been observed in some cultures of *M. oleifera* and *M. stenopetala*, there was no report on *in vitro* cultures of *M. peregrina* (16). Successful *in vitro* production of *M. peregrina* callus culture would be helpful for regeneration and micropropagation of this tree in future works.

Glucosinolates are biosynthesized from tryptophan and seven other protein amino acids including methionine and phenylalanine (17). Cysteine, as a sulphur source has the role of immediate sulphur donor in the biosynthetic pathways under normal conditions (18).

Moreover, it is proved that pathogen and herbivory- induced glucosinolate production are mediated, partially, by jasmonates, salicylic acid and ethylene, the major plant hormones related to specific defense responses (17). Therefore, providing precursor amino acids of glucosinolate biosynthetic pathway

and also elicitors in culture media could increase glucosinolate production in cell cultures.

Despite using different treatments glucosinolates hydrolyzed products were not found in callus and suspension cultures of *M. peregrina*. Some isothiocyanates were detected in *Nastirium monatum* and *Cleome chelidonii* callus and suspension cultures after feeding with 2-200 ppm L-cysteine and L-methionine or L-tryptophan (10). Also there is a report on production of isothiocyanates in *Farsetia aegyptia* suspension cultures following treatment with certain elicitors (9). However, glucosinolates were not detected in plantlet and callus cultures of rape, grown under different conditions including light, temperature, sulphur and nitrogen as well as growth regulators (19).

The disappointing results for production of glucosinolates in plant cell culture has been attributed to inadequate or lacking the factors that involved in glucosinolates biosynthesis or accumulation as well as their degradation during growth (9). Secondary metabolite synthesis and storage in plant cells almost occur in individual compartments such as secretory and accumulatory parts which are highly specialized. Since such an accumulation sites does not exist in the dedifferentiated callus cultures, very low or no secondary metabolites were obtained (20).

CONCLUSION

Generally, principal isothiocyanate constituent of seed coat and stem of *M. peregrina* was isobutyl isothiocyanate. Comparing with isothiocyanate content of other parts (seed kernel and leaf) of *M. peregrina* it seems isobutyl isothiocyanate is the main glucosinolate degradation product of aerial parts of this plant, while no isothiocyanates were found in cell cultures of *M. peregrina*. Isothiocyanates commonly present in human diet are promising cancer-preventive agents, with different mechanisms. Therefore, finding new edible sources of these compounds or their production in plant cell cultures could be medicinally important.

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