# CHAPTER II - MATERIALS AND METHODS

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## MATERIALS AND METHODS

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II.

## MATERIALS AND METHODS

This chapter describes the general procedure for initiation and maintenance of cultures employed in the present investigation. Different analytical methods adopted are also presented.

### II-1. PLANT MATERIAL

The primary source of explants used in the present investigations was from guggul tree; Commiphora wightii (Arnott) Bhandari, Family : Burseraceae. The inocula used in different experiments were as follows :-

II-1.a. Investigations regarding growth and secondary metabolites were performed with in vitro grown callus and suspension cultures derived from leaf and stem explants of

Commiphora wightii trees growing in the ravines of Mahisagar near Vasad.

II.1.b. Shoot cultures grown in vitro were raised either from the nodal segments, bearing single bud, from mature guggul trees or from aseptically grown seedlings.

## II-2. CULTURE MEDIUM

### II-2.a. Glassware

All glassware used in the present study was of either Corning or Borosil brand. Erlenmeyer flasks of 100 or 150 ml capacity and 25 x 150 mm test tubes served as culture vessels. Before use, all culture vessels and glassware used for the preparation of media or other chemical analysis were cleaned with chromic acid (Potassium dichromate 100 g, sulphuric acid 500 ml and water 1000 ml; Alexopoulos and Beneke, 1955). The acid was removed by prolonged rinsing in tap water. Following this it was washed with detergent Teepol which was later thoroughly removed by tap water. The glassware was finally rinsed with double distilled water and dried in an oven at 60°C for 1 h.

### II-2.b. Chemicals

All chemicals were of high purity grade. They were obtained from British Drug House (AnalaR grade), Loba,

E. Merck and/or Flucon (G.R. grade). Auxins, vitamins, cytokinins, antiauxins, amino acids, casein hydrolysate and the authentic samples of sterols were purchased from Sigma Chemical Company (U.S.A.). BSA [N,O-bis(trimethylsilyl acetamide)] was obtained from Fluka. Authentic sample of guggulsterone was kindly provided by Dr. Sukh Dev (Malti Chemical Co. Ltd., Nandesari, Dist. Baroda).

II-2.c. Sterilization of Glassware

Tubes, Erlenmeyer flasks, pipettes, millipore filter unit etc. were plugged with absorbant cotton. Brown papers were wrapped to protect them from condensed water during autoclaving. Petri dishes were covered with wrapping paper. Dissection instruments such as forceps, spatulas etc. were first wrapped in cotton and then with paper. The material thus prepared was sterilized at 15 lb/sq. inch for 20 min at 121°C.

II-2.d. Composition and Preparation of Media

All the media were prepared with double glass distilled water. The compositions of the basal media formulated by White (1954), Murashige and Skoog (1962), Gamborg B5 (1968), Wood and Braun (1961) are given in the Table no. II-1 (a,b,c). In White's (1954) medium instead of Ferric sulphate, Ferric citrate was added at 5 mg/l concentration. Separate stock

solutions were prepared according to the composition of the nutrient medium in glass distilled water. Stocks of inorganic salt solutions were stored at 5°C and that of vitamins and hormones at -4°C in a deep-freeze. Supplements to the basal medium which is prepared by mixing the stock solutions, were added prior to final adjustment of the volume. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or 0.1 N HCl using pH meter (Digichem, 8201 pH meter, India). The medium was solidified with SD's or E. Merck agar at 0.8 % w/v unless otherwise mentioned. After addition of agar, the medium was heated gently with constant stirring. The medium was then distributed into tubes (25 x 150 mm) in 20 ml aliquots, or into flasks (150 ml) in 30 ml aliquots. Liquid media were distributed directly in tubes or flasks. Whatman no. 1 filter paper bridges were used in the case of liquid medium in tubes to give the necessary support to the inoculum. Media were then autoclaved at 15 lb/sq. inch for 20 min. (121°C).

Heat labile compounds such as auxins, $GA_3$, Ethephon, Biotin, folic acid, etc. were sterilized by filtration through millipore membranes (0.22 $\mu$) and added to the sterile medium under aseptic conditions.

Table II-1.a: Macroelement concentrations in different basal media.

All values expressed as mg $l^{-1}$

| Chemical | Murashige and Skoog's MS (1962) | White's W (1954) | Gamborg's B5 (1968) | Wood and Braun's WB (1961) |
|---|---|---|---|---|
| $NH_4NO_3$ | 1650 | - | - | - |
| $KNO_3$ | 1900 | 80 | 2500 | 80 |
| $Ca(NO_3)_2 \cdot 4H_2O$ | - | 300 | - | 300 |
| $CaCl_2 \cdot 2H_2O$ | 440 | - | 150 | - |
| KCl | - | 65 | - | 845 |
| $MgSO_4 \cdot 7H_2O$ | 370 | 720 | 250 | 720 |
| $Na_2SO_4$ | - | 200 | - | 200 |
| $KH_2PO_4$ | 170 | - | - | - |
| $NaH_2PO_4 \cdot 2H_2O$ | - | 16.5 | 150 | 300 |
| $(NH_4)_2SO_4$ | - | - | 134 | 790 |
| $NaNO_3$ | - | - | - | 1800 |

Table II-1.b: Microelement concentration in various basal media

All values expressed in mg $l^{-1}$

| Chemical | Murashige and Skoog's MS (1962) | White's W (1954) | Gamborg's B5 (1968) | Wood and Braun's WB (1961) |
|---|---|---|---|---|
| KI | 0.83 | 0.75 | 0.75 | 0.75 |
| $H_3BO_3$ | 6.2 | 1.5 | 3.0 | 1.5 |
| $MnSO_4.4H_2O$ | 22.3 | 7.0 | 10.0 | 7.0 |
| $ZnSO_4.7H_2O$ | 8.6 | 3.0 | 2.0 | 2.7 |
| $Na_2MoO_4.2H_2O$ | 0.25 | - | 0.25 | - |
| $CuSO_4.5H_2O$ | 0.025 | 0.001 | 0.025 | - |
| $CoCl_2.6H_2O$ | 0.025 | - | 0.025 | - |
| $Na_2EDTA$ | 37.25+ | - | 37.3 | 37.25 |
| $FeSO_4.7H_2O$ | 27.95+ | - | 27.8 | 27.95 |
| Ferric citrate | - | 5* | - | - |

* Indicates difference from the original media.

\+ The $FeSO_4.7H_2O$ is dissolved in approx. 200 ml distilled water. The $Na_2EDTA$ is dissolved in approx. 200 ml distilled water separately heated and mixed (under continuous stirring) with $FeSO_4.7H_2O$ until it dissolved completely. The whole mixture was boiled for 5 min. After cooling the volume was adjusted to 1000 ml. Heating and stirring result in a more stable Fe-EDTA complex.

Table II-1.c : Organic constituents of various nutrient media

All values expressed in mg $l^{-1}$

| Chemical | Murashige and Skoog's MS (1962) | White's W (1954) | Gamborg's B5 (1968) | Wood and Braun's WB (1961) |
|---|---|---|---|---|
| Inositol | 100.0 | - | 100.0 | 100.0 |
| Nicotinic acid | 0.5 | 0.5 | 1.0 | 0.5 |
| Pyridoxine HCl | 0.5 | 0.1 | 1.0 | 0.1 |
| Thiamine HCl | 0.1 | 0.1 | 10.0 | 0.1 |
| Glycine | 2.0 | 3.0 | - | 3.0 |

## II-3. ASEPTIC TECHNIQUES

All inoculations and manipulations involving sterile cultures or media were carried out in a Laminar Flow cabinet (Klenzaids, India). Prior to use, the working surface in the Laminar Flow cabinet was wiped either with 70% alcohol or diluted Dettol. The UV light in the cabinet was also put on for 10-15 minutes to avoid contamination. The dissection instruments were kept immersed in 70% alcohol. During inoculations they were sterilized by flaming with alcohol inside the cabinet.

### II-3.a. Surface sterilization and preparation of explants

#### II-3.a. 1. Seeds

The seeds of guggul were collected from different arid and semiarid zones. Since routine sterilization procedures failed, an elaborate and rather stringent protocol was adapted for surface sterilization as under :-
The seeds were first mechanically scarified with sandpaper and washed in running tap water for 1 h. Later they were washed with 0.1% Teepol solution for 2 min. The washed seeds were treated with 10 ppm solution of Nystatin and Streptomycin in 70% alcohol for 1 min. Alcohol was washed off with distilled water. The seeds were then sterilized with 0.1% (w/v) Mercuric chloride for 20 min under vacuum in a desiccator followed by washing thoroughly with sterile

distilled water. Later the seeds were transferred to sterile distilled water and agitated for 48 h on a gyratory shaker. The seeds were sterilized once more with 0.1% (w/v) Mercuric chloride under vacuum for 10 min. The embryos were dissected out aseptically by removing the hard seed coat and planted on hormone free basal medium.

II-3.a.2. Mature tree explants

For the initiation of shoot cultures, the explants were prepared as follows :-
Cultures from adult trees were initiated from single nodal explants in the months of April to June when the plant showed fresh growth. To test the response of successive buds the terminal shoot tips were excised from the actively growing shoots. The successive nodal explants with dormant buds were inoculated on different days. The nodal explants bearing axillary buds were excised from the plants using a sharp blade and collected in water. The explants were immediately subjected to washing and inoculation procedures. They were washed first in running tap water and then with a mild detergent solution and followed by a quick dip in 70% ethanol containing nystatin and streptomycin (10 ppm). After washing out the alcohol, surface disinfection was done with 0.1% Mercuric chloride solution (w/v) for 2-5 min depending on the tenderness of the material. After repeated

washing with sterile distilled water, explants measuring 1-1.2 cm in length (approx.) were excised from the nodal segments by trimming the cut ends. Nodal explants with a single axillary bud were immediately inoculated in culture vessels.

For callus initiation, 10-20 mm stem explants and fresh leaves were collected from trees and surface sterilized according to the procedure described earlier for shoot tip cultures. Aseptically cut leaf discs (5 mm in diameter) and 5-10 mm explants of stem were inoculated onto different media for the initiation of callus.

II-3.b. Stock Cultures

Stock cultures of callus were maintained by repeatedly subculturing the healthy, fast growing callus masses on fresh medium every four weeks. These callus tissues served as inocula for the experiments.

To establish shoot cultures, the mother explant on which multiple shoots were formed, was taken out carefully into sterile Petri plates. Using a sharp blade, the callus formed, if any, was removed and the whole tissue mass with sprouted shoots was split into pieces, each bearing a single sprouted shoot and placed upright on the fresh medium. About 1/4 of the basal part of each of the explants was immersed

into the medium. Further reculturing of shoot cultures was done similarly. Care was taken to remove unwanted brown and senescenced tissue material.

For the induction of root system, well grown individual shoots were separated from the basal portion of the culture. After giving a cut in the horizontal plane, the basal end of the shoot (about 5 mm) was immersed into the semi-solid or liquid rooting medium. In liquid medium, the inoculum rested on a Whatman no. 1 filter paper bridge.

II-3.c. Suspension Culture

Cell suspensions were obtained by the transfer of friable callus masses to agitated liquid medium in 150 ml Erlenmeyer flasks. In order to achieve a high growth rate and uniform cell suspension, the medium for callus cultures was slightly modified. Healthy fine suspension was achieved by filtering through 500 $\mu$ nylon mesh. The cell suspension consists of free cells and cell aggregates ranging from 2 to 20 cells/clump (Fig. II-1).

II-3.d. Incubation

The static cultures were incubated at 25$\pm$2°C, with a photoperiod of 16 h light and 8 h dark for the duration of the experiment unless otherwise mentioned. The light

Fig. II-1 Suspension culture of Commiphora wightii

500 μ fraction consisting of free cells and cell aggregates ranging from 2 to 20 cells/ clump.

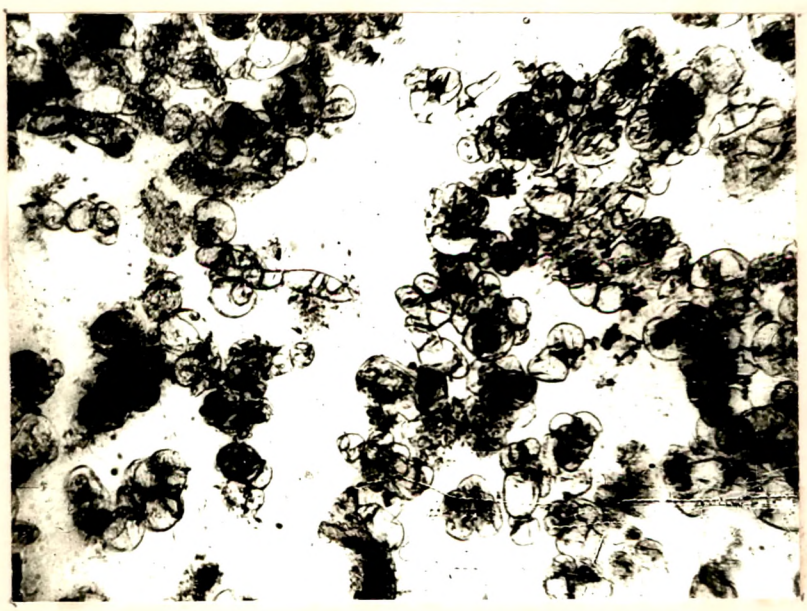


Fig. II-1

intensity was 1500 lux and was supplied by cool daylight fluorescent tubes. The suspension cultures were continuously agitated on a horizontal rotary shaker (speed 120 r.p.m.) at 25±2°C in a culture room (1000 lux, 16 h photoperiod).

## II-4. MEASUREMENT OF GROWTH

Growth measurements were made as a function of increase in fresh and dry weights of callus tissues. The growth of cell suspension cultures was monitored by measurement of fresh wt., dry wt. and packed cell volume. For growth measurements three replicate cultures were harvested at fixed intervals of time. Standard deviation was calculated to ascertain statistical significance of the growth data.

### II-4.a. Fresh Weight

In the case of callus cultures, the tissues grown on agar media were carefully transferred on previously weighed aluminium foil. Agar medium adhering to the callus masses was carefully removed to eliminate error and the weight was determined on a semimicro single pan balance. In the case of suspension cultures, the cells were harvested by suction filtration through a previously weighed sinter glass Gooch crucible and the fresh weight was measured as in the case of callus cultures.

II-4.b. Dry Weight

Dry weight of the tissue was determined by drying the weighed mass of fresh tissue to a constant weight at 60°C in an oven.

II-4.c. Packed Cell Volume (PCV)

A known volume of suspension was transferred to tapering and graduated glass centrifuge tubes. They were spinned in a centrifuge (Remi, India) for 10 min at 5000 x g. PCV is the volume of the pellet as a function of the volume of culture.

II-4.d. Studies on shoot tip cultures

Visual observations were taken at the end of 20-25 days for all the experiments on shoot formation and root induction. The number of shoots formed, the length of shoots and the leaf size were used as indices for comparison.

II-5. TRANSFER OF ROOTED PLANTLETS TO POTS

Plantlets with well grown root system were carefully removed from the culture vessel without injuring the root system. They were individually washed thoroughly in running tap water to remove excess medium or agar adhering to them. The plantlets were potted into a previously sterilized mixture of fine sand and vermiculate (1:1) and covered with

a beaker to maintain high humidity. The plantlets were watered daily with tap water and twice per week with 1/4 strength MS medium without sucrose and vitamins. The plantlets were aclimated by the removal of the beaker for a few hours daily in the beginning and then completely after 2 weeks. The pots were kept in the culture room for a few days before transferring to the green house.

## II-6. CHEMICAL ANALYSIS

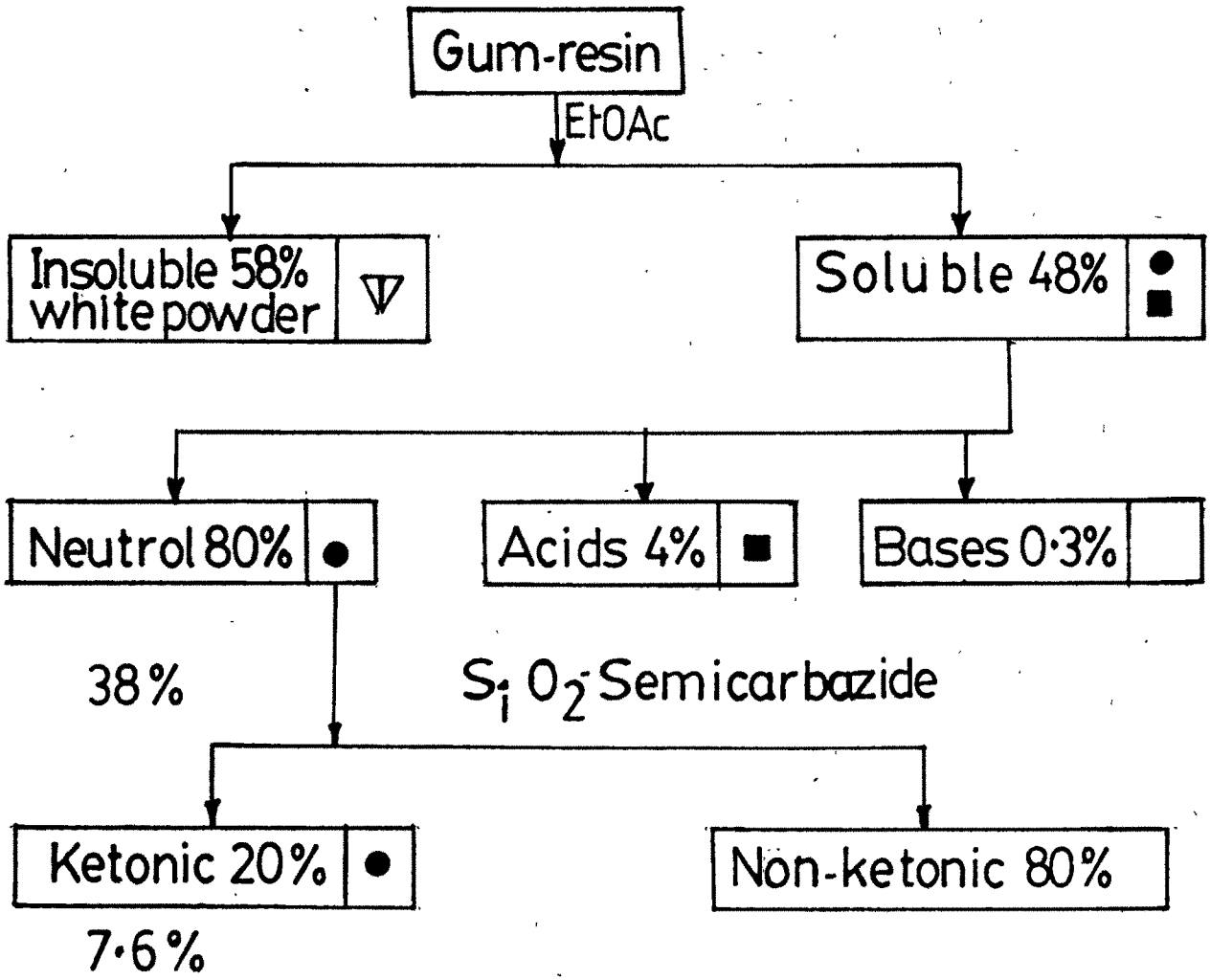
### II-6.a. Extraction and estimation of ketonic fraction and Guggulsterone Z and E

Qualitative and quantitative estimation of guggulsterones Z and E was done as described by Bajaj and Sukh Dev (1982).

Fig.II-2 explains the separation scheme followed for the extraction of ketonic fraction.

#### II-6.a.1. From gum resin

The gum resin was in the form of light to dark brown conglomerates of tears and was slightly sticky to touch and had a faint balsamic odour (Fig.II-3). The gum resin (1 g) was extracted in a mortar and pestle with 20 ml ethyl acetate. The extract was withdrawn and the operation was repeated four times - each time using 20 ml of ethyl acetate. The




■ Anti-inflammatory activity

● Hypocholesterolemic activity

▽ Toxic

Segregation of Guggul gum resin

Fig. II-2

Fig. II-3 Oleo-resin gum of Commiphora wightii.

Fig. II-3

combined ethyl acetate extracts were freed of solvent (50°C) to furnish the extract as a dark brown gum. The ethyl acetate insoluble material was an off white friable solid.

The residue was taken up in ethyl acetate (20 ml) and extracted with 3 N HCl aq. (10 ml x 2). The acid extract on basification with $NH_3$ aq. and usual work up with ethyl acetate gave basic material. The ethyl acetate layer was next extracted with 10% $Na_2CO_3$ aq. (40 ml x 2) and the carbonate extract acidified (25% v/v, $H_3PO_3$) to get acidic fraction. The neutral ethyl acetate layer was finally washed with brine (20 ml x 2) and freed of solvent to furnish neutral fraction.

A mixture of neutral fraction, 10% semicarbazide on silica gel and toluene (20 ml) was stirred and heated (60-62°C) for 14 h. After cooling (room temp.) and filtering, the silica gel was thoroughly washed with toluene (20 ml x 3). The above washed silica gel, oxalic acid aq. (10%, w/v) and toluene (40 ml) were stirred and refluxed (2.5 h) after which it was cooled and filtered. Silica gel was washed twice with ethyl acetate (20 ml). The combined solvent extract was washed with water and brine and freed of solvent to give the required ketonic fraction. The ketonic fraction was redissolved in ethanol. The UV absorbance of the extract in ethanol was measured at 241 nm (Shimadzu, UV visible

recording spectrophotometer UV-240 Graphicord). Ethanol served as blank. Percentage of guggulsterones in the sample was determined by using the formula

$$C = D\ 241/27{,}000\ b$$

where C is the molar concentration of guggulsterones; 27,000 is specific extinction coefficient and b is inside depth of the cell in centimeters which is 1(Fig.II-4).

II-6.a.2. From plant parts and callus

Plant parts such as leaves, stem and callus were first air dried. Estimation of guggulsterones was performed by the procedure described above for gum resin.

II-6.a.3. Semicarbazide on silica gel

Semicarbazide on silica gel is used for the isolation of aldehydes and ketones. It was prepared according to the procedure described by Singh et al. (1981).

Semicarbazide HCl (50 g, 0.045 mole) was added to a solution of NaOH (2.0 g, 0.05 mole) in water : methanol (1:1, 60 ml) and to the resulting clear solution, silica gel (45 g) was introduced with stirring. The whole mixture was mechanically shaked (1 h) at room temperature. Water-methanol was removed on a rotary evaporator (90°C) to get a white free flowing powder. This product was stored in a brown bottle at room temperature.

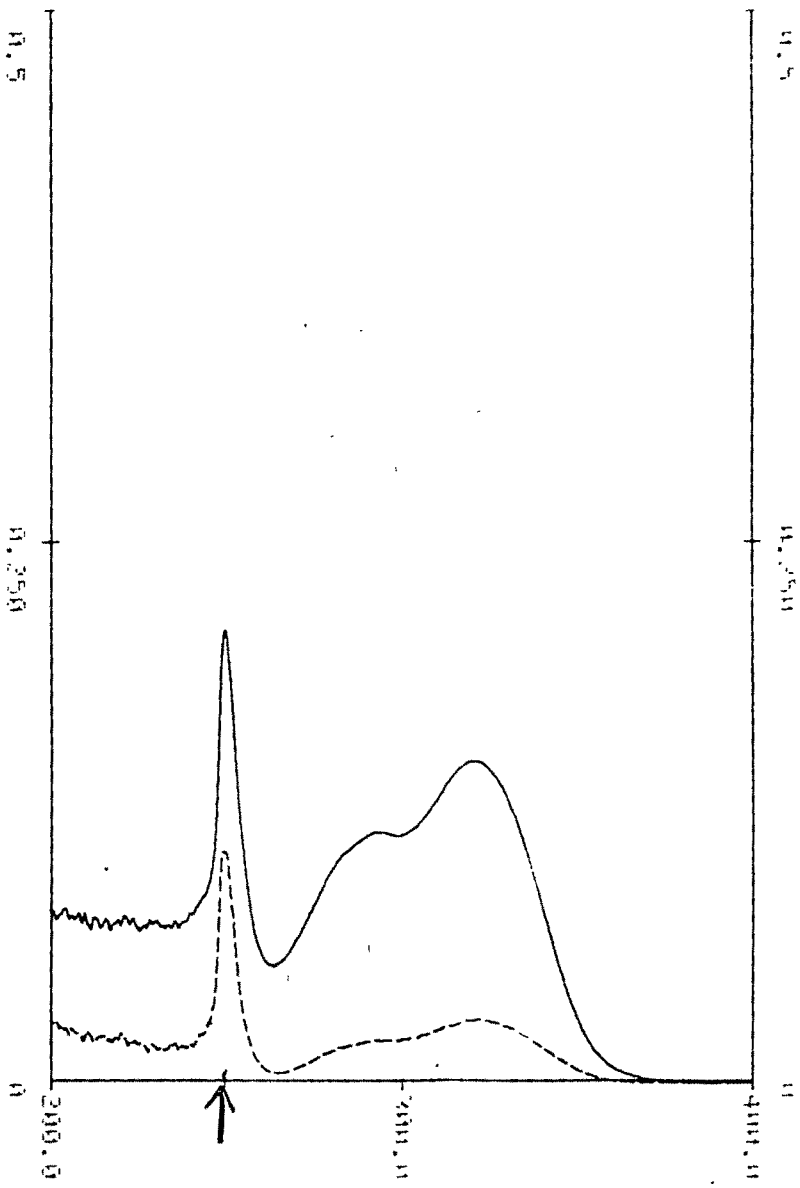


Fig. II-4 UV absorption spectrum of guggulsterones.

——— authentic

---- extract

II-6.a.4. Qualitative analysis of Guggulsterones by TLC

The guggulsterones were detected qualitatively by Thin Layer Chromatography. Glass plates (20x20 cm) were used for TLC. Slurry of silica gel G (Type 60) was used to prepare the slurry in water in a 1:2 ratio (15 g/30 ml). Chromatographic plates were prepared using a standard slurry applicator adjusted to 0.25 $\mu$. These plates were air dried and then activated at 60°C for 1 h. After the samples were spotted, the chromatograms were developed in the solvent system ethyl acetate : benzene 20:80. The samples were co-chromatographed along with authentic samples. The plates were sprayed with 2,4-Dinitrophenylhydrazine reagent. By comparing the orange spot obtained with authentic sample of guggulsterone, the presence of guggulsterones was confirmed in the ethyl acetate extract.

The 2,4-Dinitrophenylhydrazine reagent was prepared by suspending 2 g of 2,4-DNP in 100 ml methanol. 4.0 ml of conc. $H_2SO_4$ was added to it cautiously. The mixture was cooled and stored in brown bottle.

II-6.b. Determination of total $\beta$-C-3 sterols

The total $\beta$-C-3 sterols were estimated as described by Bhatt and Bhatt (1982).

Lipids were extracted from different samples using cold methanol-chloroform mixture (2:1) containing 50 mg/l butylated hydroxytoluene. The extract was filtered through suction and the residue was washed again with methanol-chloroform. The chloroform layer was separated. Chloroform was evaporated from the lipid mixture on a rotary evaporator under vacuum ( 45°C). Sterols from steryl esters and steryl glycosides were hydrolysed by alkaline (10% KOH in 95% ethanol for 30") and acid hydrolysis (0.6% $H_2SO_4$ in 95% ethanol for 2 h ) respectively in the manner suggested by Bush, Grunwald and Davis (1971). The sterol fraction was immediately dissolved in 2 ml of acetone-ethanol (1:1) to which 1 ml of a 2% hot digitonin solution in 50% ethanol was added. The mixture was kept overnight in a screw-capped vial at room temperature. Under these conditions digitonin forms an insoluble complex with 3 β sterols. The complex of digitonides was pelleted by centrifugation at 5,000 g for 15 min, washed with 2 ml acetone : ether (1:2) and repelleted. After one more washing, part of the digitonides was dissolved in 3 ml glacial acetic acid for quantitative determination by a colorimetric procedure of Zlatkies et al. (1963) using $FeCl_3.H_2SO_4$ reagent. The remaining digitonides were dissolved in 2 ml pyridine and heated at 70°C for 2 h and then left at room temperature for 12 h. Digitonin released, was precipited with ethyl ether and removed by centrifugation. Sterols

were recovered from ether layer. Ether layer was evaporated and sterols were redissolved in ethyl acetate. The regenerated sterols were silylated by addition of 200 µl BSA[N,O-bis(trimethylsilyl) acetamide] at room temperature as recommended by Grunwald (1970). At this stage a known quantity of 5α-cholastane was added as an internal standard. Finally the mixture was chromatographed on a OV 101 column (column length 2 meters and outer diameter 1/4"; glass column packed with 5% OV 101; mesh size 80-100, support cromosorb WHP.). The GC (Gas chromatograph, Chromatography and Instruments Company, Baroda) was equipped with a flame ionization detector (FID). During chromatography column temperature was adjusted to 260°C and detector and injector temperatures to 280°C. Nitrogen was used as a carrier gas at the flow rate of 30 ml/min. All biochemical analysis were done in triplicate.