CHAPTER-VII

Estrogenic activity of MIS₂ and MIS₅ from the seed extract of Melilotus indica
CHAPTER-VII

Kautilya's 'Arathshastra' describes that during Rigveda people protested against the killing of useful animals and killers were punished to death. Since long it has been observed that the human civilization mainly depends on animals for its basic needs like milk, meat, leather, wool etc. The animal, on the other hand depend on plants for food and fodder etc. The dependency of animals and human beings on plants has resulted in shortage of forage thus leading to less production of animal products. Now, it is clear that the good quality of forage results in the high production of live stock wealth to fulfill the demands of our daily needs.

The essential components like proteins, energy, minerals and vitamins etc. are provided by the plants to the animals. There are several secondary metabolites such as sterols, flavonoids, alkaloids, pterocarponoids and toxic amino acids etc. which affect on animal/human health directly or indirectly.

It has been observed from long experience and systematic experimental studies that some physiologically active constituents which are present in the forages result in disease or poor health performance or low productivity. These type of forages cause loss in livestock wealth and led to various abnormalities like premature abortions and birth defects etc. In some cases a heavy loss in the form of death of animals may occur.
The compounds isolated from plants and associated with estrogenic activity\(^1\) are known as ** PHYTOESTROGENS.** The phytoestrogens are widely distributed in plant kingdom. The first compounds of plant origin, with estrogenic properties were obtained from Palm seed kernel and Willow catkins flowers are estrone (I) and estriol (II) respectively.\(^2\) The compounds were of steroidal nature. The phytoestrogens isolated are mostly isoflavonoids,\(^3\) which occur either as aglycones or glycosides and are distributed widely in the plants.\(^4,5\)

Wenzel and Rosenberg,\(^6\) on subcutaneous injection of 4',6'-hydroxy flavone (III) found estrogenic activity. Several other compounds like Quercetin, Robinin\(^7\) and Tricin\(^8\) (IV) are found to have estrogenic activity.

Several reports on estrogenic activity of various compounds reveal that the family Leguminosae is a rich source of phytoestrogens. Genistein (IV), an estrogenic principle isolated from Trifolium subterranean by Bradbury and White\(^9\) was also found in Soja hispida,\(^10,11\) Genista tinctoria,\(^12\) Sophora japonica,\(^13,14\) Trifolium repenes,\(^15\). The coumestans are a group of compounds which are reported to possess antifungal activity. Coumestans are structurally different from sterol and isoflavone. These are biogenetically related to isoflavones.\(^16\) Among the several types of coumestans known in nature Coumestrol (V) is found to possess potent estrogenic activity. About 29 compounds were studied by Mantzer et al.\(^17,18\) and their estrogenic activities were related to coumestrol.
A number of structure activity correlations has been observed. The absence of phenolic hydroxyls at positions 7 and 8 have been found to bring inactivity. The presence of additional hydroxyl groups greatly diminish the estrogenic activity. The estrogenic activity has been evaluated on the basis of the uterine weight response of immature mice to the substance administered orally as well as intravaginally. The estrogenic activity of coumestrol was found to be due to its stilbene like structure analogous to that of diethylstilbesterol.

The above preamble has been of significant importance to develop curiosity for further investigation in the field of phytoestrogens which are responsible for causing infertility syndrome in animals but in addition are also beneficial for milk and meat production in animals grazing on these forages. As such it is evident that there is enough scope for adequate phytochemical and pharmacological investigations on the forage, Melilotus indica (N.O. Leguminosae) and hence the authoress thought it worthwhile to carry out further systematic investigations of this plant, with a view to increase the productivity of livestock wealth.
Melilotus indica\textsuperscript{19,20,21} is a small sized plant belonging to natural order Leguminosae. It is widely distributed throughout in India and is commonly known as 'Ban-methi' in Hindi.

The seeds of Melilotus indica are reported to be used for curing heart diseases, infantile diarrhoea and as anthelmintic and antipyretic. It is astringent to the bowels and also increases appetite. It is also used for curing vomiting, bronchitis, leprosy, 'vata' and piles. The plant is also reported to be used as emollient and plaster for swellings.\textsuperscript{2} The plant is extensively used for green-manuring.

The details of the plant Melilotus indica along with its significant medicinal value is reported in Chapter-II page.42 of the thesis.

**COLLECTION AND IDENTIFICATION OF PLANT MATERIAL**

The seeds of Melilotus indica were procured from M/S United Chemicals and Allied Products, Calcutta and authenticated by the Department of Botany, Dr.H.S.Gour University, Sagar. The voucher specimen no.LXXII has been deposited in the room no. 36 of the Chemistry Department of this University.
PREPARATION OF EXTRACTS

SEEDS

Air dried and powdered 4 kg seeds of plant were exhaustively, extracted with 95% ethanol in a RB flask attached with a reflux condenser on a water bath. The solvent from alcoholic extract was removed under reduced pressure and thick viscous syrup was fractionated successively with petroleum ether (60-80°C), benzene, ethyl acetate and acetone.

The extracts were concentrated under reduced pressure below 60°C and finally dried in vacuum desiccator.

SCREENING OF VARIOUS EXTRACTS FROM SEEDS OF MELILOTUS INDICA

The screening of various extracts prepared from seeds of Melilotus indica was carried out on female albino rats by uterine weight procedure.

Nine groups of female albino rats comprised of 5 rats in each group (age, 25-30 days, average weight 38g) were taken. One group was kept on normal diet as control and the other groups were fed with solvent free extracts mixed diet at the rate of 2g/rat. To ascertain complete ingestion of the test material each rat was fed individually. The rats were sacrificed after 4 days of experiment and their uteri were taken out and separated from vaginas by cutting through cervix. The surrounding tissues were removed from uteri and the intrauterine fluid was pressed out on filter paper and their fresh weights were taken. The mean uteri weights and the number of vaginal openings are recorded in Table-I.
TABLE-I

<table>
<thead>
<tr>
<th>Group NO.</th>
<th>Name of the extract given @ 2g/rat</th>
<th>Mean uterine wt.(in mg)</th>
<th>No.of vaginal openings</th>
<th>Activity inferred</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Nil</td>
<td>14.32±0.31</td>
<td>No activity was observed</td>
<td>-ve</td>
</tr>
<tr>
<td>2.</td>
<td>Petroleum ether soluble fraction of seeds</td>
<td>12.05±0.23</td>
<td>No activity was observed</td>
<td>-ve</td>
</tr>
<tr>
<td>3.</td>
<td>Benzene soluble fraction of seeds</td>
<td>15.12±0.35</td>
<td>No activity was observed</td>
<td>-ve</td>
</tr>
<tr>
<td>4.</td>
<td>Ethylacetate soluble fraction of seeds</td>
<td>27.28±0.43</td>
<td>5</td>
<td>+ve</td>
</tr>
<tr>
<td>5.</td>
<td>Acetone soluble fraction of seeds</td>
<td>25.32±0.43</td>
<td>5</td>
<td>+ve</td>
</tr>
</tbody>
</table>

CHEMICAL EXAMINATION OF ETHYLACETATE AND ACETONE SOLUBLE FRACTION OF SEEDS

In view of adequate estrogenic activity shown by ethylacetate and acetone soluble fractions of seeds, these were examined separately in detail as described in Chapter III page 77 of the thesis to get MIS$_2$ and MIS$_5$.

The EtOAc fraction on usual work up yielded MIS$_2$ as light yellow solid (from MeOH), mp 382$^\circ$C, M$^+$ 268 and analysed for C$_{15}$H$_8$O$_5$ (Found : C, 67.14; H, 2.97%). The MIS$_2$ was identified as 3,9-dihydroxy pterocarpone by various chemical methods, degradative studies and spectral analysis.

The acetone soluble fraction MIS$_5$ (25mg) was analysed for its pterocarponoidal nature only but could not be subjected to structural elucidation due to low yield.
BIOASSAY OF THE NOVEL PTEROCARPONOIDs

The novel pterocarponoids MIS$_2$ and MIS$_5$ were bioassayed on female albino rats/mice by same procedure as adopted for screening of the extracts as described on page 176.

Compound MIS$_2$ was injected intravaginally in aqueous as well as 5% EtOH:H$_2$O (V/V, 2:2) whereas MIS$_5$ was given orally with diet, due to the insoluble character of MIS$_5$. The number of vaginal openings and mean uteri weights were measured and are tabulated in Table-2.

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of rats</th>
<th>Dose in mg/rat</th>
<th>Mean uteri weight (mg)</th>
<th>No. of vaginal openings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>0.0</td>
<td>15.0±0.25</td>
<td>0</td>
</tr>
<tr>
<td>MIS$_2$</td>
<td>5</td>
<td>10.0</td>
<td>28.0±0.51</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>20.0</td>
<td>28.68±0.45</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>50.0</td>
<td>33.20±0.62</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.0</td>
<td>14.80±0.13</td>
<td>0</td>
</tr>
<tr>
<td>MIS$_5$</td>
<td>5</td>
<td>10.0</td>
<td>17.13±0.15</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>20.0</td>
<td>19.25±0.14</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>50.0</td>
<td>24.21±0.66</td>
<td>3</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

The screening of estrogenic activity of the ethyl acetate and acetone soluble fractions of seeds of
**Melilotus indica** showed that adequate estrogenic activity was associated with both the extracts by increasing uteri weight upto $27.28 \pm 0.43$ mg and $25.32 \pm 0.37$ mg respectively. As these compounds possess adequate estrogenic activity so they were further worked up separately for $\text{MIS}_2$ and was identified as **3,9-dihydroxy pterocarpone** on elucidation of structure as reported in Chapter-III page 77.

The compound $\text{MIS}_5$ responded to positive colour tests of pterocarponoids but due to low yield it could not be subjected to structural elucidation.

The results of bioassay for estrogenic activity with compounds $\text{MIS}_2$ and $\text{MIS}_5$ exhibited that $\text{MIS}_5$ showed maximum activity on oral feeding at the level of 50 mg/rat per day to weanling female albino rat with increasing uteri weight upto $24.21 \pm 0.66$mg. $\text{MIS}_2$ showed maximum activity by increasing uteri weight upto $33.18 \pm 0.62$mg on intravaginal administration in dose 50 mg/rat twice a day.

**CONCLUSION**

The above studies have thus concluded that the compounds $\text{MIS}_2$ and $\text{MIS}_5$ isolated from the seeds of **Melilotus indica** are potential phytoestrogens and may further be explored for commanding estrogenic activity.
REFERENCES