Research Article

Phytochemical Analysis of Glycosides from Leaves of *Trigonella foenum graecum*

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ABSTRACT

The objective of the experiment is to extract the Glycosides from the leaves of *Triogonella foenum graecum* and their complete phyto-chemical analysis. TLC analysis for preliminary analysis of glycosides, IR spectrum of crude glycosides extracted from the leaves of *T. Foenum graceum* plants were obtained on IR Spectrophotometer and mass spectrum were recorded by Mass Spectrometer. The crude extract after several phyto-chemical tests was observed to be a mixture of furostanol saponins. IR spectrum revealed, groups of OH, CH_3 stretching, C=C stretching and C-O-C. Less intense peaks like C-C skeletal branched chain were also obtained. The crude extract after several phytochemical tests was observed to be a mixture of furostanol saponins.

Keywords: *Trigonella foenum graecum*, Phyto-chemical analysis, IR analysis, Mass spectroscopy.

INTRODUCTION

In India, medicinal plants have been utilized as natural medicine since the days of Vedic glory. Historically Sushruta [about 400 B.C.] compiled classification of 700 herbal drugs under 37 classes in 'Sushruta Samhita' [A compendium of ancient Indian surgery][1]. Herbal drugs are considered free from side effects than synthetic one. They are less toxic, relatively cheap and more popular[2]. With the development of modern chemistry; isolation and characterization of compounds from the medicinal plants have become more accurate. They serve as complete drugs or as starting materials for the synthesis of many important drugs used in modern medicine. Therefore, it may be rightly said that medicinal herbs have played a key role in the development of modern medicine and still continues to be widely used in its original form[3].

Trigonella foenum graecum, the focus of this research has turned out to be a source of phyto-chemicals with unique chemical structures and innovative biological and pharmacological properties[4]. It is an annual self pollinating dicotyledon[5] legume which belongs to the family Papilionaceae-Leguminosae and is extensively cultivated in India, the Mediterranean region, north Africa and Yemen[6]. It has been considered the third most important seed spice in India[7]. *T. foenum graecum* plant and seeds show anti oxidant properties[8], anti-atherosclerotic[9], anti-inflammatory[10], antinociceptive[11,12], anti-ulcerogenic[13], antineoplastic effects[14] and most importantly anti-diabetic effects[1].

It is clear that current scenario demands extraction and identification of new pharmacophores from medicinally important plants[8]. Research since 1960's reveals that emphasis on isolation of native glycosides from *T. foenum graecum* seeds have been explored[15] but no research has been done in isolation and investigation of glycosides isolated from the leaves of this plant. Glycosides are organic compounds found from plants or animal sources, which on enzymatic or acid hydrolysis give one or more sugar moieties along with non sugar moiety. Glycosides play numerous important roles in living organisms. Many plants store important chemicals in the form of inactive glycosides; if these chemicals are needed, the glycosides are brought in contact with water and an enzyme, and the sugar part is broken-off, making the chemical available for use. Many such plant glycosides are used as medications.

The sugar group present in glycosides are known as the glycone and the non-sugar group as the aglycone or genin part[16]. The furostanol glycosides are known as precursors of spirostanol glycosides [steroidal saponins]. *T. foenum graecum* seed is a potential source of raw material for the steroid industry. The seeds are considered to be a potential economic source of diosgenin and yamogenin for the steroid industry which is estimated to require some 1.5 million kg of plant steroid in 1973[17].

Furostanol glycosides[18] are known as precursors of spirostanol glycosides [steroidal saponins] are of considerable importance to the pharmaceutical industry as a precursor of steroidal drugs, including corticosteroids and contraceptives[19].

For example, a furostanol glycoside, trigonelloside C is isolated from the seed[15]. The original source of diosgenin was the inedible yam tuber [genus *Dioscorea*], which contained an average of 4-6 per cent diosgenin [dw/w][20]. A minimum of three to four years growth is required before the tuber can be harvested. An alternative source of diosgenin is the aerial plant part of *T. foenum graecum*.

It can yield 1-1.5 per cent diosgenin [dw/w][21,22]. Also furostanes [steroidal sapogenins serves as biologically active materials having independent value[23-25]. Anticancer and cytotoxicity[26-31], antitumour[31-34], antiinflamatory and antioxidant[35-37], antiviral[38], antifungal and antimicrobial[39,40], molluscicidal[41-42], antihypercholesteremic[43-47] and as a plant growth stimulant[48] activities have been reported for steroidal glycosides.

Literature survey reveals that till date research focus was around the isolation of glycosides from seeds of this plant. The present investigation holds its novelty in isolation and the elucidation of furostanol glycoside from the leaves of *T. foenum graecum* plant.

MATERIALS AND METHODS

***Trigonella foenum graecum*: The Photobiont** [Source of seeds]

Seeds were collected from local nursery in Noida region and were identified morphologically. A specimen of the same was deposited at NISCAIR [Vide Voucher number: NISCAIR/RHMD/Consult/2008-09/1123/154]. These were used for cultivation in green house and further used in phytochemical analysis.

Extraction of 80 per cent Glycosides [crude] from *T. foenum graecum* leaves

Glycosides were extracted from the leaves *T. foenum graecum* plants. Phyto-chemical tests were done to determine the functional moiety or mass of the isolated glycosides from the plant samples. As given by Hardman *et al.*[49]with some modifications, following procedure was followed for glycoside extraction. 30 g of *T. foenum graecum* leaves were shade dried and grounded to powder and extracted with 80 per cent methanol [MeOH] with stirring at room temperature. The volume of solvent taken was three times the weight of leaf sample. The extract was filtered and the filtrate was distilled for MeOH at 60^0 C using vacuum. The remaining aqueous extract was extracted with *n*-hexane to remove the oily mass. Now the *n*-hexane fraction was distilled out under vacuum and remaining aqueous phase rich in mixture of glycosides was exchanged with *n*-butanol 3 times. The remaining *n*-butanol phase was distilled out under vacuum at 80^0 C. A brownish mass was obtained which was rich in mixture of glycosides. The obtained extract was subjected to column chromatography for further purification of compounds.

Column chromatography of crude glycosides extracted from leaves of *T. foenum graecum* plants

Crude glycosides were further subjected to column chromatography [CC] and eluted with $CHCl_3$:CH_3OH:H_2O [65:40:12], and further with methanol [CH_3OH] to obtain pure compounds. Silica gel for column chromatography was used as stationary phase. The flow rate used was 6 ml/min[4]. Six elutes for each solvent were taken. After TLC analysis those compounds showing similar R_f values were pooled together.

Qualitative analysis of glycosides by Thin-Layer Chromatography [TLC]

TLC analysis for glycosides obtained by column chromatography was done. For all the analysis, commercially prepared plates were used. Pre-coated silica gel 60 F TLC_{254} plate [E. Merck] of uniform thickness of 0.2 mm, 20x20 cm were used. The glycosides were applied in methanol solution of 0.1 per cent concentration at the starting line 2 cm above the lower edge of the plate. A developing chamber was used which could accommodate one or more plates and could be properly closed and sealed. The solvent system used for the same was $CHCl_3$:CH_3OH:H_2O [61:32:7] [15]. Chromatograms were developed using 10 per cent H_2SO_4 reagent[49]. A ultra-violet light chamber [Biobee, model no. SI: E-060711] suitable for observation at short [254 nm] was used for better visualization of spots. After the development of spot with the given solvent the TLC plate is air dried and sprayed with reagent or kept in Iodine chamber for better visualization. The R_f value is calculated as given[50].

Preliminary test for glycosides

Crude glycosides were subjected to TLC analysis. All the compounds separated show positive reaction with *p*-dimethylaminobenzaldehyde-HCl [Ehrlich's reagent] [49, 51].

Test for saponin as the aglycone group

About 0.5g of the powdered sample was mixed with 5 ml of distilled water and shaken vigorously on cyclomixer [Remi equipments, catalog no. CN101, serial no. BACM-209] and observed for a stable persistent froth[52].

IR Spectra for crude glycosides extracted from leaves of *T. foenum graecum* plants

IR analysis was done by IR spectrometer [Shimadzu Model 8201PC] using KBr pellets. The methodology involved mixing a small quantity of the sample along with a specially purified KBr. This powder mixture was then crushed in a pellet press in order to form a pellet through which the beam of the spectrometer could pass. This pellet was crushed to high pressures in order to ensure that the pellet is translucent.

Mass spectroscopy for crude glycosides extracted from leaves of *T. foenum graecum* plants

Crude glycosides, seen as a dark brown powder were extracted from leaves. The extract was subjected to mass spectroscopy [MALDI SYNAPT^{TM} G2 HD MS^{TM} Spectrometer from Waters] in a range of 100-600 and 600-2000. Spectra's were obtained for two samples of glycosides. Glycosides were dissolved in Chloroform: Methanol [1:1] concentration in ppm.

The liquid containing the crude glycosides of interest were dispersed by electrospray into a fine aerosol.

Because the ion formation involves extensive solvent evaporation, the typical solvents for electrospray ionization were prepared by mixing Chloroform: Methanol [1:1]. To decrease the initial droplet size, compounds that increase the conductivity [e.g. acetic acid] were customarily added to the solution. The aerosol was first sampled into the first vacuum stage of a mass spectrometer through a capillary, which was heated to aid further solvent evaporation from the charged droplets. The solvent evaporated from the charged droplet until it became unstable upon reaching its Rayleigh limit. At this point, the droplet got deformed and emitted charged jets in a process known as Coulomb fission. During the fission, the droplet lost a small percentage of its mass [1.0-2.3 per cent] along with a relatively large percentage of its charge [10-18 per cent]. Finally, small ions are liberated into the gas phase through the ion evaporation mechanism, while larger ions are formed by charged residue mechanism, which were finally detected with mass spectrometer[53, 16].

RESULTS

Extraction of 80 per cent glycosides [crude] from leaves of *T. foenum graecum* plants

The glycosides were extracted as brown colour powder [Fig. 1]. Yield of treated sample was 3.35 g and untreated sample was 2.75 g.

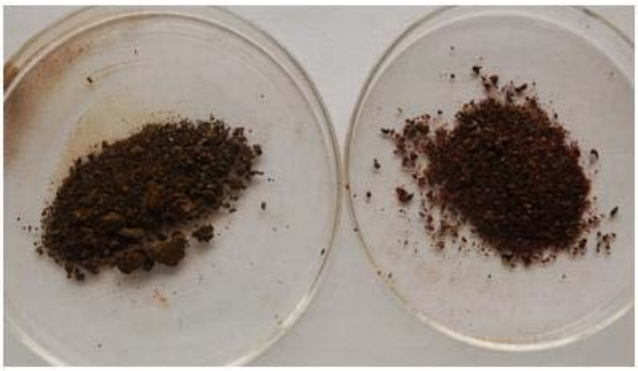

Figure 1: Glycosides powder was extracted in crude form. The extract was subjected to comparative analysis by IR, mass and antimicrobial characterization. **a**. Crude glycosides extracted from control *T. foenum graecum* leaves. **b.** Crude glycosides extracted from *P. indica* treated *T. foenum graecum* leaves

After chromatographically purifying the 80 per cent pure extracted glycosides, they were subjected to TLC analysis [$CHCl_3$:CH_3OH:H_2O [65:40:12]]. It was found that the extract contains glycosides with following R_f values 0.59, 0.46, 0.34, 0.29, 0.18, 0.52, 0.93, 0.46. From review of literature, these were found to be derivatives of furostan, also experimentally proved as all the spots gave positive reaction with Ehrilch's reagent, giving red colour spots. Presence of saponins was confirmed by observing a long lasting froth after mixing the extract in distilled water with help of vortex mixture.

IR Spectra for crude glycosides extracted from leaves of *T. foenum graecum* plants

IR analysis with KBr spectra revealed following peaks: OH [3404.18], CH_3 stretching [2929.01], C=C stretching [1628.45], C-O-C [1072.98, 1040.63]. Less intense peaks were around 1175±2 cm^{-1} for C-C skeletal branched chain. Spectrum obtained is given in Fig. 2.

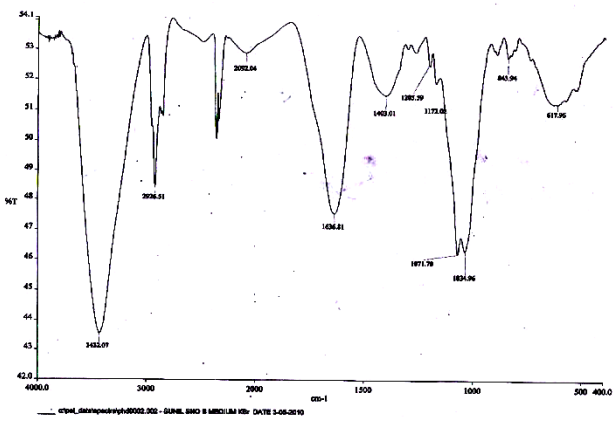

Figure 2: IR spectra of crude glycosides extracted from leaves of *T. foenum graecum* plants

Mass spectroscopy for crude glycosides extracted from leaves of *P. indica* control and treated *T. foenum graecum* plants

Mass spectroscopy was done with low unit resolution mass spectrometer [m/z 2000] in the range of 600-2000. Generally glycosides when bombarded with high ev, fragmentation occurs at the ether linkage leading mostly to loss of H_2O and sugar moiety. Spectrum obtained is shown in Fig. 3.

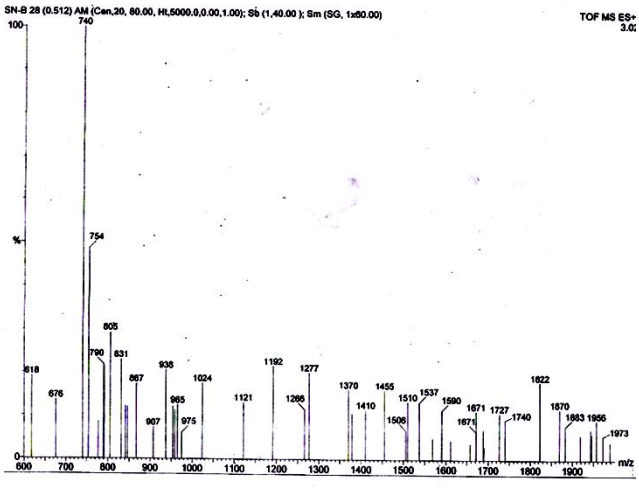

Figure 3: Mass spectra graph of crude glycosides isolated from leaves of *T. foenum graecum* plants. Range 600-2000 [m/z upto 2000]

The probable structures of mixture of glycosides showed base peak being at 716 [$C_{39}H_{52}O_{11}$] and molecular ion peak [M^+] at 1999 peak had [$C_{80}H_{125}O_{48}$]. The highest molecular ion peak for treated sample was shown at m/z 1999 but peak obtained at m/z value 1898 was

considered, based on its optimum absorption and peak intensity. The other peaks with the m/z values and probable structures were 1992 [$C_{80}H_{119}O_{48}$], 1269 [$C_{35}H_{97}O_{32}$], 800 [$C_{39}H_{56}O_{16}$], 621 [M-H-$C_6H_{12}O_6$+H_2O]$^-$. Other peaks with following m/z values were obtained and their probable structures are as follows: 1960, 1898, 1684, 1639, 1573, 1478, 1438, 1348, and 1173. The extract considered for study, being a mixture of glycosides, the exact fragmentation pattern could not be predicted. The extract obtained was also subjected to low unit resolution mass spectrometer in a range of 100-600 [m/z 600] [Fig. 4].

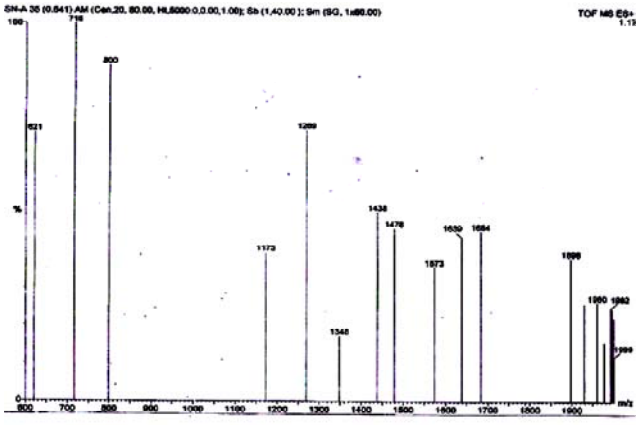

Figure 4: Mass spectra graph of crude glycosides isolated from leaves of *T. foenum graecum* plants. Range 100-600

The probable structures of mixture of glycosides were as follows: Base peak being at 187 [$C_{10}H_{19}O_3$] and [$M^{+\cdot}$] at 387 [$C_{16}H_{43}O_9$]. The highest molecular ion peak for treated sample was shown to be m/z 387, but the peak at m/z 258 was considered based on absorption and peak intensity. The other peaks with the m/z values and probable structures were 245 [$C_{13}H_{25}O_4$] and 188 [$C_{10}H_{20}O_3$]. Other peaks obtained were having following m/z values: and 384, 361, 345, 259, and 258. The extract considered for study, being a mixture of glycosides, the exact fragmentation pattern could not be predicted. Since extracted glycosides were a mixture of compounds therefore on the basis of peaks obtained at different m/z values, probable structures were deduced.

DISCUSSION

In the *T. foenum graecum* seed, diosgenin and other related steroids are present as glycosides[19]. Saponins have a characteristic chemical structure consisting of an aglycone, also called the *genin* or *sapogenin*, covalently linked to one or more monosaccharides or oligosaccharides[54, 55]. Saponins can be classified as triterpene glycoside [30 carbon], steroidal glycosides [27 carbon] or steroidal alkaloid glycosides according to the type of *genin* they contain. Lot of research was undertaken in isolation of glycosides from the seeds of *T. foenum graecum* plant. Seven furostanol glycosides which have been isolated from *T. foenum graecum* seeds were designated as Trigofoenosides A, B, C, D, E, F and G. Out of these, two new furostanol glycosides, trigofoenosides were characterised by Gupta *et al.*[4]. These were the F and G compounds. Both the compounds had shown strong IR absorption peaks in the range of 3600-3200 cm^{-1}. This was attributed due to presence of hydroxyl groups. Crude mixture of glycosides was isolated from *T. foenum graecum* leaves. Spectral graphs show similar pattern of absorbance when subjected to IR analysis as seen for furostanol glycoside isolated from seeds of this plant[49, 17,4]. Column chromatography of control and treated (with *Piriformospora indica*) sample glycosides were performed followed by TLC analysis under similar conditions as given in literature. It was found that the extract contains glycosides with following R_f values: 0.59, 0.46, 0.34, 0.29, 0.18, 0.52, 0.93, 0.46. From literature, the compounds were found to be derivatives of furostan [4, 15]. Furostanol glycosides were isolated as seen by formation of red color spots when reacted with Ehrilch's reagent[56, 57]. Also, the crude mixture of glycosides had presence of saponins as the aglycone group was also confirmed by formation of persistent froth when mixed and shaken in distilled water[52]. IR analysis with KBr pellets of control sample of glycosides was performed. The spectra revealed following peaks: OH [3432.07], CH_3 stretching [2926.51], C=C stretching [1636.81], C-O-C [1071.70, 1034.96]. Less intense peaks were around 1175 cm^{-1} for C-C skeletal branched chain. For samples treated with *Piriformospora indica*, the spectra revealed following peaks: OH [3404.18], CH_3 stretching [2929.01], C=C stretching [1628.45], C-O-C [1072.98, 1040.63]. Less intense peaks were around 1175±2 cm^{-1} for C-C skeletal branched chain. No change was observed in the functional moeity of the two samples. The glycosides samples were also subjected to mass spectroscopy with low unit resolution mass spectrometer [m/z 2000] in the range of 600-2000 and 100-600 [m/z 600]. Since extracted glycosides were a mixture of compounds therefore on the basis of peaks obtained at different m/z values, probable structures were deduced based on literature[53]. However, a comparison between treated and control samples depending on mass could not be analyzed. The exact fragmentation pattern could not be predicted.

After phytochemical isolation and evaluation of glycosides, these were assayed for their anti-microbial potential against multidrug resistant microorganisms. In recent years, uses of antimicrobial drugs in treatment of infectious diseases have developed multiple drug resistance[58] and with increase in production of new antibiotics, by pharmaceutical industry, resistance to these drugs has also increased[59]. Hence, scientists are shifting their attention to folk medicine in order to find new leads for better drugs against microbial infections. Plant materials are known as source of new anti-microbial agents, resulting in discovery of new antibacterial drugs of plant origin. A number of compounds like vincristine, quinine, salicyclic acid, eligitalis, morphine, and codeine have been derived from plants which are having enormous therapeutic potential. Still there are medicinal

plants whose properties are yet to be investigated for their phytochemical and pharmacognostical application thus giving rise to be an urgent need for identification of lead substances that are active towards resistant pathogens[60]. Three new furostanol saponins named capsicoside E [1], capsicoside F [2], and capsicoside G [5] were obtained from the seeds of *Capsicum annuum* L. var. Acuminatum along with known oligoglycosides.

CONCLUSION

There is a plethora of Indian medicinal plants which have potential therapeutic effects. Since last decade, herbal plants are gaining popularity because of lower side effect and their easy availability. Glycosides extracted from leaves of *T. foenum graecum* leaves were selected as potential for further phyto-chemical studies. It is known that furostanol glycosides obtained from *T. foenum graecum* seed serves as precursor of diosgenin. Diosgenin [[25R]-spirost-5-en-3β-ol] is of considerable importance to the pharmaceutical industry as a precursor of steroidal drugs, including corticosteroids and contraceptives. The crude extract after several phytochemical tests was observed to be a mixture of furostanol saponins. IR analysis of glycoside extracts was performed. IR spectrum revealed, groups of OH, CH_3 stretching, C=C stretching and C-O-C. Less intense peaks like C-C skeletal branched chain were also obtained. The glycosides extracted, being a mixture of compounds therefore the fragmentation pattern could not be deduced. However the base peak and $[M^+]$ were predictable from the spectra obtained. Few probable structures were written based on literature further confirming the presence of glycosides. This work requires further study relating to isolation of single compound from mixture of glycosides to predict the accurate molecular weight, base and molecular ion peak and most importantly the fragmentation pattern.

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