Bioengineering of Important Secondary Metabolites and Metabolic Pathways in Fenugreek (*Trigonella foenum-graecum* L.)

Mehrafarin A (Ph.D. Student)[1]*, Qaderi A (Ph.D. Student)[2], Rezazadeh Sh (Ph.D.)[3], Naghdi Badi H (Ph.D.)[1], Noormohammadi Gh (Ph.D.)[4], Zand E (Ph.D.)[5]

1- Department of Cultivation and Development, Institute of Medicinal Plants, ACECR, Karaj, Iran
2- Department of Plant Biotechnology, Institute of Medicinal Plants, ACECR, Karaj, Iran
3- Department of Pharmacognosy and Pharmaceutics, Institute of Medicinal Plants, ACECR, Karaj, Iran
4- Department of Agronomy, Science and Research Branch, Islamic Azad University (IAU), Tehran, Iran
5- Department of Weed Research, Iranian Plant Protection Research Institute, Tehran, Iran
*Corresponding author: Culture and Research (ACECR), Kavosh Boulevard, Supa Boulevard, 55th Kilometer of Tehran-Gazvin Freeway, Pouleh Kordan, Karaj, Iran
Tel: +98-261-4764010-19, Fax: +98-261- 4764021
Email: Mehrafarinali@hotmail.com

Receive: 21 Jul. 2010 **Acceptance:** 4 Sep. 2010

Abstract

Fenugreek (*Trigonella foenum-graecum* L.) has a long and respected history of medicinal uses in Middle East and Persian medicine. The hypocholesterolaemic and hypoglycaemic effects of fenugreek were attributed to its major steroidal sapogenin, diosgenin and its major alkaloid, trigonelline. The knowledge of diosgenin and trigonelline biosynthesis is derived from studies of cholesterol and nicotinic acid production through acetyl-CoA→ mevalonate→ isopentenyl pyrophosphate→ squalene→ lanosterol→ cholesterol→ diosgenin and quinolinic acid→ nicotinamide adenine dinucleotide→ nicotinamide→ nicotinic acid→ trigonelline pathways, respectively. This paper reviews the secondary metabolites and metabolic pathways of diosgenin and trigonelline production in fenugreek as a medicinal plant and economical crop.

Keywords: Fenugreek, Secondary metabolites, Metabolic Pathways, Diosgenin, Trigonelline

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

Introduction

Fenugreek (*Trigonella foenum-graecum* L.) is an annual crop and dicotyledonous plant belonging to the subfamily Papilionaceae, family Leguminacae (the Fabaceae) with trifoliate leaves, branched stem, white flowers, roots bearing nodules and golden yellow seeds [1] (Fig. 1). Fenugreek is an ancient crop plant. Although grown as a spice in most parts of the world, the species name "foenum-graecum" means "Greek hay" indicating its use as a forage crop in the past [1, 2]. Fenugreek is also known as one of the oldest medicinal plants recognized in recorded history [1]. Linnaeus [3] has described the species *Trigonella foenum-graecum* first. De Candolle [4] and Fazli and Hardman [5] notice that fenugreek grows wild in Punjab and Kashmir, in the desert of Mesopotamia and Persia, in Asia Minor and in some countries in Southern Europe such as Greece, Italy and Spain. De Candolle [4] believes that the origin of fenugreek should be Asia rather than Southern Europe, because if a plant of fenugreek nature was indigenous in Southern Europe it would be far more common. Many authors maintain that the direct ancestor of cultivated fenugreek is the wild *Trigonella gladiata* Ste. [2].

Fenugreek seed is an important source of steroidal sapogenins such as diosgenin which are used extensively by both pharmaceutical and nutraceutical industries. Diosgenin is often used as a raw precursor for the production of steroidal drugs and hormones such as testosterone, glucocorticoids and progesterone [5, 6]. McAnuff et al. [7] and Acharya et al. [1] reported that steroidal sapogenins are effective agents for the treatment of hypocholesterolemia, a disorder often associated with diabetes [1]. Natural diosgenin is mainly procured from the tubers of certain wild species of Mexican yam (*Dioscorea* species). However this process is both time consuming and costly, requiring several years before the yam tubers grow to a size where they possess a significant enough concentration of diosgenin to be used as a source of commercial and pharmaceutical reagents [1, 8]. Fenugreek may be a viable alternative for production of diosgenin because of its shorter growing cycle, lower production costs and consistent yield and quality [2, 9].

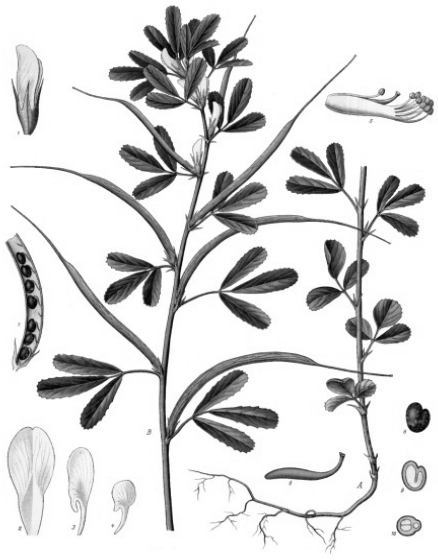

Fig. 1- ***Trigonella foenum-graecum*** **L.** [1]

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

Trigonelline [24], a methylbetaine derivative of nicotinic acid, with mild hypoglycemic [5, 33, 49] and antipellagra action [5, 9] is the main N-compound of the seeds. If the seeds are sufficiently roasted about 2/3 of trigonelline is converted into nicotinic acid [9, 43]. A higher value of c. 0.38 percent for trigonelline and c. 0.003 percent for nicotinic acid content has also been reported [28]. Trigonelline is regarded as a physiological active compound in plants inducing leaf movements [57], accumulating upon stress [8] and acting as an osmoprotectant [46]. Moreover, trigonelline has been found to function as a hormone that is involved in the control of the cell cycle in plants [56].

In biochemistry, a metabolic pathway is a series of chemical reactions occurring within a cell, catalyzed by enzymes, resulting in either the formation of a metabolic product to be used or stored by the cell, or the initiation of another metabolic pathway (then called a flux generating step). Many pathways are elaborate, and involve a step by step modification of the initial substance to shape it into the product with the exact chemical structure desired [26]. Various metabolic pathways within a cell form the cell's metabolic network. In the metabolic pathway a substrate enters depending on the needs of the cell, i.e. the specific combination of concentrations of the anabolical and catabolical end products (the energetics of the flux-generating step). Metabolic pathways include the principal chemical, mostly enzyme-dependent, reactions that an organism needs to keep its homoeostasis [26].

Characterisation of metabolic pathways is a multi-disciplinary activity. It requires the identification of metabolic intermediates and the demonstration of a plausible reaction sequence, followed by the isolation and characterization of the individual enzymes responsible [61]. A biosynthetic investigation may be stimulated or awakened as a result of interest in the pharmaceutical activity of a compound. The aim of this study is to outline current understanding of fenugreek secondary metabolism and its regulation.

Chemical Constituents

The biological and pharmacological actions of fenugreek are attributed to the variety of its constituents, namely: steroids, N-compounds, polyphenolic substances, volatile constituents, amino acids, etc [2]. Fenugreek seed contains 45-60% carbohydrates, mainly mucilaginous fiber (galactomannans), 20-30% proteins high in lysine and tryptophan, 5-10% fixed oils (lipids), pyridine alkaloids, mainly trigonelline (0.2-0.36%), choline (0.5%), gentianine and carpaine, the flavonoids apigenin, luteolin, orientin, quercetin, vitexin and isovitexin, free amino acids, such as 4-hydroxyisoleucine (0.09%), arginine, histidine and lysine, calcium and iron, saponins (0.6-1.7%), glycosides yielding steroidal sapogenins on hydrolysis (diosgenin, yamogenin, tigogenin, neotigogenin), cholesterol and sitosterol, vitamins A, B_1, C and nicotinic acid and 0.015% volatile oils (n-alkanes and sesquiterpenes) [22, 23]. In general, fenugreek contains two important chemical constituents with medicinal value; *i.e.* **1)** diosgenin, a kind of steroidal sapogenin and **2)** trigonelline, a kind of N-alkaloid. Diosgenin and trigonelline present in the seed and leaves of this legume plant contribute to anti-diabetic and hypocholesterolaemic properties attributed to the plant [2].

Mevalonate pathway and diosgenin biosynthesis

The mevalonate pathway or HMG-CoA reductase pathway or mevalonate-dependent

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

route or isoprenoid pathway, is an important cellular metabolic pathway present in all higher eukaryotes and many bacteria. The mevalonate pathway is responsible for the biosynthesis of numerous essential molecules including prenyl groups, coenzyme Q, dolichol, and sterols such as cholesterol [24]. The knowledge of steroidal biosynthesis is derived from studies of cholesterol production through Acetate→ Mevalonate→ Isopentenyl pyrophosphate→ Squalene pathway. The biosynthesis of cholesterol involves cyclization of aliphatic triterpene-squalene [20]. Cholesterol has been found to be an effective precursor for diosgenin. Fenugreek is potentially useful commercial source of diosgenin [25].

Mevalonate pathway and cholesterol biosynthesis

Mevalonate is the key precursor for synthesis of cholesterol and related isoprenoid compounds. Cholesterol is the main sterol involved in the biosynthesis of steroidal sapogenins [2]. Cholesterol is one of the isopernoids, synthesis of which start from acetyl CoA. In a long and complex reaction chain, the C_{27} sterol is built up from C_2 components. The biosynthesis of cholesterol can be divided into four sections. In the first (1), Mevalonate, a C_6 compound, arises from three molecules of acetyl CoA (Fig. 2). In the second part (2), mevalonate is converted into isopentenyl diphosphate (pyrophosphate), the "active isoprene" (Fig. 3). In the third part (3), six of these C_5 molecules are linked to produce squalene, a C_{30} compound (Fig.4). Finally (4), squalene undergoes cyclization, with three C atoms being removed, to yield cholesterol (Fig. 5). The illustration only shows the most important intermediates in biosynthesis [26].

(1) Formation of mevalonate. The conversion of acetyl CoA to acetoacetyl CoA and then to 3-hydroxy-3-methylglutaryl CoA (3-HMG CoA) corresponds to the biosynthetic pathway for ketone bodies. In this case, however, the synthesis occurs not in the mitochondria as in ketone body synthesis, but in the smooth endoplasmic reticulum. In the next step, the 3-HMG group is cleaved from the CoA and at the same time reduced to mevalonate with the help of $NADPH+H^+$, 3-HMG CoA reductase is the key enzyme in cholesterol biosynthesis. It is regulated by repression of transcription (effect: oxysterols such as cholesterol) and by interconversion (effectors: hormones) [26, 27] (Fig. 2).

(2) Formation of isopentenyl diphosphate (pyrophosphate). After phosphorylation, mevalonate is decarboxylated to isopentenyl diphosphoate, with consumption of ATP. This is the component from which all of the isoprenoids are built [26, 27] (Fig. 3).

(3) Formation of squalene. Isopentenyl diphosphate undergoes isomerization to form dimethylallyl diphosphate. The two C_5 molecules condense to yield geranyl diphosphate, and the addition of another isopentenyl diphosphate produces farnesyl diphosphate. This can then undergo dimerization, in a head-to-head reaction, to yield squalene. Farensyl diphosphate is also the starting-point for other polyisoprenoids, such as dolichol and ubiquinone [26] (Fig. 4).

(4) Formation of cholesterol. Squalene, a linear isoprenoid, is cyclized, with O_2 being consumed, to form lanosterol, a C_{30} sterol. Three methyl groups are cleaved from this in the subsequent reaction steps, to yield the end product cholesterol. Some of these reactions are catalyzed by cytochrome P450 systems [26] (Fig. 5).

The endergonic biosynthetic pathway described above is located entirely in the smooth endoplasmic reticulum. The energy needed comes from the CoA derivatives used

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

and from ATP. The reducing agent in the formation of mevalonate and squalene, as well as in the final steps of cholesterol biosynthesis, is $NADPH+H^+$ [26, 27].

The division of the intermediates of the reaction pathway into three groups is characteristic: CoA compounds, diphosphates, and highly lipophilic, poorly soluble compounds (squalene to cholesterol), which are bound to sterol carriers in the cell [26]. In general, biosynthetic enzymes of mevalonate pathway and cholesterol are showed in Table 1 [28].

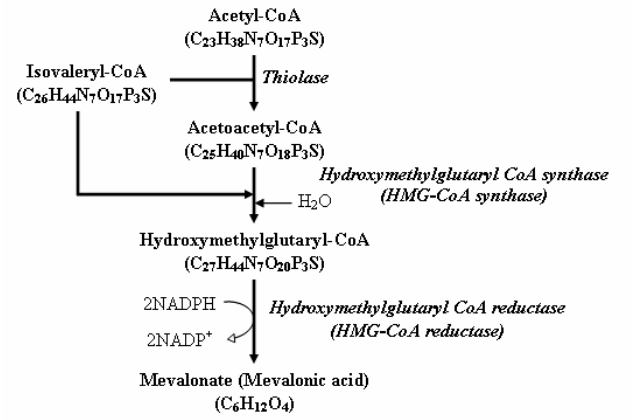

Fig. 2 - Possible metabolic pathway of biosynthesis of mevalonate from acetyl-CoA [20, 24, 26, 27]

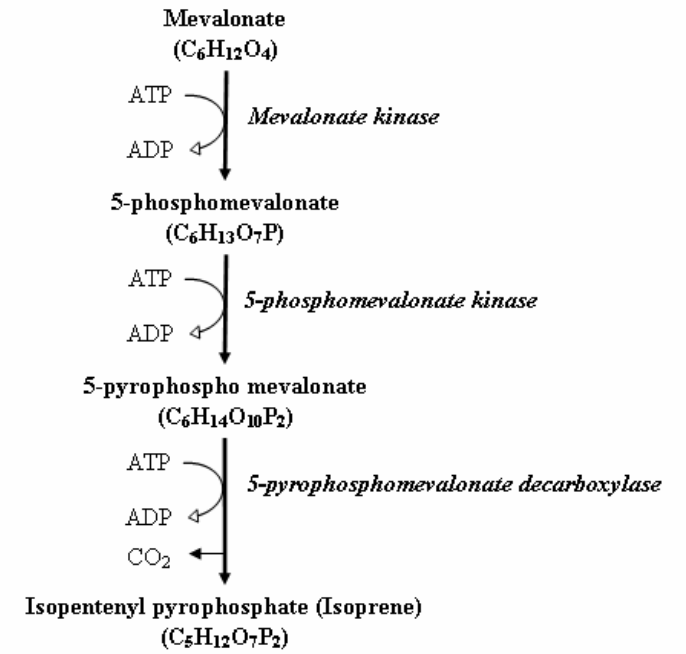

Fig. 3 - Possible Metabolic pathway of biosynthesis of isopentenyl pyrophosphate from mevalonate [20, 26, 27]

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

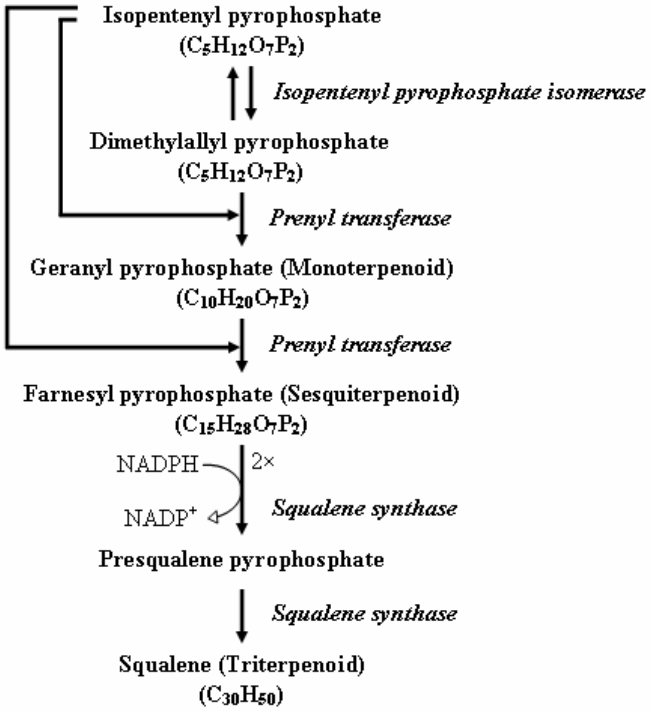

Fig. 4 - Possible metabolic pathway of biosynthesis of squalene from isopentenyl pyrophosphate [20, 26, 27]

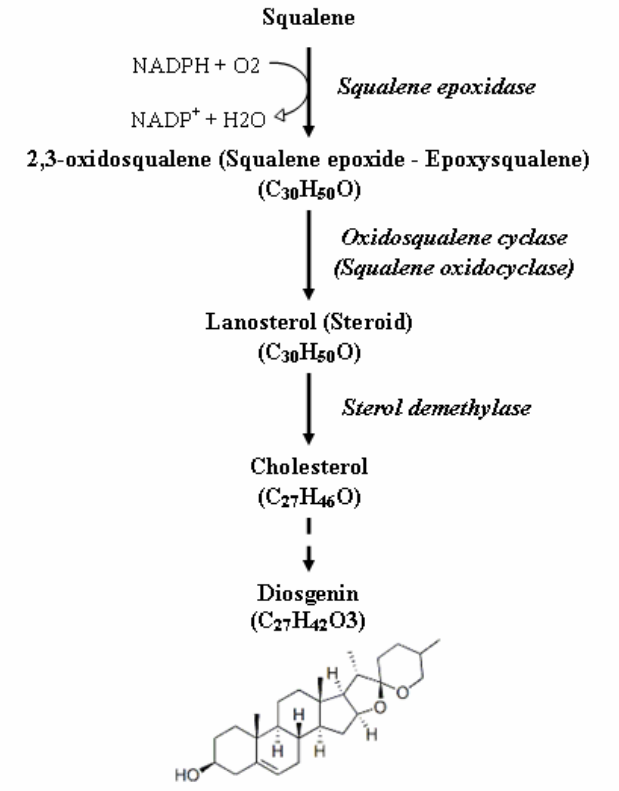

Fig. 5 - Possible metabolic pathway of biosynthesis of diosgenin from squalene [20, 26, 27, 29]

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

Table 1- Metabolic enzymes of mevalonate pathway and cholesterol biosynthesis [24, 25, 27, 28, 29]

Row	EC number*	Accepted name	Class
1	EC 2.3.3.10	*Hydroxymethylglutaryl-CoA synthase*	Transferases; Acyltransferases; Acyl groups converted into alkyl groups on transfer
2	EC 1.1.1.34**	*Hydroxymethylglutaryl-CoA reductase*	Oxidoreductases; Acting on the CH-OH group of donors; With NAD^+ or $NADP^+$ as acceptor
3	EC 2.7.1.36**	*Mevalonate kinase*	Transferases; Transferring phosphorus-containing groups; Phosphotransferases with an alcohol group as acceptor
4	EC 2.7.4.2	*Phosphomevalonate kinase*	Transferases; Transferring phosphorus-containing groups; Phosphotransferases with a phosphate group as acceptor
5	EC 4.1.1.33	*Diphosphomevalonate decarboxylase* or *pyrophosphomevalonate decarboxylase*	Lyases; Carbon-carbon lyases; Carboxy-lyases
6	EC 5.3.3.2**	*Isopentenyl-diphosphate Δ-isomerase* or *isopentenylpyrophosphate Δ-isomerase*	Isomerases; Intramolecular oxidoreductases; Transposing C=C bonds
7	EC 2.5.1.1	*Dimethylallyltranstransferase* or *prenyltransferase*	Transferases; Transferring alkyl or aryl groups, other than methyl groups
8	EC 2.5.1.21	*Squalene synthase*	Transferases; Transferring alkyl or aryl groups, other than methyl groups
9	EC 1.14.99.7**	*Squalene monooxygenase* or *squalene epoxidase*	Oxidoreductases; Acting on paired donors, with O_2 as oxidant and incorporation or reduction of oxygen. The oxygen incorporated need not be derived from O_2; Miscellaneous
10	EC 5.4.99.7	*Lanosterol synthase* or *oxidosqualene cyclase*	Isomerases; Intramolecular transferases; Transferring other groups
11	EC 1.14.13.70**	*Sterol 14-demethylase*	Oxidoreductases; Acting on paired donors, with O_2 as oxidant and incorporation or reduction of oxygen. The oxygen incorporated need not be derived from O_2; With NADH or NADPH as one donor, and incorporation of one atom of oxygen into the other donor

*Enzyme commission (EC) codes, base on recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) on the Nomenclature and Classification of Enzymes by the Reactions they Catalyse.
**Key enzyme

Regulation of cholesterol synthesis in mevalonate pathway

HMG-CoA Reductase, the rate-determining step on the pathway for synthesis of cholesterol, is a major control point. Regulation relating to cellular uptake of cholesterol will be discussed in the next class.

1. Short-term regulation. HMG-CoA Reductase is inhibited by phosphorylation, catalyzed by AMP-Dependent Protein Kinase (which also regulates fatty acid synthesis and catabolism). This kinase is active when cellular AMP is high, corresponding to when ATP is low. Thus, when cellular ATP is low, energy is not expended in synthesizing cholesterol [26, 27].

2. Long-term regulation of cholesterol synthesis is by varied formation and degradation of HMG-CoA Reductase and other enzymes of the pathway for synthesis of cholesterol.

Regulated proteolysis of HMG-CoA Reductase: Degradation of HMG-CoA Reductase is stimulated by cholesterol, by

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

oxidized derivatives of cholesterol, by mevalonate, and by farnesol (dephosphorylated farnesyl pyrophosphate). HMG-CoA Reductase includes a transmembrane sterol-sensing domain that has a role in activating degradation of the enzyme via the proteasome. (The proteasome is discussed separately in the section on protein degradation) [24, 25].

Regulated transcription: A family of transcription factors designated SREBP (sterol regulatory element binding proteins) regulate synthesis of cholesterol and fatty acids. Of these, SREBP-2 mainly regulates cholesterol synthesis. (SREBP-1c mainly regulates fatty acid synthesis.) When sterol levels are low, SREBP-2 is released by cleavage of a membrane-bound precursor protein. SREBP-2 activates transcription of genes for HMG-CoA Reductase and other enzymes of the pathway for cholesterol synthesis [24, 25, 26].

Biosynthesis of diosgenin:

Steroidal sapogenins (spirostanols) e.g. diosgenin are synthesized from cholesterol in several plants, but the intermediate biosynthetic steps have not yet been completely elucidated. Radioactive cholesterol was converted to the sapogenins diosgenin and kryptogenin. Steroidal saponins in which the side chain is held open by glycoside formation (furostanols) are naturally occurring glycosides in several plant species [29]. These glycosides are converted *in vitro* to spirostanols by the elimination of the glucose molecule at C-26 and ring closure by the action of glucosidases. These results supported the theory that in sapogenin biosynthesis oxygenation occurs first at C-26 followed by cyclization of the sterol side-chain [29]. These results also suggest that the biosynthesis of diosgenin from cholesterol proceeds via furostanol, similar to the proposed biosynthetic pathways of other spirostanols from their corresponding furostanols in various plants [25, 29].

Fenugreek sapogenins are C_{27} sterols in which the side-chain of cholesterol has undergone modification to produce either a spiroketal (spirostane saponins), e.g. dioscin, or a hemiketal (furostane saponins), e.g. protodioscin (Fig. 6). Acid hydrolysis of either dioscin or protodioscin liberates the aglycone diosgenin; the hydrolytic conversion of protodioscin into diosgenin is analogous to the biosynthetic sequence [25]. The spiroketal function is derived from the cholesterol side-chain by a series of oxygenation reactions, hydroxylating one of the terminal methyl groups and at C-16, and then producing a ketone function at C-22 (Fig. 7). This proposed intermediate is transformed into the hemiketal and then the spiroketal [25] (Fig. 7). The chirality at C-22 is fixed by the stereospecificity in the formation of the ketal, whilst the different possible stereochemistries at C-25 are dictated by whether C-26 or C-27 is hydroxylated in the earlier step. Enzymic glycosylation at the 3-hydroxyl of spirostane sapogenins has been reported, but knowledge of other steps at the enzymic level is lacking. Furostane derivatives, e.g. protodioscin (Fig. 6), can co-occur with spirostanes, and undoubtedly represent glycosylation of the intermediate hemiketal at the 26-hydroxyl. These compounds are readily hydrolysed and then spontaneously cyclize to the spiroketal [25].

Pyridine nucleotide cycle and trigonelline biosynthesis

Trigonelline, or *N*-methylnicotinic acid, is a secondary metabolite derived from pyridine nucleotides. Since it was first isolated from *Trigonella foenum-graecum* [30], this compound has been found in many plant

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

species (including pea, hemp, coffee, soybean and potatoes) [31]. Pyridine (nicotinamide adenine) nucleotides are also important nucleotides for life, because they are coenzymes for redox reactions. Many legumes produce trigonelline as a secondary metabolite derived from NAD [32]. Nicotinic acid formed from NAD via nicotinamide may be preferentially utilized for NAD formation, and the remainder may be reserved for future needs as a form of trigonelline [31] (Fig. 8).

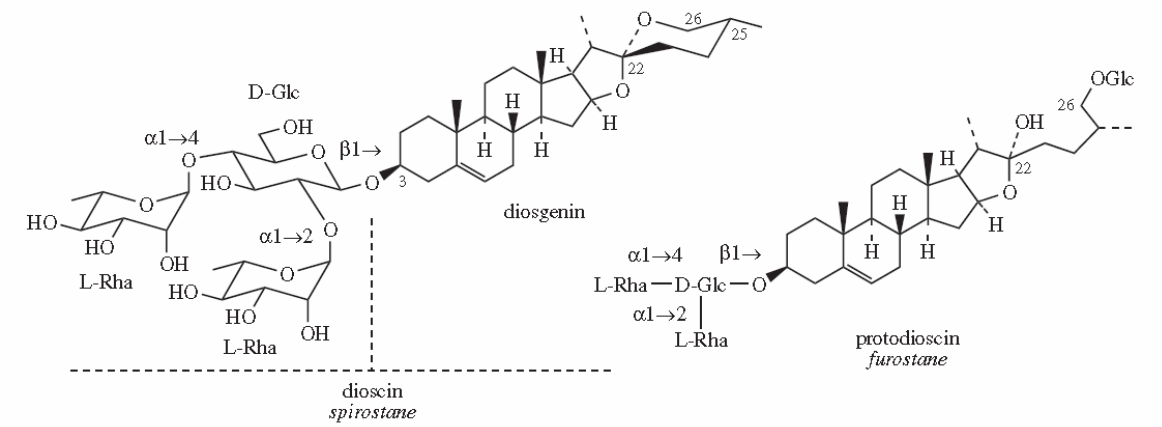

Fig. 6 - Possible metabolic steps of biosynthesis of diosgenin from cholesterol [25, 29]

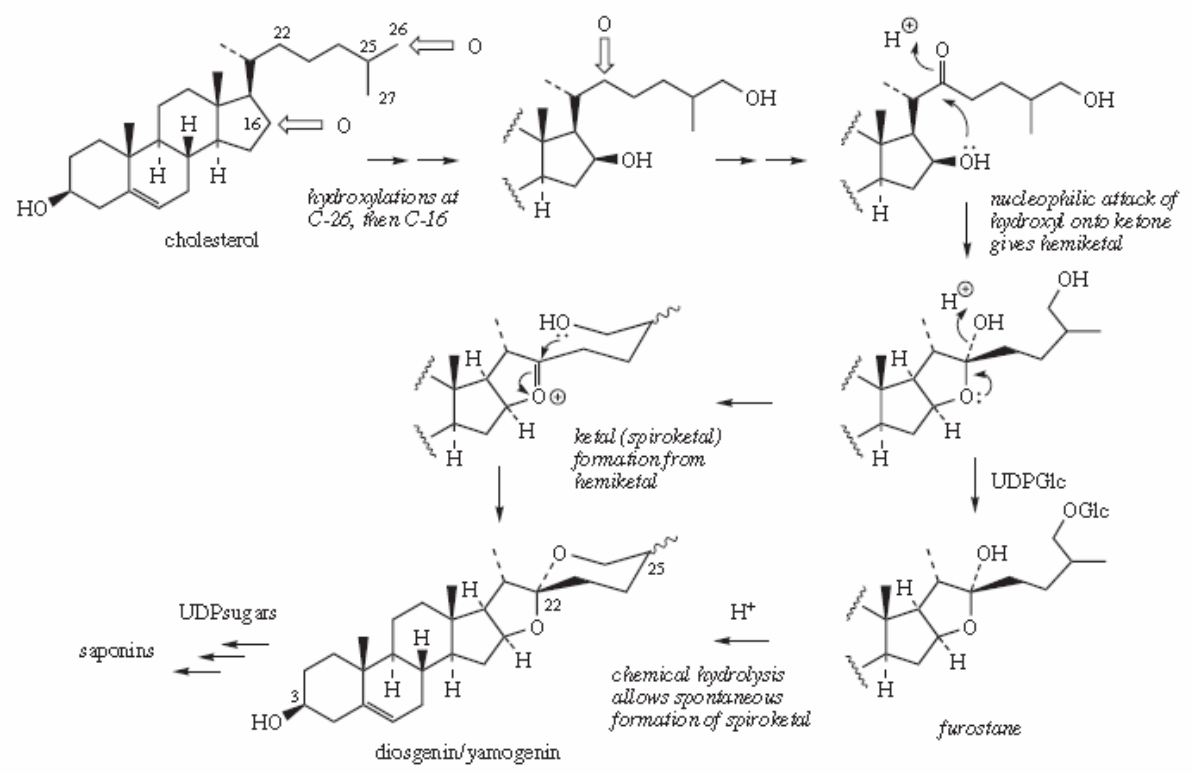

Fig. 7 - Possible metabolic steps of biosynthesis of diosgenin from cholesterol [25, 29]

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

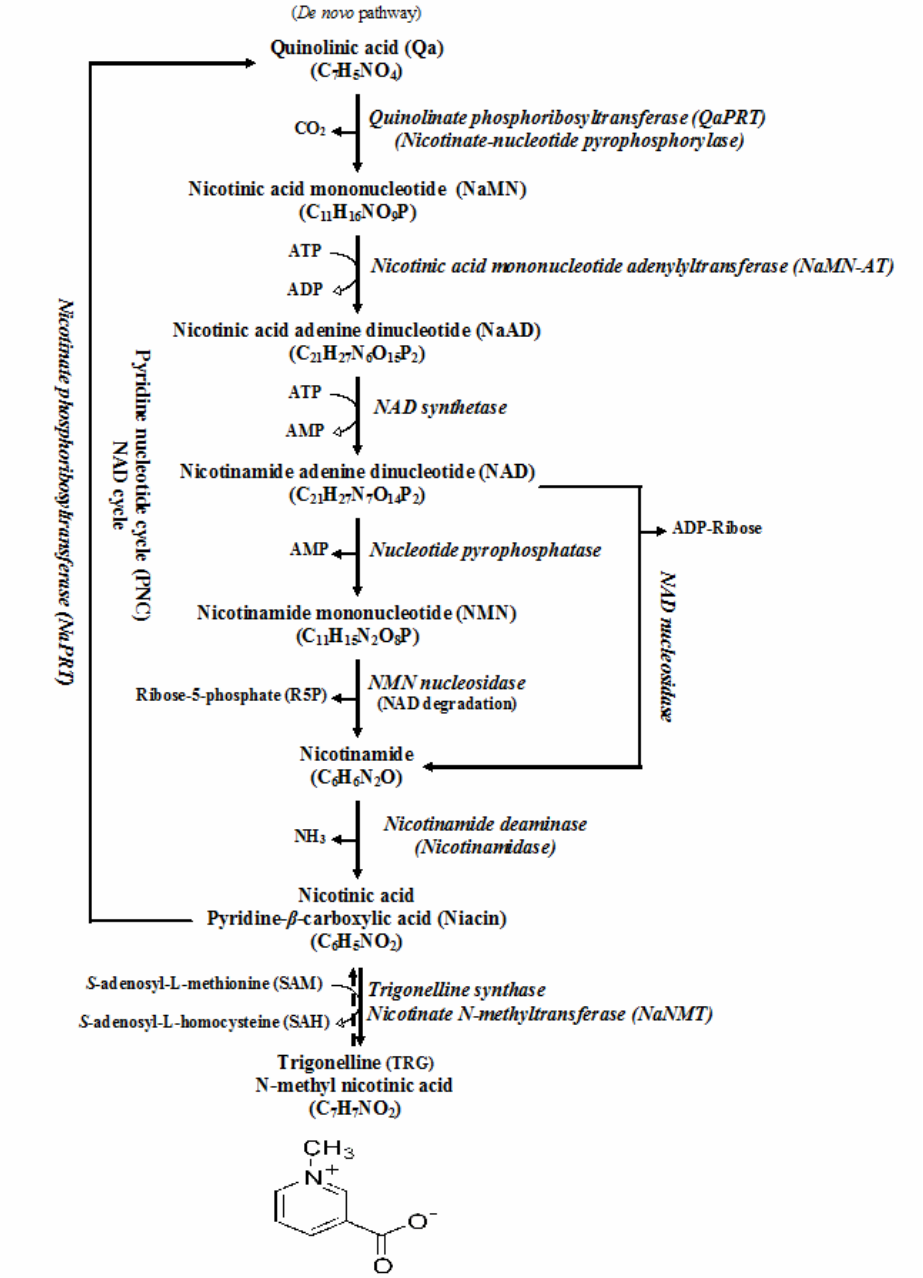

Fig. 8 - Possible metabolic pathway of biosynthesis of Trigonelline and Pyridine nucleotides [31, 32, 34, 35, 40, 41, 42, 43]

Biosynthesis of pyridine alkaloids

The direct precursor of trigonelline is nicotinic acid [33]. In plants, nicotinic acid is produced as a degradation product of NAD [34, 35] (Fig. 8). The *de novo* and salvage pathways of NAD synthesis (Fig. 8) have been investigated in several plants. Zheng and Ashihara [35] have reported that trigonelline and its metabolic synthesis from nicotinic acid are distributed in all parts of coffee seedlings. In bacteria and plants, quinolinic acid, an intermediate of the *de novo* pathway, is

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

synthesised from aspartate and triose phosphate via the so-called aspartate pathway [36, 37]. In contrast, quinolinic acid is formed in animals by a tryptophane-kynurenine pathway. A recent bioinformatic search of genome databases suggests that the tryptophane-kynurenine pathway is present in the Oryza sativa [36]. Nicotinamide and nicotinic acid formed by NAD degradation pathways are re-utilized (salvaged) for NAD synthesis [32, 38, 39]. The routes of degradation and salvage of pyridine compounds have been called the pyridine nucleotide cycle (Fig. 8).

Zheng et al. [31] studied the metabolism of quinolinic acid, nicotinamide, and nicotinic acid. Their data suggest that, in addition to the *de novo* pathway for NAD synthesis, the six membered pyridine nucleotide cycle (PNC), NAD → NMN → nicotinamide → nicotinic acid → NaMN → NaAD → NAD operates in plants [39]. Some minor pathways may also be operative in part; for example, nicotinamide may be also produced from NAD by an ADP-ribosylation reaction, and NaMN may be formed by an alternative route from nicotinic acid [31] (Fig. 8). Degradation of NAD by the pyridine nucleotide cycle is the major source of nicotinic acid, although direct nicotinic acid formation from NaMN formed by the *de novo* pyridine nucleotide biosynthesis cannot be excluded. Trigonelline is produced when nicotinic acid is in excess for pyridine nucleotide synthesis [31].

Biosynthesis of trigonelline

Trigonelline is synthesized by *S*-adenosyl-*L*-methionine (SAM) dependent nicotinate *N*-methyltransferase, which has been found in crude extracts of the pea [33] (Fig. 8). This enzyme has been purified from cultured *Glycine max* cells [40] and *Lemna paucicostata* [41]. Although nicotinic acid *N*-methyltransferase (trigonelline synthase) activity was detected in a cell-free preparation from coffee [42], no purification has been carried out. The gene encoding trigonelline synthase has not yet been cloned from any organism.

In plant, trigonelline is demethylated to nicotinic acid and utilized for NAD synthesis (degradation of trigonelline). Trigonelline demethylating activity has been found in extracts of some plant leaves, including pine leaves [43]. Metabolism of pyridine nucleotides has been investigated by some authors in leguminous seeds during germination [44, 45]. The trigonelline synthesis in pericarps is much higher than that in seeds, but its content in seeds is higher than pericarps, so that some of the trigonelline synthesized in the pericarps may be transported to seeds. Trigonelline in seeds may be utilized during germination, as its content decreases [31]. Shimizu and Mazzafera [46] investigated changes in the trigonelline content of coffee seeds during the very early stages of germination. Trigonelline accumulated in seeds is converted to nicotinic acid during germination, and is used for the NAD synthesis. In this case, trigonelline acts as a reservoir of nicotinic acid in plants. Part of the nicotinic acid formed from trigonelline is further degraded. Willeke et al. [47] stated that degradation of nicotinic acid could be observed only in cell cultures producing the sugar conjugates of nicotinic acid, and that nicotinic acid degradation does not involve free 6-hydroxynicotinic acid. However, the degradation route(s) of pyridine ring of trigonelline in plants is still unclear. In general, biosynthetic enzymes of pyridine nucleotide cycle and trigonelline are showed in Table 2 [28].

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

Table 2- Metabolic enzymes of pyridine nucleotide cycle and trigonelline biosynthesis [28, 31, 32, 35, 42]

Row	EC number*	Accepted name	Class
1	EC 2.4.2.19**	*Nicotinate-nucleotide diphosphorylase* or *quinolinate phosphoribosyltransferase*	Transferases; Glycosyltransferases; Pentosyltransferases
2	EC 2.7.7.18	*Nicotinate-nucleotide adenylyltransferase* or *nicotinate mononucleotide adenylyltransferase*	Transferases; Transferring phosphorus-containing groups; Nucleotidyltransferases
3	EC 6.3.5.1**	*NAD^+ synthase*	Ligases; Forming carbon-nitrogen bonds; Carbon-nitrogen ligases with glutamine as amido-N-donor
4	EC 3.6.1.9	*Nucleotide diphosphatase* or *nucleotide pyrophosphatase*	Hydrolases; Acting on acid anhydrides; In phosphorus-containing anhydrides
5	EC 3.2.2.14	*NMN nucleosidase*	Hydrolases; Glycosylases; Hydrolysing N-glycosyl compounds
6	EC 3.2.2.5	*NAD^+ nucleosidase*	Hydrolases; Glycosylases; Hydrolysing N-glycosyl compounds
7	EC 3.5.1.19	*Nicotinamidase* or *nicotinamide deaminase*	Hydrolases; Acting on carbon-nitrogen bonds, other than peptide bonds; In linear amides
8	EC 2.4.2.11	*Nicotinate phosphoribosyltransferase*	Transferases; Glycosyltransferases; Pentosyltransferases
9	EC 2.1.1.7**	*Nicotinate N-methyltransferase*	Transferases; Transferring one-carbon groups; Methyltransferases

*Enzyme commission (EC) codes, base on recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) on the Nomenclature and Classification of Enzymes by the Reactions they Catalyse.
**Key enzyme

Several physiological functions of trigonelline have been proposed [48], but little is known about the biosynthesis and metabolism of this compound in plants. Using pea seedlings, Evans and co-workers suggested that trigonelline is a plant hormone present in cotyledons, and that it promotes cell arrest in G_2 during cell maturation in roots and shoots [32, 49, 50]. The molecular reason for that seems to be a specific interference of trigonelline with the DNA replication process causing an elongated cell cycle and impair root elongation [51]. The G_2 cellular arrest caused by trigonelline was found to depend on the age of the organism. Older seedlings of *Pisum sativum* produce an unusual substituted pyrrole that has been identified as an endogenous control factor that could override trigonelline induced cellular arrest [52].

Cytogenetic and breeding of fenugreek

Fenugreek according to Darlington and Wylie [53] has 2n=16 chromosomes, while Joshi and Raghuvanshi [54] have investigated the presence of B-chromosomes. Singh and Singh [55] isolated five double trisomics along with primary trisomics from the progenies of autotriploids, which had 2n+1+1=8 chromosomes.

Artifical crossing of fenugreek is difficult because it is largely self-pollinated [1, 2]. Consequently selections among world accessions and mutation breeding have been advocated as the best ways to improve the crop [2], and much of the breeding with fenugreek has utilized these two approaches [56]. The chemical mutagens belong to different groups and very little is known about the action of most of them [57]. A lot of fenugreek mutants have been isolated by the treatment of dry seeds with different chemical mutagens [58, 59], while shoot apexes of fenugreek treated by colchicine produced tetraploid plants with promising economic characteristics [60]. The effect of mutagens on tissue cultures of fenugreek with UV-irradiation, ethyl methane

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

sulphonate (EMS), methyl methane sulphonate (MMS), and sodium azide (NaN_3) increased steroidal sapogenin about two- to three-fold [2, 61].

Tissue cultures

Fenugreek tissue and cell cultures have been used for either plant regeneration or for the production of secondary products of economic interest. Among these products are diosgenin and trigonelline (a saponin and an alkaloid with therapeutic properties), which are constituents of fenugreek seeds [62, 63, 64]. The development of fenugreek calli has been achieved after shoot or root culture from 4 day old seedlings upon culturing on Gamborg's B-5 modified medium supplemented with hormones. From these calli have been produced cell suspension cultures, the content of which in trigonelline was appreciably higher than that of the calli [65]. Also, for diosgenin production hair root cultures [63] and cultures from calli, which were developed from leaves, stems and roots isolated from 30 day old seedlings, have been established with *Agrobacterium rhizogenes* strain A_4 [64]. Oncina et al. [64] reported, the diosgenin levels accumulated in leaf, stem and root of fenugreek calli at 45 days (maximum production) represent 22, 10 and 27%, respectively, of the levels detected in the corresponding organs of the mother plant at 45 days.

Apart from the production of trigonelline, tissue cultures have been used for *Trigonella corniculata* L. and *Trigonella foenum-graecum* L. regeneration. In this case, calli were produced using leaves as explants. The explants were grown on Murashige and Skoog medium supplemented with casein hydrolysate or coconut milk. The first resulted in an increased number of differentiated organs per callus [2].

Regeneration of shoots have also been achieved from fenugreek protoplasts [2]. Protoplasts were isolated from the root apices of 48-h-imbibed seeds. The first divisions of root fenugreek protoplasts were observed after a 3–4 day culture and subsequent divisions gave cell colonies. However, a culture of these colonies gave only roots.

Callus cultures contain 3–4 times more trigonelline than the seeds of the plant and 12–13 times more than the roots and shoots. Even higher levels of this compound were produced by suspension cultures [2, 65]. The demand for fenugreek metabolites, mainly with a higher diosgenin and trigonelline content, prompted more directed tissue culturing efforts.

Plant growth regulators (auxins and cytokinins) are also effective triggers of secondary metabolites. Higher concentrations of NAA[1]+Kn[2] or IAA[3]+Kn promoted the yield of diosgenin in *Dioscorea bulbifera* (NAA+Kn induced much higher content. Corroborative results have been recorded in *Dioscorea deltoide*. The medium with 2,4-D favourred diosgenin production most consistently. GA[4] or Kinetin otherwise increase the steroid content in *Phaseolus aureus* and *Corylus avellana* and doubled production of diosgenin in *Solanum xanthocarpum* [66].

Conclusion

The usefulness of fenugreek production as a commercial, chemurgic, and medicinal plant is belonging to **a)** two main metabolic productions including diosgenin and trigonelline as hypocholesterolaemic and anti-diabetic components **b)** ability of this plant in increase production of secondary metabolites

1 α-Naphthelene acetic acid
2 Kinetin
3 Indole-3-acetic acid
4 Gibberlic acid

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

by biotechnological methods **c)** no side-effects comparison of synthetic diosgenin or trigonelline **d)** Short growing period (maturing about 3-5 months from sowing) with low cost production **e)** adaptation to different environments and various climates **f)** high potential of yield.

Adequate information on how secondary metabolites biosynthesis and chemical constituents can be managed for medicinal purposes needs to be developed. Metabolic engineering of natural products has been built to aim at medicinal plant improvement with the availability of modern molecular biological technologies. The identification of the mechanisms of fenugreek secondary metabolites biosynthesis in order to produce plants is appropriate for medicinal use and further research efforts. This paper reviewed the state of knowledge of mevalonate and pyridine nucleotide metabolism, with a special focus on production of diosgenin and trigonelline. Fenugreek has drawn considerable attention as a source of diosgenin and trigonelline.

In conclusion, some major techniques and methods applied for increasing of secondary metabolites content in fenugreek consist of **a)** tissue culture **b)** cell suspension culture **c)** hair root culture **d)** biological manipulations **e)** hybridisation **f)** plant growth regulators using **g)** chemical mutagens **h)** irradiation. In the future, metabolic engineering of diosgenin and trigonelline formation could therefore be a useful technology to create good quality fenugreek seeds.

However, metabolic engineering of fenugreek is an effective tool to both increase diosgenin and trigonelline yield and alter those distribution. In this order, there are some possibilities for increasing the diosgenin and trigonelline contained in the seed, either during the growing period by using different cultural techniques or during post harvest treatments by different techniques (enzymes, hormones, etc.) of germination with incubation, different conditions of incubation and fermentation by storage by the use of tissue and cell culture (static or suspension) and by biological manipulation of the steroidal and alkaloid yield. Finally, the conclusion drawn for future of fenugreek as a source of diosgenin and trigonelline is promising and economic.

Acknowledgments

We thank Nazanin Amirian and Farzaneh Akhavan for their useful assistances, comments and hard works. This work was supported by the Department of Cultivation and Development, Institute of Medicinal Plants (IMP), Academic Centre for Education Culture and Research (ACECR), in Karaj-Iran.

References

1. Acharya SN, Thomas JE, Basu SK. Fenugreek, an alternative crop for semiarid regions of North America. *Crop Sci*. 2008; 48: 841 - 53.

2. Petropoulos GA. Fenugreek, The genus Trigonella. Taylor and Francis, London and New York. 2002, pp: 1 - 255.

3. Linnaeus C. Species Plantarum, Silvius, Stockholm. 1753, pp: 1200 - 1.

4. De Candolle A. Origin of cultivated plants. Hafner, New York. 1964.

5. Fazli FRY, Hardman R. The spice, fenugreek (*Trigonella foenum-graecum* L.): Its conmmercial varieties of seed as a source

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

of diosgenin. *Tro*. *Sci*. 1968; 10: 66 - 78.

6. Raghuram TC, Sharma RD, Sivakumar B. Effect of fenugreek seeds on intravenous glucose disposition in non-insulin dependent diabetic patients. *Phytother*. *Res*. 1994; 8: 83 - 6.

7. McAnuff MA, Omoruyi FO, Morrison EYSA, Asemota HN. Plasma and liver lipid distributions in streptozotocin-induced rats fed sapogenin extract of the Jamaican bitter yam (*Dioscorea polygonoides*). *Nutr*. *Res*. 2002; 22: 1424 - 34.

8. Rosser A. The day of the yam. *Nurs*. *Times*. 1985; 81: 47 - 8.

9. Hardman R. Pharmaceutical products from plant steroids. *Trop*. *Sci*. 1969; 11: 196 - 222.

10. Karrer W. Konstitution und Vorkommen der organischen Planzenstoffe. Birkhäuser Verlag, Basel und Stuttgart. 1958, pp: 997 - 1009.

11. Bever BO, Zahnd GR. Plants with oral hypoglycaemic action. *Quart*. *J. Crude Drug Res*. 1979; 17 (3 – 4): 139 – 96.

12. Marles RJ, Farnsworth NR. Plants as sources of antidiabetic agents In: Wagnerand H and Farnsworth NR. Economic and Medicinal Plant Research. Academic Press. London. 1994, pp: 164 – 5.

13. Shani J, Goldschmied A, Joseph B, Ahronson Z, Sulman FG. Hypoglycaemic effect of *Trigonella foenum-graecum* and Lupinus termis (Leguminosae) seeds and their major alkaloids in alloxandiabetic and normal rats. *Arch. Int*. *Pharmacodyn. Ther*. 1974; 210 (10): 27 – 37.

14. Covello M. Trigonellin and nicotinic acid in *Trigonella foenum-graecum* and their relation to antipellagra activity. *Boll. Soc. Ital. Biol. Sper*. 1943; 18: 159 – 61.

15. Kühn A, Gerhard H. The trigonellin and nicotinic acid contents of semen foenugraeci. *Arch. Pharm*. 1943; 281: 378 – 9.

16. Ueda M, Yamamura S. Chemistry and Biology of Plant Leaf Movements. *Angew Chem. Int. Ed. Engl*. 2000; 39 (8): 1400 - 14.

17. Cho Y, Lightfoot DA, and Wood AJ. Trigonelline concentrations in salt stressed leaves of cultivated *Glycine max*. *Phytochem*. 1999; 52: 1235 - 8.

18. Rajasekraran LR, Aspinall D, Jones GP, Paleg LG. Stress metabolism. IX. Effect of salt stress on trigonelline accumulation in tomato. *Can. J. Plant Sci*. 2001; 81: 487 - 98.

19. Tramontano WA, Hartnett CM, Lynn DG, and Evans LS. Relationship between trigonelline concentration and promotion of cell arrest in G2 in cultured roots of *Pisum sativum*. *Phytochem*. 1989; 21 (6): 1201 - 6.

20. Khanam S. Pharmacognosy: General study of formation of secondary metabolites. Dept. of Pharmacognosy, Al-Ameen college of Pharmacy. Hosur Road, Opp. Lalbagh main Gate, Bangalore-560 027. 2007, pp: 24 - 5.

21. Walton NJ, Brown DE. Chemicals from plants, perspectives on plant secondary products. Imperial College Press. 1999, pp: 425 - 6.

22. Budavari S. The merck index: An encyclopedia of chemicals, drugs, and biologicals, 13th ed. Whitehouse Station, N.J. Merk & Co, Inc. 2001, p: 854.

23. Newall CA, Anderson LA, and Phillipson JD. Herbal medicines: A guide for healthcare professionals. The Pharmaceutical Press. London. 1996, p: 263.

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

24. Gardner RG, Hampton RY. A highly conserved signal controls degradation of 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in Eukaryotes. The Journal of Biological Chemistry. 1999; 274 (44): 31671 - 8.

25. Dewick PM. Medicinal natural products: A biosynthetic approach (3rd Edition). John Wiley & Sons, Ltd. 2009, pp: 546 - 7.

26. Koolman J, Roehm KH. Color atlas of biochemistry (3rd Edition). Thieme New York. 2005, pp: 467 - 8.

27. Liao ZH, Chen M, Gong YF, Miao ZQ, Sun XF, Tang KX. Isoprenoid biosynthesis in plants: Pathways, genes, regulation and metabolic engineering. *J. Biological Sci.* 2006; 6 (I): 209 - 19.

28. Moss GP. Recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) on the Nomenclature and Classification of Enzymes by the Reactions they Catalyse. School of Biological and Chemical Sciences, Queen Mary University of London. http://www.chem.qmul.ac.uk/iubmb/enzyme/. 2010.

29. Tal B, Tamir I, Rokem JS, Goldberg I. Isolation and characterization of an intermediate steroid metabolite in diosgenin biosynthesis in suspension cultures of *Dioscorea deltoidea* cells. *Biochem. J.* 1984; 219: 619 - 24.

30. Johns E. Ueber die alkaloide des bockshornsamens. *Ber Deut Chem Ges*. 1885; 18: 2518 - 23.

31. Zheng XQ, Nagai C, Ashihara H. Pyridine nucleotide cycle and trigonelline (N-methylnicotinic acid) synthesis in developing leaves and fruits of *Coffea arabica*. *Physiol. Plant*. 2004; 122: 401 - 11.

32. Zheng XQ, Hayashibe E, and Ashihara H. Changes in trigonelline (N-methylnicotinic acid) content and nicotinic acid metabolism during germination of mungbean (*Phaseolus aureus*) seeds. *J. Exp. Bot*. 2005; 56: 1615 - 23.

33. Joshi JG, Handler P. Biosynthesis of trigonelline. *J. Biol. Chem*. 1960; 235: 2981 - 3.

34. Wagner KG, Backer AI. Dynamics of nucleotides in plants studied on a cellular basis. In: Jeon KW, Friedlander M (eds), Int. Rev. Cytol., Academic Press. *San Diego* 1992; 134: 1 - 84.

35. Zheng XQ, Ashihara H. Distribution, biosynthesis and function of purine and pyridine alkaloids in *Coffea arabica* seedlings. *Plant Sci*. 2004; 166: 807 - 13.

36. Katoh A, Hashimoto T. Molecular biology of pyridine nucleotide and nicotine biosynthesis. *Front. Biosci*. 2004; 9: 1577 - 86.

37. Yang KS, Waller GR. Biosynthesis of the pyridine ring of ricinine from quinolinic acid glycerol and aspartic acid. *Phytochem*. 1965; 4: 881 - 9.

38. Ashihara H, Stasolla C, Yin Y, Loukanina N, Thorpe TA. *De novo* and salvage biosynthetic pathways of pyridine nucleotides and nicotinic acid conjugates in cultured plant cells. *Plant Sci*. 2005; 169: 107 - 14.

39. Wagner R, Feth F, Wagner KG. The pyridine-nucleotide cycle in tabacco: Enzyme activities for the recycling of NAD. *Planta* 1986; 167: 226 - 32.

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

40. Upmeier B, Gross W, Koster S, and Barz W. Purification and properties of S-adenosyl-l-methionine:nicotinic acid-N-methyltransferase from cell suspension cultures of *Glycine max* L. Arch. *Biochem. Biophys*. 1988; 262: 445 - 54.

41. Taguchi H, Nishitani H, Okumura K, Shimabayashi Y, Iwai K. Biosynthesis and metabolism of trigonelline in *Lemna paucicostata* 151. *Agric. Biol. Chem*. 1989; 53: 2867 - 71.

42. Taguchi H, Sakaguchi M, Yamaki K, and Shimabayashi Y. Biosynthesis of trigonelline in the coffee plant. Nippon Nogeikagaku Kaishi Journal. 1987; 61: 183 - 9.

43. Taguchi H, Shimabayashi Y. Findings of trigonelline demethylating enzyme activity in various organisms and some properties of the enzyme from hog liver. *Biochem. Biophys. Res. Commun*. 1983; 113: 569 - 74.

44. Ashihara H. Changes in activities of purine salvage and ureide synthesis during germination of black gram (*Phaseolus mungo*) seeds. *Zeitschrift fur Pflanzenphysiologie*. 1983; 113: 47 – 60.

45. Nobusawa E, Ashihara H. Purine metabolism in cotyledons and embryonic axes of black gram (*Phaseolus mungo* L.) seedlings. *International J. Biochem*. 1983; 15: 1059 – 65.

46. Shimizu MM, Mazzafera P. A role for trigonelline during imbibition and germination of coffee seeds. *Plant Biol*. 2000; 2: 605 - 11.

47. Willeke U, Heeger V, Meise M, Neuhann H, Schindelmeiser I, Vordemfelde K, Barz W. Mutually exclusive occurrence and metabolism of trigonelline and nicotinic acid arabinoside in plant cell cultures. *Phytochem*. 1979; 18: 105 - 10.

48. Minorsky PV. Trigonelline: a diverse regulator in plants. *Plant Physiol*. 2002; 128: 7 – 8.

49. Evans LS, Almeida MS, Lynn DG, Nakanishi K. Chemical characterization of a hormone that promotes cell arrest in G2 in complex tissues. *Sci*. 1979; 203: 1122 – 3.

50. Evans LS, Tramontano WA. Trigonelline and promotion of cell arrest in G2 of various legumes. *Phytochem*. 1984; 23: 1837 – 40.

51. Mazzuca S, Bitonti MB, Innocenti AM, Francis D. Inactivation of DNA replication origins by the cell cycle regulator, trigonelline, in root meristems of *Lactuca sativa*. *Planta* 2000; 211 (1): 127 - 32.

52. Lynn DG, Jaffe K, Cornwall M, Tramontano WA. Characterization of an endogenous factor controlling the cell cycle of complex tissues. *J. Am. Chem. Soc*. 1987; 109: 5859 - 61.

53. Darlington CD, Wylie AP. Chromosome atlas of flowering plants, George Allen & Unwin Ltd., London. 1945, p. 164.

54. Joshi S, Raghuvanshi SS. B-chromosomes, pollen germination in situ and connected grains in *Trigonella foenum-graecum*. *Beitr. Biol. Pf.I*. 1968; 44 (2): 161 – 6.

55. Singh D, Singh A. Double trisomics in *Trigonella foenum-graecum* L. *Crop Improvement*. 1976; 3 (1–2): 25 – 7.

56. Green JM, Sharma D, Reddy LJ, Saxena KB, Gupta SC, Jain KC, Reddy BVS, Rao MR. Methodology and progress in the I.C.R.I.S.A.T. *Pigeonpea Breeding Program*. Proc. Intern. Workshop on Pigeonpeas,

[DOR: 20.1001.1.2717204.2010.9.35.1.1]

Patancheru, Dec. 1981; pp: 437-449.

57. Auerbach C. Chemicals and their effects. Proc. Symp. on Mutation and Plant Breeding, Cornell, Nov.–Dec. 1961; 25: 585 - 621.

58. Jain SC, Agrawal M. Effect of chemical mutagens on steroidal sapogenin in *Trigonella* species. *Phytochem*. 1987; 26 (8): 2203 – 6.

59. Laxmi V, Datta SK. Chemical and physical mutagenesis in fenugreek. *Biol. Mem*. 1987; 13 (1): 64 – 8.

60. Roy RP, Singh A. Cytomorphological studies of the colchicine-induced tetraploid *Trigonella foenum-graecum*. Genet. Iber. 1968; 20 (1–2): 37 – 54.

61. Jain SC, Agrawal M. Effect of mutagens on steroidal sapogenin in *Trigonella foenum-graecum* tissue cultures. *Fitoterapia* 1994; 65 (4): 367 – 75.

62. Cerdon C, Rahier A, Taton M, Sauvaire Y. Effect of tridemorph and fenpropimorth on sterol composition in fenugreek. *Phytochem*. 1996; 41: 423 – 31.

63. Merkli A, Christen P, and Kapetanidis I. Production of diosgenin by hairy root cultures of *Trigonella foenum-graecum* L. *Plant Cell Rep*. 1997; 16: 632 – 6.

64. Oncina C, Botía JA, Del Río A, Ortuño A. Bioproduction of diosgenin in callus cultures of *Trigonella foenum-graecum* L. *Food Chem*. 2000; 70: 489 – 92.

65. Radwan SS, Kokate CK. Production of higher levels of trigonelline by cell cultures of *Trigonella foenum-graecum* than by the differentiated plant. *Planta* 1980; 147: 340 – 4.

66. Marshall JG, Staba EJ. Hormonal effects on diosgenin biosynthesis and growth in *Dioscorea deltoidea* tissue cultures. *Phyto Chem*. 1976; 15: 53 - 5.

[DOR: 20.1001.1.2717204.2010.9.35.1.1]