PART II

Studies on Gymnosporia montana (Roth), Benth.,
Syn., Maytenus emarginata (Willd.) D.Hou.
PART - II

STUDIES ON GYMNOSPORIA MONTANA (ROTH) BENTH. SYN. MAYTENUS EMARGINATA (WILLD) D.HOU.


Gymnosporia montana is a much branched, spinescent shrub or small tree, occurring throughout the arid, dry areas of India. It's systematic taxonomic position is as follows:

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Division</td>
<td>Spermatophyta</td>
</tr>
<tr>
<td>Sub-division</td>
<td>Angiospermae</td>
</tr>
<tr>
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<td>Dicotyledoneae</td>
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<tr>
<td>Sub Class</td>
<td>Polypetalae</td>
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<tr>
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</tr>
<tr>
<td>Order</td>
<td>Celastrales</td>
</tr>
<tr>
<td>Family</td>
<td>Celastraceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Gymnosporia (Wt. &amp; Am.) Benth &amp; Hook. f.</td>
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<tr>
<td>Species</td>
<td>Montana</td>
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<tr>
<td>Plant's name</td>
<td>Gymnosporia montana (Roth.) Benth.</td>
</tr>
<tr>
<td>Syn.</td>
<td>Maytenus emarginata (Willd.) D.Hou.</td>
</tr>
</tbody>
</table>

Regional Names : (333)

Ajmere : Kakra.
Bengal : Vaichigachha
Bhil : Dhatti.
Bombay : Hurmach, Malkangoni, Zekadi.
Canarese : Halumanike, Malegu, Malkanguni, Tandraja.
Central provinces : Baikal, Gajachinni
Gujaratli : Vikalo, Vikro.
Hindi : Baikal, Kngani, Tondarsajhad.
Marathi : Bharatti, Bharuli, Vekal, Vekar, Yekkadi.
Porbandar : Vikaro.
Punjab : Dajkar, Kharai, Kingaro, Mareila, Talkar.
Sanskrit : Bahuphala, Brahmapadapa, Dantakashta, Gopaghantha, Granthila, Himaka, Kantakari, Kantaki, Kantapada, Kantapatra, Kinkari, Madhuparni, Mriduphala, Padarohina, Pindara, Prithubija, Putakinkani, Ravana, Sragdaru, Sruvadrum, Sruvavriksha, Sudhavriksha, Svardkanta, Valikankata, Vikankata, Vritinkar, Vyaghrapada, Yadnavriksha, Yadhnya,
Tamil : Kattanji
Telugu : Dantausi, Danti, Gajasinni, Gechangi, Peddachintu, Peddadanta, Slinni.
Uriya : Gourokosa,
Distribution (333) : Throughout the arid, dry areas of India.

Properties and uses :

In several Ayurvedic literatures like Bhavprakash, (334) Nighantu Adarsh (335), Shaligram Nighantu (336), Vanaspati shrusti (337), Aryabishek (338), Shankar Nighantu (339), Vanaspati chandrodaya (340) it has been mentioned for various uses. It is claimed to be useful in jaundice (333, 335, 336), inflammation and rheumatic pain (333, 335-338, 340, 341), corneal opacity (335,338, 339,341), ulcers, gastrointestinal disorders, dysentery, toothache and also as a vermifuge (333, 342).

According to Shaligram Nighantu it is used in jaundice, inflammation and to cure blood disorders, Nighantu Adarsh mentions its use in kamla (jaundice). In Vanaspati shrusti the use of ripe fruit has been mentioned as blood purifier and antiinflammatory. Leaf juice is used in Pandu (anaemia) and used as an eye drop to cure corneal opacity. Bark is used to kill lice and in other infection on the head. The use of leaf juice in eye diseases particularly in opacity of cornea, inflammation and buring sensation has been mentioned in Aryabishek. In vanaspati chandradaya the use of root pulp in rheumatic pain and its gum (internally) alongwith other medicines in cholera has been advocated. Kirtikar and Basu mention the fruit as appetising and digestive and its use in jaundice and enlarged spleen. Seeds are ground to powder with turmeric and rubbed all over the body to prevent rheumatic pain from exposure to damp winds. Also gum is given alongwith other ingredients for cholera. The external application of powdered dry leaves with a little mustered oil has shown encouraging result in rickets (343).

Chemistry :

Joshi et al (344) have reported the isolation of tingenone, 3-0-acetyloleanolic acid, hexacosane, betulin, β-amyrin, hexacosanol and B-sitosterol from the leaves, stem bark and root bark of Gymnosporia montana. Akshaya Kumar et al (345) have isolated B- amyrin and n-triacontanol from the leaves and (-) epigallocatechin from the roots. Dukidol and B-amyrin
from the roots (346) and β-amyrin from the stem (347) have also been reported. Joshi et al. (348) have isolated iguesterin, pristimerin, tingenone, β-amyrin, β-sitosterol and maytenonic acid from its stem bark and iguesterin, pristimerin, tingenone, β- amyrin, and β-sitosterol from the root bark. Isolation of celacinnine, β-amyrin, β-amyrene, β-sitosterol and kaempferol have been reported from the leaves (349). A sesquiterpene pyridine alkaloid emarginatine β, and maytansine have been reported from the stem (350). Emarginatine A, another sesquiterpene pyridine alkaloid has also been isolated from this plant (351).

Several compounds have been isolated from other species of Gymnosporia (Maytenus). β-amyrin from the roots of G.oovata Laws (352), Maytansine from G.diversifolia (Gray) Maxim (353), sesquiterpenes from M.chubutensis (354), M.disticha (355) and M.canariensis (356) and triterpenoids - maitenin, pristimerin, 22-hydroxy maitenin, rigidenol and nepetricin, as well as (-) 4*-0-methyl - epigallo catechin, proanthocyanidin -A and dulcitol from the roots of M.evonymoides (357).

**Pharmacognosy :**
A perusal of the literature revealed non-availability of information on pharmacognostic characters of this plant.

**Pharmacology :**
Very few reports on pharmacological activity of Gymnosporia montana are available. Presence of antispasmodic activity has been reported by Dha‘ et al (358). Presence of a compound showing potent cytotoxicity against human KB cells (Ed$_{50}$ = 0.4 micro g/ml) has also been reported.
SECTION A: PHARMACOGNOSTIC STUDY

Gymnosporia montana (Roth.) Benth. is a large, glabrous, woody shrub or sometimes a small tree having young branches reddish to purple in colour and often spinescent at the extremities which bear leaves and flowers.

Flowers: Flowers are small, white, numerous, axillary, Cymes which are shorter than leaves, found on short tubercular branches or on the spines, are usually furcately branched; peduncles slender, solitary or fascicled, pedicels filiform, jointed below the middle, bracts small, lanceolate and acute, calyx having five lobes, broadly elliptic, oblong, rounded at the apex, ciliate, lobes fimbriate, petals five, 3 mm long, elliptic-oblong, white in colour, stamens five, filaments flattened and dilated at the base, disk fleshy, ten lobed, ovary orbicular, conlquent with the disk, style short, stigma 2-3 lobed.

Fruit: Two to three valved, globule capsule as large as a small pea about 6-7 mm in diameter, 1 to 3 celled and 1 to 2 seeds are found in each cell. Fruits are purple or nearly black when ripe. Seeds are brown, arillate arillus white, fleshy, covering the whole seed, a bumen white, cotyledons green and fleshy.

Since literature survey revealed non-availability of Pharmacognostic study on Gymnosporia montana, a detailed pharmacognostic study of the leaf and stem part and their powder was undertaken. Their pharmacognostic characters and the salient features are highlighted.

Gymnosporia montana plant is growing wildly in Rozy port, Jamnagar District, Gujarat, India. Taxonomic authenticity of the plants was confirmed (Fig. 3). Fresh leaves and stems were collected from the authentic plants in the month of December when in flowering and fruiting stage. They were then cleaned free from dust and other extraneous matter and preserved in F.A.A. mixture (95% ethyl alcohol 50 ml, glacial acetic acid 5 ml, formaldehyde 10 ml and distilled water 35 ml) and were used for pharmacognostic work. Sections were taken with the help of hand microtome. Steps for preparation of permanent slides were as follows:

i) Stained in aqueous safranin solution for half an hour and washed with distilled water.
ii) Successively passed through 30% alcohol, 50% alcohol, 70% alcohol and 95% alcohol for 15 minutes each.
iii) Stained in 1% alcoholic Malachite green for 5 minutes.
iv) Passed through absolute alcohol, two changes.
v) Two changes of xylene to clear the section and finally the slides were mounted in Canada balsam and were dried for 8 days.
Fig. 3 Gymnosporia montana plant
Fresh sections, stained in dilute aqueous Safranin solution and permanent slides were used for microscopic study and photomicrographs were taken in a Carl Zeiss, Jena Binocular microscope with photomicrographic attachments.

Surface peeling were taken from the blade (lamina) of leaf with the help of forceps, mounted and studied under microscope.

The Stomatal Index is the percentage which the number of stomata form of the total number of epidermal cells, each stomata being counted as one cell.

Stomatal Index = SX100 - (E+S), where S is the number of Stomata per unit area and E is the number of epidermal cells in the same unit area.

Stomatal index is fairly constant for any species and can be utilized as a specific character. Palisade ratio is the average number of palisade cells beneath one epidermal cell, using four contiguous epidermal cells for the count. This ratio has been shown to be sufficiently constant to serve as a diagnostic character of species belonging to the same genus.

The Stomatal index and Palisade ratio were determined according to the methods given by Wallis (359).

Dried leaf and stem powder (80 mesh) were also examined under the microscope.

1. LEAF:

I. Macroscopic characters:
Leaves are simple, alternate or clustered. They are found in the axils of spines or on small branches. They are sub-sessile, exstipulate, glabrous, coriaceous and polymorphic. Leaves are obovate, elliptic, lanceolate, oblanceolate, etc. (Fig. 4) Leaf margin is entire in the lower half and crenulate in the upper half. Leaf apex is acute, mucronate or obtuse. Leaves are 3 to 8 cm long and 1 to 3 cm broad. Petioles are reddish purple and 0.25 to 1.0 cm long.

II. Surface characters and quantitative microscopy:
Epidermal cells are angular, polygonal, smooth-walled and contain copious colouring matter (Fig. 5). Trichomes are absent both on upper and lower epidermal cells. Stomata are round, Ranunculaceous, more on lower epiderms. Calcium oxalate crystals (cluster) are abundant on lower epidermis.

The data of the quantitative microscopy has been shown in Table - 3. It can be seen from the table that the upper epidermis has a Stomatal index of 8.12 whereas the lower epidermis has a Stomatal index of 10.31. Palisade ratio is 2 to 5 with 3 to 4 observed more frequently.
Fig. 4 Gymnosporia montana leaves of variable size and shape.

Fig. 5 Photomicrograph of epidermis in surface view of G. montana leaf showing epidermal cells (EPC) and stomate (St); magnification x 400.
III). Microscopic characters:

Transverse section of leaf through mid-rib shows more or less isobilateral structure (Fig. 6). Upper epidermis of lamina is double layered, the cells of which are rectangular. The epidermis is covered by a thin, striated cuticle. Few stomata are seen here and there. Lower epidermis is also biseriate with a distinct cuticle and more stomata. Epidermal cells are round to rectangular. Certain epidermal cells contain granular material.

Mesophyll reflects a typical isobilateral nature of the leaf with two layers of palisade issue below the upper epidermis and above the lower epidermis. Cells in palisade tissue measure 35 to 56 micron in length and 10.5 to 14 micron in width. They show profuse deposits of yellowish black colouring matter. Spongy parenchyma between the upper and lower palisade tissue has loose, round to oblong parenchymatous cells, some of which enclose cluster crystals or calcium oxalate measuring 17.5 to 21 micron diameter.

The vascular bundle in the lamina portion, varying in both number and size depending upon the thickness of the veins, are horizontally arranged, round and consists of xylem and phloem elements containing colouring material at some places. Vascular bundle shows sclerenchymatous patches. Longitudinally cut xylem fibres contain cluster crystals of calcium oxalate. Longitudinally cut vessels show spiral and annular thickening.

Epidemis or mid-rib region is uniseriate followed by 3 to 4 cell layers of collenchymatous issue on either surface. The thin walled, round to flattened cells beneath this zone are parenchymatous and contain simple starch grains, rosettes and cluster crystals of calcium oxalate and deposits of colouring material. Starch grains are unique in that they do not show hilum and even striations are not distinct. Starch grains measure 3.5 to 7.0 to 10.5 micron on in diameter.
Fig. 6. Photomicrograph of transverse section of *G. montana* leaf through mid rib; magnification x 100. U.Ep.: Upper epidermis, L.Ep.: Lower epidermis, U.Pal.: Upper palisade layer, L.Pal.: Lower palisade layer, SP: Spongy parenchyma, Ph: Phloem containing colouring material, Xy: Xylem, Col.: Collenchymatous patch.
Vascular bundle in the mid-rib is crescent shaped, conjoint, collateral and surrounded by a broken ring of sclerenchymatous pericyclic fibres. Xylem vessels are narrow and xylem fibres are small, angular, radially arranged and they also contain colouring matter. Phloem, consisting of sieve tubes, companion cells and phloem parenchyma, is in the distinct curved arm of the vascular bundle. Phloem fibres are absent. Starch grains and colouring material are seen in these cells. Few thick walled cells in groups are also seen at the centre.

iv) Powder:
The leaf powder is greenish brown, slightly saline and astringent. Powder under microscope shows:

i. Cells of palisade tissue and spongy parenchyma
ii. Polygonal epidermal cells with Ranunculaceous stomata
iii. Dark colouring material in parenchymatous cells
iv. Simple, round starch grains in free and in side the cell
v. Xylem vessels with spiral and annular thickening
vi. Cluster crystals of calcium oxalate (Fig. 7).

2. STEM

I. Macroscopic characters:
Stem pieces are purplish brown, round and hard. Cut end is white and granular. Spines are straight, hard and pointed. They are modified branches and show a single node from which leaf originates. Bark is thin, has fine longitudinal wrinkles on the outer surface and its inner surface is creamy white.

II. Microscopic characters:
Transverse section of young stem (0.5 cm in diameter) is circular and exhibits cork, cortex, phloem, xylem and pith (Fig. 8).

Cork is made up of many layers of compact, thin walled, square to rectangular cells measuring 17.5 to 21 to 32 to 42 micron in length and 14 to 17.5 to 21 to 24.5 micron in width. Cortex is parenchymatous and cells are periclinaly elongated. Many cells have dark colouring matter, scattered, simple, round starch grains and isolated as well as clusters of calcium oxalate crystals.

Inner cortex has a nearly continuous ring of pericyclic fibres and few lignified, isolated, square to rectangular stone cells (Fig. 9). Phloem is present in a distinct ring and consist of sieve tubes, sieve plates, companion cells, phloem parenchyma and phloem fibres; cells in phloem region also show thick deposits of dark colouring material. Isolated, large, square to rhombooidal calcium oxalate crystals are very clearly seen in phloem region as well as in pericyclic fibres.
Fig. 7. Leaf powder of *G. montana* a) isolated cells of spongy parenchyma and palisade tissue. b) Epidermal cells in surface view without stomata. c) Epidermal cells in surface view with stomata, d) Longitudinally cut parenchymatous cells, e) Cluster crystals of calcium oxalate, f) Epidermal cells with palisade in surface view, g) Starch grains, h) Fibres - longitudinally cut i) Vessels - longitudinally cut.
Fig. 8 Photomicrograph of transverse section of stem of *G. montana* showing the distribution of tissues; magnification X32. C: Cortex, Ph: Phloem, SX: Secondary xylem, MR: Medullary rays, Pi: Pith.

Fig. 9 Photomicrograph of portion of transverse section of stem of *G. montana* showing cortex (C), pericyclic fibres (PF), phloem (Ph) and xylem (Xy).
Xylem vessels are narrow, single and radially arranged with simple as well as bordered pits. Vessels measure 525 to 600 to 750 to 900 micron in length and 30 to 60 to 70 micron in diameter. Vessels are surrounded by xylem fibres with narrow lumens. Xylem fibres are 750 to 900 to 1065 to 1200 to 1500 to 1800 micron in length and 15 to 22.5 to 30 to 45 micron broad.

Xylem and phloem are traversed by uniseriate medullary rays, the cells of which are round, parenchymatous and filled with dark colouring matter. Cells of medullary rays measure 17.5 to 35 to 52.5 to 70 micron in length and 10.5 to 17.5 to 24.5 to 35 micron in width. Pith is parenchymatous with round cells containing many round, single starch grains measuring 3.5 to 17.5 micron in diameter, dark colouring matter and few prismatic as well as cluster crystals of calcium oxalate. Cells at the centre of pith are larger than those towards the periphery (Fig. 10,11).

Older stems show annular rings in which xylem vessels towards pith are much compressed, compact and narrow.

III. Powder:
Powder of the stem is light brown in colour having no distinguishing odour and astringent, slightly sweetish later on, in taste. Powder under microscope shows:

i. Slightly elongated entire fragments of pericyclic fibres in groups as well as isolated cells of thick walls.
ii. Vessels with simple as well as bordered pits.
iii. Scattered square rectangular to rounded thick-walled stone cells in groups as well as isolated.
iv. Xylem fibres with thick, lignified walls, narrow lumen and tapering ends. Ends of some fibres are forked. The fibres have pitted thickenings.
v. Fragments of phloem elements like sieve tubes with sieve plates and companion cells and phloem parenchyma.
vi. Cells of medullary rays with dark colouring matter.
vii. Cells with dark colouring matter, starch grains and calcium oxalate crystals.
viii. Simple and compound starch grains isolated, as well as arrested in the cells. Simple starch grains are spherical, 3.3 to 6.6 to 9.9 to 16.5 micron in diameter. Striations and hilum not visible except occasional in larger simple grains. Compound starch grains are mostly of 2-3-4-5 components, grains having two components are mostly oblong and upto 16.5 micron in length.
ix. Abundant isolated, as well as in the different elements of xylem, squarish, prismatic calcium oxalate crystals (Fig. 12)

The use of various parts of Gymnosporia montana has been mentioned in Ayurvedic literature and folklore remedies. But, literature survey revealed non-availability of its pharmacognostic study. The detailed pharmacognostic study of leaf and stem of the plant was undertaken. Their pharmacognostic characters and salient features are highlighted. These characters may be taken as diagnostic features for the identification of leaf and stem of Gymnosporia montana.
Fig. 10 Photomicrograph of portion of transverse section of stem of *G. montana* showing xylem (Xy), medullary rays (MR) and pith cells containing scattered prismatic crystals of calcium oxalate (Cr); magnification x 200.

Fig. 11 Photomicrograph of portion of transverse section of stem of *G. montana* showing xylem (Xy), xylem vessels (XV) and pith (Pi); magnification x 200.
Fig. 12. Stem powder of *G. montana* a) Prismatic crystals of calcium oxalate b) Simple and compound starch grains c) Epidermal cells d) Stone cells - single and in group e) Fragment of tracheid with pitted wall, f) Grouped vessels with bordered pits - fragmented g) Thick walled angular cells containing prismatic crystals of calcium oxalate h) Fragmented pericyclic fibres i) Xylem fibres - broken.
SECTION B : PHYTOCHEMICAL SCREENING OF THE LEAF

The shade dried leaf of authenticated *Gymnosporia montana* (authentication and collection details have been described in Section A) was coarsely powdered and taken for phytochemical studies.

1. PHYSICOCHEMICAL PARAMETERS:
The sample was analysed for physicochemical parameters like ash value, acid insoluble ash, extractive values and fluorescence characters.

I. Determination of ash value:
Ash value was determined by the method as described in I.P., 1985 (360).
About 2 g of the leaf powder was accurately weighed in a tared silica crucible, incinerated in a muffle furnace at a temperature not exceeding 450°C until free from carbon, cooled and weighed. The percentage of ash, with reference to the air-dried sample, was calculated.

II. Determination of acid-insoluble ash: The ash, obtained in (i), was boiled with 25 ml of 2 N hydrochloric acid for five minutes, filtered through an ashless Whatman no. 41 filter paper, washed with hot water, ignited and weighed. The percentage of acid-insoluble ash was calculated with reference to the air-dried sample.

III. Ash analysis:
The filtrate and washings, obtained in (ii), was used for further ash analysis.

a. Determination of iron: About 2 g of ammonium chloride was added to the filtrate and heated nearly to boiling. It was made just alkaline with ammonia solution, the precipitate of ferric hydroxide was filtered and washed with hot water till free from chloride. The filtrate and washings were collected for the determination of calcium and magnesium. Precipitate of ferric hydroxide was dried, ignited and weighed as Fe$_2$O$_3$. 1g Fe$_2$O$_3$ = 0.6994g Fe

b. Determination of calcium: The filtrate obtained in (a) was treated with 2ml of concentrated hydrochloric acid and concentrated to about 200ml. To it 50ml of 10% solution of ammonium oxalate was added and boiled. To the hot solution dilute ammonia solution was added till it was slightly alkaline. After one hour the precipitate of calcium oxalate was filtered, washed with water till free from chloride and oxalate. The filtrate contains magnesium. The precipitate of calcium oxalate was dissolved in 25% sulphuric acid, the solution was heated at 70°C, and titrated with 0.1 N potassium permanganate solution. The percentage of Ca was calculated.

1ml 0.1 N KMnO$_4$ = 0.002004 g Ca.
c. Determination of Magnesium:
To the filtrate from the calcium determination, 50 ml of conc. nitric acid was added and evaporated carefully to dryness on a hot plate till only a small residue (largely magnesium saltes) was left. To it 2-3 ml of conc. hydrochloric acid and 20 ml of water were added, warmed for few minutes until the solid has dissolved. It was cooled and few drops of methyl red indicator followed by 10ml of 25% diammonium hydrogen phosphate reagent were added. Then conc. ammonia solution was added to it with stirring until the solution turns yellow. Stirring was continued for 5 minutes adding ammonia solution dropwise to keep the solution alkaline, finally 5ml conc. ammonia solution in excess was added and stirred again. The solution was kept at room temperature for at least four hours, filtered through Whatman no. 1 filter paper, washed with cold dilute ammonia solution (1:20) until free from chloride. The precipitate was dried at 110°C and weighed as magnesium ammoniumphosphate, MgNH₄PO₄, 6H₂O.

₁g MgNH₄PO₄, 6H₂O = 0.0991g Mg.

d. Determination of Sulphate:
25 ml of the acid solution (6 N HCl Solution) of ash was taken into a 250 ml beaker, heated to boiling and to it 100 ml of boiling hot barium chloride solution (2.5%) was added. After stirring vigorously it was kept in a warm place for about 30 minutes, filtered and washed until free from chloride. The precipitate was dried, ignited and weighed as barium sulphate. From weight of barium sulphate the percentage of sulphate in the sample was calculated.

₁g BaSO₄ = 0.4115 g SO₄

e. Determination of Phosphate:
25ml of the acid solution was taken into a 250 ml flask. To it 15ml conc. nitric acid was added, heated to about 70°C, 50ml of ammonium nitromolybdate solution was added and shaken vigorously. It was filtered after one hour and the precipitate was washed with 0.1% KNO₃ solution until free from acid. The precipitate was dissolved in a measured excess of standard sodium hydroxide solution and the excess sodium hydroxide was titrated with standard hydrochloric acid using phenolphthalein as indicator. The percentage of phosphate in the sample was calculated from the amount of sodium hydroxide consumed.

1ml 1 N HCl = 0.004133g Phosphate (PO₄)

f. Determination of Chloride:
A known concentration of aqueous solution of ash was prepared. To a measured volume of the aqueous solution about 2ml of conc. nitric acid and 1 ml of nitrobenzene were added and shaken vigorously. To it a known amount of 0.1 N AgNO₃ solution was added to precipitate chloride as silverchloride. It was titrated against 0.1 N KCNS solution using ferric alum as
indicator. The percentage of chloride was calculated from the amount of AgNO₃ consumed.

\[1 \text{ml } 0.1 \text{N AgNO}_3 = 0.00355 \text{g Chloride (Cl)}\]

g. Determination of sodium and potassium:
Sodium and potassium content was determined from the aqueous solution of ash with the help of Systronics Flame Photometer. Standard curves were prepared using known concentrations of standard solutions of sodium and potassium and the percentage of sodium and potassium in the sample was calculated from the standard curve.

The results of ash analysis have been shown in Table-4. It can be seen from it that in G. montana among inorganic constituents calcium was present in highest concentration followed by iron, potassium sodium and magnesium.

**TABLE - 4**
DETAILS OF ASH ANALYSIS OF GYMNOSPORIA MONTANA LEAF.

<table>
<thead>
<tr>
<th>Content</th>
<th>Value, (% W/W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash Value</td>
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<tr>
<td>Acid insoluble ash</td>
<td>0.28</td>
</tr>
<tr>
<td>Iron as Fe</td>
<td>0.47</td>
</tr>
<tr>
<td>Calcium as Ca</td>
<td>2.90</td>
</tr>
<tr>
<td>Magnesium as Mg</td>
<td>0.37</td>
</tr>
<tr>
<td>Sodium as Na</td>
<td>0.38</td>
</tr>
<tr>
<td>Potassium as K</td>
<td>0.42</td>
</tr>
<tr>
<td>Sulphate as SO₄</td>
<td>1.00</td>
</tr>
<tr>
<td>Phosphate as PO₄</td>
<td>0.28</td>
</tr>
<tr>
<td>Chloride as Cl</td>
<td>0.70</td>
</tr>
<tr>
<td>Carbonate as CO₂</td>
<td>0.99</td>
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</table>

iv. Preparation of extracts:
Six hundred grams of dried, coarsely powdered leaf of G. montana was extracted with petroleum ether (60°-80°C) by heating it for about 15-20 minutes, keeping it aside for about an hour, repeating the same for the day, left overnight and next day again the procedure was repeated. It was filtered and in the same way extraction procedure was repeated thrice. The combined petroleum ether extract was concentrated and finally evaporated to dryness. The percentage of extract (PE) obtained was calculated. The defatted leaf powder, after drying, was extracted with methanol in similar way like with petroleum-ether. The combined methanol extract was concentrated and finally evaporated to dryness. The percentage of methanol extract (TME) was calculated. A portion of methanol extract was suspended in small quantity of distilled water and extracted successively with hexane, ethyl acetate, and n-butanol. Solvents from all the fractions viz. hexane fraction (HE), ethyl acetate fraction (EAT), n-butanol fraction (BE) and the residue remaining after n-butanol extract (MAR) were removed and their percentage was calculated. The percentage of different extracts are shown in Table - 5.
EXTRACTIVE VALUES OF GYMNOSPORIA MONTANA LEAF

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Extractive value % W/W</th>
</tr>
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<tbody>
<tr>
<td>Petroleum ether extract (PE)</td>
<td>05.18</td>
</tr>
<tr>
<td>Methanol extract (TME)</td>
<td>12.16</td>
</tr>
<tr>
<td>Hexane fraction (HE)</td>
<td>03.81*</td>
</tr>
<tr>
<td>Ethyl acetate fraction (EAT)</td>
<td>06.20*</td>
</tr>
<tr>
<td>Butanol fraction (BE)</td>
<td>15.01*</td>
</tr>
</tbody>
</table>

* with reference to TME

The extracts were tested qualitatively for the presence of different groups of chemical compounds by employing standard testing methods.

1. Tests for alkaloids:
A small quantity of the extract was taken with very small amount of alcohol, rendered acidic with dilute HCl and tested with Dragendorff's reagent, Mayer's reagent and Wagner's reagent. Alkaloids give brown to red precipitate with Dragendorff's reagent, cream to whitish precipitate with Mayer's reagent and brown flocculent precipitate with Wagner's reagent.

   a. Dragendorff's reagent (Potassium-bismuth iodide):
   8 g of Bi(NO₃)₃·5H₂O was dissolved in 20 ml of nitric acid (sp. gr. 1.18) and 27.2 g of KI was dissolved separately in 50 ml water. The two solutions were mixed and allowed to stand. The supernatant was decanted off and the volume was made up to 100 ml with distilled water.

   b. Mayer's reagent (Potassium mercuric iodide):
   1.36 g HgCl₂ in 60 ml water and 5 g KI in 10 ml water were dissolved separately. The two solutions were mixed and diluted to 100 ml with distilled water. This reagent is generally the most useful of the alkaloid reagents and gives precipitate with the hydrochlorides of most alkaloids in very dilute solution.

   c. Wagner's reagent (Iodine-potassium iodide solution):
   1.27 g of iodine and 2 g potassium iodide were dissolved in 5 ml of water and the solution was diluted to 100 ml.

2. Tests for flavonoids:
   a. Shinoda's test:
   To an alcoholic solution of the extracts small quantity of magnesium and conc. HCl were added. The appearance of a pink to magenta colour indicates the presence of flavonones, flavonol and their glycosides.
b. Precipitation with basic lead acetate:
To an aliquot of aqueous solution of extracts, basic lead acetate solution was added and observed for the formation of precipitates. Basic lead acetate gives coloured precipitate with most of the flavonoid polyphenols.

3. Test for triterpenes (Liebermann-Burchard test):
To a small amount of extract in chloroform acetic anhydride was added. Next conc. sulphuric acid was added dropwise and colour formation was observed. Formation of bluish, green or violet coloured ring was considered as positive test.

4. Test for phenols:
To a small amount of extract in alcohol few drops of neutral ferric chloride solution was added and the change in colour was observed. Formation of blue, green, red or violet colour indicates the presence of Phenols.

5. Test for tannins:

a. To a small amount of extract solution few drops of ferric chloride solution (5%) was added and the colour formation was noted. Formation of deep blue black colour was considered as indication of presence of tannins.

b. To an aliquot of aqueous solution of extracts small quantity of ammonium hydroxide followed by few drops of potassium ferricyanide solution were added and observed for any colour change. Tannins produce deep red colour.

6. Test for saponins:
A small quantity of the extract was taken in water and shaken vigorously. A stable froth with honey comb structure was considered as indication for the presence of saponins.
On qualitative testing PE and TME gave positive Liebermann Burchard (L.B.) test indicating the presence of sterols, triterpenes or related compounds in the extracts. TME and BE gave strongly positive Shinoda's test and neutral ferric chloride test indicating the presence of flavonoids in it.

v. Fluorescence characters:
Ten gram leaf powder was taken and extracted successively with petroleum ether (60°-80°C), chloroform, ethyl acetate, methanol and water. The extracts were observed under short wave (254 nm) and long wave (365 nm) UV-radiation. The details of fluorescence characters were noted and have been presented in Table - 6, which shows that none of the extracts gave
fluorescence under short wave UV radiation and under long wave UV-radiation only petroleum ether and chloroform extracts gave fluorescence.

**TABLE - 6**

<table>
<thead>
<tr>
<th>Extract</th>
<th>Fluorescence in UV-light short wave</th>
<th>long wave</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>N.F.</td>
<td>Orange</td>
</tr>
<tr>
<td>Chloroform</td>
<td>N.F.</td>
<td>Dirty yellow</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>N.F.</td>
<td>N.F.</td>
</tr>
<tr>
<td>Methanol</td>
<td>N.F.</td>
<td>N.F.</td>
</tr>
<tr>
<td>Water</td>
<td>N.F.</td>
<td>N.F.</td>
</tr>
</tbody>
</table>

N.F. = No fluorescence

These parameters can be used as adjuncts to pharmacognostic characters for identifying the plant material either entire or in powder form.

2. **SEPARATION OF FREE SUGARS**

Carbohydrates occur abundantly in nature. They are preponderantly important in the vegetable kingdom. Carbohydrate residues are essential components of glycosides and are frequently present in various other biologically active substances (e.g. enzymes, co-enzymes, glycoproteins, vitamins, etc.) Sugars are carbohydrates that are sweet to the taste and soluble in water.

Application of different chromatographic methods like column chromatography, thin layer chromatography, paper chromatography, etc. have been reported for the separation of sugars. The first application of partition chromatography to sugars on columns of silica gel was published by Bell (361). The separation of sugars by paper chromatography was first described by Partridge (362, 363). Stahl and Kaltenbach (354) extended TLC to the separation of sugar mixtures. However, paper chromatography is a convenient and widely used procedure for the separation of sugars. Ascending, descending as well as circular paper chromatography can be used as a means of gaining rapid and specific information regarding the saccharide composition of a plant extract. Paper chromatographic technique has also been used for quantitative estimation of sugars (366). Block et. al. (365) have described various solvent systems and detecting agents for the separation of sugars. For extraction of sugars from the plant material 70-80 % ethanol can be used successfully (367, 368).
Paper chromatographic technique was used for the separation and detection of sugars in the leaf powder of *Gymnosporia montana*.

Leaf powder was extracted with 70% alcohol by macerating them overnight. The extract, after filtration, was concentrated. It was spotted on a Whatman no. 1 paper along with standard sugar samples. The development was conducted by descending methods using n-butanol acetic acid - water (4:1:5) as solvent system in an air-tight chamber, previously saturated with the solvent. The developed chromatogram was dried in air and sprayed with anilinephthalate spray reagent (369). Aniline phthalate spray reagent was prepared by dissolving 0.93 g aniline and 1.66 g o-phthalic acid in 100 ml n-butanol, saturated with water.

After initial drying in air, the chromatogram was heated at 110°C for 10 minutes. The Rf values of the spots were calculated and Rf values of the spots obtained in the sample were compared with those of standard sugars.

The extract showed only one brown coloured spot at Rf 0.40, which on comparison with that of standard sugars was found to be identical with the Rf value of galactose.

The results of the paper chromatogram suggest that only one free sugar, galactose is present in Gymnosporia montana leaf extract.

3. SEPARATION OF FREE AMINO ACIDS:

Amino acids are of great biological importance and play a very important role as growth prompting factors. They occur in nature both in free and bound form (peptides). In general there is a predominance of the amino acids which are not nutritionally essential for most animals. These are known to be closely linked with the metabolism of carbohydrates. They include glycine, serine, alanine, aspartic acid, glutamic acid, asparagine, glutamine and γ-aminobutyric acid, which usually represent a far higher proportion of the total free amino acids than do the corresponding residue of the total amino acid residues in ordinary proteins. There are great variations in the relative proportions of the free amino acids between species, parts of the same plant and with the physiological state of the plant (370).

The problem of amino acid separation was the starting point for the development of paper chromatography and it is used successfully for amino acid separation. Thin layer chromatography using cellulose and silica gel has also been used for the separation of amino acids (369). Use of ion-exchange chromatography and column chromatography for separation of amino acids has also been reported (371).

Among the different methods for the separation of amino acids, paper chromatography, by far is the convenient method. It gives satisfactory separation and a clear picture of the amino acid pattern in the plant. It is very useful for qualitative and rough quantitative work. Descending
paper chromatography was used by Consden et. al. (372), while Williams and Kirby (373) used ascending chromatography and several authors (374, 376) have described circular or horizontal chromatography techniques. Two dimensional paper chromatography has also been advocated for the separation of amino acids running very close to each other in one solvent.

70-80 % alcohol has been used for the extraction of free amino acids from the plant material.

At present specific reagent for detection of amino acids alone is not known. Some non-specific reagents for nitrogen-containing compounds and some which are specific for one or a few amino acids are used. The most widely used reagent for detection of amino acids is ninhydrin, the spray reagent of which is prepared by dissolving 0.3 g ninhydrin in 10 ml n-butanol and 3 ml acetic acid added to it or by dissolving 0.2 g ninhydrin in 100 ml ethanol (369). Preparation of ninhydrin spray reagent in acetone has also been reported (377). The normal method of colour development gives yellow colour to proline and hydroxyproline and violet colour to all other \( \alpha \)-amino acids. Various other spray reagents, such as alkaline bromothymol blue solution containing formaldehyde (378), aromatic aldehydes (379), l-atin (380), Folin's reagent (381) have been described for detection of amino acids.

Circular paper chromatography technique was used for the separation of amino acids in \textit{G. montana} leaf powder.

Leaf powder was extracted with 70% alcohol by macerating it overnight. It was filtered and concentrated. Then the concentrated extract was spotted on a Whatman no. 1 filter paper along with standard amino acids. Chromatogram was developed in a circular paper chromatography chamber, pre saturated with the solvent, by using butanol acetic acid-water (4:1:5) as solvent system. After development the paper was dried in air and sprayed with 0.25% ninhydrin solution in acetone followed by heating at 110°C for 10 minutes for detecting the spots. The extract gave seven spots. The \( R_f \) values of the spots of the extract were calculated and compared with those of standard amino acids. The details of the chromatogram has been recorded in Table - 7.

\begin{table}[h]
\centering
\caption{\textbf{\( R_f \) VALUES OF AMINO ACIDS OF GYMNOSPORIA MONTANA}}
\begin{tabular}{ccc}
\hline
No. & \( R_f \) Value & Colour & Amino acid \\
\hline
1. & 0.34 & Violet & Arginine \\
2. & 0.46 & Violet & Glutamic acid \\
3. & 0.59 & Violet & Alanine \\
4. & 0.67 & Yellow & Proline \\
5. & 0.75 & Violet & \( \beta \)-Aminobutyric acid \\
6. & 0.80 & Violet & Phenylalanine \\
7. & 0.86 & Violet & Unidentified \\
\hline
\end{tabular}
\end{table}
Gymnosporia montana leaf extract on paper chromatography showed the presence of seven amino acids out of which six were identified as arginine, glutamic acid, alanine, proline, γ-aminobutyric acid and phenylalanine and one with Rf value 0.86 could not be identified.

4. STUDY OF PETROLEUM ETHER EXTRACT:

The petroleum ether fractions normally contains fatty components of the plant material. Wide range of compounds such as long chain hydrocarbons, alcohols, aldehydes, fatty acids, sterols, glycerides, etc. which are widely distributed in plant and animal kingdoms, will be extracted in petroleum ether fraction. The petroleum ether fraction of G. montana leaf was examined for its fatty acid and sterol composition.

i. Fatty acid composition of G. montana leaf:
Fats and fatty oils are widely distributed in the vegetable kingdom occurring both in the vegetative and reproductive structures. The properties associated with fats and fatty oils are dependent on the nature and the extent to which the different fatty acids occur in the fatty material. Fatty acids can be divided into two groups, saturated and unsaturated. Most of the higher acids are straight chain compounds.

The unsaturated acids can be unconjugated when the double bonds are separated by one or more single bonded carbon atoms and conjugated when double bonded carbon atoms are adjacent to one another. The ultra-violet spectra will give some idea about the nature of unsaturated fatty acids.

Isolation of fatty acids can be achieved by alkaline hydrolysis of the petroleum ether extract of the sample yielding water soluble alkali salts of fatty acids. Then non-saponifiable matter can be removed by extraction with non-polar solvents like petroleum ether, benzene, ether, etc followed by liberation of mixed fatty acids from the soap by addition of mineral acids. It is generally difficult to separate higher fatty acids by simple chemical methods. Usually, they are converted into their methyl esters which can be separated by using various chromatographic techniques like adsorption thin layer chromatography, reversed phase paper chromatography and gas chromatography. Gas chromatography appears to be the most convenient and satisfactory method for analysis of fatty acid mixtures than the other methods, such as fractional distillation, low temperature crystallisation, countercurrent distribution and reversed phase chromatography because of it's speed, accuracy and especially because a complete quantitative analysis can be carried out with very small quantities of material (382).

Alkaline hydrolysis: The plant material can be saponified directly to get non-saponifiable matter (383). The plant material can also be extracted initially with petroleum ether and then the petroleum ether extract can be saponified using methanolic potassium hydroxide (369). The
fatty acids, after liberation from the saponified portion, can be converted into methyl esters by various methods like boron-trifluoride method (384), by using methanol-hydrogen chloride or methanol-sulphuric acid (369) and with diazomethane (382).

a. Gas chromatographic analysis: Fatty acid composition of Gymnosporia montana leaf was determined from its petroleum ether extract. Fatty acids were detected in the sample by converting them into volatile methyl ester and subsequent gas chromatographic analysis. Petroleum ether extract was saponified with methanolic potassium hydroxide as described by Stahl (369) and the method described by Chalvardjian et al. (385) was used for converting the fatty acids into methyl ester. About 3g accurately weighed (2.7579 g) petroleum ether extract was taken with 40 ml of 20% methanolic potassium hydroxide and kept overnight at room temperature. Next day it was refluxed for six hours and cooled. Methanol was removed, twice its volume of distilled water was added to it and extracted with ether. The combined etheral extract was washed with distilled water till neutral, dried over anhydrous sodium sulphate and the ether was evaporated to obtain unsaponifiable matter (Fraction A). The aqueous portion, left after ether extraction (saponifiable portion) was acidified with 5N sulphuric acid under etheral layer. The aqueous layer was extracted three times with ether. The combined etheral layer was washed with distilled water. The ether layer was dried over anhydrous sodium sulphate and evaporated to obtain fatty acid portion (720.80mg) (Fraction B). From this 100 mg was taken in 100 ml of a solution of absolute methanol-benzene-conc. sulphuric acid (86:10:4) mixture, refluxed on a water-bath for eight hours, cooled diluted with 75 ml water and extracted several times with hexane. Combined hexane extract was dried over sodium bicarbonate and volume was made upto 100ml. From it 10ml was taken separately, evaporated, the residue was dissolved in 0.5ml hexane and from it 10 micro litre was injected in a gas chromatograph (Netal, Omega model). Standard saturated straight chain fatty acid methyl ester samples (Sigma, U.S.A.) were also injected under the same condition.

Condition for G.C.:

| Column                          | 10 % P.E.G.A. on chromosorb W 80/100 mesh.  
|                                | (S.S.,2 meter long, 1/8 inch O.D.)        |
| Detector                       | Flame ionization detector.                |
| Carrier gas                    | Nitrogen at a flow rate of 20 ml/min.      |
| Injector temperature           | 300°C                                     |
| Detector temperature           | 220°C                                     |
| Oven temperature               | 190°C                                     |
| Chart speed                    | 1 cm/min                                  |

GLC analysis of the ester residue showed several peaks (Fig. 13). The retention time of the peaks was calculated. The area covered by individual peak and the percentage were also calculated.
Fig. 13. Gas chromatogram of fatty acid ester of *G.montana* leaf.
Area covered by individual peak = Height of that peak × Width at the centre of the height of the peak.

% of area of individual peak = \( \frac{\text{Area of that peak} \times \text{Attenuation factor}}{\text{Total area}} \) × 100

Comparison of the retention time (RT) of the peaks of the sample with that of standard samples confirmed the presence of palmitic acid, a saturated straight chain fatty acid. The details of the gas chromatogram have been recorded in Table - 8.

**TABLE - 8**

**GC OF FATTY ACID ESTERS OF GYMNOSPORIA MONTANA LEAF.**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Identity</th>
<th>Retention time in min.</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Unidentified</td>
<td>2.00</td>
<td>Traces</td>
</tr>
<tr>
<td>B</td>
<td>Unidentified</td>
<td>3.30</td>
<td>Traces</td>
</tr>
<tr>
<td>C</td>
<td>Unidentified</td>
<td>3.80</td>
<td>4.83</td>
</tr>
<tr>
<td>D</td>
<td>Unidentified</td>
<td>5.30</td>
<td>16.12</td>
</tr>
<tr>
<td>E</td>
<td>Palmitic acid</td>
<td>7.00</td>
<td>72.03</td>
</tr>
<tr>
<td>F</td>
<td>Unidentified</td>
<td>8.40</td>
<td>5.37</td>
</tr>
<tr>
<td>G</td>
<td>Unidentified</td>
<td>9.30</td>
<td>1.61</td>
</tr>
</tbody>
</table>

The gas chromatogram of fatty acid ester of *G. montana* leaf showed seven peaks indicating the presence of seven fatty acids. Among seven peaks - peak E, comprising 72.03% of the total fatty acids of *G. montana*, was identified as palmitic acid CH₃(CH₂)₁₄COOH, by comparing its retention time with that of standard palmitic acid methyl ester. The remaining six compounds could not be identified.

b. Ultra violet absorption spectra:
Conjugation of a single unsaturated linkage with a second unsaturated group gives rise to intense absorption bands throughout the region 200 to 400 nm. A conjugated dienoic system absorbs in the region about 230 nm, the trienoic conjugation results in a shift in the absorption to about 268 nm while a tetraenoic system absorbs at about 315 nm, pentaenoic at about 346 nm and hexaenoic at about 374 nm. (386). So the ultra-violet spectra can give some idea about the type of unsaturated fatty acid. Hence, the fatty acid mixture (Fraction B, as described during gas chromatographic analysis), in hexane, was also scanned through 200 to 400 nm in a Shimadzu UV-160 A, double beam recording spectrophotometer for observing the presence of unsaturated fatty acids. The spectrum has been shown in Fig. 14. It shows maximum absorption near 227 nm and and 265 nm indicating higher concentration of conjugated dienoic acid and comparatively lower concentration of trienoic acid.
Fig. 14. Ultra Violet spectrum of fatty acid of *G.montana* leaf.
Examination of unsaponifiable portion:

Sterols are widely distributed in plants particularly in seeds and leaves. The unsaponifiable portion of a plant material will contain sterols and related compounds. Various methods like fractional crystallisation, column chromatography using alumina or silicagel and digitonine precipitation have been reported (387, 388) for the isolation of sterols after the initial extraction of the plant material with organic solvents. Anjaria et al (389) have saponified the plant material directly and subsequently separated sterols from it.

In the sterol field, chromatography has become an indispensable technique for separation and purification. Among the different available methods in column, paper, thin-layer, gas chromatography, which are based on adsorption and/or partition chromatography choice must depend on the particular problem being studied, on the material at hand and on the advantages and disadvantages of the individual techniques. Often better result is obtained by combining the techniques. Due to the larger capacity, the classical column with continuous development is chosen for preparative purposes, although the resolution does not attain that of the other methods for various reasons.

The unsaponifiable portion (Fraction A- as described earlier during gas chromatographic analysis) when tested qualitatively with L.B. reagent gave positive test indicating presence of sterols and related compounds. On TLC the fraction gave several spots. So, it was subjected to column chromatography over silica gel and the course of chromatogram is given in chromatigram- I.

Chromatogram - I

The unsaponifiable portion (0.7g) was dissolved in minimum quantity of chloroform, adsorbed on small quantity of silicagel and it was dried in air. It was chromatographed over 13 g silicagel. The column was built up with hexane. 100-250 ml of solvents or solvent mixtures were used for elution.
<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Eluate</th>
<th>Quantity</th>
<th>Nature of the residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hexane</td>
<td>100 ml</td>
<td>Very small q.t. waxy residue</td>
</tr>
<tr>
<td>2</td>
<td>Petroleum ether</td>
<td>100 ml</td>
<td>Negligible q.t. waxy residue</td>
</tr>
<tr>
<td>3-8</td>
<td>Pet. ether-benzene 3:1</td>
<td>100 ml</td>
<td>No residue</td>
</tr>
<tr>
<td>9-13</td>
<td>Pet. ether-Benzene 1:1</td>
<td>100 ml</td>
<td>Fr. 10-13 cryst. residue</td>
</tr>
<tr>
<td>14-30</td>
<td>Pet. ether-Benzene 1:3</td>
<td>250 ml</td>
<td>14,15 residue; 16-22 white residue; 23-30 very small residue</td>
</tr>
<tr>
<td>31-38</td>
<td>Benzene</td>
<td>150 ml</td>
<td>Very small residue</td>
</tr>
<tr>
<td>39-44</td>
<td>Benzene-Chloroform 3:1</td>
<td>100 ml</td>
<td>Very small residue</td>
</tr>
<tr>
<td>45-50</td>
<td>Benzene-Chloroform 1:1</td>
<td>100 ml</td>
<td>Very small residue</td>
</tr>
<tr>
<td>51-56</td>
<td>Benzene-Chloroform 1:3</td>
<td>100 ml</td>
<td>Very small residue</td>
</tr>
<tr>
<td>57-66</td>
<td>Chloroform</td>
<td>200 ml</td>
<td>Very small residue</td>
</tr>
</tbody>
</table>

**Examination of the eluates:**

Eluates 1 and 2 gave very small quantity of residue, so they were not examined further.

Eluates 3-8 on evaporation gave no residue.

Eluate 9 gave very small quantity of residue and so it was not examined further.

Eluate 10-13 found to be similar (on TLC using hexane - ethyl acetate :: 85:15 gave single spot) and hence they were mixed up-compound GAU/V/PA.

Eluates 14-16 gave similar pattern (on TLC gave 2 spots, one of them being that of GAU/V/PA and so mixed together - Fraction X.

Eluates 17 and 18 gave similar pattern (on TLC gave single spot and so they were mixed together-compound GAU/V/PB.

Eluates 19-24 gave similar pattern (on TLC gave 2 spots, one of them being that of GAU/V/PB and hence mixed together - Fraction Y.

53
Eluates 25-54 gave similar pattern (on TLC gave 3 spots, one of them being that of GAU/V/PB) and hence mixed together-Fraction - Z.

Eluates 55-66 gave similar pattern and so mixed together. Since they gave very small quantity of residue they were not examined further.

Further quantity of substance GAU/V/PA and GAU/V/PB was isolated from fraction X,Y,Z by preparative TLC using hexane-ethyl acetate:: 85:15 as solvent system.

Compound GAU/V/PA: The compound was crystallized from chloroform-methanol (60 mg, m.p. 193°C). It gave single spot on TLC hexane-ethyl acetate:: 85:15, detecting reagents iodine as well as Liebermann-Burchard spray reagent. The compound was subjected to UV, I.R., NMR and Mass spectral analysis. The spectral data for the compound GAU/V/PA was as under:

i). UV:MeOH: No absorption.

$\lambda_{max}$

ii). IR: KBr cm$^{-1}$ (Fig.15)

$\delta_{max}$

3384 cm$^{-1}$ (s) -OH stretching
2860,2932 (s) -CH stretching
1467 (s) -C-O
1386,1380 (s) C-H bending, gem-dimethyl
1200 (w)
1100 (w)
1034 (m)
1000 (m)

(s= strong, m=medium w=weak)

iii). $^1$H NMR (CDCl$_3$): (Fig.16)

<table>
<thead>
<tr>
<th>$\delta$</th>
<th>Multiplicity</th>
<th>No. of protons</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.80</td>
<td>s</td>
<td>3H</td>
<td>Me</td>
</tr>
<tr>
<td>0.84</td>
<td>s</td>
<td>3H</td>
<td>Me</td>
</tr>
<tr>
<td>0.88</td>
<td>s</td>
<td>5H</td>
<td>2xMe</td>
</tr>
<tr>
<td>0.92</td>
<td>s</td>
<td>3H</td>
<td>Me</td>
</tr>
<tr>
<td>0.96</td>
<td>s</td>
<td>3H</td>
<td>Me</td>
</tr>
<tr>
<td>1.00</td>
<td>s</td>
<td>3H</td>
<td>Me</td>
</tr>
<tr>
<td>1.12</td>
<td>s</td>
<td>3H</td>
<td>Me</td>
</tr>
<tr>
<td>1.48-1.96</td>
<td>m</td>
<td></td>
<td>CH$_2$ and -C-H protons</td>
</tr>
<tr>
<td>3.24</td>
<td>dd</td>
<td>1H</td>
<td>C-3-H</td>
</tr>
<tr>
<td>5.16-5.24</td>
<td>t</td>
<td>1H</td>
<td>C-12-H</td>
</tr>
</tbody>
</table>
Fig. 15. Infrared spectrum of compound GAU/PA
Fig. 16. 1H NMR spectrum of compound GAU/V/PA
iv. Mass spectrum:

The Mass spectrum has been shown in Fig.17. It showed several fragments. Some important fragments are at:

<table>
<thead>
<tr>
<th>Fragment</th>
<th>m/e</th>
</tr>
</thead>
<tbody>
<tr>
<td>M⁺</td>
<td>426</td>
</tr>
<tr>
<td>m/e 218</td>
<td></td>
</tr>
<tr>
<td>m/e 203</td>
<td></td>
</tr>
</tbody>
</table>

The compound GAU/V/PA indicated in the IR the presence of hydroxyl group (peak at 3384 cm⁻¹), gem-dimethyl (peak at 1386, 1380 cm⁻¹). In ¹H NMR (CDCl₃) the groups appearing at δ 0.78 - 1.12, 3.24 and 5.16 - 5.24 could be identified as protons of Me, proton of C-3-H and proton of C-12 H respectively. The mass spectrum showed the M⁺ peak at 426 indicating the molecular weight of the compound to be 426. It also showed fragments at m/e 218 and 203. The molecular weight of β-amyrin is 426. Its fragmentation pattern is as follows:

\[
\text{H}_2\text{CO} + \text{CH}_3 - \rightarrow \begin{align*}
\text{C}-\text{C} \text{CH}_3 \quad \downarrow \quad \text{m/e 218} \\
\text{C} \quad \text{m/e 203}
\end{align*}
\]

This retro-Diels-Alder fragmentation leading to 'Fragment a' can be employed as a characteristic diagnostic tool for the presence of a 12-13 double bond in triterpenes of the α and β-amyrin class. Thus, β-amyrin gives fragment with m/e 218 (390).

The spectral data and m.p. supported the compound GAU/V/PA to be β-amyrin.
Fig. 17. Mass spectrum of compound GAU/V/PA
Compound GAU/V/PB:

The compound was crystallised from chloroform-methanol (40 mg, m.p. 137°C). It gave single spot on TLC (solvent system - hexane - ethyl acetate :: 85:15, detecting reagents iodine as well as Liebermann-Burchard spray reagent). UV, I.R., NMR and Mass spectral analysis of the compound was carried out and the spectral data was as under :-

i). UV : 
\[ \lambda_{\text{max}} \text{ MeOH : No absorption} \]

ii) IR : 
\[ \delta \text{ KBr cm}^{-1} \]

- 3436 cm\(^{-1}\) (s) -O-H stretching
- 2944 and 2860 (s) -C-H stretching
- 1660 and 1730 (w) C=C stretching
- 1466 and 1382 (s) C-H bending
- 1060 (s) -O-H bending
- 960 (w)

(s = strong, w=weak)

iii). \(^1\)H NMR (CDCl\(_3\)) S :

<table>
<thead>
<tr>
<th>S</th>
<th>Multiplicity</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.84 - 0.10</td>
<td>m</td>
<td>15 H, 5xCH(_3)</td>
</tr>
<tr>
<td>1. 4 - 2.28</td>
<td>m</td>
<td>-C-H2 and -C-H protons</td>
</tr>
<tr>
<td>3.4-3.7</td>
<td>Br. S.</td>
<td>1 H, -C-H-OH</td>
</tr>
<tr>
<td>5.36</td>
<td>m</td>
<td>1 H,C=C-C-H</td>
</tr>
</tbody>
</table>

No. of protons, Assignment
Fig. 18. Infrared spectrum of compound GAUV/PB
Fig. 19. $^1$H NMR spectrum of compound GAU/V/PB
Mass spectrum

The mass spectrum has been shown in Fig. 20. It showed several peaks. Some important peaks are:

- $M^+$ at 414
- m/e 329
- m/e 273
- m/e 255
- m/e 231
- m/e 213

The compound GAU/V/PB indicated in the IR the presence of -OH group (peak at 3436 cm$^{-1}$), methylene and methyl groups (peaks at 2944 and 2860 cm$^{-1}$), unsaturation (peaks at 1730 and 1660 cm$^{-1}$). In $^1$H NMR (CDCl$_3$) the groups appearing at δ 0.84 - 1.0, 1.4 - 2.28, 3.4 - 3.7 and 5.36 could be identified as protons of CH$_3$, -CH$_2$ and CH protons, protons of carbon bearing O-H group and protons of C=C-H, i.e. double bond respectively. The Mass spectrum showed $M^+$ peak at 414 indicating the molecular weight of the compound to be 414. It also showed fragments at m/e 329, 273, 255, 231 and 213. The molecular weight of β-sitosterol is 414. Its fragmentation pattern is as follows:

The spectral data and m.p. supported the compound to be β-sitosterol. The identity was further confirmed by Co-TLC with authentic B-sitosterol sample. Hence the compound GAU/V/PB was identified as β-sitosterol.
Fig. 20. Mass spectrum of compound GAU/V/PB
5. Study of methanol extract:

The methanol extract, when tested qualitatively for the presence of different group of compounds, gave positive test with L.B. reagent and positive Shinoda's test indicating the presence of triterpenes and flavonoid in it. Hence, an attempt has been made to isolate the compounds from the methanol extract. The extract was subjected to column chromatography over silica gel. The course of chromatogram has been given in chromatogram - II.

**Chromatogram - II.**

Fifteen gram methanol extract was taken in methanol, adsorbed on minimum quantity of silica gel and dried. It was chromatographed over 300g silica gel. The column was built-up with petroleum ether 60°C-80°C, and it was eluted with different solvents and solvent mixtures as shown below:

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Eluent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3</td>
<td>Petroleum ether</td>
<td>300 ml</td>
</tr>
<tr>
<td>4-10</td>
<td>Pet.ether - benzene :: 9:1</td>
<td>700 ml</td>
</tr>
<tr>
<td>11-32</td>
<td>Pet.ether-benzene :: 3:1</td>
<td>2500 ml</td>
</tr>
<tr>
<td>33-63</td>
<td>Pet.ether-benzene :: 1:1</td>
<td>1500 ml</td>
</tr>
<tr>
<td>64-84</td>
<td>Pet.ether-benzene :: 1:3</td>
<td>1000 ml</td>
</tr>
<tr>
<td>85-98</td>
<td>Benzene</td>
<td>700 ml</td>
</tr>
<tr>
<td>99-112</td>
<td>Benzene - ethyl acetate :: 3:1</td>
<td>700 ml</td>
</tr>
<tr>
<td>113-126</td>
<td>Benzene-ethyl acetate :: 1:1</td>
<td>700 ml</td>
</tr>
<tr>
<td>127-140</td>
<td>Benzene-ethylacetate :: 1:3</td>
<td>700 ml</td>
</tr>
<tr>
<td>141-154</td>
<td>Ethylacetate</td>
<td>700 ml</td>
</tr>
<tr>
<td>155-164</td>
<td>Ethyl acetate - acetone :: 3:1</td>
<td>500 ml</td>
</tr>
<tr>
<td>165-174</td>
<td>Ethyl acetate - acetone :: 1:1</td>
<td>500 ml</td>
</tr>
<tr>
<td>175-184</td>
<td>Ethyl acetate - acetone :: 1:3</td>
<td>500 ml</td>
</tr>
<tr>
<td>185-192</td>
<td>Acetone</td>
<td>400 ml</td>
</tr>
<tr>
<td>193-200</td>
<td>Acetone-butanol :: 3:1</td>
<td>400 ml</td>
</tr>
<tr>
<td>201-208</td>
<td>Acetone-butanol :: 1:1</td>
<td>400 ml</td>
</tr>
<tr>
<td>209-216</td>
<td>Acetone-butanol :: 1:3</td>
<td>400 ml</td>
</tr>
<tr>
<td>217-224</td>
<td>Butanol</td>
<td>400 ml</td>
</tr>
<tr>
<td>225-232</td>
<td>Butanol-methanol :: 3:1</td>
<td>400 ml</td>
</tr>
<tr>
<td>233-240</td>
<td>Butanol-methanol :: 1:1</td>
<td>400 ml</td>
</tr>
<tr>
<td>241-248</td>
<td>Butanol-methanol :: 1:3</td>
<td>400 ml</td>
</tr>
<tr>
<td>249-256</td>
<td>Methanol</td>
<td>400 ml</td>
</tr>
</tbody>
</table>

58
Examination of eluates:

Eluates 1 to 28 on evaporation left no or negligible residue.

Eluates 29 to 34 on evaporation left very small residue and so not examined further.

Eluate 35 to 38 on evaporation left small white residue.

Eluates 39 to 42 on evaporation left white residue which on TLC using benzene as solvent system gave similar pattern (single spot). So all these fraction were mixed together and marked as compound GAU/V-1.

Eluates 43 to 50 on TLC using benzene as solvent system gave similar pattern (two spots - one of them being that of GAU/V-1). So, they were mixed together and labelled as Fraction-M.

Eluates 51 to 53 on TLC gave similar pattern (single spot). So, they were mixed together and marked as compound GAU/V-2.

Eluates 54 to 58 on TLC gave two spots, the major one being that of GAU/V-2. So, they were mixed together and labelled as Fraction-N.

Eluates 59 to 80 on TLC gave similar pattern (six spots). So, they were mixed together and labelled as fraction - O.

Eluates 212-230 all gave dark residue on evaporation. The fractions, when tested with magnesium and conc. hydrochloric acid, gave very light pink colour indicating the presence of flavonoid in small quantity. On paper chromatography (Butanol- Acetic acid - Water ::4:1:5) they gave similar pattern but with trailing and no clear separation.

Eluates 231-248 all gave mezenta colour with magensium and conc.hydrochloric acid indicating the presence of flavonoid. On paper chromatography they gave similar pattern and hence mixed together. This fraction was labelled as Fraction-Q.

By preparative TLC of fraction M,N,O,P further quantity of compounds GAU/V-1 and GAU/V-2 were isolated.

A portion of Fraction Q was hydrolysed with 2 M HCl by refluxing for 5 hours. It was cooled and extracted successively with ether and ethyl acetate. The aqueous portion was concentrated and subjected to paper chromatography, along with standard sugar samples, using butanol- acetic acid - water (4:1:5) as solvent system. Aniline phthalate spray reagent was used for detection. It showed three spots of which two were identified as xylose and glucose (major spot) and the third spot, which was faint, could not be identified.
Fraction Q on paper chromatography (solvent system: butanol - acetic acid - water ::4:1:5) gave two yellow spots. So, the extracts were spotted in band and paper chromatography was carried out in preparative scale. After development the spots were marked, paper was cut and extracted with alcohol. The extracts were evaporated to dryness and the residues were marked as GAU/V-5 and GAU/V-6.

**Compound GAU/V-1:**

The compound GAU/V-1 gave positive test with Liebermann-Burchard reaction. The compound was crystallised (30 mg, m.p. 211°C). It gave single spot on TLC (solvent system benzene and benzene-ethyl acetate ::2:1, detecting reagent iodine vapour as well as sulphuric acid). The compound was subjected to IR, NMR and Mass spectral analysis. The spectral data was as under:

i). \( \text{IR : } K\text{Br cm}^{-1} \) (Fig.21)

\[
\begin{array}{c|c|c}
\text{cm}^{-1} & \text{Assignment} \\
\hline
3440 & \text{(br) } -\text{OH stretching} \\
2930 and 2860 & \text{(s) } -\text{C-H stretching} \\
1710 & \text{(m)} \\
1490 and 1460 & \text{(m)} \\
1360 & \text{(s)} \\
1145 & \text{(s)} \\
860 & \text{(s)} \\
\end{array}
\]

s=strong m=medium, br = broad

ii). \( ^1\text{H NMR (CDCl}_3\text{)} \)

\[
\begin{array}{c|c|c|c}
\text{No. of protons} & \text{Multiplicity} & \text{Assignment} \\
\hline
3 \text{H} & 0.72 & s \\
3 \text{H} & 0.88 & s \\
3 \text{H} & 0.92 & s \\
3 \text{H} & 0.96 & s \\
6 \text{H} & 1.00 & s \\
3 \text{H} & 1.04 & s \\
3 \text{H} & 1.18 & s \\
\text{methylene and methine proton s.} & 1.36-2.36 & m \\
1 \text{H} & 3.20 & \text{dd} \\
\end{array}
\]

\( \text{Assignment: } \text{Me, 2x Me, Me, C-3-H} \)
Fig. 21. Infrared spectrum of compound GAU/V-1.
Fig. 22. $^1$H NMR spectrum of compound GAUV-1.
Ill). Mass spectrum:

The mass spectrum of the compound has been presented in Fig. 23. Following peaks can be seen in the figure:

\[ \text{M}^+ 426 \]
\[ m/e 232 \]

The IR of the compound GAU/V-1 has indicated the presence of -OH group (peak at 3440 cm\(^{-1}\)), -CH\(_3\) group (peak at 2930 and 2860 cm\(^{-1}\)).

In \( ^1\)H NMR (CDCl\(_3\)) the groups appearing at \& 0.72-1.18, 1.36-2.36 and 3.20 could be identified as protons of -CH\(_3\), methylene and methine protons and C\(_3\)-H proton. There is no signal between \& 4.0-6.0 supporting the presence of a 13,18 double bond. In Mass spectrum the M\(^+\) peak at 426 indicates the molecular weight of the compound to be 426. The molecular weight of \( \delta \)-amyrin is 426. It also showed fragment at m/e 232. The fragmentation of \( \delta \)-amyrin is as follows:

\[ \text{M}^+ 426 \rightarrow \left[ \begin{array}{c} \text{HO} \\ \text{CH} \_3 \end{array} \right] + \left[ \begin{array}{c} \text{CH} \_3 \\ \text{CH} \_3 \end{array} \right] \]

The fragment at m/e 232 is the characteristic fragment of \( \delta \)-amyrin.

The spectral data and m.p. supported the compound GAU/V-1 to be \( \delta \)-amyrin.

Compound GAU/V-2:

The compound (5 mg.) gave single spot on TLC (solvent system - Benzene and detection with iodine vapour), the IR and NMR spectra of the compound were taken. The details of spectral analysis are as follows:
Fig. 10. Mass spectrum of compound GAU/V-1.
IR : KBr cm$^{-1}$
max
3500-3600 cm$^{-1}$ (br.)
2930 cm$^{-1}$ (m)
2860 cm$^{-1}$ (m).

$^1$H NMR : (CDCl$_3$) $\delta$: (Fig. 24).
0.85 (t, protons of -C-H$_3$)
2.2 (s, protons of -C-H$_2$)

At $\delta$ 3.2 the signal is not clear.

In NMR the signal at $\delta$ 3.2 may be an -OH since there is indication of –OH in IR (broad peak at 3500-3400 cm$^{-1}$). The spectrum also indicates the presence of -CH$_3$ and -CH$_2$ group. It may be a straight chain compound. Further studies are necessary for its characterisation. But due to paucity of compound, further work could not be undertaken.

**Compound GAU/V-5**

Useful information on the structure of flavones and flavonols can be obtained from their Ultra violet spectra in methanol and in presence of certain spectral shift reagents(391, 392). Flavonols and flavones, in methanol, exhibit two major absorption peaks in the region 240 to 400 nm. These are Band-I usually between 300-360 nm associated with absorption due to the B-ring cinnamoyl system, and Band-II usually between 240-280 nm associated with absorption involving the A-ring benzoyl system (391).

Hence, the UV spectra of the compound GAU/V-5 in methanol and after addition of certain reagents were recorded in a Shimadzu UV-visible double beam recording spectrophotometer (model UV 160A).
Fig. 24. 1H NMR spectrum of compound GAU/V-2.
A reference solution was prepared by extracting a piece of blank chromatographic paper from the paper chromatogram of flavonoid fraction in methanol.

Small amount of compound GAU/V-5 was dissolved in methanol to prepare a stock solution of the flavonoid. Then following steps were followed:

a. The methanol spectrum of the flavonoid solution was recorded.
b. Two drops of 2 M NaOH solution was added to 'a' and the spectrum was recorded.
c. Six drops of aluminium chloride solution (5% in methanol) were added to the flavonoid solution and the spectrum was recorded immediately.
d. There drpos of HCl solution (prepared by mixing 5 ml of conc. HCl with 10 ml distilled water) was added to 'c' and immediately the spectrum was recorded.
e. Excess anhydrous sodium acetate was added withshaking to the cuvette containing stock flavonoid solution and the spectrum was recorded.
f. Sufficient powdered anhydrous boric acid powder to give a saturated solution was added with shaking to 'e' and the spectrum was recorded.

The UV spectrum of the compound GAU/V-5 in methanol has been presented in Fig.25 A. It exhibits spectral maxima at 355.5 nm, 258.5 nm and 232.5 nm. As mentioned earlier, flavones and flavonols exhibit two major absorption peaks in the region 240-400 nm, Band I between 300-380 nm and Band II between 240-280 nm. The compound GAU/V-5 showed absorption peaks at 355.5 nm (Band - I) and 258.5 nm (Band II), suggesting the compound to be a flavone/flavonol.

The compound gave 50 nm bathochromic shift (Band I) with NaOH (absorption peak at 405 nm) (Fig.25 B.), indicating the presence of a free hydroxyl group at C-4'. The compound gave no shift in Band I with aluminium chloride and aluminium chloride-hydrochloric acid reagents while there was bathochromic shifts of 9.5 mm in Band I in presence of sodium acetate and in presence of sodium acetate - boric acid reagent the spectrum was similar to that in methanol alone. The UV spectra of flavones and flavonols containing free 7-hydroxy group exhibit a diagnostic 5-20 nm bathochromic shift of Band II in the presence of sodium acetate(391). The compound exhibited 9 nm bathochromic shift in Band II (absorption at268 nm) in presence of sodium acetate which indicates the presence of a free hydroxyl group at C-7. Analysis of the UV spectra suggest the compound GAU/V-5 to be a flavonoid with free hydroxyl groups at C-4' and C-7.

**Compound GAU/V-6 :**

The UV spectra of the compound GAU/V-6 in methanol alone and after addition of aluminium chloride, aluminium chloride - hydrochloric acid and sodium hydroxide reagents were recorded (Fig.26 A,B) The UV spectrum of the compound in methanol exhibited an intense peak at 263.5
Fig. 25A. Ultra violet spectrum of compound GAUV-5 in methanol.

| 222.2 | 1.629  |
| 258.8 | 1.664  |
| 322.2 | 1.270  |

---

\( \text{PEAK} \rightarrow \text{VALLEY} \rightarrow \text{PEAK} \)
Fig. 25 B. Ultra violet spectrum of compound GAU/V-5 in presence of NaOH.
Fig. 26 A Ultra violet spectrum of compound GAU/V-6 in methanol.
Fig. 26 B. Ultra violet spectrum of compound GAU/V-6 in presence of AlCl3.
Fig. 26 C. Ultra violet spectrum of compound GAU/V-6 in presence of AlCl₃/HCl.
Fig. 28 D. Ultra violet spectrum of compound GAU-56 in presence of NaOH.
nm. After addition of aluminium chloride there was a bathochromic shift of 10.5 nm (absorption at 274 nm) and in presence of aluminium chloride - hydrochloric acid reagent it exhibited a peak at 271 nm, i.e. a bathochromic shift of 7 nm (relative to the spectrum in methanol). After addition of NaOH also there was a bathochromic shift in Band II (absorption at 285.5 nm).

Isoflavones, flavanones and dihydroflavonols exhibit an intense Band II absorption with only a shoulder or low intensity peak representing Band-I. The band II absorption of a isoflavones (in methanol) usually occurs in the region 245-270 nm. Band II in the UV spectra of 5-hydroxyisoflavones undergoes a 10 -14 nm bathochromic shift (relative to the spectrum in methanol) in the presence of AlCl₃/HCl. The spectra of isoflavones lacking a free 5-hydroxyl group are unaffected by this reagent (391). The compound GAU/V-6 (in methanol) exhibited Band II absorption at 263.5 nm which showed bathochromic shift of 7 nm in the presence of AlCl₃/HCl reagent suggesting it to be a 5-hydroxyisoflavone.
SECTION C

EVALUATION FOR ANTIINFLAMMATORY AND HEPATOPROTECTIVE ACTIVITIES.

In several Ayurvedic literature Gymnosporia montana has been mentioned to be useful in jaundice, inflammation and rheumatic pain. As mentioned earlier in aims and objectives, different extracts of G. montana leaf were studied for antiinflammatory and hepatoprotective activities.

Extracts: The details of preparation of extracts have been mentioned in Section B. PE, TME, EAT, BE and MAR were used for the studies. A fire suspension of extracts was prepared using 3% tween 80 and diluted to requisite concentration with distilled water.

Experimental Animals: Experimental animals, i.e., Swiss albino mice and Charles foster strain albino rats were from the animal house facility attached to the Institute of Post Graduate Teaching and Research, Gujarat Ayurved University, Jamnagar. Rats and mice were maintained on Lipton's Gold Mohur rat pellet feed and tap water was given ad libitum. The animals were kept under ideal husbandry conditions, exposed to natural day and night cycles. Each group consisted of 6 animals of either sex. Control group received equal quantity of vehicle used for the preparation of the extracts.

Statistical analysis: For statistical analysis Students T test for unpaired data was used.

1. ANTIINFLAMMATORY ACTIVITY:

The antiinflammatory activity in the extracts (PE, TME, EAT, BE and MAR) was evaluated by noting the effect of their prior treatment on 1% carrageenin induced rat hind paw oedema as described by Winter et al. (393). Rats of either sex, weighing between 120 to 170 g were used for the study. Food and water were available to the animals till the start of the experiment. The extracts (200 mg/kg) were administered by intraperitoneal injection 45 minutes prior to carrageenin injection. Phenylbutazone, 100 mg/kg body weight, administered group served as reference standard while control group received equal volume of 3% tween 80. Tap water, 2ml/100 g body weight, was administered to each rat along with the drug to ensure uniform hydration in the animals and to minimise variation in oedema formation. Oedema was induced by injecting 0.1ml freshly prepared 1% carrageenin (Sigma-type I) in sterile saline solution, into subplantar tissue of the right hind paw. Paw volume was measured before and three hours after carrageenin injection with the help of a plethysmograph as described by Bhatt et al (394). Fig. 27 shows the plethysmograph. The initial level of the fluid was adjusted and set to zero each time prior to paw immersion. The paw was immersed in water exactly upto a mark on the skin upto the level of lateral malleolus and the increased level of
Fig. 27. Plethysmograph set up for measuring paw volume in rats.
TABLE - 9
EFFECT OF G.MONTANA LEAF EXTRACTS ON CARRAGEENIN HIND PAW OEDEMA IN RATS

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg kg⁻¹)</th>
<th>Volume of paw oedema (ml) Mean±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>0.35±0.16</td>
</tr>
<tr>
<td>PE</td>
<td>200</td>
<td>0.42±0.06</td>
</tr>
<tr>
<td>TME</td>
<td>200</td>
<td>0.41±0.08</td>
</tr>
<tr>
<td>EAT</td>
<td>200</td>
<td>0.46±0.09</td>
</tr>
<tr>
<td>BE</td>
<td>200</td>
<td>0.53±0.11</td>
</tr>
<tr>
<td>MAR</td>
<td>200</td>
<td>0.46±0.12</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>100</td>
<td>0.10±0.04*</td>
</tr>
</tbody>
</table>

* p < 0.05

It can be seen from the Table that none of the extracts evaluated could suppress carrageenin induced hind paw oedema indicating lack of antiinflammatory activity. Thus the results of the study did not provide pharmacological basis to the therapeutic application of G.montana leaf in the inflammatory disorders.

2. HEPATOPROTECTIVE ACTIVITY
Hepatoprotective activity in the extracts was assessed by employing two methods. Preliminary screening was carried out by noting the effect of extracts on pentobarbitone sleeping in CCl₄ administered mice. Extracts, found to produce significant protection in primary screening, were further evaluated by noting their effect on CCl₄ induced changes on certain biochemical parameters and liver cytoarchitecture.

I. Pentobarbitone sleeping time:

The effect of extracts on pentobarbitone (PBN) sleeping time (dose 30 mg kg⁻¹) after CCl₄ injection in mice was noted as described by Bhargava et al (289). Mice of either sex, weighing between 25-30 g were used for the study. Food and water were freely available during the experiment.

Initial PBN sleeping time was noted with 30 mg kg⁻¹ dose. The extracts, PE, TME, EAT, BE
and MAR - 100 and 200 mgkg\(^{-1}\), were administered by intraperitoneal injection for seven consecutive days. Control groups received equal volume of 3% tween 80. On the seventh day CCl\(_4\) (50% V/V in liquid paraffin) was given by i.p. injection (2 mlkg\(^{-1}\)) to all the groups and PBN (30 mgkg\(^{-1}\)) sleeping time was noted 24 hours later. Decrease in the duration of PBN sleeping time in extract treated groups compared to vehicle control group was considered as index of protection.

II. Effect on liver weight and volume:

Effect of extract on liver weight and volume was noted. Volume was measured by liquid displacement method.

III & Iv. Effect on CCl\(_4\) Induced changes in biochemical parameters and liver cytoarchitecture.

Methanol extract (TME) was found to be effective in PBN sleeping test. Hence, it was further evaluated by noting its effect on CCl\(_4\) induced changes in transaminase activity and various biochemical parameters in serum and liver in rats as described by Dwivedi et al. (298). Effect of the extracts on CCl\(_4\) induced changes in liver cytoarchitecture was also studied.

Rats of either sex, weighing between 140-190 g were used for the study. The extract, TME 100 and 200 mgkg\(^{-1}\) was administered by intraperitoneal route for ten consecutive days. Control group received equal volume of 3% tween 80. Carbon tetrachloride was injected i.p. in the dose of 0.7 mlkg\(^{-1}\) on third, sixth and tenth days. Rats were sacrificed one hour after last CCl\(_4\) injection by cervical dislocation, blood was collected from the neck blood vessels, serum separated and stored at 4°C till estimation was carried out. Liver was carefully dissected out, extraneous tissue was cleaned off and then wet weight and volume of the liver were noted.

Part of the liver was fixed in Bouin's solution for microtome sectioning. For determination of enzyme activities liver was homogenised in distilled water at 4°C and the estimations were carried out within 24 h of homogenate preparation. Following biochemical parameters were estimated in serum and liver.

a. Estimation of total protein: Protein content in serum was estimated by using Biuret method (396) whereas that in liver homogenate was estimated by using Lowry's method (397).

i. Biuret method:

**Principle:**
The CO-NH group in the protein molecule reacts with copper sulphate in alkaline medium to give purple colour which is then measured at 540 nm.
Reagents:

**Biuret reagent** - 4.25 g potassium sodium tartrate (KNaC$_4$H$_2$O$_6$, 4H$_2$O), 1.5 g cupric sulphate (CuSO$_4$, 5H$_2$O) and 2.5 g potassium iodide were dissolved in about 500 ml distilled water. 4 g sodium hydroxide was dissolved in this solution and the volume was made up to one litre.

**Procedure**:

To 0.1 ml serum 4 ml Biuret reagent was added, mixed by swirling and allowed to stand for 30 minutes at room temperature. Absorbance was measured at 540 nm against blank prepared by using 0.1 ml distilled water instead of serum. The protein concentration in the sample was calculated from a standard curve obtained by using different concentrations of standard solution (Bovine serum albumin) instead of sample.

**ii. Lowry's method**:

**Principle**:
The final colour, which is measured at 750 nm, is a result of (i) Biuret reaction of protein with copper in alkaline medium and (ii) reduction of the phosphomolybdic phosphotungstic reagent by the tyrosine and tryptophan present in the treated protein.

**Reagents**:

1. 4% Sodium carbonate.
2. 0.5% CuSO$_4$.5H$_2$O in 1% potassium sodium tartrate.
3. Alkaline copper solution - 50 ml of reagent (1) was mixed with 2 ml of reagent (2). (Prepared freshly).
4. 0.1 N NaOH
5. **Diluted Folin's reagent** - Folin-Ciocalteau reagent was diluted with an equal volume of 0.1 N NaOH.

**Folin-Ciocalteau reagent**:

100 g sodium tungstate, 25 g sodium molybdate and 100 ml conc. HCl were taken in a 2000 ml round bottom flask and the mixture was refluxed gently for 10 hours. To it 150 g lithium sulphate, 50 ml water and few drops of bromine were added, excess bromine was removed by boiling for 15 min, cooled, diluted to 1 lit and filtered.

**6. Standard protein solution** - The standard protein solution was prepared with bovine serum albumin to contain a concentration of 100 µg protein/ml of the solution.
Procedure:

To the sample (0.5 ml) necessary amount of 0.1 N sodium hydroxide solution was added to make the volume 1.5 ml. To this 1.5 ml alkaline copper solution was added, mixed and allowed to stand for 10 minutes. Then 0.15 ml of diluted Folin’s reagent was added with continuous shaking, allowed to stand for half an hour, 4 ml of 0.1 N NaOH was added and the absorbance was measured at 750 nm against a blank prepared by using equal volume of distilled water instead of sample.

A standard curve was prepared by using different concentration of standard protein solution and the amount of protein in the sample was calculated from standard curve.

b. Estimation of Glutamic oxaloacetic transaminase (GOT) or Aspartate transaminase (L-Aspartate, 2-Oxoglutarate amino-transaminase, EC 2.6.1.1) activity:

GOT activity was estimated in serum and liver homogenate by using the method of Reitman and Frankel (398).

Principle: Transamination is the process in which an amino group is transferred from an \( \alpha \)-amino acid to an \( \alpha \)-keto acid and as a result different \( \alpha \)-amino acids and \( \alpha \)-keto acids are formed. The determination of GOT activity is based on the transamination of aspartic acid to \( \alpha \)-ketoglutaric acid. GOT catalyses the following reaction:

\[
\alpha\text{-Oxoglutarate} + \alpha\text{-aspartate} \rightleftharpoons \text{L-Glutrate} + \text{Oxaloacetate}.
\]

Oxaloacetate, so formed, is coupled with 2,4- dinitrophenyl hydrazine to give the corresponding hydrazone, which gives brown colour in alkaline medium. The colour is measured at 510 nm.

Reagents:

1. Phosphate buffer (0.1 M, pH 7.4) - 11.3 g dry anhydrous disodium hydrogen phosphate and 2.7 g dry anhydrous potassium dihydrogen phosphate were dissolved in distilled water. Then volume was made up to one litre with water, pH was checked and stored at 4°C.

2. GOT substrate (200 mM d-l-aspartic acid, 2 mM \( \alpha \)-keto-glutaric acid) 13.3 g d-l aspartic acid was dissolved in minimum amount of 1 N sodium hydroxide and a solution of pH 7.4 was produced. To it 0.146 g ketoglutaric acid was dissolved by adding a little more sodium hydroxide solution. The pH was adjusted to 7.4 and volume was made up to 500 ml with phosphate buffer. It was stored at 15°C.
3. Dinitrophenyl hydrazine (DNPH) reagent (1 mM) 19.8 mg dinitrophenyl hydrazine was dissolved in 10 ml conc. hydrochloric acid and volume was made up to 100 ml with distilled water. It was stored at 15°C.

4. Sodium hydroxide 0.4 N-16 g sodium hydroxide in 1000 ml distilled water.

5. Stock pyruvate standard (200 mM) 220 mg sodium pyruvate was dissolved in 100 ml phosphate buffer. It was stored at 15°C.


Procedure: Sample (0.05 ml serum/0.1 ml liver homogenate) was added to 0.5 ml substrate, mixed and incubated at 37°C in a thermostatic water bath for one hour. The reaction was arrested by adding 0.5 ml DNPH reagent and the tubes were kept at room temperature for 20 minutes. Then 5 ml 0.4 N sodium hydroxide solution was added and the absorbance at 510 nm was measured after 10 minutes. A set of control tubes were also processed in a similar way except that the sample was added after arresting the reaction with DNPH reagent. Blank was prepared by using 0.1 ml water instead of sample. A standard curve was prepared using pyruvate standard at concentrations ranging from 0.2 - 10 micromoles and from it the concentrations of pyruvate released in the samples were obtained.

c. Estimation of Glutamic pyruvate transaminas (GPT) or Alanine transaminase (1-Alanine, 2-oxoglutarate aminotransferase, EC 2.6.1.2) activity:

GPT activity in serum and tissue homogenates were also estimated by Reitman and Frankel method (398).

Principle:
GPT catalyzes the following reaction:

Ketoglutarate + L-Alanine <=> L-Glutamate + Pyruvate.

Pyruvate, so formed, is coupled with 2, 4- dinitrophenyl hydrazine to give the corresponding hydrazone, which in alkaline medium gives brown colour and the absorbance is measured at 510 nm.

Reagents:

1. Phosphate buffer (0.1 M, pH 7.4)
2. GPT substrate (200 mM dl-alanine, 2mM ketoglutarate) 9 g alanine was dissolved in 90 ml
distilled water with the addition of 1 N sodium hydroxide to adjust the pH to 7.4. To this solution 0.146 g ketoglutaric acid was added and dissolved by adding a little more sodium hydroxide and adjusting the pH to 7.4. Volume was made up to 500 ml with phosphate buffer.

3. 2, 4- Dinitrophenyl hydrazine (DNPH) reagent (1mM).
4. Sodium hydroxide 0.4 N.
5. Stock pyruvate standard (200 mM).
6. Working pyruvate standard (4 mM).

**Procedure:**

0.5 ml substrate was added to sample (0.1 ml tissue homogenate/0.05 ml serum) and incubated at 37°C for thirty minutes. Then the reaction was terminated by the addition of 1 ml DNPH reagent and kept at room temperature for 20 minutes. Then 5 ml of 0.4 N sodium hydroxide solution was added to it and the absorbance was measured at 510 nm after 10 minutes against reagent blank prepared in similar way by using equal volume of water instead of sample. Similarly a set of control was also processed where the sample was added after addition of DNPH reagent. The concentration of pyruvate released was obtained from the standard curve of pyruvate.

d. Acid phosphatase activity (Orthophosphoric acid monoester phosphohydrolase, (E.C. 3.1.3.2)):

Acid phosphatase activity in serum and liver homogenate was estimated according to the procedure described by King and King (399).

**Principle:**
The enzymes phosphatases catalyse splitting of phosphoric acid from certain monophosphoric esters thereby releasing inorganic phosphate.

Disodium phenylphosphate is used as the substrate and phenol released by enzymatic hydrolysis is measured.

**Reagents:**

1. Citric acid - Sodium citrate buffer (pH 4.9) - 21 g citric acid was dissolved in water and to it 188 ml 1 N sodium hydroxide was added and volume was made up to 500 ml with distilled water. The pH was adjusted to 4.9 with the help of sodium hydroxide and hydrochloric acid. It was kept in refrigerator.

2. Substrate Disodium phenyl phosphate - 1.09 g disodium phenyl phosphate was dissolved in water and volume was made up to 500 ml. It was quickly heated just to boil, cooled, little chloroform was added and kept in refrigerator.
3. Buffer substrate - Equal volumes of citric acid - sodium citrate buffer and substrate disodium phenyl phosphate were mixed.

4. Sodium hydroxide (0.5 M) -2 g sodium hydroxide in 100 ml distilled water.

5. Sodium bicarbonate (0.5 M) 2.1 g sodium bicarbonate in 50 ml distilled water.

6. Aminoantipyrine - o.6 g Aminoantipyrine in 100 ml distilled water.

7. Potassium ferricyanide - 2.4 g potassium ferricyanide in 100 ml distilled water.

8. Standard phenol solution -100 mg phenol was dissolved in 100 ml of 0.1 N hydrochloric acid.

Procedure:
2 ml buffer substrate was taken in two sets of tubes. Then to one tube (Test) 0.1 ml serum/liver homogenate was added and both the tubes were incubated at 37°C for one hour. Then 1 ml of 0.5 N sodium hydroxide followed by 1 ml of 0.5 M sodium bicarbonate were added to both the tubes. Then 0.1 ml serum/liver homogenate was added to the other set of tubes (Control). 1 ml of aminoantipyrine and 1 ml of potassium ferricyanide reagent were added to all the tubes and immediately absorbance was measured at 520 nm. Similarly standard (in different concentrations) and standard blank were run by using 1.1 ml buffer substrate and 1 ml standard phenol (for standard) and 1.1 ml buffer substrate and 1 ml distilled water (for standard blank) and standard curve was prepared. The concentration of phenol in the samples was obtained from standard curve.

e. Estimation of Alkaline phosphatase activity: Alkaline phosphatase activity was estimated in serum and liver homogenate (400).

Principle:

Phenylphosphate is converted to inorganic phosphate and phenol by the enzyme alkaline phosphatase at pH 10.0. Phenol, thus formed, reacts in alkaline medium, with aminoantipyrine in the presence of potassium ferricyanide, an oxidising agent, to form a complex of orange-red colour, which is measured at 510-520 nm. The intensity of the colour is proportional to alkaline phosphatase activity.

Reagents:

1. Solution A - Sodium carbonate - sodium bicarbonate buffer (0.1 M) - 3.18 g anhydrous sodium carbonate and 1.88 g sodium bicarbonate were dissolved in 500 ml water.
2. Solution B (substrate) - Disodium phenyl phosphate (0.001 M) - 1.09 g disodium phenyl phosphate was dissolved in water and volume made upto 500 ml. The solution was heated quickly just to boil, cooled, little chloroform was added and kept in a refrigerator.

3. Buffered substrate for use (pH 10.0) - Equal volumes of solution A and solution B were mixed and pH was checked.

4. Sodium hydroxide (0.5 N) - 2g sodium hydroxide was dissolved in 100 ml water.

5. Sodium bicarbonate (0.5 N)-2.1 g sodium bicarbonate was dissolved in 50 ml water.

6. Aminoantipyrine - 0.5 g aminoantipyrine in 100 ml water.

7. Potassium ferricyanide - 2.4 g potassium ferricyanide in 100 ml water.

8. Standard phenol solution - 100 mg phenol was dissolved in 100 ml 0.1 N hydrochloric acid.

Procedure:

2 ml buffer substrate was taken in two set of tubes and placed in a water bath at 37°C for a few minutes. Then to one tube (test) 0.1 ml sample (serum/liver homogenate) was added and incubated at 37°C, for exactly 15 minutes. The tubes were removed from the water bath and 0.8 ml of 0.5 N sodium hydroxide and 1.2 ml of 0.5 N sodium bicarbonate were added to both the tubes. 0.1 ml sample (serum/liver homogenate) was added to the second tube (blank). Then 1 ml aminoantipyrine reagent and 1 ml potassium ferricyanide solutions were added to both the tubes and the absorbance was measured at 520 nm. Similarly, standard and standard blank were run by using 1.1 ml buffer substrate and 1 ml standard phenol (for standard) and 1.1 ml buffer substrate and 1 ml water (for standard blank).

f. Estimation of serum Acid glycoprotein (orosomucoid, seromucoid):

Serum orosomucoid level was estimated by the method described by Winzler (401).

Principle:

After removing heat coagulable proteins with perchloric acid, the orosomucoid which remains in solution is precipitated by phosphotungstic acid and estimated by determining its carbohydrate content by reaction with orcinol - sulphuric acid reagent or its nitrogen content by Kjeldahl - nesslerization, or its tyrosin content using the Folin-Ciocalteu reagent. In the present method tyrosine content using Folin-Ciocalteu reagent was measured.
Reagents:

1. Sodium chloride solution - 9 g sodium chloride was dissolved in 1000 ml distilled water.

2. Perchloric acid, (1.8 M) - 28 ml perchloric acid (72 %) was diluted to 200 ml with distilled water.

3. Phosphotungstic acid 50 g/lit. in 2 mol/lit. HCl.

4. Perchloric acid, (600 mM/lit)-1.8 M perchloric acid was diluted to 1 to 3.

5. Phenol reagent of Folin and Ciocalteu- 100g sodium tungstate and 25 g sodium molybdate were dissolved in about 700 ml distilled water in a 2 liter round bottom flask. Then 50 ml syrupy 85% phosphoric acid and 100 ml conc. hydrochloric acid were added and refluxed for 10 hours. 150 g lithium sulphate, 50 ml water and few drops of bromine were added, cooled, volume made upto one liter and filtered.

6. Sodium carbonate solution - 200g sodium carbonate was dissolved in 1000 ml distilled water.

7. Standard tyrosine solution - 100 mg/lit. tyrosine in 100 mM/lit hydrochloric acid.

Procedure: 0.2 ml serum was added to 4.8 ml sodium chloride solution followed by 2.5 ml of 1.8 M. perchloric acid dropwise with shaking. After 10 minutes it was filtered through Whatman no. 50 filter paper. To 5 ml filtrate 1 ml phosphotungstic acid solution was added, mixed and after 10 minutes centrifuged at 2000 rpm for 10 minutes and decanted. The precipitate was washed with phosphotungstic acid solution and diluted 1 to 3 with 600 mM/lit. perchloric acid. Again centrifuged and the supernatant was decanted. Then 1 ml sodium carbonate solution was added to dissolve the precipitate and 3.5 ml distilled water and 0.5 ml phenol reagent were added, placed in a water bath at 37°C for 15 minutes and the absorbance was measured at 680 nm. Similarly, a standard was prepared by using 0.5ml tyrosine solution, 1 ml sodium carbonate solution, 3 ml water and 0.5 ml phenol reagent. Absorbance of both standard and test were measured against a blank containing 3.5 ml water, 1 ml sodium carbonate solution and 0.5 ml phenol reagent.

Calculation:

Since 100 g orosomucoid contains 4.2 g tyrosine, the factor for converting g tyrosine to g orosomucoid is 23.8. So, serum orosomucoid (g/lit).

\[
\text{Reading of unknown} \quad \frac{0.05}{0.33} \times 23.8
\]
For estimation of various lipid components of liver, it is first extracted from the tissue

**Extraction of lipids in liver:**
2 g liver was homogenised with 40 ml (2 X 20 ml) chloroform - methanol (2:1) mixture. It was filtered through Whatman no. 41 filter paper, the filter paper was washed with chloroform - methanol (2:1) mixture, to it 10 ml calcium chloride solution (0.02 %) was added, mixed thoroughly and kept overnight in stoppered container in cold to allow the layers to separate into clear solutions. Next day, the upper methanol layer was removed. The lower chloroform layer, which contains lipid fraction was dried over anhydrous sodium sulphate and the volume was made upto 25 ml with chloroform. Aliquots of this were used for the estimation of cholesterol, triglyceride, total lipids and phospholipids.

g. Estimation of Triglyceride :
Triglyceride level was estimated in serum and liver homogenate (395). Principle - Triglyceride is saponified to free glycerol with KOH. Glycerol moiety is oxidised to formaldehyde which is condensed with ammonia and 2,4-pentanedione (acetyl acetone) to produce 3,5-diacetyl 1,4-dihydro toluidine having yellow colour and the absorbance is measured at 405 nm.

**Reagents -**

1. Alumina (neutral - chromatography grade)- The alumina was washed with water, dried in an oven at 100°-110°C overnight, cooled and stored in a desiccator.

2. Saponification reagent - 5g potassium hydroxide was dissolved in 60 ml distilled water and 40 ml isopropanol was added to it.

3. Sodium metaperiodate reagent - To 77 g of anhydrous ammonium acetate in 700 ml distilled water, 60 ml glacial acetic acid and 650 mg sodium metaperiodate were added, dissolved and diluted to 1 lit with distilled water.

4. Acetyl acetone reagent - 0.75 ml acetyl acetone was added to 20 ml isopropanol, mixed well, to it 80 ml distilled water was added and mixed again.

5. Stock standard solution - 4 mg/ml of triolein.
Procedure:

Serum: 0.1 ml
Liver: 1 ml aliquot of lipid fraction was taken and evaporated to dryness.

Serum/liver samples were taken and volume was made up to 4 ml with isopropanol. Mixed well and 400 mg of washed alumina was added. It was shaken for 15 minutes and centrifuged. 2 ml of supernatant was taken, to it 0.6 ml of saponification reagent was added and incubated at 60°-70°C for 15 minutes. After cooling 1 ml sodium metaperiodate solution was added and mixed well. Then 0.5 ml acetyl acetone reagent was added, mixed again and incubated at 50°C for 30 minutes. After cooling the absorbance was measured at 405 nm. Similar procedure was followed for blank and standard.

h. Estimation of Total Lipids:

Total lipid was estimated by sulphophosphovanillin reaction (402) by taking 0.1 ml serum/residue from 0.5 ml lipid fraction.

Principle:

Heating the sample in presence of sulphuric acid probably forms carbon-carbon double bonds (C=C) in many of those lipids which do not already possess them (by a dehydration type of reaction), and reacts with these carbon-carbon double bonds to form intermediate compounds which react afterwards giving a colour when vanillin and phosphoric acid are added and the absorbance is measured at 540 nm.

Reagents:

1. Phosphovanillin reagent - 1.2 g vanillin was dissolved in 200 ml water with constant stirring. To it 800 ml conc. phosphoric acid was added, mixed and stored in a dark bottle.

2. Conc. H₂SO₄

Procedure:

Two ml conc. H₂SO₄ was added to the sample (0.1 ml serum/residue from 0.5 ml lipid fraction of liver), heated on a boiling water bath for 10 minutes and cooled. From it an aliquot (0.1 ml for liver and 0.4 ml for serum) was taken (for blank equal volume of conc. H₂SO₄ was taken), 6 ml phosphovanillin reagent was added to it, mixed well, incubated at 37°C for 15 minutes and the absorbance was measured at 540 nm against the blank. A standard curve was prepared by using suitable concentration of triolein.
I. Estimation of Total Cholesterol:

Total cholesterol was estimated in serum and liver homogenate by taking 0.1 ml serum and residue of 0.5 ml lipid fraction.

Principle:
Cholesterol in acetic acid solution gives a red colour with ferric chloride and sulphuric acid and the colour is measured at 500 nm.

Reagents:
1. Acetic acid.
2. Ferric chloride solution - 0.05 % solution of ferric chloride, hexahydrate in acetic acid.
3. Conc. sulphuric acid.
4. Stock cholesterol standard - 100 mg/100 ml acetic acid.
5. Cholesterol standard for use - Stock solution diluted with ferric chloride- acetic acid reagent to get different concentrations.

Procedure:
To serum/lipid sample 10 ml ferric chloride solution was added, kept for 15 minutes for the proteins to flocculate and centrifuged. From the supernatant 5 ml was taken and for blank 5 ml ferric chloride solution was taken. To it 3 ml conc. sulphuric acid was added, mixed thoroughly, kept for 20 - 30 minutes and the absorbance was measured at 500 nm against the blank. A standard curve was prepared by using different concentrations of standard cholesterol.

j. Estimation of Phospholipids:

Phospholipid content was estimated in lipid fraction by employing the following procedure.

Principle:
The organic phospholipid phosphorus is converted to inorganic phosphorus which reacts with ammonium molybdate to form phosphomolybdic acid which on reduction and reaction with 1,2,4- aminonaphthosulphonic acid (ANSA) forms a stable blue colour and it's absorbance is measured at 660 nm.

Reagents:
1. 2.5 % Ammonium molybdate - 25 g ammonium molybdate was dissolved in about 200 ml water. It was transferred to a one litre vol. flask containing 300 ml 10 N H2SO4 and the volume was made up with distilled water.
2. Fiske-Subba Row reagent - 0.5 g 1,2,4-aminonaphthalene-sulphonic acid was added to 195 ml of 15 % sodium bisulphite solution. To it 5 ml of 20 % sodium sulphite was added, mixed well, filtered and stored in an amber coloured bottle (stable for 4 weeks).

3. Standard phosphorus solution - 0.351 g monopotassium phosphate was dissolved in 10 ml of 10 N H\textsubscript{2}SO\textsubscript{4} and the volume was made up to one litre with distilled water. Concentration of this solution was 0.4 mg phosphorus/5ml.

**Procedure:**

0.5 ml of lipid fraction was evaporated and the residue was used for the estimation. To the residue 0.5 ml of 10 N H\textsubscript{2}SO\textsubscript{4} was added and heated in an oven at 150°C-160°C for at least three hours. Then two drops of fuming nitric acid was added and again heated in the oven for at least one and a half hours more to complete the combustion. Sample was cooled and to it 4.6 ml ammonium molybdate solution was added followed by 0.2 ml Fiske-Subba Row reagent. It was covered with marble, heated for 7 minutes in boiling water bath, cooled and absorbance was measured at 660 nm. Similar procedure was followed for blank and standard. The values of phosphorus were expressed as the phospholipid (lecithin) by multiplying by a factor of 25.

k. Estimation of Liver Glycogen:

Glycogen content in liver was estimated by employing the following procedure (395).

**Principle:**

Glycogen is hydrolysed to glucose, which is then estimated by using anthrone reagent.

**Reagents:**

1. 30 % Potassium hydroxide.

2. 95 % Ethanol and 60 % ethanol.

3. 2 N Sulphuric acid.

4. Anthrone reagent - 180 ml conc. sulphuric acid was added slowly to 70 ml distilled water to prepare 72 % H\textsubscript{2}SO\textsubscript{4}. Then 0.125 g anthrone and 2.5 g thiourea were added to this 250 ml of 72 % H\textsubscript{2}SO\textsubscript{4}.

5. Standard stock glucose solution - 1 mg/ml in distilled water.
Procedure:
Liver was taken out rapidly, excess of blood was removed by blotting between folds of filter paper, from it 1 g was taken into a stoppered tube and to it 2 ml 30% KOH was added. The content of the tubes were digested in a boiling water bath for one and a half hours, cooled in ice cold water, 4.5 ml 95% ethanol was added, heated just to boiling and left overnight in cold. Next day the tubes were centrifuged, the precipitate was dissolved in 5 ml warm water, 10 ml 95% ethanol was added to reprecipitate glycogen and centrifuged. The precipitate was washed several times with 60% ethanol, 2 ml 2 N H₂SO₄ was added and hydrolysed in boiling water bath for 3-4 hours. It was cooled, neutralised with NaOH, volume was made to 10 ml and filtered. 0.2 ml filtrate was taken (0.2 ml distilled water for blank), to it 5 ml anthrone reagent was added with stirring, covered with marble and kept in boiling water bath for 15 minutes. Tubes were cooled to room temperature and the absorbance was measured at 620 nm against a blank. A standard curve was prepared by using standard glucose solution. Amount of glucose in the sample was calculated from the standard curve.

To assess the effect of extracts on CCl₄ induced histopathological changes in liver in rats, liver was excised out, cleaned of extraneous tissue and fixed in Bouin's solution, and processed for microtome sectioning following standard techniques (395). Tissue sections of 5 micron thickness were taken and stained with haematoxylin and eosin. Stained tissue sections were observed under binocular microscope at various magnifications. Changes in liver cytoarchitecture were noted down. Photomicrographs were taken in a Carl Zeiss, Jena Binocular microscope with photomicrographic attachments.
RESULTS AND DISCUSSION

i. Effect on PBN sleeping time in CCl₄ treated mice:
The data have been presented in Table - 10. TME produced significant antagonism of CCl₄ induced prolongation at 200 mgkg⁻¹ dose. At this dose level only 3/6 mice slept in comparison to control group in which 6/6 mice slept. The antagonism observed with 100 mgkg⁻¹ dose was not statistically significant. PE did not inhibit CCl₄ induced prolongation of PBN sleep. In BE treated group shortening of duration of PBN sleep was observed but it was not statistically significant. However only 4/6 at 100 mgkg⁻¹ and 5/6 at 200 mgkg⁻¹ dose slept. MAR did not produce significant effect at both the dose levels studied. The result of the primary screening indicates possibility of presence of significant hepatoprotective activity with ME. BE seems to possess weak to moderate hepatoprotective activity.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mgkg⁻¹)</th>
<th>PBN sleeping time (min.)</th>
<th>No of animal losing righting reflex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Initial</td>
<td>After CCl₄</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>36.00 ± 3.90</td>
<td>220.39 ± 80.00</td>
</tr>
<tr>
<td>PE</td>
<td>100</td>
<td>39.20 ± 3.69</td>
<td>360.80 ± 98.34</td>
</tr>
<tr>
<td>PE</td>
<td>200</td>
<td>43.31 ± 9.61</td>
<td>430.08 ± 98.73</td>
</tr>
<tr>
<td>TME</td>
<td>100</td>
<td>48.11 ± 8.71</td>
<td>195.18 ± 76.12</td>
</tr>
<tr>
<td>TME</td>
<td>200</td>
<td>35.60 ± 8.71</td>
<td>060.14 ± 13.40*</td>
</tr>
<tr>
<td>BE</td>
<td>100</td>
<td>46.71 ± 7.89</td>
<td>130.46 ± 95.00</td>
</tr>
<tr>
<td>BE</td>
<td>200</td>
<td>51.20 ± 8.82</td>
<td>140.84 ± 81.35</td>
</tr>
<tr>
<td>MAR</td>
<td>100</td>
<td>38.39 ± 6.18</td>
<td>172.75 ± 27.81</td>
</tr>
<tr>
<td>MAR</td>
<td>200</td>
<td>22.00 ± 2.65</td>
<td>148.50 ± 22.50</td>
</tr>
</tbody>
</table>

* P < 0.05

II. Effect on liver weight and volume:
The data have been presented in Table - 11. Both liver weight and volume significantly increased in CCl₄ treated group in comparison to normal control group. Marginal increase in liver weight and slight increase in liver volume, at 100 mgkg⁻¹ dose level, was observed in extract treated groups.
TABLE-11
EFFECT OF G. MONTANA LEAF EXTRACT ON LIVER WEIGHT AND VOLUME IN CCl₄ TREATED RATS

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver weight g/100 g body wt.</th>
<th>Liver Volume ml/100 g body wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Mean±SEM</td>
</tr>
<tr>
<td>Control I</td>
<td>3.20±0.13</td>
<td>2.98±0.15</td>
</tr>
<tr>
<td>(Normal)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control II</td>
<td>4.44±0.42*a</td>
<td>3.83±0.29*a</td>
</tr>
<tr>
<td>(CCl₄)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol ext.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>4.82±0.25</td>
<td>4.33±0.47</td>
</tr>
<tr>
<td>200</td>
<td>4.03±0.31</td>
<td>4.08±0.18</td>
</tr>
</tbody>
</table>

*a* P < 0.05 in comparison to control I.

III. Biochemical parameters:

a. Effect on total protein content:
The data have been presented in Table-12. As could be observed from it - marginal decrease in serum total protein content was noted in CCl₄ treated group in comparison to normal control. Statistically non-significant decrease was observed in extract treated group. Total protein content was significantly increased in liver homogenate from CCl₄ treated group in comparison to normal control. Extract pretreatment significantly inhibited CCl₄ induced increase in liver protein content.

TABLE - 12
EFFECT OF G. MONTANA LEAF EXTRACT ON SERUM AND LIVER PROTEIN CONTENT IN CCl₄ TREATED RATS

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose mg/kg⁻¹</th>
<th>Total protein content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum g/100 ml</td>
<td>Liver mg/g wet tissue</td>
</tr>
<tr>
<td></td>
<td>Mean±SEM</td>
<td>Mean±SEM</td>
</tr>
<tr>
<td>Control I</td>
<td>5.95±0.37</td>
<td>3.09±0.20</td>
</tr>
<tr>
<td>(Normal)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control II</td>
<td>5.50±0.55</td>
<td>6.06±0.35***a</td>
</tr>
<tr>
<td>(CCl₄)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol ext.</td>
<td>100</td>
<td>4.99±0.21</td>
</tr>
<tr>
<td></td>
<td>5.15±0.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.55±0.15***b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.37±0.26***b</td>
<td></td>
</tr>
</tbody>
</table>

***a* P < 0.001 in comparison to normal control ***b P < 0.001 in comparison to CCl₄ control
b. Effect on GOT activity:
Marked elevation in serum GOT activity was observed in CCl4 treated group in comparison to normal control. This elevation in GOT activity was significantly inhibited by extract pretreatment (Table-13). Similar elevation in GOT activity was observed in liver homogenate obtained from CCl4 treated rats. In extract treated group significant inhibition of GOT activity in comparison to CCl4 control was noted.

**TABLE -13**

EFFECT OF G. MONTANA LEAF EXTRACT ON SERUM AND LIVER GOT ACTIVITIES IN CCl4 TREATED RATS.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Serum GOT activities</th>
<th>Liver GOT activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μ mole pyruvate/min/lit. at 37°C</td>
<td>n mole pyruvate/min/mg protein at 37°C</td>
</tr>
<tr>
<td>Control I</td>
<td></td>
<td>83.55±7.11</td>
<td>1027.33±156.26</td>
</tr>
<tr>
<td>(Normal)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control II</td>
<td>100</td>
<td>215.26±12.31***a</td>
<td>1940.80±74.39***a</td>
</tr>
<tr>
<td>(CCl4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol ext.</td>
<td>100</td>
<td>93.82±24.30**b</td>
<td>971.83±42.97***c</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>85.89±12.26**b</td>
<td>783.40±97.69***c</td>
</tr>
</tbody>
</table>

***a p < 0.001 in comparison to normal control
**b p < 0.01 in comparison to CCl4 control
***c p < 0.001 in comparison to CCl4 control.

c. Effect on GPT activity:

Significant elevation of GPT activity in both serum and liver homogenate was observed in CCl4 treated group in comparison to normal control. Decrease in serum GPT activity was noted with lower dose of extract, however, no decrease could be noted at higher dose level. Significant decrease in GPT activity of the liver homogenate was observed in extract treated groups. The data have been shown in Table -14.
TABLE - 14

EFFECT OF G. MONTANA LEAF EXTRACT ON SERUM AND LIVER GPT ACTIVITIES IN CCl₄ TREATED RATS.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>Serum GPT activities</th>
<th>Liver GPT activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg kg⁻¹</td>
<td>µ mole pyruvate/ min/lit. at 37°C</td>
<td>n mole pyruvate/min/mg protein at 37°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>Control I</td>
<td>1</td>
<td>18.10±3.58</td>
<td>1228.28±113.95</td>
</tr>
<tr>
<td>(Normal)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control II</td>
<td>100</td>
<td>112.55±13.80***a</td>
<td>2270.80±158.46***a</td>
</tr>
<tr>
<td>(CCl₄)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol ext.</td>
<td>100</td>
<td>73.01±15.07</td>
<td>1306.72±96.06**b</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>115.69±25.63</td>
<td>1146.69±44.37**b</td>
</tr>
</tbody>
</table>

***a p < 0.001 in comparison to normal control
**b p < 0.01 in comparison to CCl₄ control

d. Effect on acid phosphatase activity:

The data on the effect of extracts on acid phosphatase (ACPase) activity in serum and liver homogenate have been presented in Table-15. Increase in ACPase activity was observed in both serum and liver homogenate of CCl₄ treated rats. Extract pretreatment decreased CCl₄ induced elevation in serum ACPase activity. However, increase in ACPase activity was noted in liver homogenate of extract treated rats. But the changes observed were not statistically significant.

TABLE - 15

EFFECT OF G. MONTANA LEAF EXTRACT ON SERUM AND LIVER ACID PHOSPHATASE ACTIVITY IN CCl₄ TREATED RATS.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>Acid phosphatase activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg kg⁻¹</td>
<td>Serum</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n mole phenol/dl/ min at 37°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean ±SEM</td>
</tr>
<tr>
<td>Control I</td>
<td>1</td>
<td>24.94±4.85</td>
</tr>
<tr>
<td>(Normal)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control II</td>
<td>100</td>
<td>62.36±14.50*a</td>
</tr>
<tr>
<td>(CCl₄)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol ext.</td>
<td>100</td>
<td>31.35±10.89</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>40.89±16.49</td>
</tr>
</tbody>
</table>

*a p < 0.05 in comparison to normal control
e. Effect on alkaline phosphatase activity:

The data have been summarised in Table-16. Statistically non-significant decrease in alkaline phosphatase (ALPase) activity was observed in both serum and liver homogenate of CCl₄ administered rats in comparison to normal control. The liver ALPase activity was further decreased in extract treated groups, however, the decrease was not statistically significant.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Serum Alkaline phosphatase activities</th>
<th>Liver Alkaline phosphatase activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n mole phenol/litre/ min at 37°C</td>
<td>n mole phenol/mg/min at 37°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean±SEM</td>
<td>Mean±SEM</td>
</tr>
<tr>
<td>Control I</td>
<td></td>
<td>3984.50±952.73</td>
<td>33.26±8.26</td>
</tr>
<tr>
<td>(Normal)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control II</td>
<td></td>
<td>3605.40±956.04</td>
<td>19.27±6.46</td>
</tr>
<tr>
<td>(CCl₄)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol ext.</td>
<td>100</td>
<td>3136.17±470.64</td>
<td>17.43±7.26</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>4672.50±1406.93</td>
<td>12.88±6.59</td>
</tr>
</tbody>
</table>

f. Effect on orosomucoid level in serum:

Orosomucoid level in serum increased significantly in CCl₄ treated group in comparison to normal control. Extract pretreatment inhibited CCl₄ induced elevation in serum orosomucoid level, but the inhibition was not dose dependent and was significant only at lower dose level (100 mg/kg⁻¹). The data have been presented in Table -17.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Serum orosomucoid level (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean±SEM</td>
</tr>
<tr>
<td>Control I</td>
<td></td>
<td>0.71±0.05</td>
</tr>
<tr>
<td>(Normal)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control II</td>
<td></td>
<td>0.98±0.14</td>
</tr>
<tr>
<td>(CCl₄)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol ext.</td>
<td>100</td>
<td>0.66±0.05*b</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0.76±0.11</td>
</tr>
</tbody>
</table>

* b p < 0.05 in comparison to CCl₄ control group.
g. Effect on triglyceride level:
As could be observed from the data presented in Fig. 28 A, B, marked decrease in serum triglyceride level was observed in CCl₄ administered group in comparison to normal group. The effect of extract on serum triglyceride level was not dose dependent. At lower dose level (100 mg kg⁻¹) moderate inhibition was observed but the effect noted at higher dose level (200 mg kg⁻¹) was not significant. Triglyceride level in liver increased significantly in CCl₄ treated group in comparison to control group. This CCl₄ induced increase in triglyceride level was prevented by pretreatment with extracts.

Serum Triglyceride content

NOR CON - 339.13±46.19, CCl₄ CON - 79.85±14.50, 
TME 100 - 160.66±15.88, TME 200 - 86.28±24.00

* p < 0.05  *** p < 0.001

Fig. 28 A: EFFECT OF G. MONTANA EXTRACT ON SERUM TRIGLYCERIDE CONTENT IN CCl₄ TREATED RATS.
Liver Triglyceride Content

mg/g wet tissue

NOR CON - 2.81±0.30, CCl₄ CON - 3.97±0.32,
TME 100- 2.54±0.25, TME 200 - 2.87±0.44.

Fig. 28 B: EFFECT OF Q. MONTANA EXTRACT ON LIVER TRIGLYCERIDE CONTENT IN CCl₄ TREATED RATS.

h. Effect on total lipid:

The data have been presented in Table - 18. Marked decrease in serum total lipid level and significant increase in total lipid content of the liver was observed in CCl₄ treated group in comparison to normal control. Extract pretreatment lead to significant antagonism of CCl₄ induced decrease in serum total lipid level. The extract at 100 mgkg⁻¹ dose showed statistically nonsignificant inhibition of CCl₄ induced increase in total lipid content of the liver. At 200 mgkg⁻¹ dose no effect could be observed.
TABLE - 18

EFFECT OF G.MONTANA LEAF EXTRACT ON SERUM AND LIVER TOTAL LIPID IN CCl₄ TREATED RATS

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose mg/kg⁻¹</th>
<th>Serum Total lipid</th>
<th>Liver Total lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n#g'1</td>
<td>mg/100 ml Mean ±SEM</td>
<td>mg/g wet tissue Mean ± SEM</td>
</tr>
<tr>
<td>Control I</td>
<td>(Normal)</td>
<td>575.39±45.38</td>
<td>05.78±0.50</td>
</tr>
<tr>
<td>Control II</td>
<td>(CCl₄)</td>
<td>051.98±03.67***a</td>
<td>12.48±3.21</td>
</tr>
<tr>
<td>Methanol ext. 100</td>
<td>100</td>
<td>384.95±64.47**b</td>
<td>09.51±0.65</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>530.87±88.01***c</td>
<td>11.57±1.11</td>
</tr>
</tbody>
</table>

***a p < 0.001 in comparison to normal control
***c p < 0.001 in comparison to CCl₄ control.
**b p < 0.01 in comparison to CCl₄ control

I. Effect on total cholesterol: The data on the effect of extracts on total cholesterol levels have been presented in fig.29 A,B Similar to the effect noted on total lipid, marked decrease in serum total cholesterol level and significant increase in total cholesterol level in liver were observed in CCl₄ administered group in comparison to normal control. Extract administration not only inhibited CCl₄ induced decrease in serum total cholesterol level but elevated it significantly in comparison to normal control group. Similarly, the extract treatment inhibited CCl₄ induced increase in total cholesterol level of liver.

Serum

Cholesterol content

NOR CON - 93.44±5.12, CCl₄ CON - 59.14±6.92,
TME 100-137.16±9.84, TME 200 - 130.76±24.00.
Fig. 29 A : EFFECT OF G.MONTANA EXTRACT ON SERUM CHOLESTEROL CONTENT IN CCl₄ TREATED RATS.
Liver
Cholesterol Content

**mg/g wet tissue**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose ( \text{mg/kg} )</th>
<th>Liver phospholipid ( \text{mg/g wet tissue} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control I</td>
<td></td>
<td>16.75±1.84</td>
</tr>
<tr>
<td>(Normal)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control II</td>
<td></td>
<td>03.93±0.44*</td>
</tr>
<tr>
<td>(( \text{CCI}_4 ))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol ext.</td>
<td>100</td>
<td>08.32±0.76**</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>10.31±1.20</td>
</tr>
</tbody>
</table>

**a** \( p < 0.001 \) in comparison to control I  **b** \( p < 0.001 \) in comparison to control II

**Fig. 29 B.** : EFFECT OF G.MONTANA EXTRACT ON LIVER CHOLESTEROL CONTENT IN \( \text{CCI}_4 \) TREATED RATS.

**J. Effect on phospholipid content in the liver**:

The data on the effect of extracts on phospholipid content of the liver has been presented in Table - 19. Injection of \( \text{CCI}_4 \) caused significant decrease in phospholipid content of the liver. This decrease was significantly inhibited by pretreatment of rats with the extract.
k. Effect on liver glycogen:

The data on the effect of extracts on liver glycogen content have been summarised in Table - 20. Significant decrease in liver glycogen content was observed in CCl₄ treated rats in comparison to normal rats. This liver glycogen depletion effect of CCl₄ was antagonised to some extent by pretreatment with extracts, especially at lower dose level (100 mgkg⁻¹). However, the effect was not statistically significant.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mgkg⁻¹)</th>
<th>Liver glycogen content (mg/g wet tissue)</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control I</td>
<td></td>
<td>4.70±0.74</td>
<td></td>
</tr>
<tr>
<td>(Normal)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control II</td>
<td></td>
<td>2.29±0.58*</td>
<td></td>
</tr>
<tr>
<td>(CCl₄)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol ext.</td>
<td>100</td>
<td>4.34±1.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>3.51±0.85</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05

iv. Histopathological study:

Figure. 30 A, B show section of liver obtained from a normal rat showing normal cytoarchitecture- normal parenchymal cells showing nucleus in the centre of the cell.

Section of liver obtained from CCl₄ control rats showed presence of marked centrilobular necrosis, degeneration and vacuolisation of parenchymal cells. Many cells showed eccentric nuclei, some of them showed more than one nucleus and there are areas in which many nucleus could be seen without any cell boundary. (Fig.31 A,B)

Section of liver obtained from lower dose (100 mgkg⁻¹) methanol extract administered group showed the following cytoarchitecture: Necrosis of the parenchymal cells was observed but it was much less in both severity and extent in comparison to CCl₄ control rats. Vacuolated cells were very few and degenerative changes were also less. The overall extent of liver injury was significantly less in comparison to CCl₄ control (Fig.32 A,B).
Fig. 30 A. Photomicrograph of section of liver from normal control rat. H & E; magnification x 32. CV : Central vein, S : Hepatic sinusoid, PC : Parenchymal cells. (Note : Normal cytoarchitecture).

Fig. 30 B. Same as in Fig. 30 A but magnification X 200 (Note : Normal cytoarchitecture).
Fig. 31 A. Photomicrograph of section of liver from CCl₄ treated control rats. H & E; magnification X 100. NA: Necrotic area, VC: Vacuolated cell. (Note: Marked degenerative changes, centrilobular necrosis and vacuolated cells).

Fig. 31 B. Same as in Fig. 31 A but at higher magnification (X 200) (Note: Degenerative changes, necrosis (NA), vacuolated cells (VC), presence of many nuclei without cell boundary(\(^\text{\textsuperscript{*}}\)).
Fig. 32 A. Photomicrograph of section of liver from rats administered CCl₄ and TME (100 mg kg⁻¹) of *G. montana*. H & E, magnification x 100. NA: Necrotic area. (Note: Centrilobular necrosis, vacuolisation of cell not seen. Compare with fig 31 A).

Fig. 32 B. Same as in Fig. 32 A but at higher magnification (x 200). CV: Central vein, NA: Necrotic area. [Note: Necrotic changes and multinucleated area (^)].
Liver section obtained from rats belonging to higher dose (200 mg kg\(^{-1}\)) methanol extract group showed only few patches of necrotic areas; there was slight dilation of liver sinusoids, no vacuolation could be observed in parenchymal cells. The extent of liver injury was minimum in comparison to CCl\(_4\) control group. (Fig.33 A,B)

Liver is one of the most important organs in the body. It performs many varied functions that are vital for the maintenance of internal chemical environment in the body. Any derangement in its function will have grave implications to the survival of that animal. The basic functions of liver may be divided into (1) vascular functions, (2) excretory and secretory functions, (3) metabolic functions and (4) detoxification. Vascular functions are blood storage, taking part in body's immune mechanism through its reticuloendothelial system. Bile formation and excretion of bile into intestine are the secretory functions. It is the key organ concerned with metabolism of carbohydrate, protein, lipid, mineral and vitamins. It is the main detoxication organ in the body. Besides, it is also involved in the formation of fibrinogen, prothrombin, heparin and erythrocyte destruction. Injury to the liver due to any cause leads to derangement of the above functions.

At present, no drug is available for the treatment of liver diseases such as viral hepatitis in modern therapeutic armamentarium. On the other hand number of remedies of plant origin are in use for treating liver disorders, since centuries, in traditional medical systems like Ayurveda. Plant isolates like silymarin and picroliv are found to be efficacious in treating different types of experimental liver disorders (404). Reports on efficacy of silymarin in experimental liver injury lead to intensified evaluation of plant products as possible remedies in hepatic disorders.

Gymnosporia montana is one of the many medicinal plants advocated for the treatment of liver disorders in Ayurveda (333, 335, 336). Besides this, it also has strong folklore advocacy as a very useful remedy for liver disorders. The present study was undertaken to screen the extracts of the plant for hepatoprotective activity against CCl\(_4\) induced liver injury in primary screening test. The extracts found to be effective in it were subjected to a detailed investigations to understand the probable mechanism of action by noting their effect on number of parameters that are supposed to be altered during hepatic injury. Evidently, the main aim was to provide experimental basis to the therapeutic claim and to analyse the extracts for major phytochemical fractions with a view to isolate them.

The approach adopted was as follows: (1). Preliminary screening in CCl\(_4\) induced liver injury in mice by noting the effect of extracts on CCl\(_4\) induced prolongation of pentobarbitone (PBN) sleeping time, which also serves as a parameter to assess the effect on functional integrity of liver cells.
Fig. 33 A. Photomicrograph of section of liver from rats administered CCl₄ and higher dose (200 mgkg⁻¹) of TME of G.montana. (H & E, magnification x 100) NA : Necrotic area, S : Hepatic sinusoid. (Note : Very much reduced necrotic changes, no vacuolisation of hepatic cell but increase in the size of hepatic sinusoid. Compare with Fig. 31 A.)

Fig. 33 B. Same as in Fig. 33 A but at higher magnification (x 200).
Effect of extracts on CCl₄ induced changes in liver in rats was studied. This involved assessment of three different types of parameters:

a. Effect on morphological parameters like liver weight, liver volume and body weight.
b. Effect on biochemical parameters like transaminases, phosphatase, orosomucoid, liver glycogen and lipid content of serum and liver.
c. Effect on histopathological changes caused by CCl₄:

Five extracts of the leaf viz., PE, TME, EAT, BE, and MAR were evaluated for hepatoprotective activity by assessing their effect on pentobarbitone (PBN) induced sleep in CCl₄ treated mice. Of the five extracts evaluated, TME was found to possess comparatively better hepatoprotective effect. Hence, it was evaluated in detail by noting its effect on several parameters. Of the remaining extracts hepatoprotective effect was also noted with butanol fraction of methanol extract (BE), though less in comparison to TME.

The liver damage may manifest in the following manner (294, 405)

Once fibrosis sets in liver tissue, it is very difficult to reverse it to the original normal level. It is possible that the drugs having the capability of enhancing proliferative activity of the liver parenchymal cells may have profound modulatory effect on liver damage caused by different causative factors. The parenchymal cells are primarily responsible for the metabolic functions of the liver. They have comparatively more regenerative and reserve capacity in comparison to other cells. These cells are also extremely sensitive to deleterious effect of hypoxia, toxin and infection.
Liver damage caused by carbontetrachloride (CCl₄) is reported to be similar in many respects to the liver injury seen during viral hepatitis (294). Hence, it is used as a medium to induce liver injury to study the hepatoprotective effect of test drugs.

It is now generally accepted that hepatic injury noted with CCl₄ is due to formation of trichlormethyl (CCl₃) radical from CCl₄ after its metabolism by drug metabolising enzymes of the liver (406). However, the exact mechanism through which cell injury occurs is not yet clear (407). CCl₄ is metabolised by a specific ferrous cytochrome P-450; cytochrome 450 of 5.1.6 Kd. is reported to be the enzyme involved (407). The metabolism of CCl₄ by the mixed function oxidases leads to the genesis of 3 factors which may be responsible for the lethal cell injury (407). They are: (1) formation of free radicals, (2) formation of electrophilic products and (3) generation of activated oxygen species. During the initial reaction carbon-chlorine bond in CCl₄ is cleaved leading to the release of chloride ion and formation of CCl₃. CCl₃ is very reactive and may undergo many reactions. It may covalently bind to microsomal membrane lipids and proteins causing alteration in the functions of the membrane. It can cleave a hydrogen atom from unsaturated lipids to produce chloroform and initiate oxygen-consuming lipid peroxidation. Lipid peroxidation generates variety of toxic products which may be lethal to cells. CCl₃ radicals attack polyunsaturated fatty acid and form lipid peroxides. This is accompanied by concurrent formation of free radicals. Free radicals cause cytotoxicity through peroxidation as well as covalent linkages with biomolecules (406). CCl₄, or its metabolites also inhibit the ability of the liver microsomes to actively sequester calcium ions (Ca⁺⁺) (407). Inhibition of calcium sequestration by the endoplasmic reticulum causes elevation of cytosol free calcium concentration, which would mediate damage to the cell membrane. All these changes indicate that CCl₄ administration leads to discrete changes in the composition, function and structure of endoplasmic reticulum (407). Alteration in the phospholipid component of cell membrane has also been reported (407). CCl₄ administration renders phosphatidyl serine, a type of cell membrane phospholipid, more susceptible to peroxidation. Thus it seems that CCl₄ induce cell toxicity through complex processes mainly involving cell membrane.

CCl₄ administration in rats induces significant morphological, biochemical and histopathological changes in liver. Effect of extracts was evaluated on these changes.

Morphological changes:
Results of the study reveal that TME did not influence liver weight and volume significantly indicating that it has no significant influence on CCl₄ induced morphological changes in the liver. It is not clear why no inhibition of CCl₄ induced morphological changes was shown by the extract inspite of significant antagonism of CCl₄ induced biochemical and histopathological changes. Similar type of result have been reported by other workers also (294, 405).
Biochemical alterations:

a. Effect on protein content in liver and serum:
The results of the study show that CCl₄ did not produce any significant change in serum protein content. However, significant increase was noted in liver protein content. This indicates that there is either increased protein biosynthesis or (and) decrease in its catabolism.

Extract treatment antagonised the CCl₄ induced increase in liver protein content. The probable site of action may be at endoplasmic reticulum, since CCl₄ produces its effect through binding to it. The exact mechanism through which liver protein content increases and the mechanism of modulatory effect of extract remains to be elucidated.

b. Effect on transaminase activity:
Transamination of amino acids is one of the important reaction taking place in liver parenchymal cells. Though different types of transaminases are present, Glutamic oxaloacetic transaminase (GOT) and Glutamic pyruvate transaminase (GPT) are important from the point of view of functional status of parenchymatous cells (278). In parenchymal cell disease their concentration in serum markedly increases. Extensive tissue destruction also leads to increased level of these enzymes in serum. GOT activity is reported to raise more in myocardial damage and GPT activity in liver damage (408). In CCl₄ liver injury increase in transaminase activity in serum is mainly due to release of these intracellular enzymes from damaged parenchymal cells. Increase noted in liver may be due to alterations in the permeability of cell membrane and increased synthesis or decreased destruction of the enzyme(409). In the present study also significant elevation of both GOT and GPT activity was noted both in serum and liver homogenate. Extract treatment significantly antagonised increase in GOT activity in serum and liver homogenate. GPT activity was also antagonised in both serum and liver homogenate. However, at higher dose level the decrease noted in serum was not significant. This type of dose independent nature of activity is common with extracts and it may be due to presence of multiple components in extracts often with opposite effect. The decrease in transaminase activity in serum in extract treated group may be indicative of decrease in the destruction of liver parenchymal cells with consequent decrease in their release. The extracts may also be modulating increased synthesis, decreased enzyme degradation or increased cell permeability to these two enzymes.

c. Effect on acid phosphatase activity:

Acid phosphatase is mainly confined to lysosomal granules. Measurement of ACPase activity is employed as a marker for lysosomal membrane disruption. Its increase after CCl₄ administration may be indicative of cell destruction with consequent lysosomal labilization. The result of the present study show that ACPase activity in serum and liver homogenate increase significantly after CCl₄ administration. This result is in conformity with
earlier report (298). The extract decreased serum ACPase activity, but the decrease was not statistically significant. In liver homogenate, elevation in ACPase activity was noted. The extract may not have significant effect on ACPase release from the cells. It also does not seem to inhibit the activity of the released enzyme. Thus, unlike the previous reports the present study could not find correlation between ACPase activity and liver protection.

d. Effect on alkaline phosphatase activity:

Alkaline phosphatase concentration in serum is reported to increase significantly in obstructive jaundice in comparison to the concentration normally found in hepatic jaundice (278). The increase may be due to impairment of the excretion of the enzyme through bile. Normally ALPase is taken up from blood by the parenchymal cell and excreted into the bile. Therefore, any inhibition to flow of bile will cause back flow of this enzyme from liver to blood. In addition, in some abnormal conditions liver may form excessive alkaline phosphatase and release it to the blood. Contrary to the earlier reports (298), the result of the present study did not show significant elevation in serum ALPase activity. The reason for this discrepancy is difficult to ascertain with the present data and would require further elaborative studies. Statistically non-significant decrease in liver ALPase activity was noted in CCl4 treated group. Extract treatment caused further decrease in ALPase activity. Since, CCl4 induces hepatocellular toxicity, ALPase activity may not be prominent.

e. Effect on orosomucoid level in serum:

Orosomucoid, also known as a1-acid glycoprotein, is an acute phase protein. Acute phase proteins are a group of proteins which collectively raise during inflammation including hepatitis. In the present study increase in orosomucoid level in serum was noted, but the increase was not significant. Extract treatment decreased orosomucoid level but the decrease was significant only at lower dose level. The intensity of CCl4 induced inflammation may not have been of sufficient intensity to cause a significant elevation in serum orosomucoid level, but tendency towards increase was noted. It is also possible that CCl4 induced toxicity might have crippled the organ incapacitating it from reacting in normal way. Decrease in orosomucoid level in extract treated group may reflect comparatively low level of hepatitis in this group and may be indicative of mild liver injury.

f. Effect on total lipid, cholesterol and triglyceride level in serum and liver:

Liver plays an important role in lipid metabolism also. It facilitates digestion and absorption of lipids through production of bile, which contains cholesterol and bile salts (408). It also contains enzyme systems required for synthesizing and oxidising fatty acids, for synthesizing triglycerides, phospholipids, plasma lipoproteins and enzymes for converting fatty acids to ketone bodies (408). The major lipid components in the plasma like cholesterol and
triglyceride do not circulate in free state but are transported in the form of complexes with lipoproteins (410). Any disturbances in lipoprotein metabolism is reflected in the lipid profile of plasma and liver. Liver has a major role in the metabolism of lipoprotein. Hence, derangement in its activity leads to alteration in the lipid profile of plasma.

Administration of CCl₄ caused marked decrease in serum total lipids and increased liver total lipid content. Reduction in serum total lipid content may be due to incomplete absorption from intestine and mobilization of lipids from liver.

Like total lipid, serum cholesterol decreased significantly and liver cholesterol content increased in CCl₄ treated groups. Decrease in serum cholesterol may be due to alterations in its synthesis in liver, since liver is the main site of cholesterol synthesis (408). Impairment of its transport from liver to serum may also contribute to the decreased level. Serum triglyceride level also decreased markedly and liver triglyceride content increased after CCl₄ treatment. Decrease in serum level may be due to impairment of their synthesis in liver. Inhibition of their transport from liver to serum may also contribute to this.

Accumulation of lipid in liver may be due to several causative factors. The most important among them are increased level in free fatty acid in plasma as a result of mobilization of fat from adipose tissue or from the hydrolysis of lipoprotein chylomicron triglycerides by lipoprotein lipase in extrahepatic tissues (408). This leads to increased uptake and esterification in liver. Since, production of lipoprotein does not keep pace with increased free fatty acids level, triglyceride and cholesterol accumulate in the liver (408). The second reason for fat accumulation in liver may be due to metabolic block in the production of lipoproteins, especially its apoprotein part (408). In the present study significant decrease in total lipids, cholesterol and triglyceride levels in serum after CCl₄ administration was noted. This indicates that the first mechanism, i.e., mobilization of fat from extrahepatic source is not involved in the lipid accumulation observed. It is likely that it is due to impairment of synthesis of lipoproteins. The extract treatment significantly reversed the derangement noted in lipid profile of both liver and serum. The exact mechanism of this reversal is not clear. It is possible that the reversal is a result of antagonism of CCl₄ induced liver cell damage. In addition, the extract may have modulatory effect on lipid metabolism.

g. Effect on phospholipid content of liver:
CCl₄ treatment lead to marked depletion of phospholipid content in the liver. The exact mechanism of the depletion is not clear. As already mentioned, CCl₄ administration increases the susceptibility of certain types of phospholipids to peroxidase attack and this may result in enhancement of their breakdown. This effect combined with impairment in their synthesis due to damage to liver parenchymal cells may cause decrease in phospholipid content of the liver. The extract treatment prevented CCl₄ induced decrease in phospholipid content of the liver. The exact mechanism is not clear. Prevention of CCl₄
induced injury to liver parenchymal cells may be one of the mechanisms. It would be interesting to study the effect of extract on phospholipid metabolism and to assess whether it antagonises the \( \text{CCI}_4 \) induced susceptibility of certain types of phospholipids to peroxidase attack.

**h. Effect on liver glycogen:**

Glycogen is a polysaccharide in the animal body. Though its formation occurs in most of the body tissues, it is mainly formed in liver and muscle. It is normally stored in cells for short-term energy requirements. Its formation is also deranged in \( \text{CCI}_4 \) induced liver injury. In the present study injection of \( \text{CCI}_4 \) caused significant decrease in liver glycogen content. The decrease might have been due to derangement in the activity of the enzymes involved in glycogen synthesis and enhanced glycogen utilization. It is also possible that inhibition of lipid utilization in \( \text{CCI}_4 \) induced hepatitis may promote breakdown of glycogen for metabolic energy requirement (409). Extract treatment antagonised \( \text{CCI}_4 \) induced depletion of liver glycogen. But, the inhibition was statistically significant only at lower dose level. From the available data it is not possible to pin point the exact mechanism of action of inhibition of glycogen depletion. It may be reflective of general hepatoprotective effect of the extract with resultant decrease in liver glycogen depletion or the extract may have modulatory effect on glycogen synthesis and its breakdown.

Analysis of the result presented above clearly reveals that methanol extract of the defatted leaf of Gymnosporea montana possess significant hepatoprotective effect. It showed significant effect in primary screening test and restored majority of the altered biochemical parameters studied. Results of the histopathological studies provide unequivocal evidence for the presence of hepatoprotective effect.

From the present data it is not possible to ascertain the exact mechanism through which hepatoprotective effect is produced.

As already discussed, \( \text{CCI}_4 \) induced toxicity is due to damage to liver cell membrane, as a result of peroxidation of lipid component of cell membrane leading to alteration in cell permeability. Generation of toxic free radicals and inhibition of cytosol calcium sequestration are the other mechanisms ascribed to \( \text{CCI}_4 \).
The extract may be exerting its beneficial effect through the following mechanisms:

1. By increasing the stability of cell membrane.
2. By lowering the lipid peroxidation.
3. By scavenging the released free radicals or by enhancing the activity of super oxide dismutase, which acts as a naturally occurring scavenger of free radicals (411).
4. It may be interfering with the effect of CCl₄ on cytosol calcium sequestration.
5. It may be conditioning the hepatic cell, to cause accelerated regeneration of parenchymal cells (294).

It would be interesting and worthwhile to evaluate the extracts in test systems representing the above mechanism to arrive at a definitive conclusion.