CHAPTER - II

MATERIAL AND METHODS

The present work deals with the pharmacognosy of 14 plants as shown in the following table:

Name of the plant and market name of the drug	Collected from	Date of collection	Part studied
1. Glinus lotoides Linn.* (Ficoideae)	Road side pits Ahmedabad.	May, June 1957	Entire
2. Glinus oppositifolius L.* (Ficoideae)	Chandola lake, Ahmedabad.	April-May 1959	Stem and leaf; partly root also
3. Mollugo nudicaulis Lamk.* (Ficoideae)	From fields, Ahmedabad.	September-October, 1959 & 1960	Entire
4. Mollugo cerviana (L.) Ser.* (Ficoideae)	Fields, Ahmedabad purchased from Madras market	October - November, 1958	Entire
5. Gisekia pharnaceoides L. (Ficoideae)	M.G. Science College garden, Ahmedabad.	December-January, 1959.	Entire

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Name of the plant and market name of the drug	Collected from	Date of collection	Part studied
6. Primula denticulata Smith(Primulaceae)	Gulmarg,Kashmir, (alt. 9000 - 10000 ft.)	May, 1959	Entire
7. Anagallis arvensis L. (Primulaceae)	Fields, Ahmedabad	March-April 1959, 1960	Entire
8. Dodonaea Viscosa *@ L. (Sapindaceae)	Pharmacognosy Garden, Ahmedabad	May-June, 1960 1961	Entire
9. Smilax china L. (Liliaceae) 'Chobchini'	Central Drugs Laboratory, Calcutta and also purchased from Ahmedabad Market	-	Rhizone
10. Capparis moonii Weight(Capparidaceae) 'Rudanti'	R.H.Wats & Sons, Khandala; Unichem Laboratories, Bombay, and Himalaya Drug Co., Bombay.	-	Fruit
11. Zanthoxylum rhetsa DC.(Rutaceae)	Karwar(Maharashtra State)	-	Stem - bark

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Name of the plant and market name of the drug	Collected from	Date of collection	Part studied
12. Merremia emarginata@ Hallier. (Convolvulaceae) ' Brahmi '	University campus, Ahmedabad	August-September, 1958 & 1960	Entire
13. Withania somnifera Dunal (Solanaceae) 'Asvaghandha' or ' asan ' or ' asgund'	Pharmacognosy garden, Ahmedabad; Central Drugs Laboratory, Calcutta, and Ahmemedabad market	-	Entire
14. Naregamia alata£ Wight (Meliaceae) ' Goanese or Portugese or country ipecac' and ' Nilanarakam '	Delhi and Trivendrum markets; Entire plants from Trivendrum	-	Entire

* Antibacterial activity has also been studied

£ Phyto-chemistry has also been studied

@ Pharmacology has also been studied.

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Leaf, petiole, stem and root pieces from different levels were fixed in formaline-acetic-alcohol (Chamberlain, 1932). The material was dehydrated and imbedded in paraffin in the usual way. Sections were cut at 15-20 μ thickness as per requirements. Safranin and fast green were used for staining purposes in most cases. In case of rhizome of *Smilax china*, pericarp of *Capparis moonii* and the stem-bark of *Zanthoxylum rhetsa*, the material was first soaked in a mixture of water, alcohol and glycerine (equal parts) for about ten days. Free-hand sections were first cleared with chloral hydrate before preparing permanent mounts.

Maceration was carried out according to Schultze's method (Trease, 1952). Leaf constants viz. stomatal index, palisade ratio and vein-islet numbers were determined according to Wallis (1955).

Evaluation of saponin :

There are at present, no satisfactory chemical methods for the quantitative estimation of saponin. Estimation is done by different methods depending on frothing, surface tension, viscocity, haemolysis or toxicity to lower organisms. It may be mentioned that the methods are relative and there is no relation or

parallelism between the different methods.

Several methods are in use for the estimation of saponin. In the present work, saponin has been estimated by two methods viz. (1) Froth number (Kofler in Wasicky, et al., 1936) and (2) Haemolytic index (Wasicky, et al., 1936). They gave fairly satisfactory results.

Method 1 :

Froth number is determined according to the method of Kofler (Wasicky et al.,1936). It is the dilution of the drug extract corresponding to 1 g. of the drug which gives 1 cm. high froth, when 10 c.c. of such diluted extract is shaken vigorously for 15 seconds and allowed to stand for 15 minutes in a test tube having a diameter of 16 mm. For the determination, aqueous extracts of definite concentration are prepared by heating the drug for half an hour with 100 c.c. of distilled water in a flask on a boiling water bath. During this period, the flask is covered with a funnel to minimise evaporation. If the extract is acedic, it is neutralised with 5% sodium carbonate solution. The extract is filtered, cooled to room temperature and adjusted to 100 c.c. with distilled water.

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Ten test tubes of equal internal diameter (i.e. 16 mm.) are selected and 1, 2, 3, ...0 c.c. portions of distilled water are added to these test-tubes in order to make the final volume of 10 c.c. in each test-tube (Plate I, 1).

Each test-tube in succession is then shaken for 15 seconds and kept for 15 minutes. The concentration in the test-tube showing 1 cm. high froth is used for calculation of the froth number.

For the sake of illustration, the procedure with the roots of Glinus lotoides may be described. With a 1% extract of the drug, 1 cm. high froth is observed in the 5th test-tube (Plate I, 1) chich contains 5 c.c. of 1% extract, or 0.05 g. of the drug in 10 c.c. The froth number is then $\frac{10 \times 1}{0.05} = 200$

Method 2 :

Haemolytic Index : It is the concentration of the saponin i.e. drug containing saponin which causes complete haemolysis of erythrocytes. For the determination of haemolytic index following procedure (Wasicky, et al., 1936) is used.

Blood of freshly slaughtered ox is

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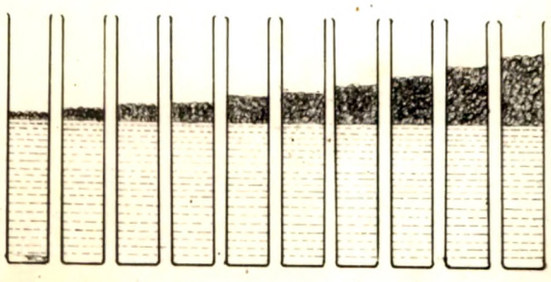
collected in the evening from slaughter house, defabrinated immediately and kept in ice overnight. Next morning, the blood is placed in refrigerator. Some blood is withdrawn each time from stored blood for the experiments by a sterilised pipette. It is then diluted before use, (1 : 50) with physiological saline solution. The diluted blood is then ready for experiments.

Extracts of the powder (40 mesh) are prepared by weighing a definite quantity of the drug and extracting with 100 c.c. of isotonic buffer solution of pH 7.33. The extracts of different pH are prepared similarly by using different isotonic buffer solutions. The extraction is carried out by heating the drug for 30 minutes on a boilding water bath. It is then filtered hot, cooled to room temperature and the final volume is adjusted to 100 c.c.

The buffer solutions are prepared by using modified buffer solutions of Sörenson (as given in Layman and Sprowls, 1955), as given in the following table :

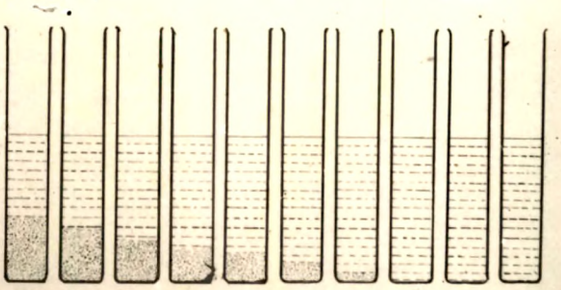
FROTH NUMBER OF ROOTS OF GLINUS LOTOIDES, LINN.



TEST TUBE NO.	1	2	3	4	5	6	7	8	9	10
1% EXTRACT	1 c.c.	2 c.c.	3 c.c.	4 c.c.	5 c.c.	6 c.c.	7 c.c.	8 c.c.	9 c.c.	10 c.c.
DISTILLED WATER	9 c.c.	8 c.c.	7 c.c.	6 c.c.	5 c.c.	4 c.c.	3 c.c.	2 c.c.	1 c.c.	0 c.c.

Fig. 1

HÆMOLYTIC INDEX OF ROOTS OF GLINUS LOTOIDES, LINN.



TEST TUBE NO.	1	2	3	4	5	6	7	8	9	10
(1:50) BLOOD	5·0 c.c.	5·0 c.c.	5·0 c.c.	5·0 c.c.	5·0 c.c.	5·0 c.c.	5·0 c.c.	5·0 c.c.	5·0 c.c.	5·0 c.c.
ADJUSTING SOLUTION	4·5 c.c.	4·5 c.c.	4·5 c.c.	4·5 c.c.	4·5 c.c.	4·5 c.c.	4·5 c.c.	4·5 c.c.	4·5 c.c.	4·5 c.c.
0·5% EXTRACT	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.

Fig. 2

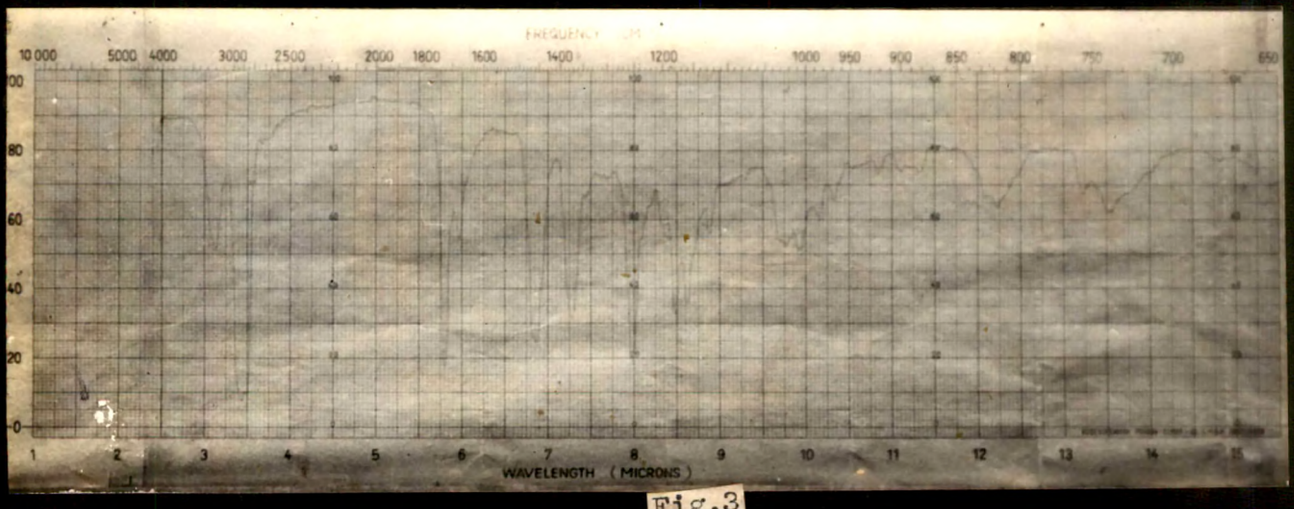


Fig. 3

P L A T E - I

Fig. 1. A diagram showing Froth number

Fig. 2. A diagram showing haemolytic index.

Fig. 3. Infra-red spectrum of the acetate derivative of sapogenin obtained by hydrolysis of saponin isolated from the roots of *Glinus lotoides* Linn.

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TABLE - I

Buffer Solutions

pH	Quantity of M/15 NaH_2PO_4	Quantity of M/15 Na_2HPO_4	Percentage of NaCl to make isotonic
5.91	90 c.c.	10 c.c.	0.52%
6.24	80 c.c.	20 c.c.	0.51%
6.47	70 c.c.	30 c.c.	0.50%
6.64	60 c.c.	40.c.c.	0.49%
6.81	50 c.c.	50 c.c.	0.48%
6.98	40 c.c.	60 c.c.	0.46%
7.17	30 c.c.	70 c.c.	0.45%
7.38	20 c.c.	80 c.c.	0.44%
7.73	10 c.c.	90 c.c.	0.43%
8.04	5 c.c.	95 c.c.	0.42%

Ten clear dry and transparent test tubes are selected for each experiment and are numbered 1, 2, 3 ..... 10. 5.0 c.c. of blood diluted (1 : 50) as mentioned above is used in all test tubes. 4.5 c.c. of adjusting buffer solution is added in the 1st test tube, 4.0 c.c. in 2nd test tube, 3.5 c.c. in 3rd test tube, ... 0.0 c.c. in 10th test tube respectively (Plate I, 2).

The contents of the test tubes are mixed by gently shaking (taking care that no frothing occurs) immediately after adding the solutions and also 15 minutes

after the first shaking. The test tubes are then kept for 12 hours.

In the determination of the haemolytic index the time for which the saponin is allowed to act is of importance. Some saponins (especially digitonin) act very rapidly, others (especially primula saponins) are much slower. With the slow saponins, the action is delayed by lowering the temperature. A temperature of 15°- 20° c. should be maintained and it should not be allowed to drop below 10° c.

After some hours, some test tubes show partial haemolysis while some show complete haemolysis. The test tubes having no sediments of R.B.C. and having uniform red coloured solution were taken as showing complete haemolysis. The extracts of the drug are so adjusted that the complete haemolysis is always found in the test tube No.6 onwards.

As a standard for haemolytic index determination, no substance is ideal. Generally, 'saponin pure Merck' has been used as a standard. Jaretzky (1936) mentions that using 'saponin pure Merck', no uniform results are obtained and recommends digitonin as a standard. Digitonin has therefore, been used as a standard here. Digitonin has the haemolytic index 195,000 so the results were corrected by multiplying with the factor $\frac{195,000}{125,000} = 1.56$.

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Besides digitonin, primulic acid (Jaretzky, 1936), and non-saponins like desoxycholic acid (Runge, 1952), sodium oleate (Fisher, 1952), sodium lauryl sulphate (Ruyssen, 1952) etc. have also been recommended as standards. The results, using crystalline digitonin are satisfactory.

The haemolytic index in case of roots of Glinus lotoides is calculated as follows:-

As shown in Plate I, figure 2, test tube No.7 shows partial haemolysis, while test tube No.8 shows complete haemolysis. 8th test tube contains 4.0 c.c. of 0.5% extract in 10 c.c. of the blood suspension, or $\frac{(4.0 \times 0.5)}{100}$ = i.e. 0.02 g. of the drug in the test tube.

Therefore, 0.02 g : : 1.0 g :::: 10 c.c. } i.e. $\frac{10 \times 1}{0.02} = 500$ H.I.

The above result is corrected by multiplying with the factor (1.56) as shown above. The corrected value of H.I. is then 780.

At a latter stage, the values of haemolytic index are compared with those of senega root and quillaia bark; this is necessitated as digitonin available in the market after the first stock is exhausted, was found to be impure and gave unsatisfactory results.

Antibacterial activity :

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Leaves of Glinus lotoides Linn. and aerial parts of G.oppositifolius Linn., Mollugo nudicaulis Lamk., M. cerviana (L), Ser. and Dodonaea viscosa Linn. were collected when the plants were in flowering. The materials were dried at room temperature under shade. Aqueous and alcoholic extracts were then prepared as follows:-

(A) Aqueous extract : The drug was powdered in the grinding mill and passed through 40 (B.S.) mesh sieve. The powdered drug was imbibed with chloroform water and set aside for 4 hours. It was then placed in a percolator and macerated with sufficient chloroform water for 24 hours and then percolated. The first portion was reserved and the percolation continued till complete exhaustion. The first and the subsequent portions of the percolate were concentrated under reduced pressure and the concentration was finally adjusted so as to represent 250 mg. of the drug in 1 ml. of the extract.

(B) Alcoholic extract : Alcoholic extract was prepared by complete exhaustion of the powdered drug in a soxhlet extractor with 90% (v/v) alcohol. Finally, the alcohol strength was adjusted to 70%. The final concentration was adjusted so as to represent 250 mg. of the drug in 1 ml. of the extract.

The antibacterial activity of aqueous and alcoholic extracts was studied by cup-plate method (Patel and Chemburkar, 1958) on the following organisms :

(1) Micrococcus pyogenes var. aureus
(2) Micrococcus pyogenes var. albus
(3) Micrococcus pyogenes var. citreus
(4) Bacillus megatherium
(5) Bacillus mycoides
(6) Bacillus subtilis
(7) Sarcina lutea
(8) Escherichia coli
(9) Salmonella typhi
(10) Salmonella paratyphi 'A'
(11) Salmonella paratyphi 'B'
(12) Salmonella, paratyphi 'C'
(13) Proteus vulgaris
(14) Pseudomonas aeruginose

Cup plate method : Petridishes having 14 cm. diameter were used. 40 c.c. of nutrient agar, melted and cooled was added in the petridish to get base layer. It was allowed to set completely. 8 c.c. of nutrient agar, melted and cooled at 45°, and inoculated with 0.5 c.c. of 24 hours' old culture of test organisms was poured carefully in the petridish to form a seed layer without disturbing the base layer. The layer was allowed to set completely. Cups were then bored with a sterilised cork borer No.5. 0.2 c.c. of the aqueous and alcoholic extracts as prepared above were carefully pipetted in agar cups; 70% alcohol was used as control which did not give any inhibition.

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The petridishes were refrigerated for one hour and then incubated at 37° for 16 - 18 hours. The diameter of the zone of inhibition was then measured. Mean of three readings ~~were~~ was then taken for each of the organisms.