Chapter - 9

Isolation and Identification of steroidal sapogenins compounds
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The present study pertains to production, isolation and identification of steroidal sapogenins compounds from root, stem and flower of *Cistanche tubulosa* Wight. (Orobanchaceae) and *Orobanche aegyptiaca* Pers. Syn. (Orobanchaceae).

INTRODUCTION

Steroidal sapogenins are chemically triterpenoid glycosides or glycosides of steroids with a spiroketal side chain. These are widely distributed in plant kingdom. Sapogenins are widely used in the field of medicine as they are the main precursors of many medicinally useful steroidal hormones such as sex hormones. Hence, attempts have been made to furnish information regarding steroidal sapogenins from *C. tubulosa* and *O. aegyptiaca*.

MATERIAL AND METHODS

Fully matured and healthy plants selected for the present investigation of *Cistanche tubulosa* and *Orobanche aegyptiaca* were collected from outskirts area of Bikaner from villages i.e. Naal and Khara. Plant parts viz. stem, root and flowers of both plant species were separately dried at 100°C for 15 minutes to inactivate the enzymes and then at 60°C until a constant weight was achieved. The dried and powdered samples were used for extraction of steroidal sapogenins compounds.

EXTRACTION PROCEDURE

Each of the dried samples were hydrolysed with 30% (v/v) hydrochloric acid. The hydrolysed test samples were washed separately with distilled water till neutrality. Test samples thus obtained were dried and Soxhlet extracted in benzene (Nag et al., 1979). Each of the benzene extracts
of the various test samples was separately dried in vacuo and taken up in chloroform for further analysis of its steroidal sapogenins.

THIN LAYER CHROMATOGRAPHY (TLC) QUALITATIVE:

Each of the crude extracts along with the references sapogenins (diosgenin, hecogenin, cryptogenin, tigogenin and yamogenin) was dissolved in chloroform and applied separately on silica gel ‘G’ coated and activated glass plates. These glass plates were developed in an organic solvent mixture of hexane:acetone, (8:2). The developed glass plates were air dried and visualized under UV light showed four fluorescent spots in each of the test samples which on spraying with 50% sulphuric acid and subsequent heating at 100°C for 10 minutes showed spots coinciding with that of their standard reference compounds as hecogenin, tigogenin, cryptogenin and diosgenin (Rf 0.23, 0.40, 0.20 and 0.42) respectively. Coloured spots were observed in all the test samples of the plant species; hecogenin (dark yellow, Rf 0.23), tigogenin (brown, Rf 0.40), cryptogenin (pinkish brown, Rf 0.20) and diosgenin (brownish green Rf 0.42). A few other solvent systems [benzene:ethyl acetate, (85:15); acetone:benzene, (1:2); hexane:ethyl acetate, (3:1); benzene:ethyl acetate, (3:1)] were also tried, but hexane:acetone, (8:2) gave excellent results in the present investigation.

PREPARATIVE THIN LAYER CHROMATOGRAPHY (PTLC)

Each of the extracts along with the standard reference steroids sapogenins were applied separately on thickly (0.3mm to 0.4mm) silica gel ‘G’ coated and activated glass plates. The plates were developed in an organic solvent mixture of hexane:acetone, (8:2). The developed plates were air dried and visualized under UV light. Four fluorescent spots (Rf 0.23, 0.40, 0.20, 0.42) corresponding with those of the standard reference samples of hecogenin, tigogenin, cryptogenin and diosgenin in test samples were
separately marked and collected along with silica gel from unsprayed plates. Each of the mixture was eluted with cholorform, the elutes were dried in vacuo, crystallized separately with methanol and chloroform. Each of the crystallized isolates from all the samples tested was subjected to colorimetry (for quantitative estimations), melting point (melting point apparatus, Toshniwal, India) and infra red spectral (Perkin-Elmer, 337, Grating, Infra-red spectrophotometer, using nujol or potassium bromides pellets) studies along with their respective standard reference steroidal sapogenins.

QUANTITATIVE ESTIMATION OF SAPOGENINS

Steroidal sapogenins were estimated following the spectrophotometric method of Sanchez et al., (1972). Standard stock solutions of diosgenin, hecogenin, cryptogenin and tigogenin were separately prepared in chloroform out of which various concentrations were made ranging from 10µg to 120µg and applied separately on silica gel coated and activated glass plates along with a parallel run of blank. These glass plates were run in solvent mixture of hexane:acetone, (8:2), air dried and kept in a chamber saturated with iodine vapours. The resulting coloured spots were marked and the plates were kept in an oven at 100°C for 15 minutes so as to evaporate the excess of iodine. Spots of diosgenin, hecogenin, cryptogenin and tigogenin and blank zones from the parallel run were separately scrapped along with the absorbent eluted with 5 ml of methanol and then centrifuged. From each of the samples 4ml of aliquots was taken and evaporated to dryness on a water bath. To each of the resulting residue, 4ml of 80% methanolic sulphuric acid was added and kept for 2 hrs. Absorbance from each of the known samples were measured on a spectronic – 20- colorimeter (Bausch and Lomb set at 405 nm against a blank (80% methanolic sulphuric acid) and a regression curve of various concentrations against optical densities was computed which followed the Beer’s law.
Absorbance from each of the unknown samples was also taken in a similar manner and their concentrations were determined (mg/gdw) by comparing with those of their standard curves. Five such replicates of each of the samples were examined and the mean values taken (SE <0.05).

RESULTS AND DISCUSSION

Presence of diosgenin, hecogenin, cryptogenin and tigogenin from all the samples were confirmed by co-chromatography (diosgenin Rf 0.42; hecogenin Rf 0.23, cryptogenins Rf 0.20 and tigogenin Rf 0.40) mp (207-208°C; 264-266°C and 203-204°C respectively) and mmp (undepressed). The presence of diosgenin was further confirmed by superimposable IR spectra of the isolates and reference compounds.

Diosgenin was detected in all the plant parts of O.aegyptiaca, it was detected 2.46 mg/gdw in the root, 1.49 mg/gdw in the stem and 4.44 mg/gdw in the flower. It was observed maximum 5.46 mg/gdw in the flower of C.tubulosa and minimum 1.49 mg/gdw in the stem of O.aegyptiaca, whereas, it was absent in the stem and root of C.tubulosa. (Table 9.1 and Fig. 9.1).

Cryptogenin was only present in the flower of C.tubulosa 2.23 mg/gdw. It was completely absent in O.aegyptiaca and other plant parts of C.tubulosa. (Table 9.1 and Fig. 9.1).

Hecogenin was present in all the plant parts of C.tubulosa. It was observed 1.29 mg/gdw in the root, 2.30 mg/gdw in the stem, and 1.47 mg/gdw in the flower. Hence, it was observed maximum in the stem. Hacogenin was detected only in the stem of O.aegyptiaca (0.31mg/gdw). (Table 9.1 and Fig. 9.1).

Tigogenin was present in the root, stem of C.tubulosa and in the stem of O.aegyptiaca (1.52 mg/gdw, 3.53 mg/gdw and 2.55 mg/gdw respectively). It was observed maximum in the stem of C.tubulosa 3.53 mg/gdw, minimum in the root of C.tubulosa 1.52 mg/gdw. (Table 9.1 and Fig. 9.1).
Steroidal sapogenins have been reported from a number of plant species. *Dioscorea*, *Agave* and *Yucca* have been reported among the promising source with regard to their valuable steroidal sapogenins. Diosgenin, an important sapogenin has been reported from various species of *Dioscorea* (Staba, 1977; Singh et al., 1979; Khanna et al., 1980a; Mandal and Chaterjee, 1985). Nag et al., (1979) reported the presence of diosgenin in *Tribulus terrestris* and *T. alatus*. Singh and Nag (1981) have also reported the diosgenin from plant parts of *Peganum harmala*.

There are several reports on the presence of steroidal sapogenins by some researchers like diosgenin has been reported from the tissue cultures of a number of plant species, *Dioscorea deltoidea*, (Kaul and Staba, 1968; Abroshnikova et al., 1971; Chaturvedi and Srivastava, 1976), *D. tokora*, (Tomita et al., 1970); *D. composita* and *D.speculiflora*, (Mehta and Staba, 1970); *Daucus carota*, (Khanna et al., 1977b); *Dioscorea floribunda*, (Khanna et al., 1980a); *Lycium barbarum*, (Grover, 1984); *Tribulus alatus*, (Jit, 1985) and many other plant species. Diosgenin with other sapogenins such as gitogenin and tigogenin has been reported from *Trigonella foenum-graecum* (Khanna and Jain, 1973a). Hecogenin, gitogenin with tigogenin have been reported from static and submerged cultures of *Agave wightii* (Sharma and Khanna, 1980).

Nag, (1986) has reviewed and Khanna, (1987a,b) has also reported a number of steroidal sapogenins from the tissue cultures of various plants. Mathur, (1987) screened the *Zygophyllum simplex* both in vivo and in vitro for the steroidal contents and reported the presence of diosgenin, hecogenin and tigogenin with 2 sterols (β-sitosterol and stigmasterol).

Some sapogenins can mimic or regulate steroid hormones or hormone precursors. *Dioscorea* spp. which contains considerable amounts of the sapogenin-diosgenin can be converted into corticosteroids, dehydroepiandrosterone (DHEA), estrogen and progesterone in laboratory (Araghiniknam, 1996). Fenugreek (*Trigonella foenum-graecum*) is richer in
diosgenin and other saponins. Its therapeutic effect has been studied by Sharma et al., 1996. The most celebrated saponins are ginsenosides from Korean ginseng (*Panax ginseng*). Ginseng has been promoted as an aphrodisiac, studied by Wang and Lee, 1998.

Diosgenin, a major raw material for the commercial production of steroidal contraceptives and sex hormones is useful in several medical therapies and is principally obtained from *Dioscorea* spp. (Mandal and Chatterjee, 1985; Ravishankar and Grewal, 1986; Bishop and Yokata, 2001). It has also been reported from other plant species like *Fagonia cretica* and *Lycium barbarum* (Harsh and Nag, 1981; Harsh, 1982), *Smilax zeylanica* (Kar and Sen, 1984b), *Lycium barbarum* (Grover, 1984), *Asparagus* species (Kar and Sen, 1984a; Kar, 1985), *Seetzenia orientalis* (Sethia, 1988) and *Zygophyllum simplex* (Mathur, 1988).

Singh (1989) detected diosgenin, hecogenin, cryptogenin and tigogenin from plant parts and cultures of *Abutilon pannosum* and *Ocimum americanum*. cryptogenin and diosgenin were found in tissue cultures of *Fagonia cretica* (Kapoor, 1991). Diosgenin was also reported from excised root cultures of *Dioscorea floribunda* (Sengupta et al., 1985) tissue cultures of *Lycium barbarum* (Mukhi, 1995), *Gossypium* cultivars (Kaur, 1997; Goyal, 1997), *Lycium barbarum* (Verma, 1998); *Dioscorea floribunda* (Dixit and Banerji et al., 2000); *Vigna aconitifolia* (Tyagi, 2002).

In recent years, a great deal of interest has been given to the role of plant sterols in the protection from cancer. The mechanisms by which phytosterols influence cell growth and apoptosis of tumor cells require additional study. The interaction between phytosterols and other phytochemicals such as phytoestrogen, vitamin E and antioxidants, which have also been shown to protect from cancer, should be explored. In the present study also, four steroidal sapogenins diosgenin, hecogenin, cryptogenin and tigogenin has observed in the parasitic plants of arid zone of
Rajasthan i.e. *C.tubulosa* and *O.aegyptiaca* Hence, they are good source of economically important steroidal sapogenins.
Fig. 9.1 Steroidal Sapogenins composition in *C. tubulosa* and *O. aegyptiaca* (mg/gdw)

<table>
<thead>
<tr>
<th>PLANTS PARTS</th>
<th>Diosgenin</th>
<th>Cryptogenin</th>
<th>Hecogenin</th>
<th>Tigogenin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ct R = <em>C. tubulosa</em> Root</td>
<td>-</td>
<td>-</td>
<td>1.29</td>
<td>-</td>
</tr>
<tr>
<td>Ct S = <em>C. tubulosa</em> Stem</td>
<td>-</td>
<td>-</td>
<td>2.30</td>
<td>-</td>
</tr>
<tr>
<td>Ct F = <em>C. tubulosa</em> Flower</td>
<td>5.46</td>
<td>2.23</td>
<td>1.47</td>
<td>-</td>
</tr>
<tr>
<td>Oa R = <em>O. aegyptiaca</em> Root</td>
<td>2.46</td>
<td>-</td>
<td>-</td>
<td>0.31</td>
</tr>
<tr>
<td>Oa S = <em>O. aegyptiaca</em> Stem</td>
<td>-</td>
<td>0.31</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oa F = <em>O. aegyptiaca</em> Flower</td>
<td>-</td>
<td>2.55</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 9.1 Sapogenins composition (mg/gdw)* of *Cistanche tubulosa* and *Orobanche aegyptiaca*

<table>
<thead>
<tr>
<th>Sapogenins</th>
<th><em>Cistanche tubulosa</em></th>
<th><em>Orobanche aegyptiaca</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Root</td>
<td>Stem</td>
</tr>
<tr>
<td>Diosgenin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cryptogenin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hecogenin</td>
<td>1.29</td>
<td>2.30</td>
</tr>
<tr>
<td>Tigogenin</td>
<td>1.52</td>
<td>3.53</td>
</tr>
</tbody>
</table>

*mg/gram dry weight