CHAPTER 3

RESULTS AND DISCUSSION

3. Results and Discussion

In this chapter the various results obtained from various investigations carried out are compiled. An attempt has also been made to discuss these results in order to provide convincing reasons for the studies performed.

3.1 Pharmacognostic studies

3.1.1 Macroscopic evaluation

Aerial parts of *P. daemia*:

The leaves appear to be broadly ovate, or deeply cordate. Leaves have peculiar odour stems are pale green, fibrous, hard, and covered with hairs and hollow in the nature. Flowers are axillary, long peduncled, corymbose clusters, dull white, tinged with purple. Fruits are follicles, lanceolate, long pointed covered with soft spines, containing numerous seeds with tuft of silky hairs. Seeds are pale yellowish green when fresh, brown when dried and are oval shaped in nature. The photographs of the plant and aerial parts are shown in Figure 3 (a-d).

Roots of *B. montanum*:

Root pieces are almost cylindrical, straight or ribbed with secondary roots, 0.2-4 cm thick and up to 10 cm or more in length, tapering at one end, tough, externally brown; surface, rough due to longitudinal striations, transverse cracks and scars of rootlets; internally cream-coloured; transversely smoothened root shows thin, brown bark and yellowish-white central core. The photographs of the plant and roots are shown in Figure 4(a, b)

These features will assist as identifying characters for the selected drugs on preliminary basis.

3.1.2 Microscopic evaluation

The microscopic features of transverse sections of different organs of *P. daemia* are as follows.

A. TS of leaf:

The transverse section of the leaf was divided into two regions, lamina and midrib region.

1. Lamina: Single layer of upper epidermal cells covered with cuticle and the epidermal cells are rectangular in the nature. Upper epidermis shows the pre-

Figure3: Photographs of *P. daemia*

Figure 4: Photographs of *B. montanum*

sence of multicellular trichomes (3-5 cells) which are uniseriate rarely biseriate. Occasionally unicellular covering trichomes are also seen. Trichomes are unlignified in nature. Below the upper epidermis are present a single layer of palisade cells which are narrow and elongated in the nature containing chloroplasts. Below the palisade there is a presence of spongy parenchyma. Interspersed with spongy parenchyma there can be seen latex tubes. Next appears lower epidermis which is similar to the upper epidermis. Distributed in spongy parenchyma there are lignified vascular elements. Both upper and lower epidermis shows the presence of anomocytic stomata.

2. Mid rib: Upper and lower epidermis also extends into midrib region where as palisade do not extend into the midrib region. Below the upper epidermis and above the lower epidermis (3-5 layers in case of upper epidermis, 2-3 layers in the case of lower epidermis) are present patches of collenchyma. In the centre of the midrib there present well developed bicollateral vascular bundle which is of arc shaped. The photo micrograph of transverse section of leaf was shown in Fig.5 (a-c)

B. TS of stem:

1. Epidermis: Epidermis consists of single layer of epidermal cells with thick cuticle. Numerous covering trichomes which are uniseriate, multicellular and biseriate multicellular which are unlignified are seen. Some of the cells of covering trichomes are collapsed in nature there by forming collapsed celled trichomes. Apex of the trichomes is blunt in the nature.

2. Cortex: Outer cortex about 2-6 layers consists of cortical parenchyma that are small oval to polyhedral shaped cells. Inner cortex which is many layered consists of large oval to irregular cortical parenchyma. Distributed in the cortex region there is a presence of a ring of latex tubes.

3. Phloem: Phloem is present towards both sides of xylem and contains colourless, irregular parenchyma.

4. Xylem: It forms a continuous band there by appearing like a ring and contains xylem vessels and scanty xylem parenchyma.

5. Pith: Large and made of thin walled unlignified big polygonal to round parenchyma cells with intercellular spaces. A large hole is present in the

centre of the pith through which the stems are hollow. Some of the pith cells show brownish matter. Medullary rays that are uniseriate are present in the xylem and extended up to outer phloem. In the xylem region medullary rays are lignified in the nature. Rosettes of calcium oxalate crystals are observed in cortex, phloem parenchyma, and medullary rays of phloem region.

The photo micrograph of transverse section of stem was shown in Figure 5(d).

C. TS of root:

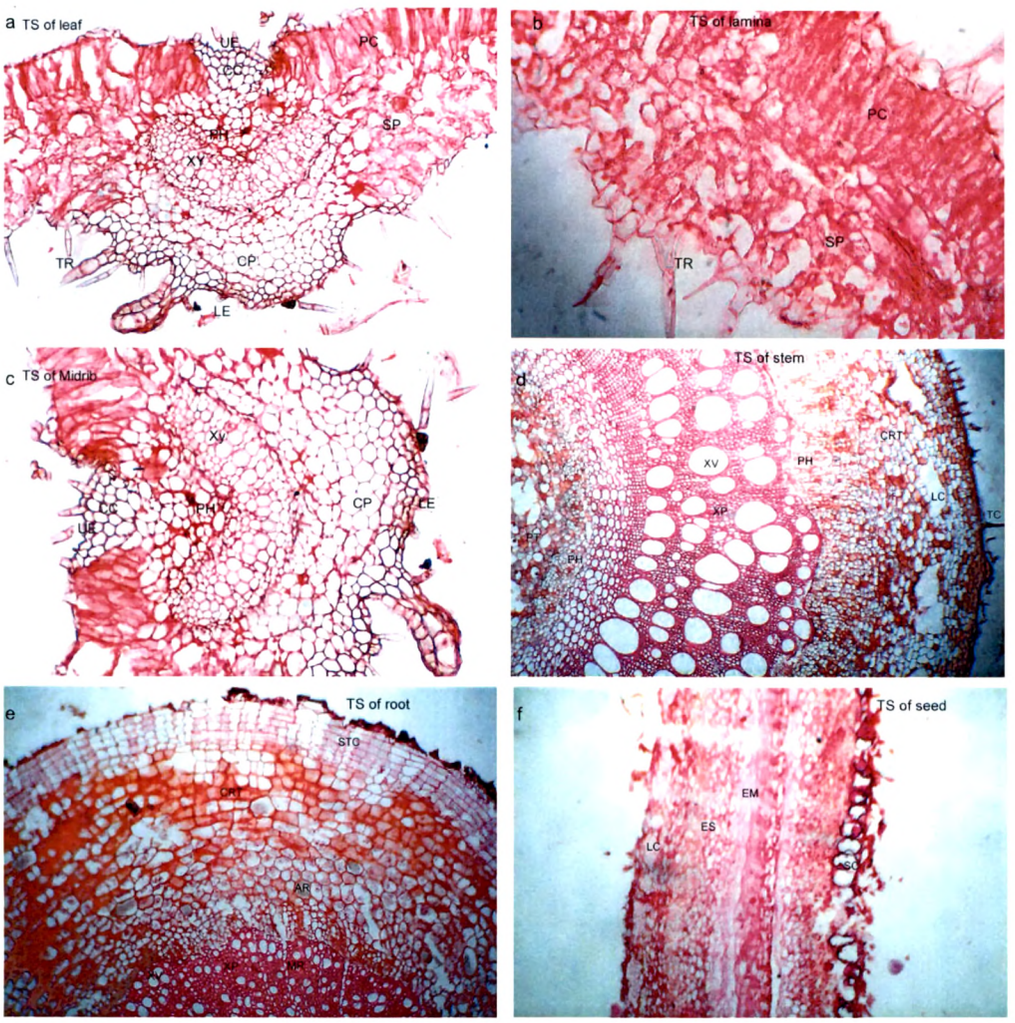
1. Periderm: Divided into Cork, Phellogen, and Phelloderm.
    a. Cork: It is of 7 – 8 layered with tangentially elongated cells, lignified and arranged in stratified manner.
    b. Phellogen & Phelloderm: Absent
2. Cortex: It is many layered, cells are polygonal to rectangular is nature. Some of the cells of cortex show acicular raphides. Distributed in the cortex region there is a presence of groups of (2–5 cells) stone cells.
3. Phloem: Consists of thin walled phloem parenchyma, phloem fibres are absent. The cells appear to be polyhedral to rectangular. The phloem is traversed by medullary rays which are unlignified in the phloem and are lignified in xylem.
4. Xylem: Consists of xylem vessels and thick walled xylem parenchyma (scanty parenchyma).Vessels appear to be single. Metaxylem is towards centre.
5. Medullary rays: Medullary rays are uniseriate in nature and extend up to phloem, lignified in the xylem region, unlignified in phloem region.

The photomicrograph of transverse section of root was shown in Figure 5(e).

D. TS of seed:

Seed appears to be compressed in nature with concave and convex surfaces. Seed coat consisting of a single layer surrounds the seed. A single layer of cells which are large and irregular in nature are seen below the convex surface. On both the surfaces seed coat cells are covered with hairs. Below the seed coat on concave side there is a presence of laticiferous ducts.

Figure 5: Microscopy of transverse sections of *P. daemia.*



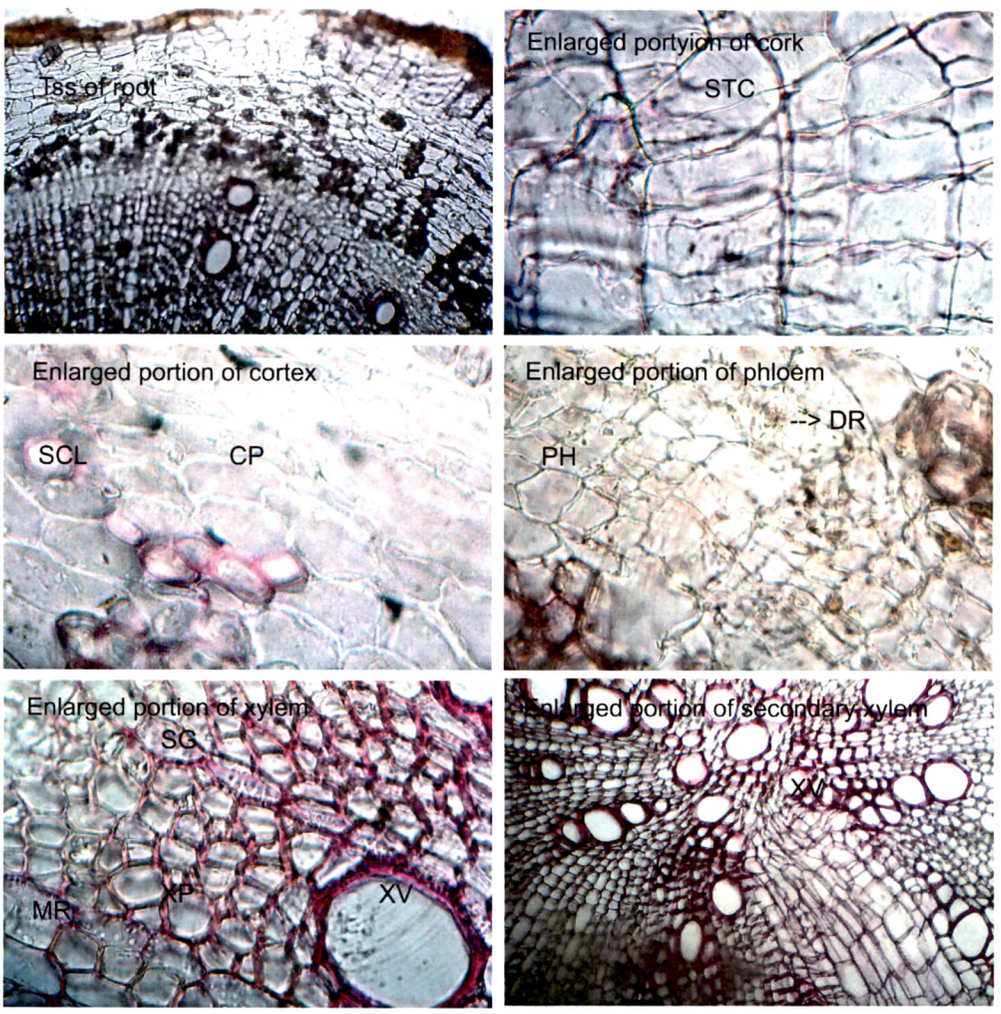
UE: upper epidermis, LE: lower epidermis, SP: spongy parenchyma, PC: palisade cells, TR: trichomes, PH: phloem, XY: xylem, XP: xylem parenchyma, XV: xylem vessels, CRT: cortex, CP: cortical parenchyma, MR: medullary rays, STC: stratified cork, PT: pith, ES: endosperm, EM: embryo, LC: latex ducts/canals, SC: seed coat.

Below the seed coat there is a presence of hilum which is many layered with small cells that are round to oval in the shape. Next follows the endosperm consisting of large polyhedral to oval shape containing oil globules. At the centre of the seed below endosperm appears embryo containing thick walled

oval to rectangular cells. The photo micrograph of transverse section of seed was shown in Figure 5 (f).

Microscopy of the transverse sections of roots of *B. montanum* showed the following characters.

Figure 6: Microscopy of transverse sections roots of *B. montanum.*



STC: stratified cork, SCL: sclereids (stone cells), CP: cortical parenchyma, PH: phloem, DR: druses (rosettes), MR: medullary rays, XP: xylem parenchyma, XV: xylem vessels, SG: starch grains.

The cross section of a matured root is circular in outline and has cork tissue towards the exterior. Cork is of 5-18 layered and consists of tangentially

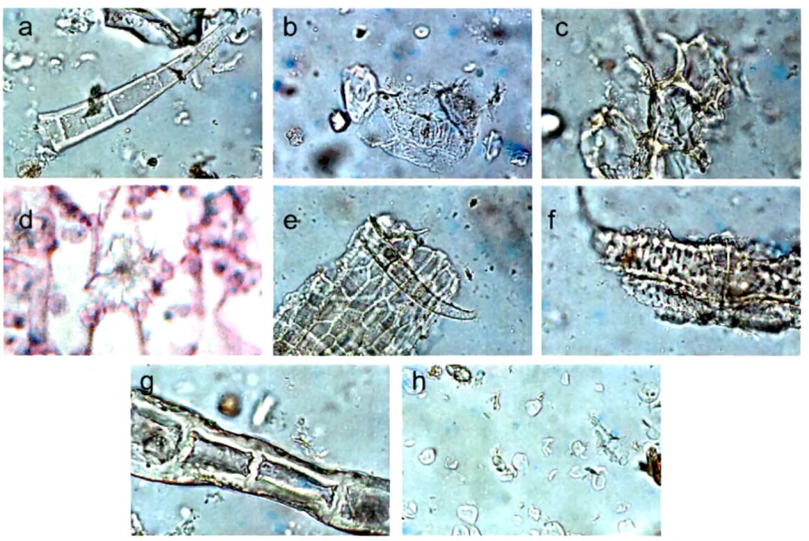
elongated, thick walled and lignified brick shaped cells and brown coloured. Some cells of cork contain red colouring matter. This is followed by phellogen consisting of a few layers. The cortex is predominant; 2-10 layered and consists of oval to elliptical, tangentially elongated cells. The cortex is characterised by the presence of starch containing cells, stone cells and cells containing druses. Cortical fibres are also present in this region. The phloem consists of usual elements, traversed by uni to biseriate medullary rays. Some phloem cells also contain starch grains. Xylem consists of vessels, tracheids, a few containing reticulate thickening. Xylem fibres are slightly thick walled with narrow lumen. Uni to biseriate medullary rays are seen in xylem region. Rosette crystals of calcium oxalate and starch grains are present only in cortex and phloem regions. Starch grains are solitary and in groups, simple, round to oval in nature. The photomicrograph of transverse section of root was shown in Figure 6 (a-f).

Microscopy of powdered mounts of the aerial parts of *P. daemia* shows multicellular, uniseriate covering trichomes which are unlignified, collapsed covering trichomes, epidermal cells, cortical parenchyma, xylem vessels and rosettes of calcium oxalate crystals. The photomicrographs are shown in Figure 7(a-h).

Microscopy of powdered mounts of the roots of *B. montanum* shows fragments of cork more or less rectangular, druses (rosette) of calcium oxalate crystals, xylem fibres, xylem vessels with reticulate thickening and starch grains solitary and occasionally in groups. The photomicrographs are shown in Figure 8 (a-f).

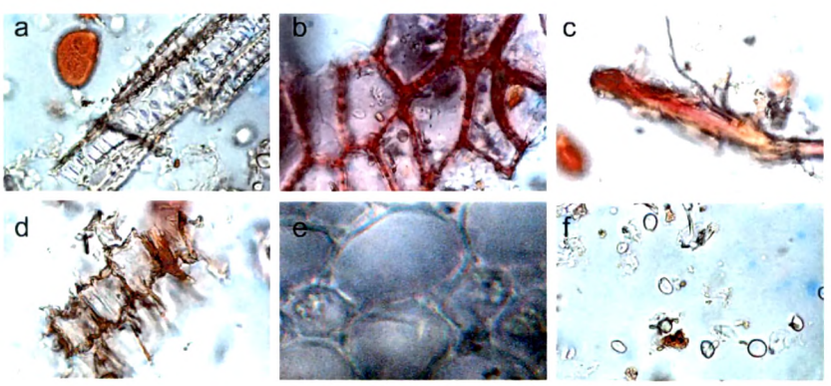
The presence of uni to multi cellular covering trichomes, collapsed covering trichomes, biseriate covering trichomes, rosettes and acicular raphides types of calcium oxalate crystals, stratified cork and anomocytic stomata forms the important diagnostic features of the *P. daemia*. Similarly presence of rosettes of calcium oxalate crystals, stratified cork, stone cells forms the important diagnostic features of the roots of *B. montanum* which helps in the identification and evaluation.

Figure7: Powder microscopy of aerial parts of *P. daemia*



a: covering trichome, b: anomocytic stomata, c: cortical parenchyma, d: rosette calcium oxalate crystal, e: epidermal cells, f: xylem vessels, g: collapsed celled covering trichome, h: starch grains.

Figure 8: Powder microscopy of aerial parts of *B. montanum*



a: Xylem vessels, b: xylem parenchyma, c: xylem fibres, d: cork, e: rosette or druses of calcium oxalate crystals, f: starch grains.

3.2 Proximate analysis

The results of proximate analysis of aerial parts of *P. daemia* and roots of *B. montanum* are recorded in Table 13.

Proximate analysis helps to set up certain standards for the crude drugs in order to avoid batch to batch variations and also to judge their quality and purity.

Table 13: Proximate analysis of aerial parts of *P. daemia* and roots of *B. montanum*

Parameter	*P. daemia*	*B. montanum*
Foreign organic matter	3.48% w/w	0.6% w/w
Total ash	10.16% w/w	6.72% w/w
Acid insoluble ash	1.65% w/w	1.36% w/w
Water soluble ash	2.27% w/w	1.83% w/w
Alcohol soluble extractive	4.82% w/w	3.28% w/w
Water soluble extractive	3.60% w/w	3.39% w/w
Loss on drying	6.09% w/w	4.03% w/w
Manganese	43.07 ppm	64.65 ppm
Zinc	99.84 ppm	43.75 ppm
Copper	08.81 ppm	10.71 ppm
Lead	41.14 ppm	32.50 ppm
Cadmium	Nil	Nil
Foaming index	Less than 100	Less than 100
Total phenolic content	10.89	11.61
Total flavonoid content	8.31	8.94

Total ash value was found to be more for the aerial parts of *P. daemia*. Similarly alcohol soluble extractive also found to be more in both the plants selected i.e. 4.82 and 3.28% w/w respectively. These determinations provide an idea regarding the conditions of procurement and storage of the drug and also probable content of various inorganic metal ions as well as the nature of the constituents present. More ash value indicates the presence of more inorganic matter.

Foaming index provides the data regarding the foaming ability of the aqueous decoction of the plant material because of its saponin contents. Now

in present study both, the *P. daemia* and *B. montanum* were found to contain foaming index less than 100.

Contamination of medicinal plants with heavy metals can attributed to the environmental pollution and traces of pesticides. However some of these ions like manganese, copper, zinc etc. are also required to acts as catalysts and co-enzymes in metabolic reactions of the growing plants. Therefore the estimation of various heavy metals present in the crude drugs help not only to fix standards for their identification, but also provide means to detect possible adulterations.

The phenolic content of ethanol extract of *P. daemia* and methanol extract of *B. montanum* was found to be 10.89% w/w and 11.61% w/w respectively representing the presence of various phenolic compounds like poly phenols, flavonoids, phenolic acids etc.

From the calibration curve of the quercetin, the concentrations of the flavonols and flavones in the ethanol extract of *P. daemia* and methanol extract of *B. montanum* are found to be 1.76% w/w and 1.43% w/w respectively. From the calibration curve of naringenin, the amount of flavanones in the ethanol extract of *P. daemia* and methanol extract of *B. montanum* was found to be 6.55% w/w and 7.51% w/w respectively.The sum of the values obtained from these two methods was considered as total flavonoid content and are found to be 8.31% and 8.94% w/w respectively for *P. daemia* and *B. montanum*.

3.3 Phytochemical studies

3.3.1 Successive solvent extraction

The results of successive solvent extraction of aerial parts of *P. daemia* and roots of *B. montanum* are recorded in Table 14.

The successive solvent extraction of the drug with solvents of increasing polarity generally results in the separation of the constituents according their polarity. The non-polar constituents are extracted in solvents like petroleum ether, and benzene being non-polar; semi- polar constituents are extracted in chloroform and acetone being semi-polar; while the polar and highly polar constituents are found in ethanol and water. Thus, the values of

successive solvent extraction provide an idea regarding the presence of various non-polar, semi-polar and polar constituents. In presence studies on aerial parts of *P. daemia* the extractive values of ethanol (10.54% w/w) and petroleum ether (5.18% w/w) were in considerably high in comparison to the values of other extracts. Similarly in roots of *B. montanum* the extractive values of ethanol (6.46% w/w) and water (2.06% w/w) were in higher side.

Table 14: Successive solvent extraction of aerial parts of *P. daemia* and roots of *B. montanum*

Solvent	*P. daemia*		*B. montanum*	
	Colour and Consistency	**Extractive value**	**Colour and Consistency**	**Extractive value**
Petroleum ether	Yellowish green and viscous semi-solid	5.18	Yellowish and greasy	0.67
Benzene	Dark brown and viscous solid	1.05	Greenish yellow and viscous	0.39
Chloroform	Yellowish brown and viscous semi-solid	0.66	Brownish and viscous	0.15
Acetone	Yellowish green and viscous semi - solid	2.63	Brownish and semi-solid	1.44
Ethanol (95%)	Brownish and viscous semi-solid	10.54	Brownish and semi-solid	6.46
Chloroform Water	Brownish and solid	1.22	Brown and solid	2.06

3.3.2 Qualitative evaluation of successive extracts

The results obtained from the qualitative evaluation of successive extracts of aerial parts of *P. daemia* and roots of *B. montanum* are recorded in Table 15.The results indicated the presence of cardenolides, phenolic compounds and flavonoids, phytosterols, saponins, carbohydrates, amino

acids and fixed oils in aerial parts of *P. daemia* and phenolic compounds and flavonoids, terpenoids and steroids, sterols, saponins, carbohydrates, amino acids and fixed oils in roots of *B. montanum*. The presence of these compounds was further confirmed by thin layer chromatographic studies.

Table 15: Qualitative evaluation of successive extracts

Constituents	*P. daemia*						*B. montanum*					
	P	B	C	A	E	W	P	B	C	A	E	W
Alkaloids	-	-	-	-	-	-	-	-	-	-	-	-
Cardenolides	-	+	+	+	-	-	-	-	-	-	-	-
Phenolics and flavonoids	-	-	-	+	+	-	-	-	-	+	+	+
Anthracene glycosides	-	-	-	-	-	-	-	-	-	-	-	-
Terpenoids and steroids	+	+	-	-	-	-	+	+	+	-	-	-
Sterols	+	-	-	-	-	-	+	-	-	-	-	-
Saponins	+	-	-	-	-	-	+	-	+	-	-	-
Amino acids	-	-	-	-	+	+	-	-	-	-	+	+
Carbohydrates	-	-	-	-	+	+	-	-	-	-	+	+
Fixed oils and fats	+	-	-	-	-	-	+	-	-	-	-	-
Volatile oils	Absent						Absent					

P: Petroleum ether, B: Benzene, C: Chloroform, A: Acetone, E: Ethanol, W: Chloroform water, -: Negative, +: Positive.

3.3.3 TLC of the extracts obtained in successive extraction

The extracts obtained in the successive solvent extraction process were subjected to thin layer chromatographic studies (TLC) in order to confirm the results obtained from qualitative tests. The results obtained from TLC of the successive extracts obtained form aerial parts of *P. daemia* and roots of *B. montanum* are recorded in Table 16 and 17 respectively.

P. daemia: Terpenoids are detected in petroleum ether and benzene extracts. Cardenolides are detected in benzene, chloroform and acetone extracts. Flavonoids are detected in acetone and ethanol extracts. Sterols are detected in petroleum extract. Ethanol and aqueous extracts showed the presence of carbohydrates and amino acids.

Table 16: TLC studies on successive extracts of aerial parts of *P. daemia*

Constituents	PE	BE	CE	AE	EE	CW
Cardenolides	Not detected	0.35, 0.40, 0.53, 0.62	0.10, 0.13 0.20, 0.35 0.40, 0.53 0.62	0.13, 0.52 0.62	Not detected	Not detected
Terpenoids	0.07, 0.16, 0.26, 0.36, 0.42, 0.54, 0.61, 0.66, 0.78, 0.92	0.07, 0.16, 0.26, 0.36, 0.78.	Not detected	Not Detected	Not detected	Not detected
Flavonoids and Phenolics	Not detected	Not detected	Not detected	0.15, 0.25, 0.32, 0.42 0.54, 0.65 0.77, 0.83 0.95	0.15, 0.25 0.32, 0.42 0.54, 0.65	Not detected
Alkaloids	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Sterols	0.80, 0.82, 0.91.	Not detected	Not detected	Not detected	Not detected	Not detected
Amino acids	Not detected	Not detected	Not detected	Not detected	0.15, 0.22, 0.30, 0.43.	0.05, 0.15, 0.25
Carbohydrates	Not detected	Not detected	Not detected	Not detected	0.20, 0.33, 0.48	0.08, 0.20.

PE: petroleum extract; BE: benzene extract; CE: chloroform extract; AE: acetone extract; EE: ethanol extract; CW: aqueous extract.

B. montanum: Terpenoids are detected in petroleum ether, benzene and chloroform extracts. Steroids are detected in benzene and chloroform extracts. Petroleum ether extract showed the presence of sterol. Acetone, ethanol and aqueous extracts showed the presence of flavonoids. Carbohydrates and amino acids are detected in ethanol and aqueous extracts.

These studies provide the lead to select the extracts obtained with non-polar, semi-polar and polar nature of solvents for further detailed investigations and correspondingly the selected drugs were subjected to selective solvent extraction. The detection of these constituents also gave an

idea about the constituents present which may be responsible for the particular activity observed in these plants.

Table 17: TLC studies on successive extracts of roots of *B. montanum*

Constituents	PE	BE	CE	AE	EE	CW
Terpenoids	0.15, 0.21, 0.32, 0.40, 0.51, 0.60, 0.75, 0.88	0.15, 0.21, 0.32, 0.41	0.15, 0.21, 0.40	Not detected	Not detected	Not detected
Steroids	Not detected	0.53, 0.68, 0.87	0.53, 0.68	Not detected	Not detected	Not detected
Alkaloids	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Cardenolides	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Flavonoids and Phenolics	Not detected	Not detected	Not detected	0.12, 0.23, 0.33, 0.41, 0.48, 0.53, 0.61, 0.74, 0.8 , 0.90	0.12, 0.23, 0.33, 0.41, 0.48, 0.53, 0.61	0.23, 0.12
Sterols	0.72	Not detected	Not detected	Not detected	Not detected	Not detected
Amino acids	Not detected	Not detected	Not detected	0.26, 0.48	0.09, 0.19, 0.26, 0.35, 0.48, 0.55	0.26, 0.35 0.48, 0.55
Carbohydrates	Not detected	Not detected	Not detected	Not detected	0.18, 0.36, 0.41	0.18, 0.36 0.41

PE: petroleum extract; BE: benzene extract; CE: chloroform extract; AE: acetone extract; EE: ethanolic extract; CW: aqueous extract.

3.3.4 Preparation of selective extracts

The percentage yields of ethanol (EE) and aqueous extracts (AE) of aerial parts of *P. daemia* are found to be 13.9% w/w and 4.23% w/w respectively. Similarly the percentage yields of methanol (ME) and aqueous extracts (AE) of roots of *B. montanum* were found to be 6.25% w/w. and 2.74% w/w respectively.

3.3.5 Fractionation of bio-active extracts

The active EE of aerial parts of *P. daemia* and ME of roots of *B. montanum* were further subjected for fractionations in order to determine activity guided fractions of the plants.

Fractionation of EE from aerial parts of *P. daemia*:

The yields of chloroform (CFEE) and 95% ethyl alcohol (EFEE) fractions obtained from the EE were found to be 2.46% and 71.78% respectively. The fractionation of EFEE with benzene (BFEFEE) chloroform (CFEFEE), acetone (AFEFEE) and ethanol (EFEFEE) resulted 1.72%, 3.55%, 26.81% and 47.12% yield respectively.

Fractionation of ME from roots of *B. montanum*:

The yields of chloroform (CFME) and methanol (MFME) fractions obtained from the ME were found to be 12.06% and 68.86% respectively. Further fractionation of MFME with ethyl methyl ketone (EMKMFME) and methanol (MFMFME) resulted 8.47% and 73.44% yield respectively.

This fractionation of biologically active extracts will not only help in production of biologically active fractions, but also give an idea regarding the type of constituents responsible for the activity, which can be further subjected for isolation of biologically active molecules with ease.

3.3.6 TLC studies on the extracts and their fractions

The results of TLC studies of EE, CFEE, EFEE, CFEFEE, AFEFEE and EFEFEE obtained from the aerial parts of *P. daemia*; ME, CFME, MFME, EMKMFME and MFMFME obtained form the roots of *B. montanum* are as shown in Table 18 and 19 respectively. EE, EFEE, AFEFEE and EFEFEE were found to contain phenolics and flavonoids. Cardenolides were detected in EE, CFEE, EFEE, BFEFEE, CFEFEE and AFEFEE. Terpenoids are detected in EE and CFEE.

ME and CFME were found to contain phenolics, flavonoids and terpenoids and steroids. MFME and MFMFME were found to contain phenolics, flavonoids and terpenoids. EMKMFME was found to contain phenolics and flavonoids.

Table 18: TLC studies on the extracts and their fractions of *P. daemia*

Constituents	EE	CFEE	EFEE	BFEFEE	CFEFEE	AFEFEE	EFEFEE
Flavonoids and Phenolics	0.15, 0.25, 0.32, 0.42, 0.54, 0.65, 0.77, 0.83, 0.95.	Not detected	0.15 0.25 0.32 0.42 0.54 0.65 0.77 0.83 0.95	Not detected	Not detected	0.15, 0.25, 0.32, 0.42, 0.54, 0.65, 0.77, 0.83, 0.95	0.15, 0.25, 0.32, 0.42,
Cardenolides	0.10, 0.13, 0.20, 0.35, 0.40, 0.53, 0.62.	0.10 0.13 0.20 0.35 0.40 0.53 0.62	0.10 0.13 0.20 0.35	0.10 0.13 0.20 0.35	0.10 0.13 0.20 0.35	0.12, 0.20.	Not detected
Terpenoids	0.07, 0.19, 0.29, 0.41, 0.80.	0.07 0.19 0.29 0.41 0.80	Not detected	Not detected	Not detected	Not detected	Not detected

EE: Ethanol extract, CFEE: Chloroform fraction of EE, EFEE: Ethanol fraction of EE, BFEFEE: Benzene fraction of EFEE, CFEFEE: Chloroform fraction of EFEE, AFEFEE: Acetone fraction of EFEE, EFEFEE: Ethanol fraction of EFEE.

`1Table 19: TLC studies on the extracts and their fractions of *B. montanum*

Constituents	ME	CFME	MFME	EMKMFME	MFMFME
Flavonoids and Phenolics	0.12, 0.23, 0.33, 0.41, 0.48, 0.53, 0.61, 0.74, 0.87, 0.91.	0.54, 0.91	0.12, 0.23, 0.33, 0.41, 0.48, 0.53, 0.61, 0.74, 0.87, 0.90.	0.40, 0.47, 0.55, 0.60, 0.75, 0.64 0.87, 0.92, 0.97	0.13, 0.23, 0.33, 0.40, 0.47, 0.54
Terpenoids	0.15, 0.22, 0.31, 0.42, 0.51, 0.61, 0.75, 0.88.	0.15, 0.22, 0.31, 0.42, 0.51, 0.62, 0.75, 0.88.	0.15, 0.22.	Not detected	0.15, 0.22, 0.30
Steroids	0.53, 0.68, 0.87	0.53, 0.68, 0.87	Not detected	Not detected	Not detected

ME: Methanol extract, CFME: Chloroform fraction of ME, MFME: Methanol fraction of ME, EMKMFME: Ethyl methyl ketone fraction of MFMF, MFMFME: Methanol fraction MFME.

The results give an idea regarding distribution of constituents based on the polarity as these were subjected to fractionation using the solvents of different polarities.

3.4 Biological screening of extracts, fractions and sub-fractions

3.4.1 Acute toxicity studies

The ethanol extract (EE), aqueous extract (AE), ethanol fraction of ethanol extract (EFEE), acetone and ethanol sub-fractions of ethanol extract (AFEFEE and EFEFEE) of *P. daemia* were subjected to acute toxicity determinations as per OECD guidelines. None of these showed mortality even at the dose level of 2000 mg/kg and therefore considered safe.

The methanol extract (ME), aqueous extract (AE), methanol fraction of methanol extract (MFME), ethyl methyl ketone and methanol sub-fractions of methanol extract (EMKMFME and MFMFME) from *B. montanum* did not show mortality even at the dose level of 2000 mg/kg and therefore considered safe.

All the extracts were investigated for hepatoprotective activity in rats using different models.

3.4.2 Hepatoprotective activity

The extracts and fractions of both the plants selected were subjected to hepatoprotective activities in vivo while the sub-fractions were subjected to both in vivo and in vitro studies.

3.4.2.1 Hepatoprotective activity in vivo

All the extracts were administered at dose levels of 100, 200 and 300 mg/kg, where as their fractions were administered at dose levels of 50, 150 and 250 mg/kg; while the sub-fractions at dose levels of 50, 100, 150 mg/kg. Silymarin being positive control was administered at dose level of 100 mg/kg.

Hepatoprotective activity of selective extracts

Ethanol extract (EE) and aqueous extract (AE) of aerial parts of *P. daemia* and ME and AE of roots of *B. montanum* were subjected to the preliminary evaluation of hepatoprotective activity in vivo, against CCl_4-induced toxicity by assessing them through biochemical parameters and histopathological observations. The selected extracts were tested at fixed dose level of 200 mg/kg p.o. while the silymarin at dose level of 200 mg/kg p.o. was used as a positive control.

Effect of aerial parts of *P. daemia* against CCl_4-induced hepatotoxicity:

CCl_4 intoxication in normal rats elevated the serum levels of GOT (141.83 ± 29.46 to 345.33 ± 34.36), GPT (117.30 ±14.44 to 249.02 ± 37.36), ALKP (332.50 ± 21.59 to 455.0 ± 19.66), TBL (1.23 ± 0.19 to 2.37 ± 1.16), and CHL (92.85 ± 7.86 to 129.45 ± 25.82); where as decreased the levels of TPTN (5.91 ± 0.51 to 3.21 ± 0.24) ALB (3.90 ± 0.26 to 1.98 ± 0.17) significantly ($p<0.05$) when compared to control, indicating acute hepato cellular damage and biliary obstruction leading to necrosis. The rats treated with EE and silymarin, showed a significant ($p<0.05$) decrease in all the elevated GOT, GPT, ALKP, TBL and CHL levels and significant ($p<0.05$) increase in TPTN and ALB levels. The rats treated with AE have shown significant decrease in the levels of GOT and CHL and increase in the levels of ALB. The results are recorded as shown in Table 20 and Figure 9.

Histopathological examination of liver sections of control group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein (Figure 9a). Disarrangement of normal hepatic cells with intense centrilobular necrosis and vacuolization were observed in CCl_4-intoxicated liver (Figure 9b). The sections of the rat liver treated with EE and intoxicated with CCl_4 (Figure 9e), were less vacuoled without necrosis similar to those in case of silymarin (Figure 9c), suggesting the protective effect of the extract. The visible changes observed (Figure 9d) in sections of rat liver treated with AE and intoxicated with CCl_4, were on the lower side compared to those observed in EE treated rat liver sections.

The percentage restoration of various biochemical parameters in case of silymarin, EE and AE against CCl_4 are as shown in Table 21 and Graph 7. EE at a selected dose level offered maximum protection.

Table 20: Effect of EE and AE of *P. daemia* against CCl_4-induced hepatotoxicity in rats

GROUP	GOT (IU/L)	GPT (IU/L)	ALKP (IU/L)	TBL (mg/dl)	CHL (mg/dl)	TPTN (g/dl)	ALB (g/dl)
Control	141.83 ± 29.46	117.30 ± 14.44	332.50 ± 21.59	1.23 ± 0.19	92.85 ± 7.86	5.91 ± 0.51	3.90 ± 0.26
CCl_4	345.33 ± 34.36	249.02 ± 37.36	455.00 ± 19.66	2.37 ± 1.16	129.45 ± 25.82	3.21 ± 0.24	1.98 ± 0.17
Silymarin	140.33 ± 28.03*	115.50 ± 19.98*	352.50 ± 24.95*	1.07 ± 0.16*	63.36 ± 6.26*	5.27 ± 0.55**	3.26 ± 0.18**
AE	173.83 ± 30.56*	217.16 ± 31.47	396.17 ± 27.36	1.53 ± 0.20	71.27 ± 8.92*	4.27 ± 0.54	2.33 ± 0.21**
EE	169.67 ± 29.73*	127.00 ± 17.54*	358.33 ± 19.90*	1.16 ± 0.30*	88.70 ± 13.38*	5.25 ± 0.56**	3.40 ± 0.33**
F calculated	7.98	6.07	4.50	2.99	7.00	4.69	10.99
DV	112.11	94.54	84.12	1.04	35.70	1.82	0.30

Values are mean ± SEM of six animals, F theoretical = 2.76 ($p<0.05$). AE: Aqueous extract: EE: Ethanol extract.

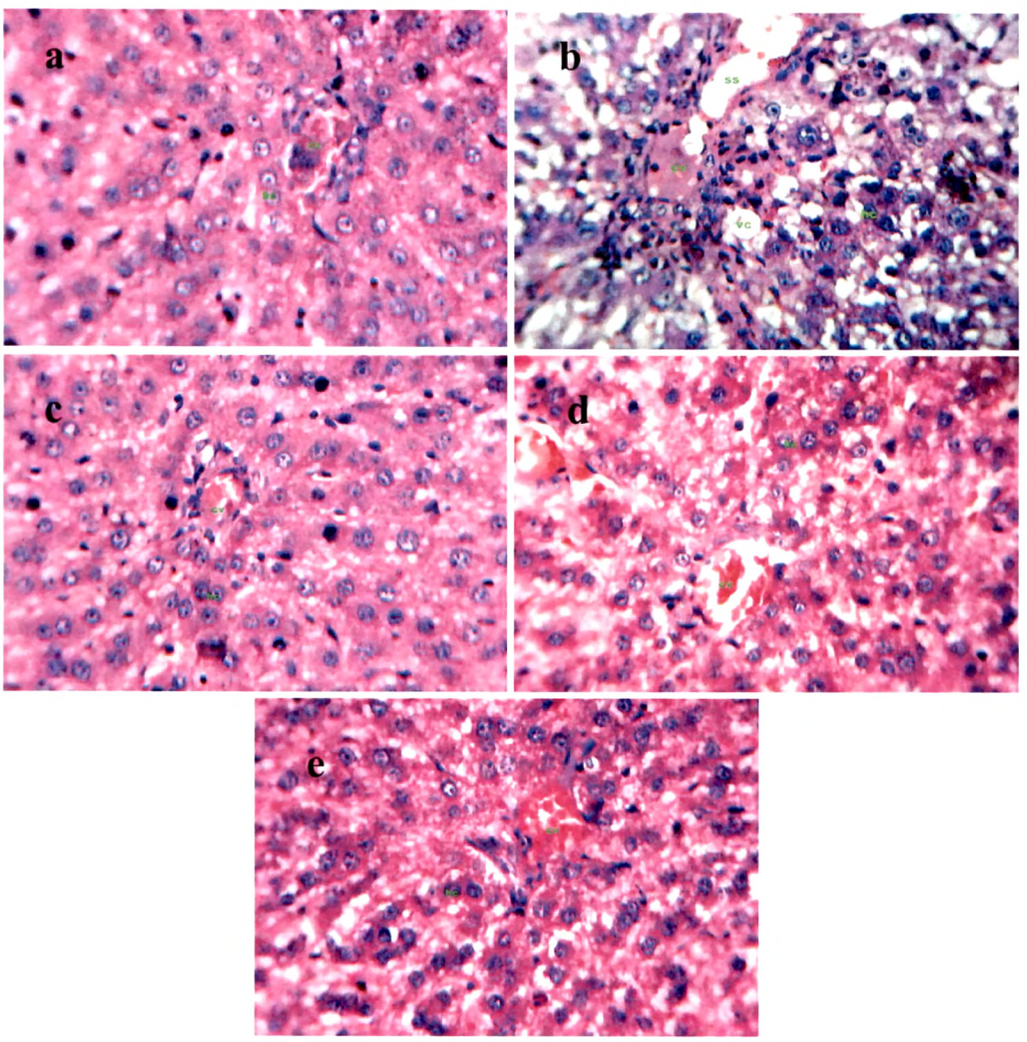
* Significant reduction compared to CCl_4 ($p<0.05$). ** Significant increase compared to CCl_4 ($p<0.05$). DV: Dunnett value

Table 21: Percentage restoration of various biochemical parameters by EE and AE against CCl_4-induced toxicity

GROUP	GOT	GPT	ALKP	TBL	CHL	TPTN	ALB
Silymarin (200 mg/kg)	100.45	101.36	83.64	114.02	180.42	76.28	66.66
EE (200 mg/kg)	86.07	92.01	78.88	106.12	111.24	75.54	73.95
AE (200 mg/kg)	84.03	24.18	48.01	73.67	158.83	39.25	18.23

AE: Aqueous extract: EE: Ethanol extract.

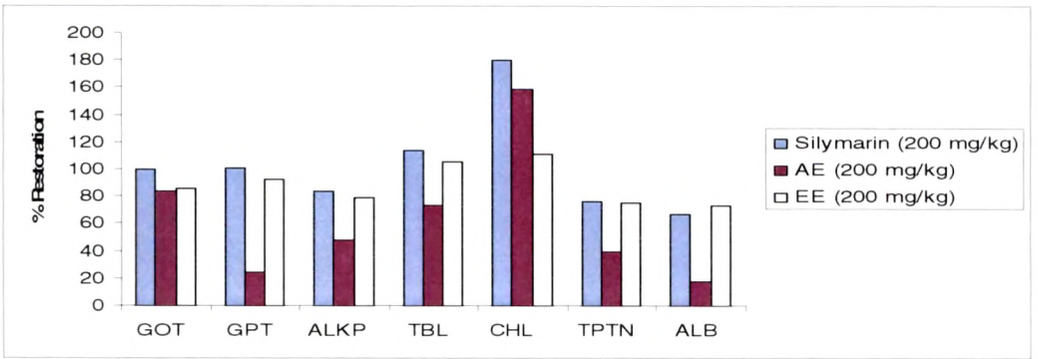
Figure 9: Photomicrographs representing effect of EE and AE against CCl_4-induced hepatotoxicity in rats



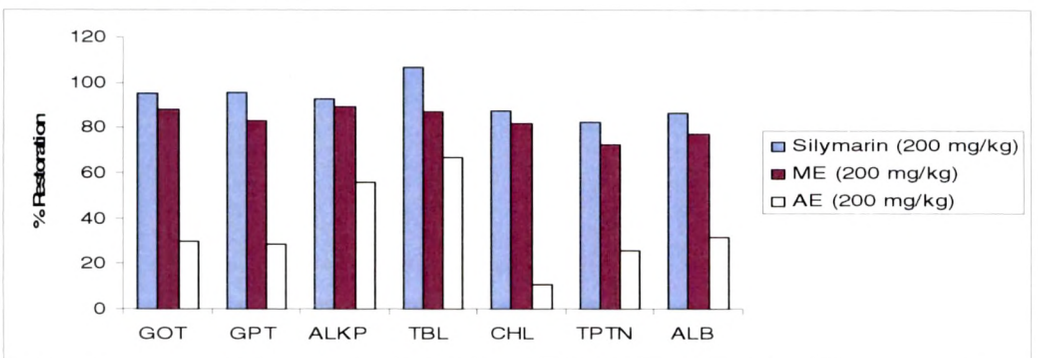
a: Normal rat liver section; b: Liver section of the rat intoxicated with CCl_4; c: Liver section of the rat treated with silymarin and intoxicated with CCl_4; d: Liver section of the rat treated with AE and intoxicated with CCl_4; e: Liver sections of the rat treated with EE and intoxicated with CCl_4. Eosin-Haematoxylin stain. 400X

cv: central vein, vc: vacuole, ss: sinusoidal spaces, hc: hepatocytes.

Graph 7: Percentage restoration of various parameters by EE and AE against CCl_4-induced hepatotoxicity



Graph 8: Percentage restoration of various parameters by ME and AE against CCl_4-induced hepatotoxicity

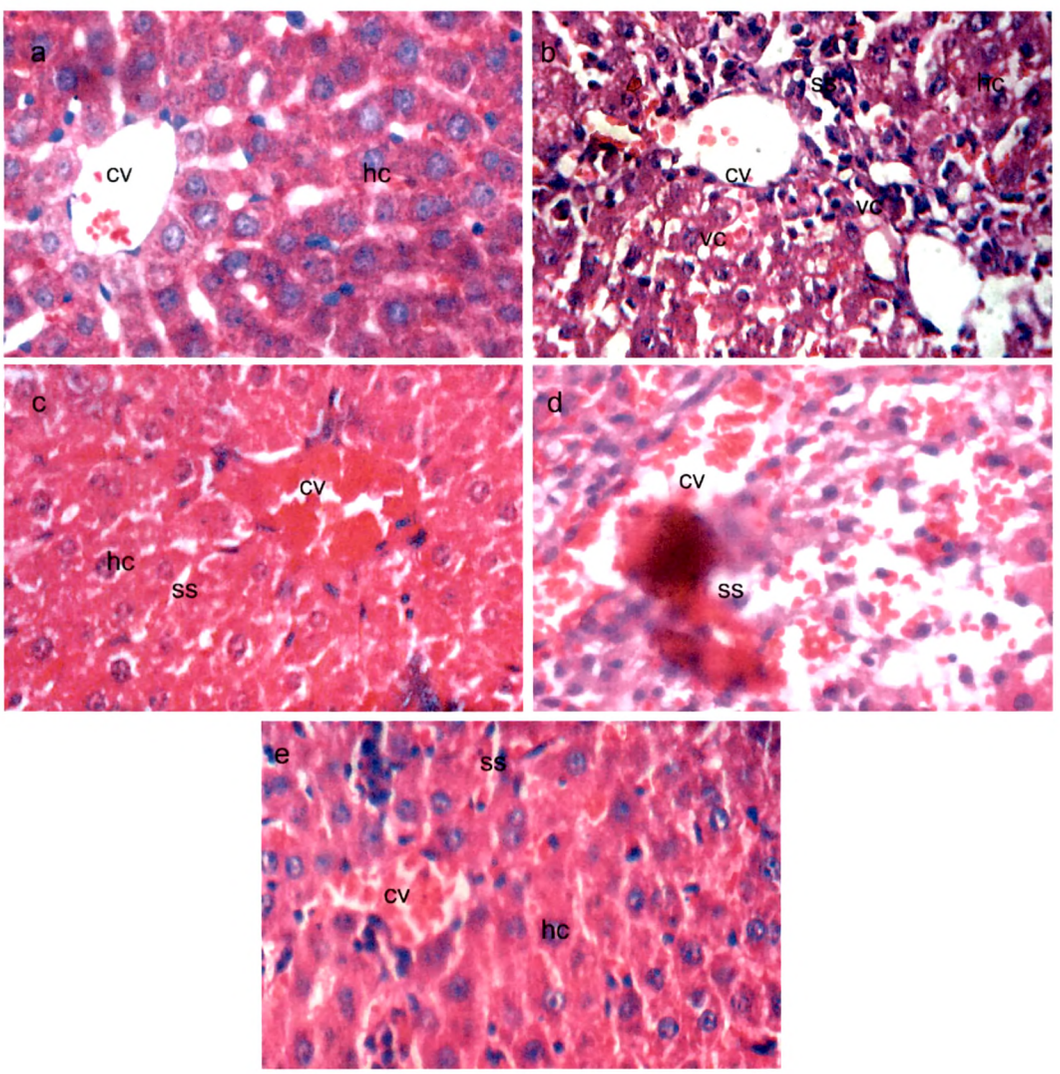


Effect of roots of *B. montanum* against CCl_4-induced hepatotoxicity:

Elevation in the levels of GOT (120.17 ± 7.01 to 335.50 ±33.91), GPT (100.17 ± 4.60 to 291.01 ± 17.31), ALKP (285.50 ± 21.35 to 451.16 ±33.91), TBL (1.35 ± 0.21 to 2.73 ±0.45), and CHL (113.50 ± 12.61 to 200.50 ±28.04) were observed in normal rats due to CCl_4 intoxication; where as decrease in the levels of TPTN (7.80 ± 0.37 to 3.91 ± 0.33) ALB (4.38 ± 0.26 to 1.95 ± 0.13) were observed significantly ($p<0.05$) when compared to control, indicating acute hepato cellular damage leading to necrosis. The rats treated with ME and silymarin, showed a significant ($p<0.05$) decrease in all the elevated GOT, GPT, ALKP, TBL and CHL levels and significant ($p<0.05$) increase in TPTN and ALB levels. The rats treated with AE have not shown

significant ($p<0.05$) changes in the altered levels of various biochemical parameters. The results are represented in Table 22 and Figure 10.

Figure 10: Photomicrographs representing effect of ME and AE against CCl_4-induced hepatotoxicity in rats



a: Normal rat liver section; b: Liver section of the rat intoxicated with CCl_4; c: Liver section of the rat treated with silymarin and intoxicated with CCl_4; d: Liver section of the rat treated with AE and intoxicated with CCl_4; e: Liver sections of the rat treated with ME and intoxicated with CCl_4. Eosin-Haematoxylin stain. 400X

cv: central vein, vc: vacuole, ss: sinusoidal spaces, hc: hepatocytes.

Table 22: Effect of ME and AE of *B. montanum* against CCl_4-induced hepatotoxicity in rats

GROUP	GOT (IU/L)	GPT (IU/L)	ALKP (IU/L)	TBL (mg/dl)	CHL (mg/dl)	TPTN (g/dl)	ALB (g/dl)
Control	120.17±7.01	100.07±4.60	285.50±21.35	1.35±0.21	113.50±12.61	7.80±0.37	4.38±0.26
CCl_4	335.50±33.91	291.01±17.31	451.16±33.80	2.73±0.45	200.50±28.04	3.91±0.33	1.95±0.13
Silymarin	13.067±6.93*	108.17±8.64*	296.83±25.45*	1.26±0.14*	124.17±12.36*	7.13±0.28**	4.06±0.27**
ME	145.83±4.52*	132.16±6.86*	302.67±33.47*	1.53±0.23*	129.50±13.51*	6.75±0.27**	3.83±0.16**
AE	270.83±17.24	235.81±31.36	357.63±42.68	1.81±0.22	191.17±20.68	4.92±0.49	2.72±0.23
F calculated	29.95	25.44	4.54	4.82	4.90	20.49	21.47
DV	65.16	62.23	118.76	1.00	67.92	1.33	0.81

Values are mean ± SEM of six animals, F theoretical = 2.76 ($p<0.05$). AE: Aqueous extract: ME: Methanol extract.

* Significant reduction compared to CCl_4 ($p<0.05$). ** Significant increase compared to CCl_4 ($p<0.05$). DV: Dunnett value.

Table 23: Percentage restoration of various parameters by ME and AE against CCl_4-induced toxicity

GROUP	GOT	GPT	ALKP	TBL	CHL	TPTN	ALB
Silymarin (200 mg/kg)	95.04	95.62	93.06	106.51	87.78	82.75	86.82
ME (200 mg/kg)	88.01	83.07	89.53	86.95	81.65	72.98	77.36
AE (200 mg/kg)	30.01	28.85	56.21	66.66	10.72	25.95	31.68

AE: Aqueous extract: ME: Methanol extract.

Histopathological examination revealed the disarrangement of normal hepatic cells with intense centrilobular necrosis and vacuolization in CCl_4-intoxicated rat liver sections (Figure 10b), compared to normal rats (Figure 10a). The sections of the rat liver treated with ME followed by CCl_4 intoxication (Figure 10e), showed less vacuolization and without necrosis as observed with the case of silymarin treated rat liver sections (Figure 10c), suggesting the protective effect of the extract. The AE was not able to alter the visible changes occurred due to CCl_4 intoxication (Figure 10d).

The percentage restoration of various biochemical parameters showed by silymarin, EE and AE against CCl_4 are represented in Table 23 and Graph 8. ME at selected dose level i.e. offered maximum protection.

From the Table 20 and Figure 9 of *P. daemia*; Table 22 and Figure 10 of *B. montanum*, it is evident that the EE of *P. daemia* and ME of *B. montanum* reduced almost all the elevated biochemical parameters due to the CCl_4 intoxication and reduced levels of total proteins and albumin. The reduction is attributed to the damage produced and localised in the endoplasmic reticulum which results in the loss of cytochrome P_{450} leading to its functional failure with a decrease in protein synthesis and accumulation of triglycerides. CCl_4 intoxication also inhibits the synthesis of the bile acids from cholesterol in liver or derived from plasma lipids, and increases the cholesterol level. Suppression of cholesterol levels suggest the inhibition of the synthesis of bile acids was reversed by the extract. Reduction of elevated levels of GOT and GPT to normal is an indication of stabilisation of plasma membrane as well as repair of hepatic tissue damages caused by CCl_4. Reduction of ALKP levels with concurrent depletion of raised bilirubin level suggests the stability of the biliary function during injury with CCl_4. The rise of values of protein and albumin levels, suggests stabilization of endoplasmic reticulum leading to protein synthesis. The protective effects exhibited by EE of *P. daemia* and ME of *B. montanum* were comparable significantly to the effect exhibited by silymarin.

The histological examination of the sections of rat liver revealed that the normal liver architecture was disturbed by hepatotoxin intoxication. In the

liver sections obtained from the rats treated with EE of *P. daemia* and ME of *B. montanum* and intoxicated with hepatotoxin the normal cellular architecture was retained as observed with silymarin treated rat liver sections, there by confirming the protective effect of the extracts.

Hepatoprotective activity of selective active extracts and their fractions

The active EE and its fraction EFEE of aerial parts of *P. daemia*, ME and its fraction MFME of roots of *B. montanum* were subjected to hepatoprotective activity against carbon tetrachloride, paracetamol and thioacetamide induced toxicities in rats. The EE and ME were tested for activity at dose levels of 100, 200 and 300 mg/kg p.o. where as EFEE and MFME were tested at dose levels of 50, 150 and 250 mg/kg p.o. respectively. The results were compared with those of silymarin (100 mg/kg) as standard hepatoprotective agent

CCl_4-induced hepatotoxicity

Effect of aerial parts of *P. daemia*:

CCl_4 intoxication in normal rats elevated the serum levels of GOT (103.50 ± 5.07 to 314.17 ±20.64), GPT (90.16 ± 7.86 to 247.00 ±20.01), ALKP (217.83 ± 10.56 to 442.33 ± 24.56), TBL (1.21 ± 0.14 to 3.01 ± 0.32), and CHL (108.12 ± 6.54 to 290.47 ±34.61); where as decrease in the levels of TPTN (6.93 ± 0.71 to 2.95 ± 0.28) ALB (4.55 ± 0.22 to 1.95 ± 0.13) significantly ($p<0.05$) indicating acute hepato cellular damage and biliary obstruction. The rats treated with EE at dose levels of 200 and 300 mg/kg; EFEE at dose levels of 150 and 250 mg/kg showed a significant ($p<0.05$) restoration of altered biochemical levels due to CCl_4 intoxication, similar to that observed in silymarin treated group. The activities exhibited by EE and EFEE were statistically ($p<0.05$) similar. The results obtained are recorded in Table 24 and Figure 11.

Table 24: Effect of EE and EFEE of *P. daemia* on CCl_4-induced hepatotoxicity in rats

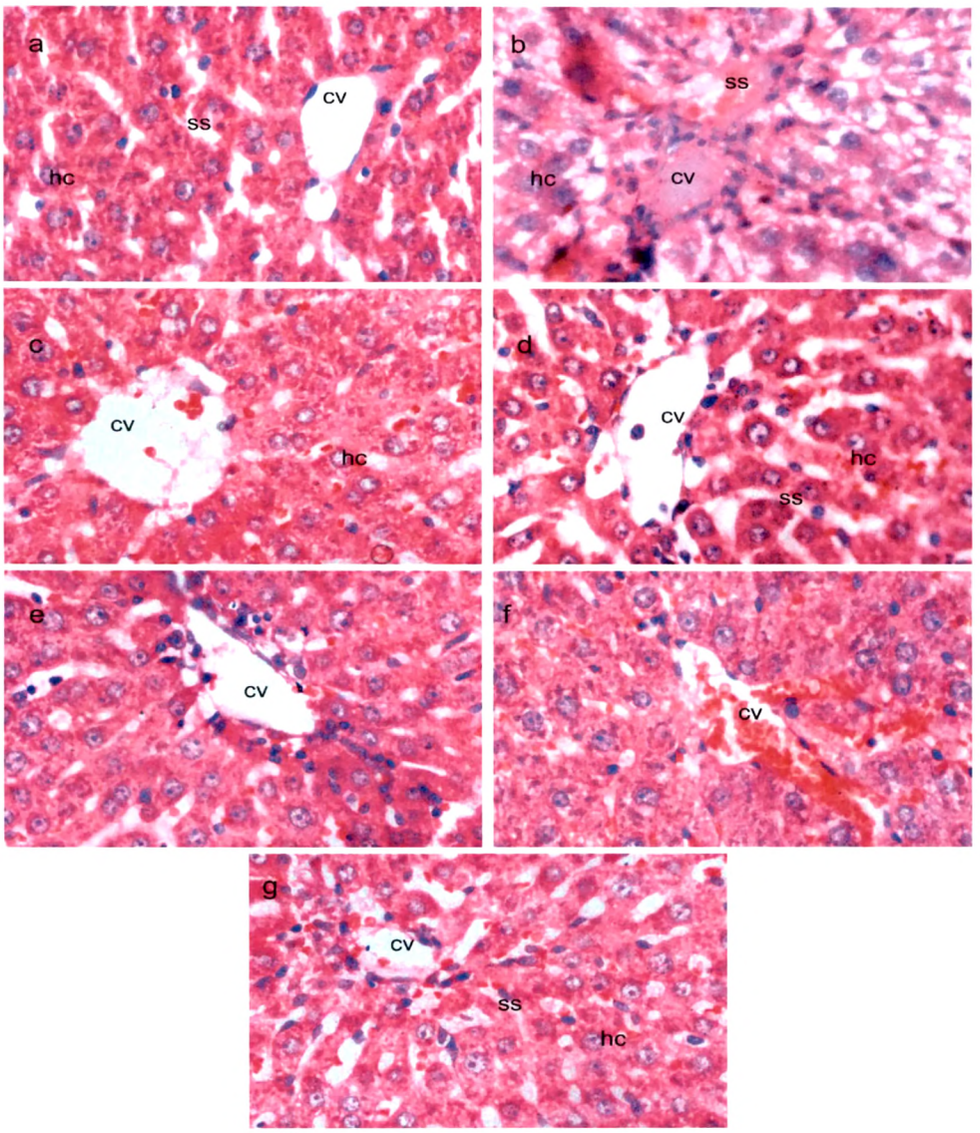
GROUP	GOT (IU/L)	GPT (IU/L)	ALKP (IU/L)	TBL (mg/dl)	CHL (mg/dl)	TPTN (g/dl)	ALB (g/dl)
Control	103.50 ± 5.07	90.16 ± 7.86	217.83 ± 10.56	1.21 ± 0.14	108.12 ± 6.54	6.93 ± 0.71	4.55 ± 0.22
CCl_4	314.17 ± 20.64	247.00 ± 20.01	442.33 ± 24.56	3.01 ± 0.32	290.47 ± 34.61	2.95 ± 0.28	1.95 ± 0.13
Silymarin	107.00 ± 5.48*	104.17 ± 10.05*	212.82 ± 10.77*	1.50 ± 0.18*	113.33 ± 7.62*	7.10 ± 0.67**	4.12 ± 0.27**
EE 1	300.33 ± 17.07	211.33 ± 32.11	386.17 ± 29.22	2.43 ± 0.35	276.50 ± 34.03	3.81 ± 0.34	2.23 ± 0.23
EE 2	132.67 ± 7.66*	135.50 ± 7.07*	252.33 ± 16.71*	1.80 ±0.24*	129.00 ± 8.50*	5.80 ± 0.41**	3.40 ± 0.25**
EE 3	104.50 ± 6.09*	118.17 ± 8.64*	228.53 ± 13.74*	1.65 ± 0.21*	116.83 ± 3.67*	6.83 ± 0.78**	3.83 ± 0.16**
EFEE 1	294.83 ± 23.01	199.67 ± 31.18	362.67 ± 28.63	2.35 ± 0.34	250.50 ± 24.00	3.46 ± 0.37	2.51 ± 0.24
EFEE 2	122.33 ± 7.69*	129.33 ± 6.50*	240.50 ± 15.01*	1.67 ± 0.23*	133.17 ± 6.62*	6.05 ± 0.46**	3.98 ± 0.29**
EFEE 3	109.31 ± 5.34 *	111.33 ± 8.24*	223.17 ± 12.49*	1.57 ± 0.20*	111.33 ± 7.31*	6.78 ± 0.45**	3.72 ± 0.26**
F Calculated	55.23	9.74	20.40	4.80	17.04	9.87	14.76
DV	50.09	68.97	75.35	1.01	73.76	2.05	0.92

Data represents the mean ± SEM of six animals. F theoretical = 2.18 ($p<0.05$).

EE 1, EE 2 and EE 3: Ethanol extract 100, 200 and 300 mg/kg; EFEE 1, EFEE 2 and EFEE 3: Ethanol fraction of ethanol extract 50, 150 and 250 mg/kg..; DV: Dunnett value.

* Significant reduction compared to CCl_4 ($p<0.05$). ** Significant increase compared to CCl_4 ($p<0.05$).

Figure 11: Photomicrographs representing effect of EE and EFEE against CCl_4-induced hepatotoxicity in rats



a: Normal rat liver section; b: Liver section of the rat intoxicated with CCl_4; c: Liver section of the rat treated with silymarin and intoxicated with CCl_4; d: Liver section of the rat treated with EE 200 mg/kg and intoxicated with CCl_4; e: Liver sections of the rat treated with EE 300 mg/kg and intoxicated with CCl_4; f: Liver section of the rat treated with EFEE 150 mg/kg and intoxicated with CCl_4; g: Liver section of the rat treated with EFEE 250 mg/kg and intoxicated with CCl4. Eosin-Haematoxylin stain. 400X. cv: central vein, vc: vacuole, ss: sinusoidal spaces, hc: hepatocytes.

Table 25: Percentage restoration of various parameters showed by EE and EFEE against CCl_4-induced hepatotoxicity

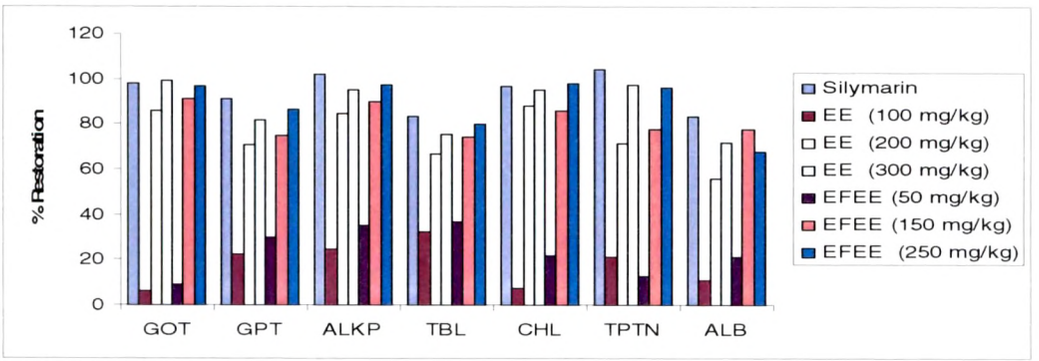
GROUP	GOT	GPT	ALKP	TBL	CHL	TPTN	ALB
Silymarin	98.19	90.98	102.13	83.80	97.07	104.24	83.45
EE (100 mg/kg)	06.56	22.71	24.99	32.21	07.65	21.60	10.76
EE (200 mg/kg)	86.03	71.02	84.55	67.21	88.48	71.59	55.76
EE (300 mg/kg)	99.38	82.06	95.41	75.54	95.14	97.46	72.30
EFEE (50 mg/kg)	09.17	30.14	35.44	36.66	21.90	12.81	21.53
EFEE (150 mg/kg)	90.93	74.95	89.81	74.43	86.20	77.87	78.07
EFEE (250 mg/kg)	97.09	86.42	97.52	79.99	98.16	96.21	68.07

EE: Ethanol extract; EFEE: Ethanol fraction of ethanol extract.

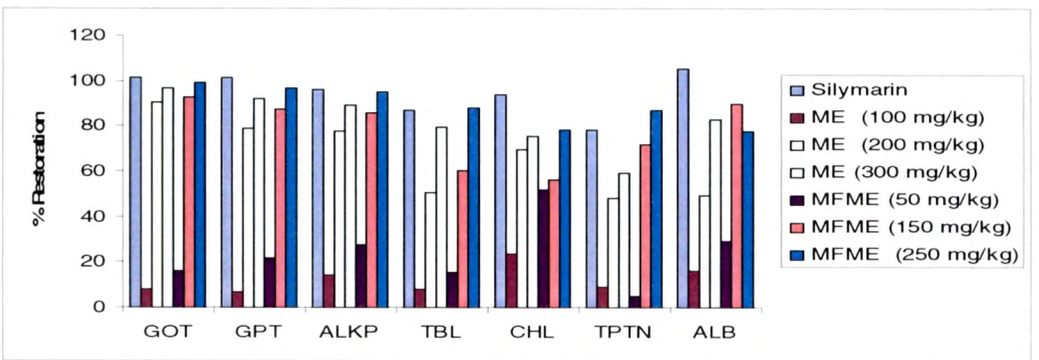
Histopathological examination of rat liver sections of control group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein (Figure 11a), which are affected due to CCl_4-intoxication (Figure 11b) leading to intense centrilobular necrosis and vacuolization. Treatment with EE (200 and 300 mg/kg) and EFEE (150 and 250 mg/kg) followed by CCl_4 intoxication resulted the absence of necrosis, vacuolization and lesser degree of disarrangement and degeneration of hepatocytes (Figure 11d-g) indicating marked protective activity similar to that of observed in case of silymarin treated group (Figure 11c).

The percentage restoration of various biochemical parameters showed by EE and EFEE at various dose levels against CCl_4-induced-hepatotoxicity are represented in Table 25 and Graph 9. Though the activity increased in a dose dependant manner, EE at a dose level of 300 mg/kg offered maximum protection. Similarly EFEE at dose level of 250 mg/kg exhibited maximum protection.

Graph 9: Percentage restoration of various parameters by EE and EFEE against CCl_4-induced hepatotoxicity



Graph 10: Percentage restoration of various parameters by ME and MFME against CCl_4-induced hepatotoxicity



Effect of roots of *B. montanum*:

The results of the effect of roots of *B. montanum* against CCl_4-induced hepatotoxicity are shown in Table 26 and Figure 12.

Intoxication of normal rats with CCl_4 elevated the serum levels of GOT (101.33 ± 5.07 to 309.50 ± 20.95), GPT (92.33 ± 7.63 to251.83 ± 20.34), ALKP (220.33 ± 10.62 to 435.50 ± 24.17),TBL (1.15 ± 0.13 to3.11 ± 0.32), and CHL (105.67 ± 6.03 to313.33 ± 24.94); where as decrease in the levels of TPTN (7.13 ± 0.26 to3.62 ± 0.21) ALB (4.17 ± 0.21 to2.25 ± 0.20) significantly ($p<0.05$) indicating acute hepato cellular damage. The rats which received ME 200 and 300 mg/kg; MFME 150 and 250 mg/kg showed a significant ($p<0.05$) restoration of altered biochemical levels due to CCl_4 intoxication, as observed

Table 26: Effect of ME and MFME of *B. montanum* on CCl_4-induced hepatotoxicity in rats

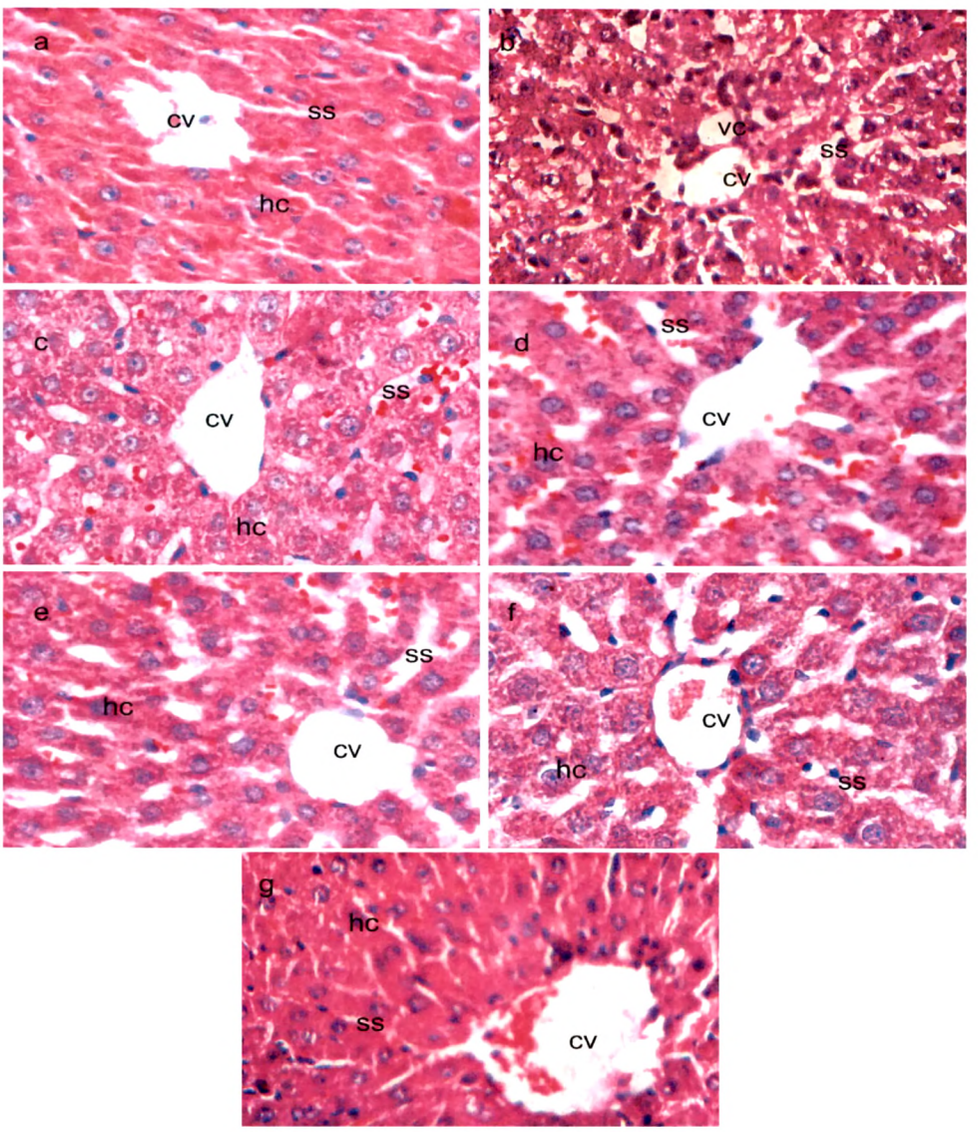
GROUP	GOT (IU/L)	GPT (IU/L)	ALKP (IU/L)	TBL (mg/dl)	CHL (mg/dl)	TPTN (g/dl)	ALB (g/dl)
Control	101.33 ± 5.07	92.33 ± 7.63	220.33 ± 10.62	1.15 ± 0.13	105.67 ± 6.03	7.13 ± 0.26	4.17 ± 0.21
CCl_4	309.50 ± 20.95	251.83 ± 20.34	435.50 ± 24.17	3.11 ± 0.32	313.33 ± 24.94	3.62 ± 0.21	2.25 ± 0.20
Silymarin	97.83 ± 4.69*	90.33 ± 7.17*	225.67 ± 13.48*	1.40 ± 0.19*	116.83 ± 6.22*	6.38 ± 0.63**	4.28 ± 0.19**
ME 1	292.67 ± 18.67	240.67 ± 20.11	403.83 ± 23.74	2.95 ± 0.32	264.17 ± 13.28	3.30 ± 0.16	2.56 ± 0.24
ME 2	121.33 ± 6.51*	126.33 ± 8.53*	266.33 ± 11.15*	2.12 ± 0.14*	167.67 ± 13.37*	5.33 ± 0.16**	3.20 ± 0.19**
ME 3	107.17 ± 4.98*	105.50 ± 8.09*	240.83 ± 10.74*	1.55 ± 0.12*	156.33 ± 13.49*	5.70 ± 0.57**	3.85 ± 0.28**
MFME 1	275.33 ± 15.61	217.00 ± 14.93	374.67 ± 20.67	2.80 ± 0.32	204.67 ± 7.86	3.81 ± 0.17	2.81 ± 0.19
MFME 2	116.31 ± 5.37*	112.67 ± 6.51*	248.50 ± 10.47*	1.92 ± 0.13*	195.50 ± 7.39*	6.15 ± 0.64**	3.98 ± 0.17**
MFME 3	103.17 ± 5.11*	98.33 ± 7.46*	228.17 ± 11.28*	1.38 ± 0.12*	150.17 ± 13.32*	6.67 ± 0.54**	3.75 ± 0.17**
F Calculated	64.77	29.64	28.38	11.41	26.86	11.41	10.68
DV	45.11	48.46	63.03	0.86	50.87	1.65	0.89

Data represents the mean ± SEM of six animals. F theoretical = 2.18 ($p<0.05$).

ME 1, ME 2 and ME 3: Methanol extract 100, 200 and 300 mg/kg; MFME 1, MFME 2 and MFME 3: Methanol fraction of methanol extract 50, 150 and 250 mg/kg..; DV: Dunnett value.

* Significant reduction compared to CCl_4 ($p<0.05$). ** Significant increase compared to CCl_4 ($p<0.05$).

Figure 12: Photomicrographs representing effect of ME and MFME against CCl_4-induced hepatotoxicity in rats



a: Normal rat liver section; b: Liver section of the rat intoxicated with CCl_4; c: Liver section of the rat treated with silymarin and intoxicated with CCl_4; d: Liver section of the rat treated with ME 200 mg/kg and intoxicated with CCl_4; e: Liver sections of the rat treated with ME 300 mg/kg and intoxicated with CCl_4; f: Liver section of the rat treated with MFME150 mg/kg and intoxicated with CCl_4; g: Liver section of the rat treated with MFME 250 mg/kg and intoxicated with CCl_4.Eosin-Haematoxylin stain. 400X. cv: central vein, vc: vacuole, ss: sinusoidal spaces, hc: hepatocytes.

Table 27: Percentage restoration of various parameters showed by ME and MFME against CCl_4-induced hepatotoxicity

GROUP	GOT	GPT	ALKP	TBL	CHL	TPTN	ALB
Silymarin	101.60	101.74	96.52	87.24	94.32	78.63	105.72
ME (100 mg/kg)	08.07	06.71	14.56	08.16	23.59	09.12	16.14
ME (200 mg/kg)	90.32	79.06	77.81	50.51	69.91	48.71	49.47
ME (300 mg/kg)	97.11	92.19	89.54	79.59	75.36	59.25	83.32
MFME (50 mg/kg)	16.41	21.94	27.98	15.81	52.16	05.41	29.16
MFME (150 mg/kg)	92.73	87.67	86.02	60.71	56.55	72.07	90.19
MFME (250 mg/kg)	99.03	96.70	95.37	88.26	78.31	86.89	78.12

ME: Methanol extract; MFME: Methanol fraction of methanol extract.

in case of silymarin treated groups. Further the activities exhibited by ME and MFME wee statistically ($p<0.05$) similar.

The sections of the rat liver showed that the normal cellular architecture (Figure 12a) was affected due to CCl_4-intoxication (Figure 12b) leading intense centrilobular necrosis and vacuolization. Treatment with ME(200 and 300 mg/kg) and MFME (150 and 250 mg/kg) followed by CCl_4 intoxication resulted he absence of necrosis, vacuolisation and less disarrangement and degeneration of hepatocytes (Figure 12d-g) indicating marked protective activity similar to that observed in silymarin treated group (Figure 12c).

The percentage restoration of various biochemical parameters showed by ME and MFME at various dose levels against CCl_4-induced hepatotoxicity are recorded in Table 27 and Graph 10. ME at a dose level of 300 mg/kg offered maximum protection by restoring the altered parameters. The MFME at dose level of 250 mg/kg exhibited maximum protection.

Paracetamol-induced hepatotoxicity

Effect of aerial parts of *P. daemia*:

Paracetamol intoxication (3 g/kg p.o.) induced a marked increase in the serum levels of GOT (108.17 ± 4.78 to 448.50 ± 29.81), GPT (100.67 ± 2.82 to 314.83 ± 36.45), ALKP (205.67 ± 5.25 to 604.83 ± 47.19), TBL (0.92 ± 0.11 to 3.98 ± 0.48), and CHL (120.17 ± 6.27 to 347.50 ± 31.26); and decrease in

the levels of TPTN (6.15 ± 0.19 to 2.41 ± 0.36) and ALB (4.58 ± 0.18 to 1.53 ± 0.36) when compared to normal rats indicating acute centrilobular necrosis. The groups of rats which received EE at dose levels of 200 and 300 mg/kg p.o. and EFEE at dose levels of 150 and 250 mg/kg p.o. showed a significant decrease ($p<0.05$) in almost all the elevated levels of biochemical parameters and significant ($p<0.05$) increase in depleted TPTN and ALB levels similar to that observed in the case of rats of silymarin treated group. There was no significant ($p<0.05$) difference in the activity, exhibited by EE at dose levels of 200 and 300 mg/kg; EFEE at dose levels of 150 and 250 mg/kg. The activity exhibited by EE was statically similar to the activity exhibited by EFEE. The results obtained are shown in Table 28 and Figure 13.

Histological examination of liver sections of rats of control group, revealed normal cellular architecture (Figure 13a) while those intoxicated with paracetamol (3 g/kg p.o.) showed disarrangement and degeneration of normal hepatic cells with intense centrilobular necrosis and bridged necrosis, characterised by bands of necrosis linking one central vein to another, sinusoidal haemorrhages and dilatation (Figure 13b). Treatment with EE (200 and 300 mg/kg) and EFEE (150 and 250 mg/kg) followed by paracetamol intoxication resulted the absence of necrosis, sinusoidal dilation and lesser degree of disarrangement and degeneration of hepatocytes (Figure 13d-g) indicating marked protective activity similar to that observed in silymarin treated rat liver sections (Figure 13c).

The percentage restoration of various biochemical parameters showed by EE and EFEE at various dose levels against paracetamol-induced hepatotoxicity are represented in Table 29 and Graph 11. The maximum percentage restoration, in the levels of GOT, GPT and ALB was observed with EE at dose level of 300 mg/kg, where as in the levels of ALKP, TBL, CHL and TPTN at dose level of 200 mg/kg. EFEE at a dose level of 150 mg/kg afforded highest percentage restoration in the levels of ALKP, TBL, TPTN and ALB, while at dose level of 250 mg/kg in the levels of GOT, GPT and CHL.

Table 28: Effect of EE and EFEE of *P. daemia* on paracetamol-induced hepatotoxicity in rats

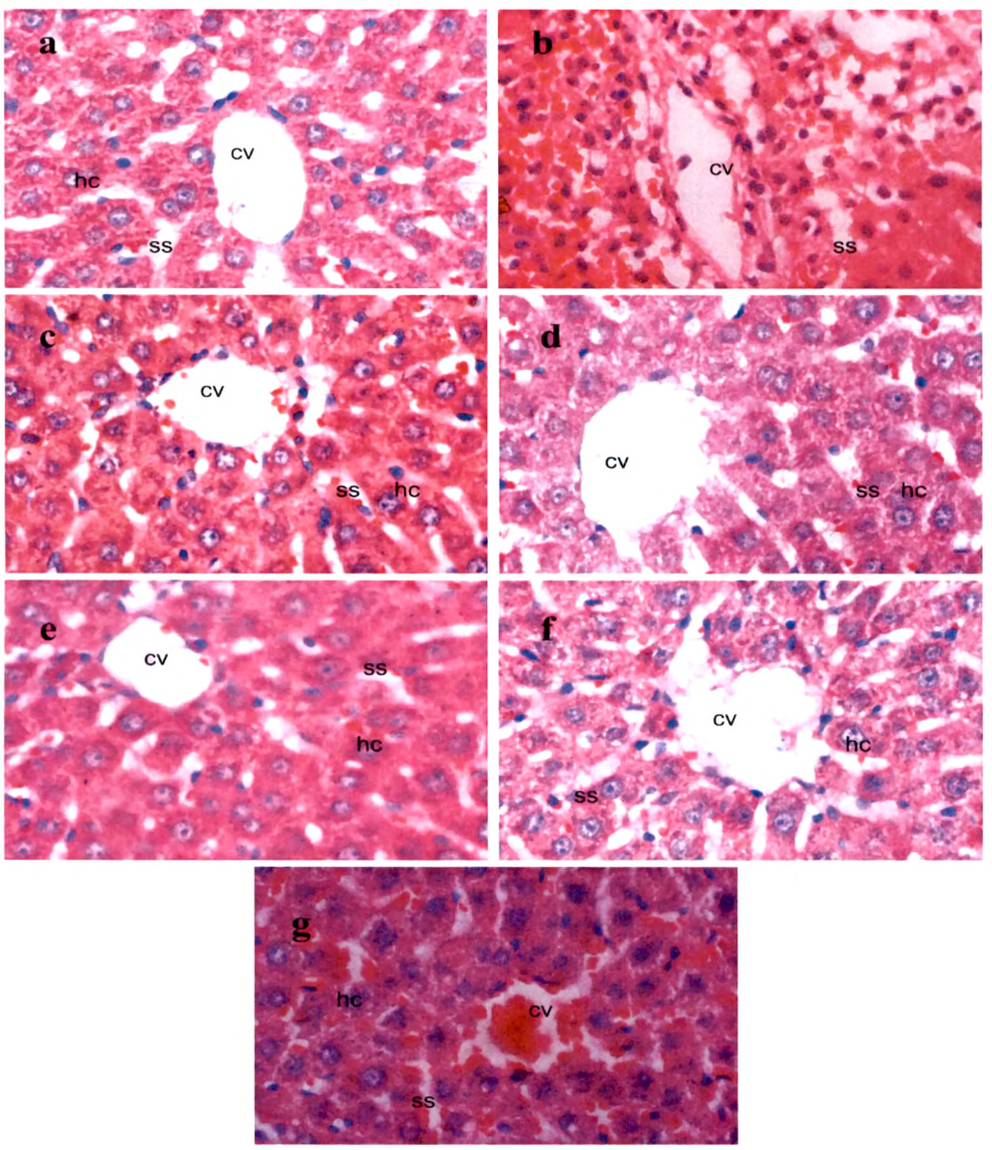
GROUP	GOT (IU/L)	GPT (IU/L)	ALKP (IU/L)	TBL (mg/dl)	CHL (mg/dl)	TPTN (g/dl)	ALB (g/dl)
Control	108.17 ± 4.78	100.67 ± 2.82	205.67 ± 5.25	0.92 ± 0.11	120.17 ± 6.27	6.15 ± 0.19	4.58 ± 0.18
Paracetamol	448.50 ± 29.81	314.83 ± 36.45	604.83 ± 47.19	3.98 ± 0.48	347.50 ± 31.26	2.41 ± 0.36	1.53 ± 0.36
Silymarin	119.67 ± 12.58*	102.67 ± 10.16*	221.33 ± 16.82*	1.03 ± 0.16*	127.67 ± 10.48*	6.42 ± 0.49**	4.53 ± 0.89**
EE 1	389.67 ± 61.71	268.33 ± 27.22	540.00 ± 53.53	2.83 ± 0.29	276.83 ± 40.72	2.52 ± 0.26	2.13 ± 0.47
EE 2	148.33 ± 61.71*	129.50 ± 19.45*	247.67 ± 20.15*	1.38 ± 0.23*	141.83 ± 16.91*	5.63 ± 0.50**	3.95 ± 0.68**
EE 3	135.50 ± 16.24*	117.50 ± 9.45*	277.16 ± 49.42*	1.60 ± 0.21*	200.50 ± 24.87*	4.75 ± 0.59**	4.35 ± 0.53**
EFEE 1	392.17 ± 51.13	284.17 ± 32.14	479.17 ± 72.32	3.01 ± 0.36	303.83 ± 43.73	3.23 ± 0.27	2.73 ± 0.62
EFEE 2	155.00 ± 24.51*	139.50 ± 21.52*	278.50 ± 127.51*	1.67 ± 0.34*	160.67 ± 24.86*	6.70 ± 0.70**	5.05 ± 0.69**
EFEE 3	138.50 ± 15.95*	121.83 ± 11.42*	312.83 ± 33.67*	1.82 ± 0.33*	152.16 ± 16.17*	6.10 ± 0.49**	4.23 ± 0.30**
F calculated	19.96	15.88	12.92	11.71	09.91	13.87	04.74
DV	122.37	85.36	162.12	1.17	105.10	1.81	2.21

Data represents the mean ± SEM of six animals. F theoretical = 2.18 ($p<0.05$).

EE 1, EE 2 and EE 3: Ethanol extract 100, 200 and 300 mg/kg; EFEE 1, EFEE 2 and EFEE 3: Ethanol fraction of ethanol extract 50, 150 and 250 mg/kg; DV: Dunnett value.

* Significant reduction compared to hepatotoxin ($p<0.05$). ** Significant increase compared to hepatotoxin ($p<0.05$).

Figure 13: Photomicrographs representing effect of EE and EFEE against paracetamol-induced hepatotoxicity in rats



a: Normal rat liver section; b: Liver section of the rat intoxicated with paracetamol; c: Liver section of the rat treated with silymarin and intoxicated with paracetamol; d: Liver section of the rat treated with EE 200 mg/kg and intoxicated with paracetamol; e: Liver sections of the rat treated with EE 300 mg/kg and intoxicated with paracetamol; f: Liver section of the rat treated with EFEE 150 mg/kg and intoxicated with paracetamol; g: Liver section of the rat treated with EFEE 250 mg/kg and intoxicated with paracetamol. Eosin-Haematoxylin stain. 400X.

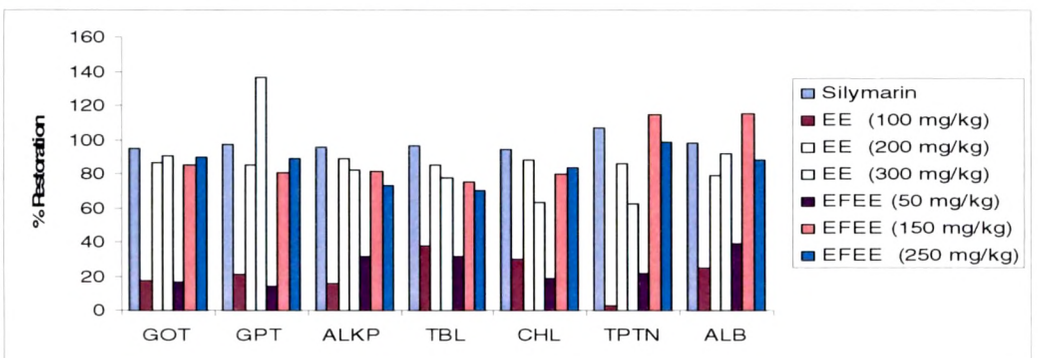
cv: central vein, vc: vacuole, ss: sinusoidal spaces, hc: hepatocytes.

Table 29: Percentage restoration of various parameters showed by EE and EFEE against paracetamol-induced hepatotoxicity

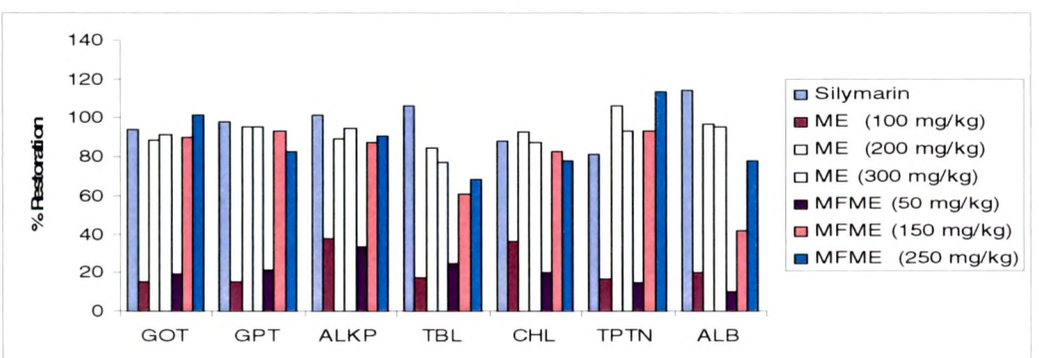
GROUP	GOT	GPT	ALKP	TBL	CHL	TPTN	ALB
Silymarin	95.36	97.59	95.87	96.37	94.52	107.18	98.34
EE (100 mg/kg)	17.06	21.39	16.20	37.57	30.38	02.94	25.24
EE (200 mg/kg)	87.04	85.25	89.29	84.94	88.43	86.07	79.32
EE (300 mg/kg)	90.77	136.77	81.91	77.75	63.21	62.54	92.43
EFEE (50 mg/kg)	16.33	14.10	31.41	31.68	18.77	21.91	39.33
EFEE (150 mg/kg)	85.11	80.65	81.58	75.46	80.33	114.67	115.38
EFEE (250 mg/kg)	89.90	88.78	73.00	70.56	83.99	98.63	88.50

EE: Ethanol extract; EFEE: Ethanol fraction of ethanol extract.

Graph 11: Percentage restoration of various parameters by EE and EFEE against paracetamol-induced hepatotoxicity



Graph 12: Percentage restoration of various parameters by ME and MFME against paracetamol-induced hepatotoxicity



Effect of roots of *B. montanum*:

Significant ($p<0.05$) increase in the serum levels of GOT (115.67 ± 4.80 to 462.50 ± 20.25), GPT (76.83 ± 2.52 to 317.17 ± 27.42), ALKP (185.50 ± 4.37 to 539.83), TBL (1.22 ± 0.15 to 5.07 ± 0.68) and CHL (95.67 ± 4.33 to 313.83 ± 31.93); decrease in the levels of TPTN (5.72 ± 0.21 to 2.45 ± 0.22) and ALB (4.47 ±0.38 to 1.53 ± 0.17) occurred in normal rats upon intoxication with paracetamol (3 g/kg p.o.). ME at dose levels of 200 and 300 mg/kg, MFME at dose levels of 150 and 250 mg/kg exhibited a significant ($p<0.05$) decrease ($p<0.05$) in all the elevated biochemical levels and significant ($p<0.05$) increase in depleted TPTN and ALB levels as observed in case of silymarin treated group. The activity exhibited by ME and MFME was statistically ($p<0.05$) similar. The results obtained are shown in Table 30 and Figure 14.

Histological examination of liver sections of rats of control group revealed normal cellular architecture (Figure 14a) which was affected by paracetamol intoxication as evidenced by disarrangement and degeneration of hepatic cells with intense centrilobular necrosis and bridged necrosis, sinusoidal haemorrhages and dilatation (Figure 14b). Treatment with ME (200 and 300 mg/kg) and MFME (150 and 250 mg/kg) followed by paracetamol intoxication resulted the absence of necrosis, sinusoidal dilation and lesser degree of disarrangement and degeneration of hepatocytes (Figure 14d-g) indicating marked protective activity as observed in case of liver sections of silymarin treated group rats (Figure 14c).

The percentage restoration of various biochemical parameters showed by ME and MFME at various dose levels against paracetamol-induced hepatotoxicity are represented in Table 31 and Graph 12. Among the dose levels tested with ME, the maximum percentage restoration in GOT, GPT and ALKP levels were obtained with 300 mg/kg, while TBL, CHL, TPTN and ALB levels with 300 mg/kg. Similarly among the dose levels tested with MFME, the maximum percentage restoration in GOT, ALKP, TBL, TPTN and ALB levels with 250 mg/kg, while the GPT and CHL with 150 mg/kg.

Table 30: Effect of ME and MFME of *B. montanum* on paracetamol-induced hepatotoxicity in rats

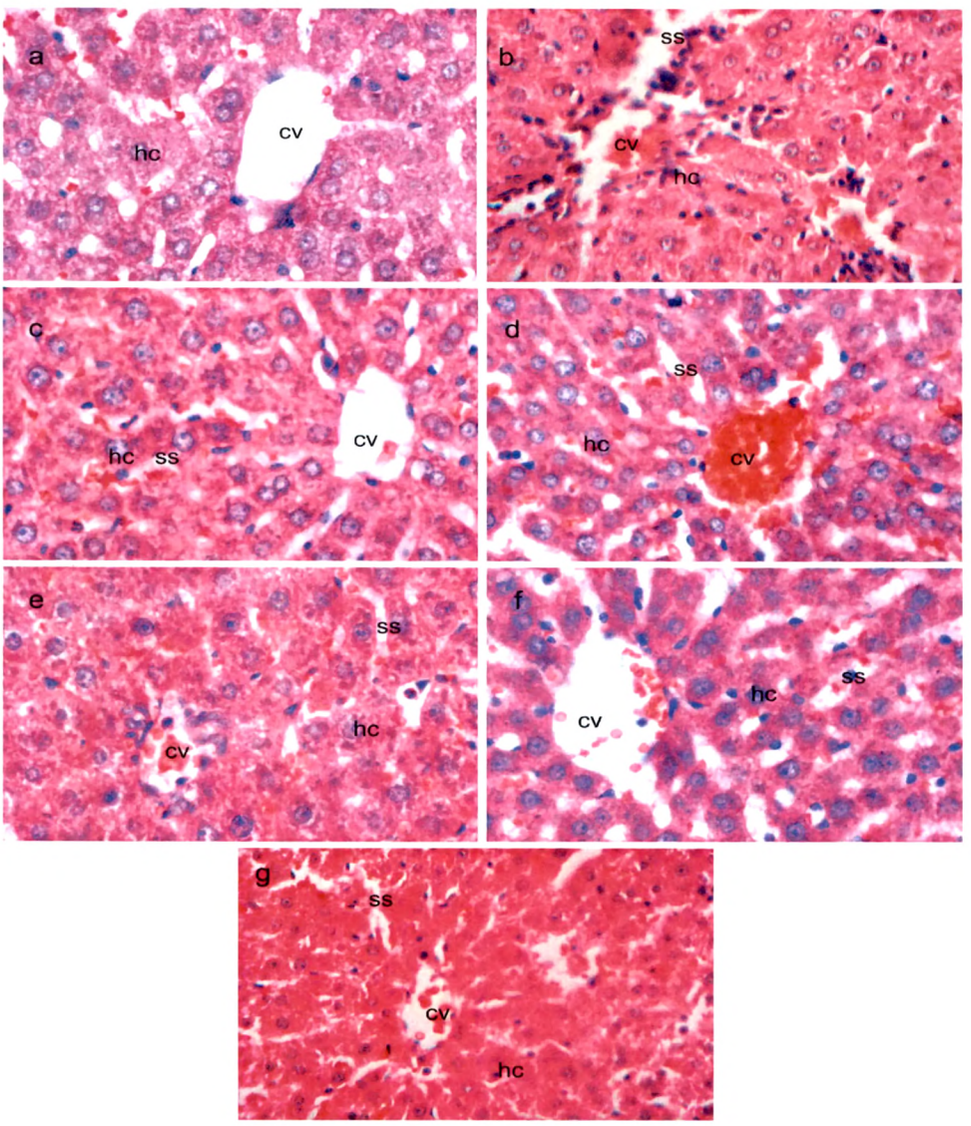
GROUP	GOT (IU/L)	GPT (IU/L)	ALKP (IU/L)	TBL (mg/dl)	CHL (mg/dl)	TPTN (g/dl)	ALB (g/dl)
Control	115.67 ± 4.80	76.83 ± 2.52	185.50 ± 4.37	1.22 ± 0.15	95.67 ± 4.33	5.72 ± 0.21	4.47 ± 0.38
Paracetamol	462.50 ± 20.25	317.17 ± 27.42	539.83 ± 33.60	5.07 ± 0.68	313.83 ± 31.93	2.45 ± 0.22	1.53 ± 0.17
Silymarin	137.69 ± 12.98*	83.50 ± 4.19*	175.67 ± 6.60*	0.98 ± 0.36*	117.17 ± 8.24*	5.11 ± 0.30**	4.90 ± 0.35**
ME 1	408.66 ± 36.18	281.00 ± 25.72	405.17 ± 53.93*	4.40 ± 0.65	232.66 ± 24.26	3.01 ± 0.27	2.13 ± 0.24
ME 2	156.03 ± 17.82*	90.17 ± 10.01*	220.00 ± 15.89*	1.80 ± 0.36*	107.83 ± 12.92*	5.93 ± 0.75**	4.38 ± 0.47**
ME 3	148.00 ± 12.04*	89.16 ± 9.51*	201.01 ± 23.65*	2.10 ± 0.47*	119.50 ± 10.00*	5.50 ± 0.41**	3.75 ± 0.78**
MFME 1	395.50 ± 48.55	265.33 ± 30.62	418.67 ± 59.09	4.12 ± 0.85	269.00 ± 40.26	2.93 ± 0.33	1.83 ± 0.37
MFME 2	151.50 ± 22.17*	94.83 ± 8.12*	227.17 ± 18.71*	2.71 ± 0.61*	129.33 ± 16.15*	5.51 ± 0.55**	2.75 ± 0.63
MFME 3	113.00 ± 20.90*	119.50 ± 20.23*	214.67 ± 15.02*	2.42 ± 0.49*	139.66 ± 14.98*	6.67 ± 0.73**	3.83 ± 0.81**
F calculated	33.16	29.14	17.34	6.89	14.47	9.91	5.79
DV	98.20	72.04	123.76	02.16	83.27	01.82	02.02

Data represents the mean ± SEM of six animals. F theoretical = 2.18 ($p<0.05$).

ME 1, ME 2 and ME 3: Methanol extract 100, 200 and 300 mg/kg; MFME 1, MFME 2 and MFME 3: Methanol fraction of methanol extract 50, 150 and 250 mg/kg; DV: Dunnett value.

* Significant reduction compared to paracetamol ($p<0.05$). ** Significant increase compared to paracetamol ($p<0.05$).

Figure 14: Photomicrographs representing effect of ME and MFME against paracetamol-induced hepatotoxicity in rats



a: Normal rat liver section; b: Liver section of the rat intoxicated with paracetamol; c: Liver section of the rat treated with silymarin and intoxicated with paracetamol; d: Liver section of the rat treated with EE 200 mg/kg and intoxicated with paracetamol; e: Liver sections of the rat treated with EE 300 mg/kg and intoxicated with paracetamol; f: Liver section of the rat treated with EFEE 150 mg/kg and intoxicated with paracetamol; g: Liver section of the rat treated with EFEE 250 mg/kg and intoxicated with paracetamol. Eosin-Haematoxylin stain. 400X.

cv: central vein, vc: vacuole, ss: sinusoidal spaces, hc: hepatocytes.

Table 31: Percentage restoration of various parameters showed by ME and MFME against paracetamol-induced hepatotoxicity

GROUP	GOT	GPT	ALKP	TBL	CHL	TPTN	ALB
Silymarin	94.20	98.14	101.96	106.21	88.49	81.34	114.61
ME (100 mg/kg)	15.61	15.19	37.71	17.39	36.52	17.12	20.41
ME (200 mg/kg)	88.87	95.34	89.55	84.92	92.70	106.41	96.92
ME (300 mg/kg)	91.21	95.76	94.87	77.13	87.44	93.26	95.50
MFME (50 mg/kg)	19.43	21.77	33.92	24.67	20.17	14.67	10.20
MFME (150 mg/kg)	90.19	93.38	87.54	61.28	83.02	93.57	41.49
MFME (250 mg/kg)	101.35	83.02	91.04	68.82	78.37	113.57	78.22

ME: Methanol extract; MFME: Methanol fraction of methanol extract.

Thioacetamide-induced hepatotoxicity

Effect of aerial parts of *P. daemia*:

Administration of thioacetamide (100 mg/kg i.p.) induced a marked increase in the serum levels of GOT (117.17 ± 8.66 to 554.00 ± 42.48), GPT (76.67 ± 3.78 to 292.50 ± 15.49), ALKP (214.50 ± 11.30 to 504.00 ± 41.47), TBL (1.18 ± 0.14 to 4.88 ± 0.61), and CHL (99.83 ± 3.75 to 350.67 ± 19.17); and decrease in the levels of TPTN (6.83 ± 0.29 to 2.97 ± 0.32) and ALB (5.33 ± 0.26 to 2.50 ± 0.18), indicating parenchymal cell necrosis. Significant decrease ($p<0.05$) in all the elevated levels of biochemical parameters and significant ($p<0.05$) increase in depleted TPTN and ALB levels was observed with the groups of rats which received EE at dose levels of 200 and 300 mg/kg and EFEE at dose levels of 150 and 250 mg/kg, as observed in case of silymarin treated group. There was no significant ($p<0.05$) difference between the activity shown by EE at dose levels of 200 and 300 mg/kg. Similarly EFEE at dose levels of 150 and 250 mg/kg. Results of thioacetamide-induced hepatotoxicity are shown in Table 32 and Figure 15.

Normal cellular architecture with distinct hepatic cells, sinusoidal spaces, and central vein (Figure 15a) was observed with histological examination of liver sections of control group. The examination of liver sections of rats administered with TAA showed hepatic cells with severe toxicity characterised by centrilobular necrosis along with various gradation of fatty changes comprising of tiny to large sized vacuoles, disarrangement of

hepatic cells with blood pooling in sinusoidal spaces (Figure 15b). The liver sections of the rats administered with 200 and 300 mg/kg p.o. of EE (Figure 15d,e) and 150 and 250 mg/kg of EFEE (Figure 15f,g) followed by TAA intoxications, showed lesser degree of visible changes similar to that observed in case of silymarin treated rat liver sections (Figure 15c) there by suggesting the protective effect of the extracts.

The percentage restoration of various biochemical parameters showed by EE and EFEE at various dose levels against thioacetamide-induced hepatotoxicity are represented in Table 33 and Graph 13. The maximum percentage restoration, in the levels of GPT, ALKP, TBL, CHL and TPTN was observed with EE at dose level of 300 mg/kg, while GOT and ALB with EE at dose level of 200 mg/kg. EFEE at a dose level of 150 mg/kg offered maximum percentage restoration in the levels of GOT, TBL, and ALB, while GPT, ALKP, CHL, and TPTN at dose level of 250 mg/kg.

Table 32: Effect of EE and EFEE of *P. daemia* on thioacetamide-induced hepatotoxicity in rats

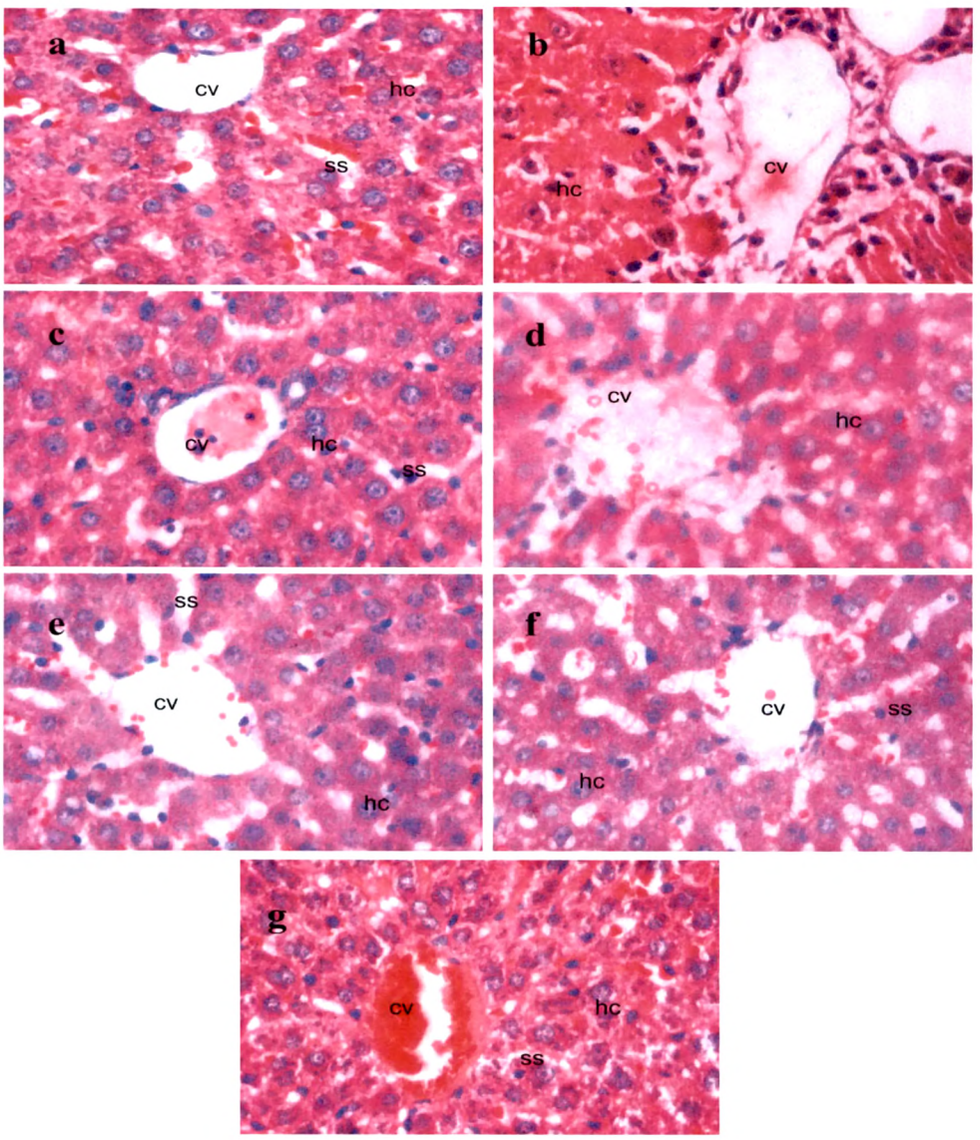
GROUP	GOT (IU/L)	GPT (IU/L)	ALKP (IU/L)	TBL (mg/dl)	CHL (mg/dl)	TPTN (g/dl)	ALB (g/dl)
Control	117.17 ± 8.66	76.67 ± 3.78	214.50 ± 11.30	1.18 ± 0.14	99.83 ± 3.75	6.83 ± 0.29	5.33 ± 0.26
Paracetamol	554.00 ± 42.48	292.50 ± 15.49	504.00 ± 41.47	4.88 ± 0.61	350.67 ± 19.17	2.97 ± 0.32	2.50 ± 0.18
Silymarin	128.67 ± 12.89*	71.50 ± 5.22*	259.83 ± 14.48*	1.58 ± 0.24*	120.50 ± 7.49*	6.05 ± 0.48**	4.75 ± 0.26**
EE 1	505.16 ± 32.63	88.67 ± 4.87	481.67 ± 35.67	4.55 ± 0.53	286.17 ± 23.59	2.88 ± 0.23	2.67 ± 0.35
EE 2	181.67 ± 13.31*	273.67 ± 16.02	289.17 ± 21.41*	1.75 ± 0.31*	138.83 ± 8.45*	5.97 ± 0.36**	5.30 ± 0.41**
EE 3	187.67 ± 13.31*	81.00 ± 5.03*	274.50 ± 10.15*	1.62 ± 0.21*	130.67 ± 6.82*	5.98 ± 0.75**	5.20 ± 0.25**
EFEE 1	523.83 ± 36.44	286.83 ± 12.13	484.50 ± 42.87	4.28 ± 0.61	303.83 ± 19.77	3.28 ± 0.32	2.85 ± 0.27
EFEE 2	175.66 ± 24.95*	94.33 ± 5.96*	435.83 ± 58.43*	1.85 ± 0.31*	153.17 ± 19.27*	5.80 ± 0.61**	5.68 ± 0.48**
EFEE 3	180.33 ± 26.71*	83.17 ± 4.04*	347.83 ± 30.18*	1.91 ± 0.27*	137.67 ± 10.60*	5.92 ± 0.42**	5.35 ± 0.24**
F calculated	50.19	117.04	11.20	12.79	40.27	11.76	17.09
DV	102.19	36.62	130.91	1.62	58.20	1.77	1.25

Data represents the mean ± SEM of six animals. F theoretical = 2.18 ($p<0.05$).

EE 1, EE 2 and EE 3: Total ethanol extract 100, 200 and 300 mg/kg; EFEE 1, EFEE 2 and EFEE 3: Ethanol fraction of total ethanol extract 50, 150 and 250 mg/kg; DV: Dunnett value.

* Significant reduction compared to thioacetamide ($p<0.05$). ** Significant increase compared to thioacetamide ($p<0.05$).

Figure 15: Photomicrographs representing effect of EE and EFEE against thioacetamide-induced hepatotoxicity in rats



a: Normal rat liver section; b: Liver section of the rat intoxicated with thioacetamide; c: Liver section of the rat treated with silymarin and intoxicated with thioacetamide; d: Liver section of the rat treated with EE 200 mg/kg and intoxicated with thioacetamide e: Liver sections of the rat treated with EE 300 mg/kg and intoxicated with thioacetamide; f: Liver section of the rat treated with EFEE 150 mg/kg and intoxicated with thioacetamide; g: Liver section of the rat treated with EFEE 250 mg/kg and intoxicated with paracetamol. Eosin-Haematoxylin stain. 400X.

cv: central vein, vc: vacuole, ss: sinusoidal spaces, hc: hepatocytes.

Table 33: Percentage restoration of various parameters showed by EE and EFEE against thioacetamide-induced hepatotoxicity

GROUP	GOT	GPT	ALKP	TBL	CHL	TPTN	ALB
Silymarin	97.82	101.66	84.23	89.16	89.76	79.77	79.49
EE (100 mg/kg)	11.23	93.76	07.70	08.92	25.15	---	06.01
EE (200 mg/kg)	85.63	08.66	74.12	84.57	82.61	77.70	98.92
EE (300 mg/kg)	84.25	97.29	79.17	88.08	85.80	77.95	95.39
EFEE (50 mg/kg)	06.93	02.61	06.72	16.21	18.26	08.03	12.36
EFEE (150 mg/kg)	87.01	91.15	23.51	81.87	77.02	73.29	112.34
EFEE (250 mg/kg)	85.94	96.29	53.87	80.24	83.07	76.41	100.69

EE: Total ethanol extract; EFEE: Ethanol fraction of total ethanol extract.

Effect of roots of *B. montanum*:

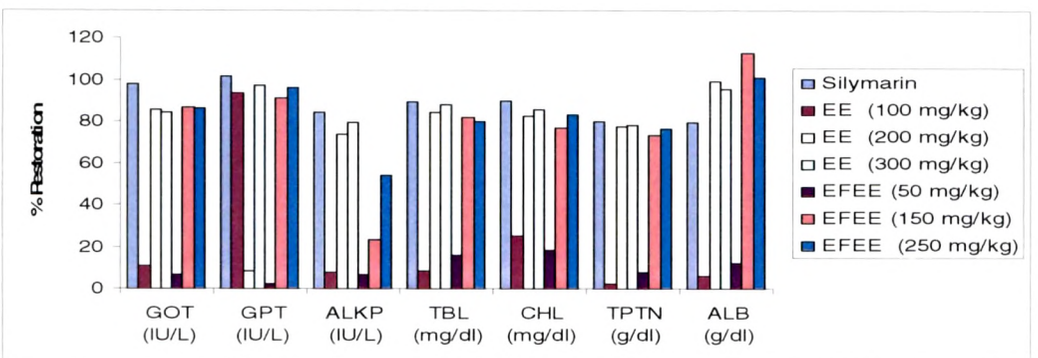
Significant ($p<0.05$) increase in the serum levels of GOT (110.83 ± 3.28 to 427.67 ± 26.48), GPT (83.50 ± 3.39 to 279.67 ± 29.69), ALKP (195.83 ± 4.51 to 496.67 ± 29.18), TBL (1.81 ± 0.31 to 3.98 ± 0.52) and CHL (110.50 ± 3.61 to 310.33 ± 31.89); decrease in the levels of TPTN (5.93 ± 0.34 to 2.70 ± 0.41) and ALB (4.82 ±0.26 to 2.53 ± 0.28) occurred in normal rats upon intoxication with paracetamol (3 g/kg p.o.). ME at dose levels of 200 and 300 mg/kg, MFME at dose levels of 150 and 250 mg/kg showed significant decrease ($p<0.05$) in all the elevated biochemical levels and significant ($p<0.05$) increase in depleted TPTN and ALB levels similar to that observed in case of silymarin treated group. The activity exhibited by ME and MFME was statistically ($p<0.05$) similar. The results obtained are shown in Table 34 and Figure 16.

Histological examination of liver sections of rats of control group revealed normal cellular architecture (Figure 16a) which was affected by thioacetamide intoxication as evidenced by disarrangement and degeneration of hepatic cells with intense centrilobular necrosis and vacuolisation, sinusoidal haemorrhages and dilatation (Figure 16b). Treatment with ME (200 and 300 mg/kg) and MFME (150 and 250 mg/kg) followed by thioacetamide intoxication resulted the absence of necrosis, sinusoidal dilation and less disarrangement and degeneration of hepatocytes (Figure 16d,e and Figure

16f,g) indicating marked protective activity as observed in case with liver sections of silymarin treated group rats (Figure 16c).

The percentage restoration of various biochemical parameters showed by ME and MFME at various dose levels against thioacetamide-induced hepatotoxicity are shown in Table 35 and Graph 14. The maximum percentage restoration in GOT, GPT, ALKP, TPTN and ALB levels are obtained with 300 mg/kg of ME, while TBL and CHL levels with 200 mg/kg. Similarly among the dose levels tested with MFME, the maximum percentage restoration in GPT, ALKP, TBL, CHL, TPTN and ALB levels with 250 mg/kg, while the GOT with 150 mg/kg.

Graph 13: Percentage restoration of various parameters by EE and EFEE against thioacetamide-induced hepatotoxicity



Graph 14: Percentage restoration of various parameters by ME and MFME against thioacetamide-induced hepatotoxicity

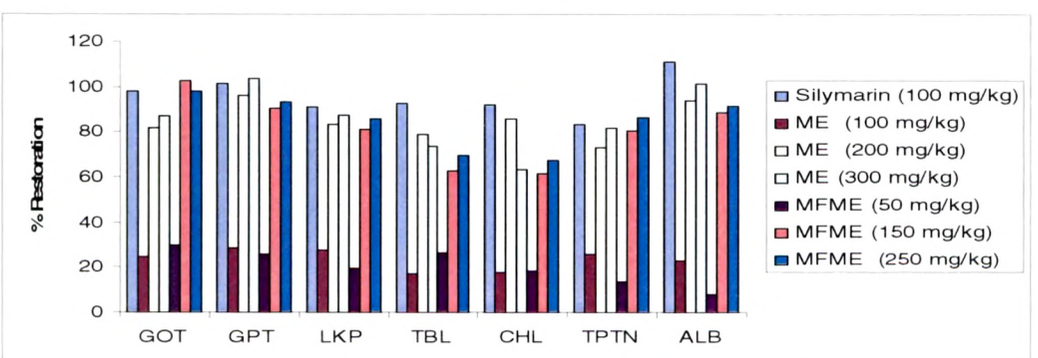


Table 34: Effect of ME and MFME of *B. montanum* on thioacetamide-induced hepatotoxicity in rats

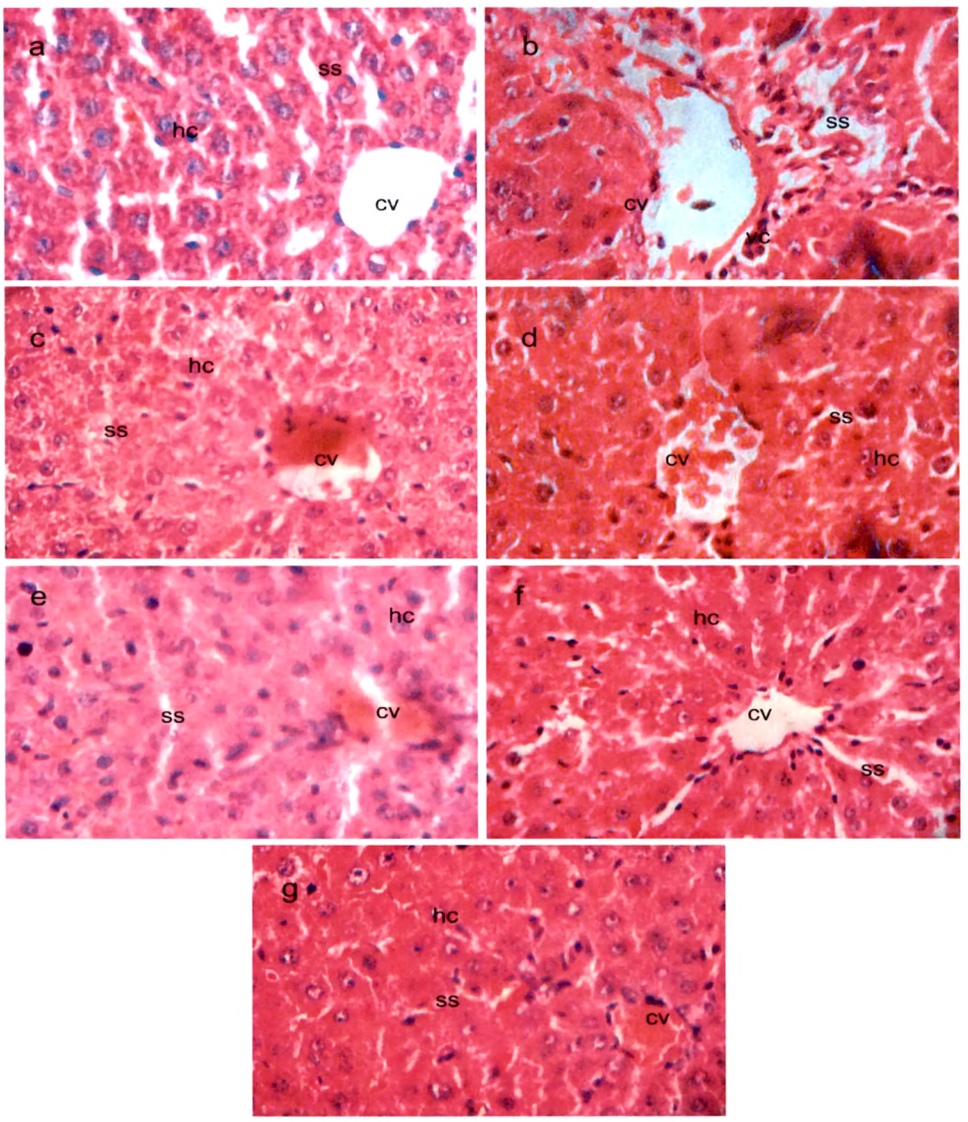
GROUP	GOT (IU/L)	GPT (IU/L)	ALKP (IU/L)	TBL (mg/dl)	CHL (mg/dl)	TPTN (g/dl)	ALB (g/dl)
Control	110.83 ± 3.28	83.50 ± 3.39	195.83 ± 4.51	1.81 ± 0.31	110.50 ± 3.61	5.93 ± 0.34	4.82 ± 0.26
Thioacetamide	427.67 ± 26.48	279.67 ± 29.69	496.67 ± 29.18	3.98 ± 0.52	310.33 ± 31.89	2.70 ± 0.41	2.53 ± 0.28
Silymarin	121.17 ± 13.53*	80.50 ± 6.78*	220.83 ± 25.35*	1.96 ± 0.21*	126.00 ± 16.01*	5.41 ± 0.38**	5.08 ± 0.72**
ME 1	350.33 ± 30.64*	222.67 ± 28.34	413.00 ± 51.06	3.60 ± 0.27	274.00 ± 32.75	3.53 ± 0.56	3.06 ± 0.46
ME 2	172.17 ± 23.23*	90.33 ± 5.92*	243.67 ± 21.85*	2.27 ± 0.18*	138.33 ± 21.19*	5.07 ± 0.67**	4.68 ± 0.71**
ME 3	153.17 ± 15.75*	76.17 ± 7.81*	231.50 ± 21.84*	2.38 ± 0.14*	183.50 ± 27.03*	5.35 ± 0.77**	4.85 ± 0.38**
MFME 1	334.16 ± 26.62*	228.33 ± 22.65	437.17 ± 45.89	3.40 ± 0.35	273.17 ± 20.56	3.15 ± 0.46	2.72 ± 0.23
MFME 2	107.34 ± 5.83*	102.17 ± 9.13*	250.16 ± 33.71*	2.62 ± 0.28*	187.17 ± 21.65*	5.31 ± 0.51**	4.57 ± 0.73**
MFME 3	121.66 ± 6.36*	96.00 ± 10.57*	236.17 ± 11.69*	2.47 ± 0.34*	174.83 ± 27.29*	5.50 ± 0.40**	4.63 ± 0.58**
F calculated	41.11	21.85	13.52	6.73	8.96	5.27	3.76
DV	76.16	65.89	120.05	01.14	93.91	02.03	02.06

Data represents the mean ± SEM of six animals. F theoretical = 2.18 ($p<0.05$).

ME 1, ME 2 and ME 3: Methanol extract 100, 200 and 300 mg/kg; MFME 1, MFME 2 and MFME 3: Methanol fraction of methanol extract 50, 150 and 250 mg/kg; DV: Dunnett value.

* Significant reduction compared to thioacetamide ($p<0.05$). ** Significant increase compared to thioacetamide ($p<0.05$).

Figure 16: Photomicrographs representing effect of ME and MFME against thioacetamide-induced hepatotoxicity in rats



a: Normal rat liver section; b: Liver section of the rat intoxicated with thioacetamide; c: Liver section of the rat treated with silymarin and intoxicated with thioacetamide; d: Liver section of the rat treated with ME 200 mg/kg and intoxicated with thioacetamide e: Liver sections of the rat treated with ME 300 mg/kg and intoxicated with thioacetamide; f: Liver section of the rat treated with MFME 150 mg/kg and intoxicated with thioacetamide; g: Liver section of the rat treated with MFME 250 mg/kg and intoxicated with paracetamol. Eosin-Haematoxylin stain. 400X.

cv: central vein, vc: vacuole, ss: sinusoidal spaces, hc: hepatocytes

Table 35: Percentage restoration of various parameters showed by ME and MFME against thioacetamide-induced hepatotoxicity

GROUP	GOT	GPT	ALKP	TBL	CHL	TPTN	ALB
Silymarin	98.08	101.57	91.02	93.08	92.16	83.87	111.39
ME (100 mg/kg)	24.75	29.07	27.61	17.51	18.16	25.68	23.13
ME (200 mg/kg)	81.76	96.56	83.49	78.79	86.00	73.35	93.86
ME (300 mg/kg)	87.34	103.78	87.51	73.72	63.41	82.01	101.29
MFME (50 mg/kg)	29.92	26.18	19.63	26.72	18.58	13.92	08.29
MFME (150 mg/kg)	102.50	90.52	81.34	62.66	61.58	80.77	89.06
MFME (250 mg/kg)	97.92	93.67	85.96	69.58	67.75	86.66	91.68

ME: Methanol extract; MFME: Methanol fraction of methanol extract.

Hepatoprotective activity of sub-fractions

The AFEFEE and EFEFEE from active EFEE of *P. daemia* and EMKMFME and MFMFME from active MFME of *B. montanum* were subjected for hepatoprotective activity against CCl_4, paracetamol and thioacetamide induced toxicities. The sub-fractions selected were used for hepato protective activity in vivo at dose levels of 50, 100 and 150 mg/kg p.o. The results were compared with silymarin (100 mg/kg), a standard hepatoprotective agent.

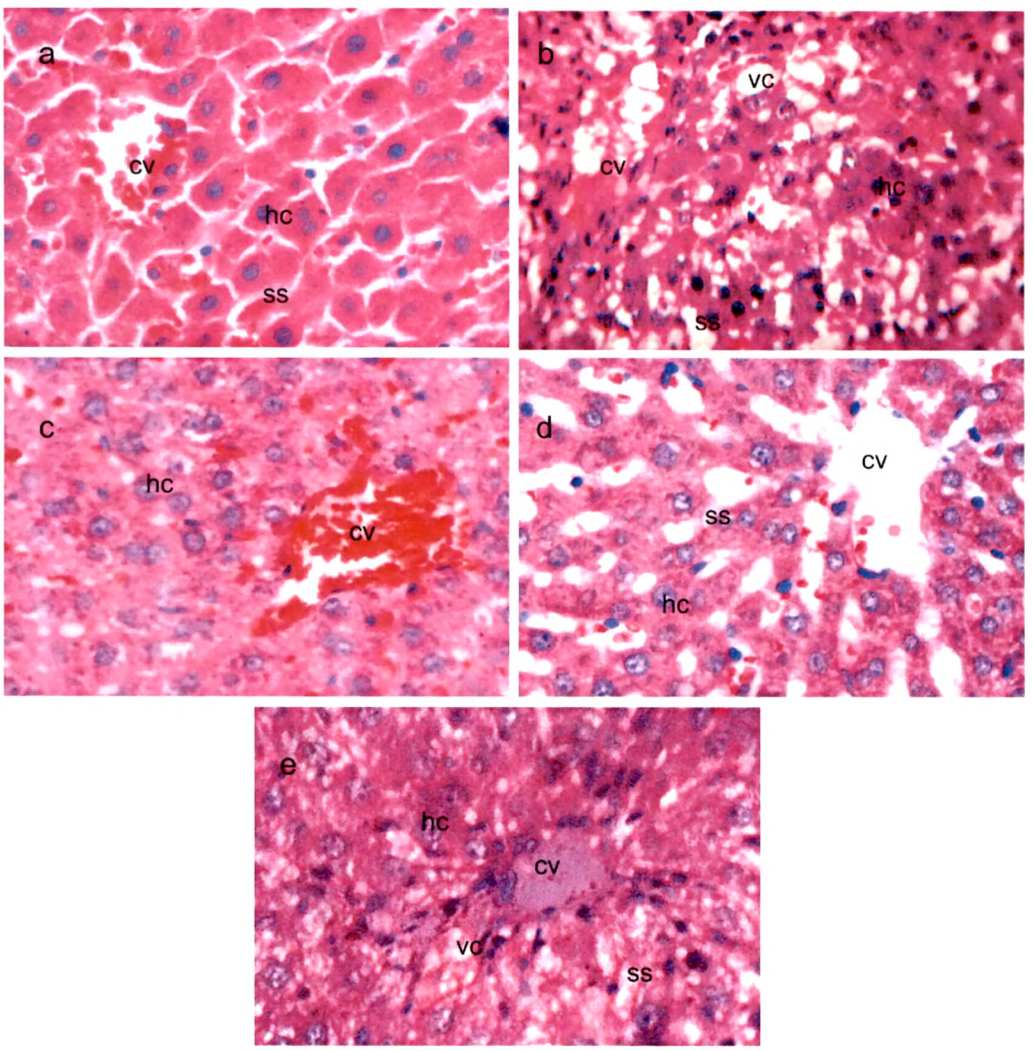
CCl_4-induced hepatotoxicity

Effect of aerial parts of *P. daemia*

CCl_4 intoxication in normal rats elevated the serum levels of GOT (128.17 ± 5.09 to 283.34 ± 30.13), GPT (102.50 ± 2.82 to 242.17 ± 36.45), ALKP (200.33 ± 5.25 to 424.50 ± 47.19), TBL (1.16 ± 0.12 to 2.96 ± 0.34), and CHL (101.34 ± 2.45 to 231.83 ± 27.04); decreased the levels of TPTN (7.10 ± 0.25 to 2.26 ± 0.39) and ALB (3.98 ± 0.14 to 1.60 ± 0.28) significantly ($p<0.05$) indicating acute hepato cellular damage. The rats that received 150 mg/kg of AFEFEE showed a significant decrease ($p<0.05$) in the elevated GOT, GPT, ALKP, TBL and CHL levels and significant ($p<0.05$) increase in reduced TPTN and ALB levels similar to that observed in silymarin treated group of rats. The rats which received EFEFEE have not shown significant ($p<0.05$) changes in the altered biochemical levels. The activities showed by AFEFEE (150 mg/kg) and silymarin against CCl_4-induced hepatotoxicity statistically similar. The results of CCl_4-induced hepatotoxicity are shown in Table 36 and Figure 17.

Histopathological examination of liver sections of control group rats showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein (Figure 17a). Disarrangement of normal hepatic cells with necrosis and vacuolization are observed in CCl_4-intoxicated liver (Figure 17b). The liver sections of the rat treated with 150 mg/kg of AFEFEE followed by CCl_4 intoxication (Figure 17d), showed less vacuolization without necrosis and overall no visible changes observed similar to that observed in silymarin treated rat liver sections (Figure 17c), suggesting the protective effect of the extract. EFEFEE was not able to restore the altered changes (Figure 17e).

Figure 17: Photomicrographs representing effect of AFEFEE and EFEFEE against CCl_4-induced hepatotoxicity in rats



a: Normal rat liver section; b: Liver section of the rat intoxicated with CCl_4; c: Liver section of the rat treated with silymarin and intoxicated with CCl_4; d: Liver section of the rat treated with AFEFEE 150 mg/kg and intoxicated with CCl_4; e: Liver sections of the rat treated with EFEFEE 150 mg/kg and intoxicated with CCl_4. Eosin-Haematoxylin stain. 400X. cv: central vein, vc: vacuole, ss: sinusoidal spaces, hc: hepatocytes.

The percentage restoration of various biochemical parameters showed by AFEFEE and EFEFEE at various dose levels against CCl_4-induced hepatotoxicity are shown in Table 37 and Graph 15. AFEFEE at dose level of 150 mg/kg afforded maximum percentage restoration.

Table 36: Effect of AFEFEE and EFEFEE of *P. daemia* on CCl_4-induced hepatotoxicity in rats

GROUP	GOT (IU/L)	GPT (IU/L)	ALKP (IU/L)	TBL (mg/dl)	CHL (mg/dl)	TPTN (g/dl)	ALB (g/dl)
Control	128.17 ± 5.09	102.50 ± 2.82	200.33 ± 5.25	1.16 ± 0.12	101.34 ± 2.45	7.10 ± 0.25	3.98 ± 0.14
CCl_4	283.34 ± 30.13	242.17 ± 36.45	424.50 ± 47.19	2.96 ± 0.34	231.83 ± 27.04	2.26 ± 0.39	1.60 ± 0.28
Silymarin	125.00 ± 11.83*	103.83 ± 10.16*	194.34 ± 16.82*	1.35 ± 0.23*	106.17 ± 6.98*	6.53 ± 0.42**	4.35 ± 0.21**
AFEFEE 1	262.67 ± 28.05	227.33 ± 27.22	374.67 ± 53.53	2.40 ± 0.41	213.83 ± 22.75	2.83 ± 0.46	2.26 ± 0.26
AFEFEE 2	226.50 ± 31.13	170.00 ± 19.45	290.17 ± 20.15*	2.01 ± 0.42	156.67 ± 27.56	4.95 ± 0.84**	3.13 ± 0.37**
AFEFEE 3	132.16 ± 15.52*	99.50 ± 9.45*	204.83 ± 49.42*	1.50 ± 0.29*	111.33 ± 10.48*	6.95 ± 0.53**	3.95 ± 0.36**
EFEFEE 1	276.17 ± 28.13	235.00 ± 32.14	348.00 ± 72.32	2.35 ± 0.34	209.83 ± 18.58	2.76 ± 0.41	2.03 ± 0.35
EFEFEE 2	262.17 ± 29.58	205.50 ± 21.52	311.50 ± 27.51*	2.45 ± 0.40	193.00 ± 19.37	3.52 ± 0.51	2.36 ± 0.38
EFEFEE 3	263.00 ± 30.03	259.83 ± 11.42	315.01 ± 33.67*	2.06 ± 0.41	208.33 ± 27.61	3.33 ± 0.70	2.78 ± 0.31
F calculated	7.54	5.83	15.36	2.94	6.68	13.29	9.98
DV	97.96	106.46	81.93	1.35	78.94	2.08	1.21

Data represents the mean ± SEM of six animals. F theoretical = 2.18($p<0.05$).

AFEFEE 1, 2 and 3: AFEFEE 50, 100 and 150 mg/kg; EFEFEE 1, 2 and 3: EFEFEE 50, 100 and 150 mg/kg; DV: Dunnett value

*Significant reduction compared to CCl_4 ($p<0.05$). ** Significant increase compared to CCl_4 ($p<0.05$)

Table 37: Percentage restoration of various parameters showed by AFEFEE and EFEFEE against CCl_4-induced hepatotoxicity

GROUP	GOT	GPT	ALKP	TBL	CHL	TPTN	ALB
Silymarin (100 mg/kg)	101.33	98.22	101.27	89.43	95.50	88.21	115.52
AFEFEE (50 mg/kg)	13.23	10.54	21.92	31.11	13.68	11.77	27.73
AFEFEE (100 mg/kg)	36.37	51.24	59.11	52.77	57.12	55.57	64.27
AFEFEE (150 mg/kg)	96.75	101.29	96.65	81.10	91.58	96.89	98.72
EFEFEE (50 mg/kg)	04.58	05.09	33.66	33.88	16.72	10.33	18.06
EFEFEE (100 mg/kg)	13.54	26.03	49.72	28.33	29.51	26.03	31.92
EFEFEE (150 mg/kg)	13.01	12.53	48.17	49.99	17.86	22.11	49.57

AFEFEE: Acetone sub-fraction of ethanol extract; EFEFEE: Ethanol sub-fraction of ethanol extract.

The percentage restoration of various biochemical parameters showed by AFEFEE and EFEFEE at various dose levels against CCl_4-induced hepatotoxicity are shown in Table 37 and Graph 15. AFEFEE at dose level of 150 mg/kg p.o exhibited maximum percentage restoration.

Effect of roots of *B. montanum*:

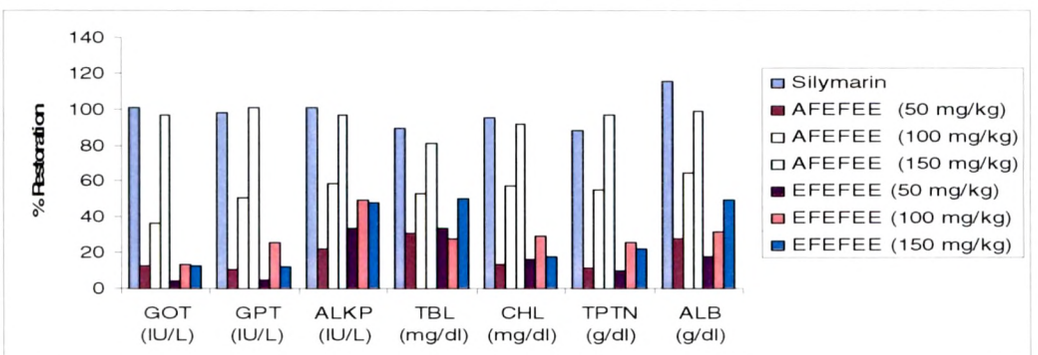
Significant ($p<0.05$)increase in the serum levels of GOT (118.17 ± 4.87 to 299.50 ± 21.12), GPT (104.67 ± 3.87 to 384.00 ± 15.45), ALKP (191.83 ± 8.39 to 436.50 ± 31.76), TBL (1.48 ± 0.19 to 4.36 ± 0.57) and CHL (95.50 ± 5.50 to 205.50 ± 16.97); decrease in the levels of TPTN (6.53 ± 0.57 to 2.76 ± 0.45) and ALB (4.10 ± 0.41 to 2.01 ± 0.19) occurred in normal rats upon intoxication with CCl_4. MFMFME at dose levels of 150 mg/kg, exhibited a significant decrease ($p<0.05$) in the elevated biochemical levels and significant ($p<0.05$) increase in depleted TPTN and ALB levels as observed with silymarin group. EMKMFME at a dose level of 150 mg/kg also restored the altered biochemical parameters, due to CCl_4 intoxication except ALB significantly ($p<0.05$) as observed with MFMFME. There is significant ($p<0.05$) difference in the activities exhibited by EMKMFME and silymarin. The results obtained are shown in Table 38 and Figure 18.

Histological examination of liver sections of rats of control group revealed normal cellular architecture (Figure 18a) which was affected by CCl_4

intoxication as evidenced by disarrangement and degeneration of hepatic cells with centrilobular necrosis and vacuolisation (Figure 18b). Treatment with MFMFME 150 mg/kg, followed by CCl_4 intoxication resulted the absence of necrosis and less disarrangement and degeneration of hepatocytes (Figure 18d) indicating marked protective activity as observed with silymarin treated group (Figure 18c). Though less visible changes were seen in the liver sections of the rats treated with EMKMFME 150 mg/kg (Figure 18e) the intensity was less.

The percentage restorations of various biochemical parameters showed by MFMFME and EMKMFME at various dose levels against CCl_4-induced hepatotoxicity are shown in Table 39 and Graph 16. The maximum percentage restoration was obtained with MFMFME 150 mg/kg.

Graph 15: Percentage restoration of various parameters by AFEFEE and EFEFEE against CCl_4-induced hepatotoxicity



Graph 16: Percentage restoration of various parameters by EMKMFME and MFMFME against CCl_4-induced hepatotoxicity

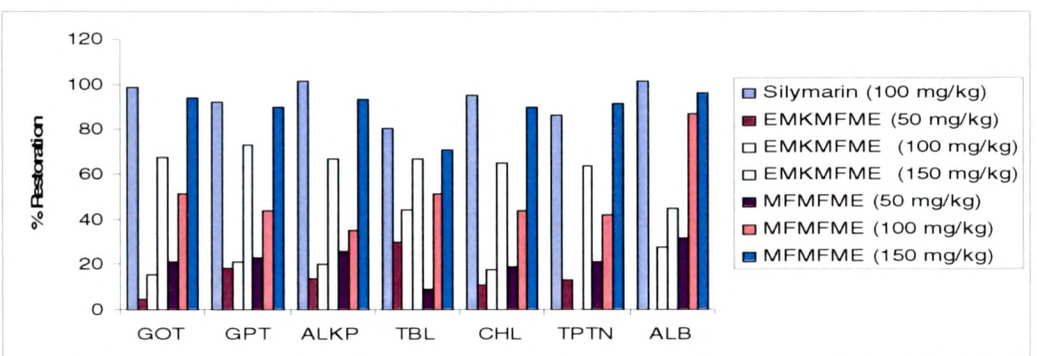
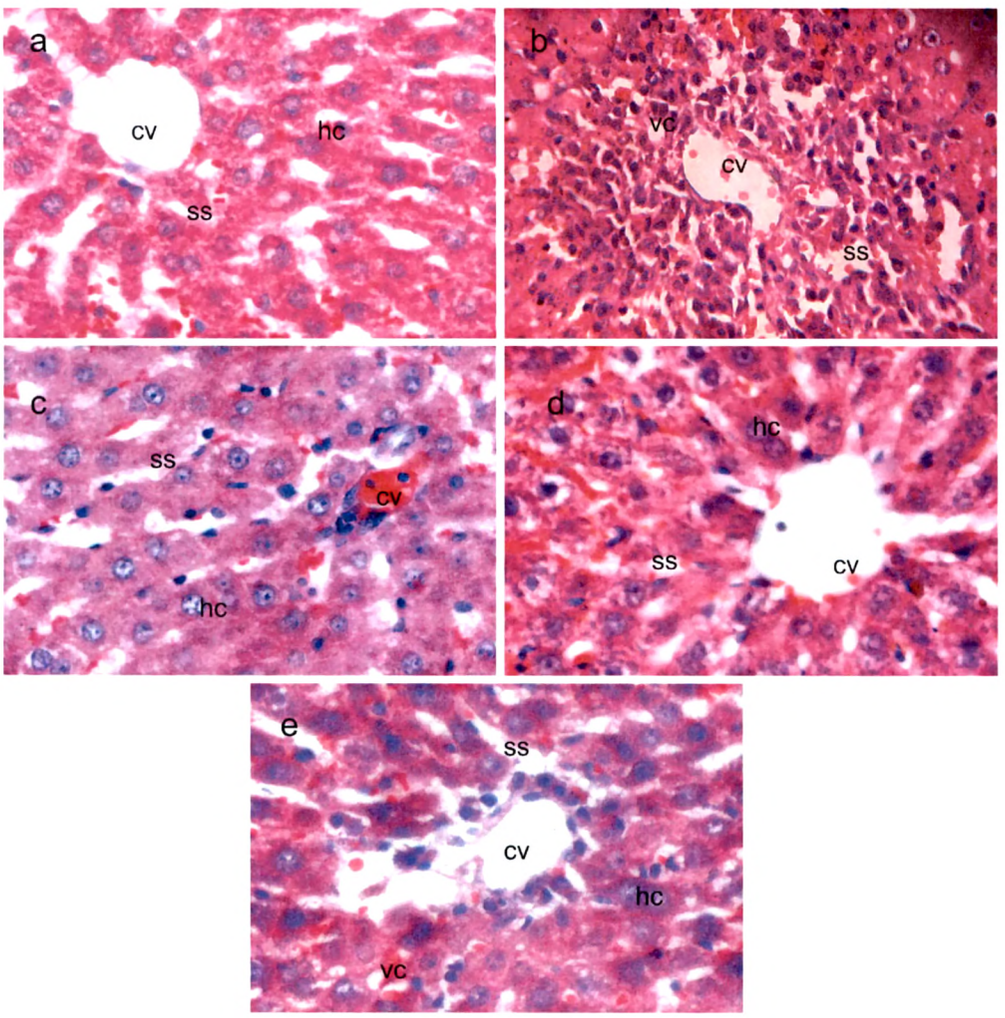


Figure 18: Photomicrographs representing effect of EMKMFME and MFMFME against CCl_4-induced hepatotoxicity in rats



a: Normal rat liver section; b: Liver section of the rat intoxicated with CCl_4; c: Liver section of the rat treated with silymarin and intoxicated with CCl_4; d: Liver section of the rat treated with MFMFME 150 mg/kg and intoxicated with CCl_4; e: Liver sections of the rat treated with EMKMFME 150 mg/kg and intoxicated with CCl_4. Eosin-Haematoxylin stain. 400X.
cv: central vein, vc: vacuole, ss: sinusoidal spaces, hc: hepatocytes.

Table 38: Effect of EMKMFME and MFMFME of *B. montanum* on CCl_4-induced hepatotoxicity in rats

GROUP	GOT (IU/L)	GPT (IU/L)	ALKP (IU/L)	TBL (mg/dl)	CHL (mg/dl)	TPTN (g/dl)	ALB (g/dl)
Control	118.17 ± 04.87	104.67 ± 03.87	191.83 ± 08.99	1.48 ± 0.19	95.50 ± 05.50	6.53 ± 0.57	4.10 ± 0.41
CCl_4	299.50 ± 21.12	384.00 ± 15.45	436.50 ± 31.76	4.36 ± 0.57	205.50 ± 16.97	2.76 ± 0.45	2.01 ± 0.19
Silymarin	120.33 ± 04.28*	120.50 ± 05.71*	188.17 ±10.14*	2.03 ± 0.20	100.83 ± 06.81	6.03 ± 0.58	4.13 ± 0.38
EMKMFME 1	291.50 ± 10.47	331.83 ± 29.92	403.00 ± 35.49	3.50 ± 0.54	193.50 ± 19.13	3.26 ± 0.56	1.96 ± 0.27
EMKMFME 2	271.33 ± 10.26	323.50 ± 18.03	387.83 ± 19.45	3.08 ± 0.61	185.81 ± 22.41	2.53 ± 0.47	2.48 ± 0.28
EMKMFME 3	176.50 ± 20.06*	175.17 ± 09.29*	273.50 ± 19.26*	2.43 ± 0.26*	134.17 ± 06.81*	5.18 ± 0.50**	2.95 ± 0.37
MFMFME 1	260.67 ± 14.14	317.67 ± 20.26	372.83 ± 21.61	4.10 ± 0.69	184.67 ± 13.91	3.56 ± 0.48	2.67 ± 0.33
MFMFME 2	206.50 ± 08.30*	258.17 ± 27.74*	350.66 ± 31.99*	2.88 ± 0.25	157.50 ± 16.91	4.35 ± 0.45	3.83 ± 0.24
MFMFME 3	129.00 ± 08.85*	127.33 ± 07.18*	209.17 ± 14.57*	2.31 ± 0.23*	106.82 ± 06.95*	6.21 ± 0.45**	4.02 ± 0.56
F calculated	34.86	36.61	17.55	04.65	09.34	09.27	06.59
DV	49.60	69.17	91.12	01.73	55.25	01.98	01.36

Data represents the mean ± SEM of six animals. F theoretical = 2.18 ($p<0.05$). Silymarin: Silymarin 100 mg/kg

EMKMFME 1, 2 and 3: EMKMFME 50, 100 and 150 mg/kg; MFMFME 1, 2 and 3: MFMFME 50, 100 and 150 mg/kg; DV: Dunnett value

*Significant reduction compared to CCl_4 ($p<0.05$). ** Significant increase compared to CCl_4 ($p<0.05$)

Table 39: Percentage restoration of various parameters showed by EMKMFME and MFMFME against CCl_4-induced hepatotoxicity

GROUP	GOT	GPT	ALKP	TBL	CHL	TPTN	ALB
Silymarin (100 mg/kg)	98.54	92.22	101.81	80.89	95.25	86.72	101.42
EMKMFME (50 mg/kg)	04.40	18.25	13.73	29.85	10.92	13.26	---
EMKMFME (100 mg/kg)	15.49	21.17	20.15	44.44	17.89	---	27.48
EMKMFME (150 mg/kg)	67.65	73.09	66.83	67.01	64.91	64.17	44.96
MFMFME (50 mg/kg)	21.35	23.21	26.11	09.02	18.95	21.21	31.57
MFMFME (100 mg/kg)	51.15	44.04	35.19	51.38	43.60	42.16	87.06
MFMFME (150 mg/kg)	93.77	89.83	93.21	71.25	89.78	91.49	96.15

EMKMFME: Ethyl methyl ketone sub-fraction of total methanol extract; MFMFME: Methanol sub-fraction of total methanol extract.

Paracetamol-induced hepatotoxicity

Effect of aerial parts of *P. daemia*

Paracetamol intoxication (3 g/kg p.o.) induced a marked increase in the serum levels of GOT(118.67 ± 3.32 to 277.83 ± 23.09) , GPT (105.33 ± 5.62 to 248.83 ± 38.08), ALKP (208.67 ± 7.99 to 437.17 ± 30.95), TBL (1.13 ± 0.13 to 3.03 ± 0.22), and CHL (117.67 ± 3.59 to 293.83 ± 30.07); decrease in the levels of TPTN (7.73 ± 3.59 to 1.96 ± 0.36) and ALB (4.47 ± 0.27 to 2.20 ± 0.11) indicating acute centrilobular necrosis. The group of rats administered with AFEFEE at dose levels of 150 mg/kg p.o. showed a significant ($p<0.05$) restoration in the altered biochemical parameters due to paracetamol intoxication similar to that observed in silymarin treated group. The rats treated with EFEFEE have not shown significant changes in the elevated and depleted biochemical parameters except TPTN. The results are shown in Table 40 and Figure 19.

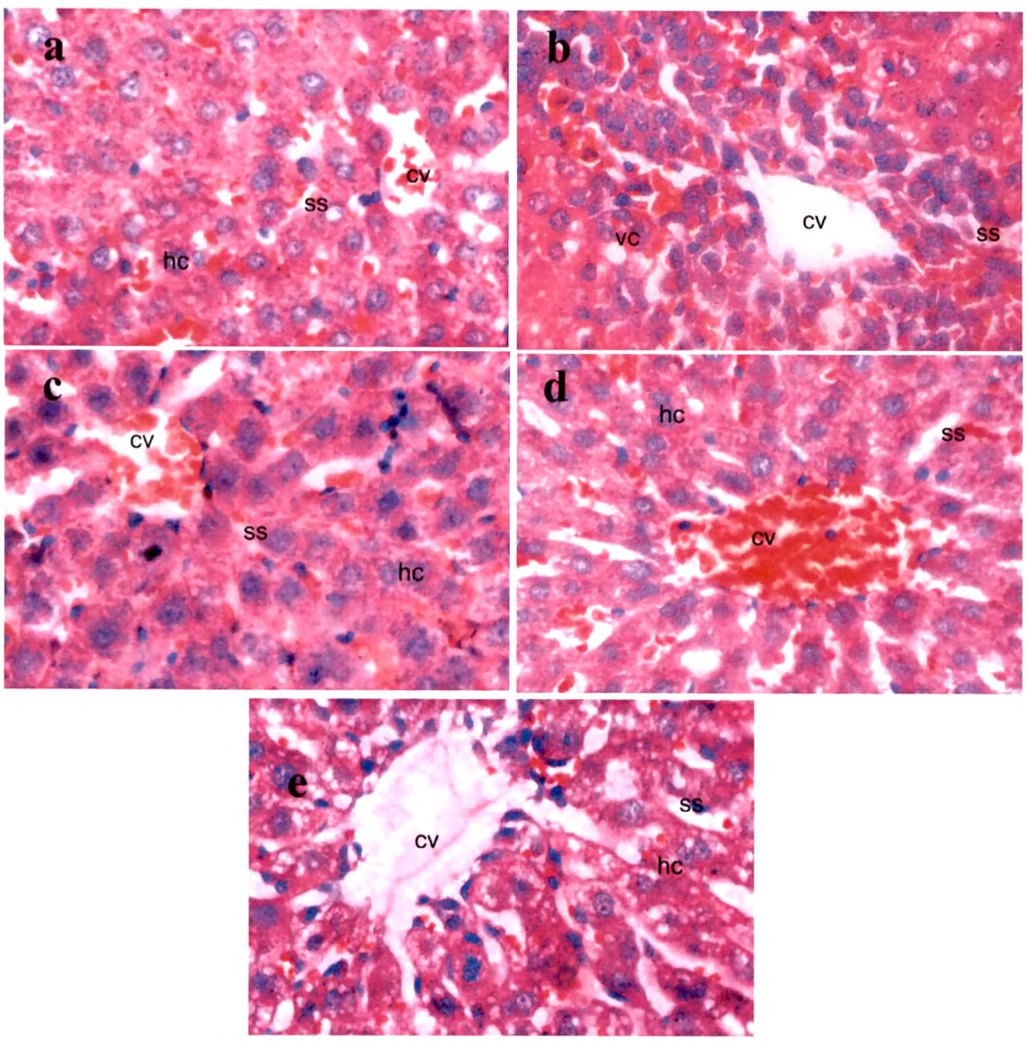
Histological examination of liver section of rats of control group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces, and central vein (Figure 19a). Histopathological examination of liver sections of rats administered with paracetamol (3 g/kg p.o.) showed disarrangement and degeneration of normal hepatic cells with intense centrilobular necrosis extending to mid zone and bridged necrosis, sinusoidal haemorrhages and dilatation (Figure 19b). Treatment with AFEFEE at dose level of 150 mg/kg

(Figure 19d) followed by paracetamol intoxication resulted the absence of necrosis, sinusoidal dilation and less disarrangement and degeneration of hepatocytes indicating marked protective activity similar to that observed in silymarin treated rat liver sections (Figure19c). Though visible changes were seen in the rats treated with EFEFEE and intoxicated with paracetamol (Figure 19e), the intensity was on lower side compared to AFEFEE treated rat liver sections.

The percentage restoration of various biochemical parameters showed by AFEFEE and EFEFEE at various dose levels against paracetamol-induced hepatotoxicity are shown in Table 41 and Graph 17. AFEFEE at dose level of 150 mg/kg exhibited maximum percentage restoration.

From Table 40, it is evident that AFEFEE at dose level of 150 mg/kg reduced significantly the elevated parameters induced by paracetamol. Further the sub-fraction increased the levels of TPTN and ALB indicating the hepatoprotective activity as stimulation of protein synthesis accelerates the regeneration process and the production of liver cells. Histopathological studies reveal that the centrilobular necrosis extending to mid zone and bridged necrosis are well reversed by the fractions thus supplementing the results of biochemical studies.

Figure 19: Photomicrographs representing effect of AFEFEE and EFEFEE against paracetamol-induced hepatotoxicity in rats



a: Normal rat liver section; b: Liver section of the rat intoxicated with paracetamol; c: Liver section of the rat treated with silymarin and intoxicated with paracetamol; d: Liver section of the rat treated with AFEFEE 150 mg/kg and intoxicated with paracetamol; e: Liver sections of the rat treated with EFEFEE 150 mg/kg and intoxicated with paracetamol. Eosin-Haematoxylin stain. 400X.

cv: central vein, vc: vacuole, ss: sinusoidal spaces, hc: hepatocytes.

Table 40: Effect of AFEFEE and EFEFEE of *P. daemia* on paracetamol-induced hepatotoxicity in rats

GROUP	GOT (IU/L)	GPT (IU/L)	ALKP (IU/L)	TBL (mg/dl)	CHL (mg/dl)	TPTN (g/dl)	ALB (g/dl)
Control	118.67 ± 3.32	105.33 ± 5.62	208.67 ± 7.99	1.13 ± 0.13	117.67 ± 3.59	7.73 ± 0.36	4.47 ± 0.27
Paracetamol	277.83 ± 23.09	248.83 ± 38.08	437.17 ± 30.95	3.03 ± 0.22	293.83 ± 30.07	1.96 ± 0.36	2.20 ± 0.11
Silymarin	112.50 ± 7.36*	107.50 ± 9.15*	202.50 ± 26.41*	1.67 ± 0.29*	127.00 ± 17.18*	6.86 ± 0.41**	4.80 ± 0.60**
AFEFEE 1	255.00 ± 41.59	228.00 ± 33.89	401.00 ± 32.93	2.53 ± 0.25	263.17 ± 32.29	2.63 ± 0.40	2.33 ± 0.55
AFEFEE 2	180.17 ± 27.81	186.67 ± 18.41	346.83 ± 38.95	2.11 ± 0.28	258.67 ± 32.41	5.03 ± 0.66**	3.21 ± 0.21
AFEFEE 3	124.33 ± 12.25*	102.33 ± 6.16*	214.17 ± 26.21*	1.37 ± 0.34*	122.83 ± 7.10*	6.72 ± 0.53**	4.30 ± 0.46**
EFEFEE 1	249.17 ± 20.88	223.83 ± 32.82	392.34 ± 31.20	2.52 ± 0.27	255.00 ± 31.07	2.90 ± 0.49	3.50 ± 0.30
EFEFEE 2	232.88 ± 41.17	220.33 ± 36.19	374.83 ± 23.36	2.10 ± 0.35	246.50 ± 20.62	2.80 ± 0.57	2.51 ± 0.21
EFEFEE 3	209.00 ± 30.71	215.83 ± 33.44	343.00 ± 42.21	2.06 ± 0.36	250.17 ± 22.92	3.88 ± 0.43**	2.71 ± 0.28
F calculated	5.82	4.92	9.06	4.35	8.52	20.25	7.04
DV	103.84	106.02	119.01	1.13	94.80	1.87	1.45

Data represents the mean ± SEM of six animals. F theoretical = 2.18($p<0.05$).

AFEFEE 1, 2 and 3: AFEFEE 50, 100 and 150 mg/kg; EFEFEE 1, 2 and 3: EFEFEE 50, 100 and 150 mg/kg; DV: Dunnett value

*Significant reduction compared to paracetamol ($p<0.05$). ** Significant increase compared to paracetamol ($p<0.05$)

Table 41: Percentage restoration of various parameters showed by AFEFEE and EFEFEE against paracetamol-induced hepatotoxicity

GROUP	GOT	GPT	ALKP	TBL	CHL	TPTN	ALB
Silymarin (100 mg/kg)	102.50	97.17	100.91	71.57	93.42	84.91	114.47
AFEFEE (50 mg/kg)	14.15	14.03	15.55	26.31	17.16	11.61	05.72
AFEFEE (100 mg/kg)	60.54	42.54	38.84	48.41	19.68	53.20	44.47
AFEFEE (150 mg/kg)	95.17	100.74	95.89	87.36	95.76	82.49	92.46
EFEFEE (50 mg/kg)	17.76	16.96	19.27	26.84	21.74	16.29	57.23
EFEFEE (100 mg/kg)	27.86	19.32	26.80	48.94	26.50	14.55	13.64
EFEFEE (150 mg/kg)	42.67	22.42	40.49	51.05	24.45	33.27	22.45

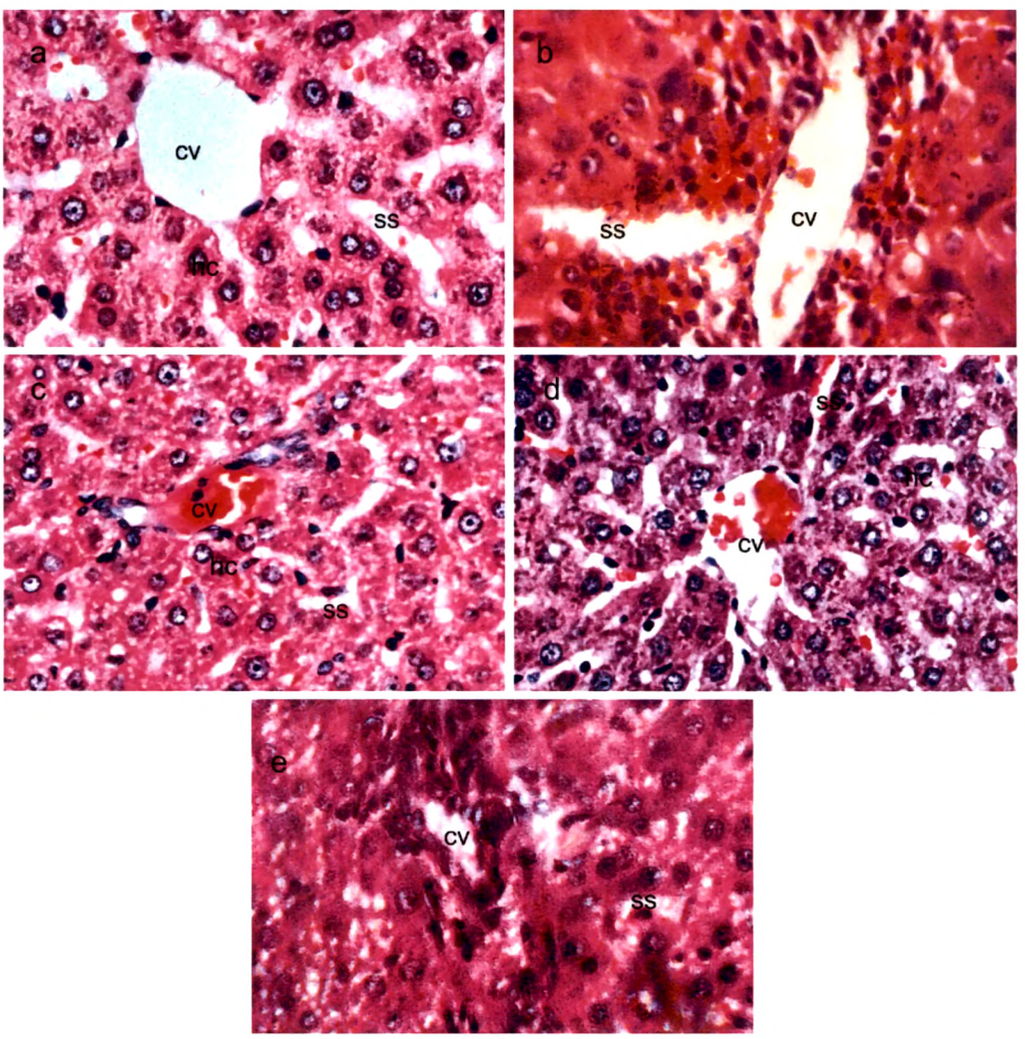
AFEFEE: Acetone sub-fraction of ethanol extract; EFEFEE: Ethanol sub-fraction of ethanol extract.

Effect of roots of *B. montanum*

Significant ($p<0.05$) increase in the serum levels of GOT (125.33 ± 2.98 to 243.16 ±16.31), GPT (79.50 ± 3.68 to 157.83 ± 9.87), ALKP (192.17 ± 5.59 to 396.17 ±14.71), TBL (1.17 ± 0.12 to 3.05 ± 0.44) and CHL (99.67 ± 3.45 to 216.34 ± 25.93); decrease in the levels of TPTN (5.65 ± 0.23 to 2.38 ± 0.34) and ALB (3.60 ± 0.25 to 1.93 ± 0.43) occurred in normal rats upon intoxication with paracetamol. MFMFME at dose levels of 150 mg/kg, exhibited a significant decrease ($p<0.05$) in all the elevated biochemical levels and significant ($p<0.05$) increase in depleted TPTN and ALB levels similar to that observed in silymarin treated group. EMKMFME at a dose level of 150 mg/kg also restored the altered biochemical parameters, due to paracetamol intoxication significantly ($p<0.05$) as observed with MFMFME. There is significant ($p<0.05$) difference in the activities exhibited by EMKMFME and silymarin i.e. the activities exhibited by EMKMFME and silymarin are not similar statistically ($p<0.05$). The results obtained are shown in Table 42 and Figure 20.

Histological examination of liver sections of control group revealed normal cellular architecture (Figure 20a) which was disturbed by paracetamol intoxication as evidenced by disarrangement and degeneration of hepatic cells with centrilobular necrosis and bridged necrosis (Figure 20b). Treatment with MFMFME 150 mg/kg, followed by paracetamol intoxication resulted the

Figure 20: Photomicrographs representing effect of EMKMFME and MFMFME against paracetamol-induced hepatotoxicity in rats



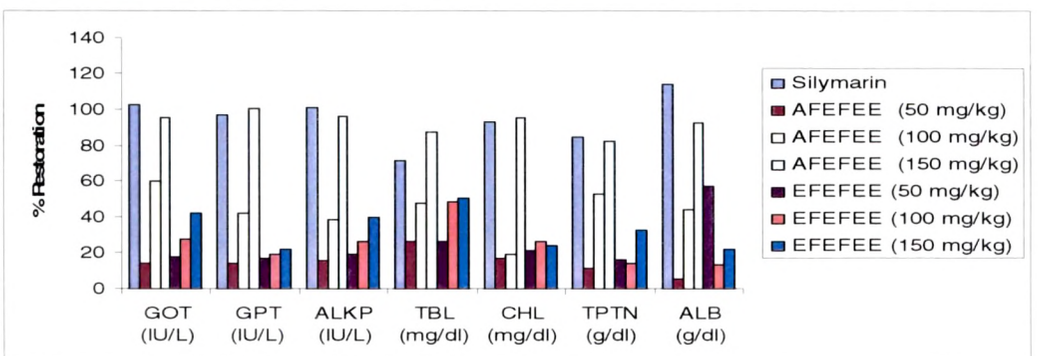
a: Normal rat liver section; b: Liver section of the rat intoxicated with paracetamol; c: Liver section of the rat treated with silymarin and intoxicated with paracetamol; d: Liver section of the rat treated with MFMFME 150 mg/kg and intoxicated with paracetamol; e: Liver sections of the rat treated with EMKMFME 150 mg/kg and intoxicated with paracetamol. Eosin-Haematoxylin stain. 400X.

cv: central vein, vc: vacuole, ss: sinusoidal spaces, hc: hepatocytes.

absence of necrosis and less disarrangement and degeneration of hepatocytes (Figure 20d) indicating marked protective activity similar to that observed in silymarin treated rat liver sections (Figure 20c). Though less visible changes are seen in the liver sections of the rats treated with EMKMFME 150 mg/kg followed by intoxication with paracetamol (Figure 20e) the intensity was less.

The percentage restoration of various biochemical parameters showed by MFMFME and EMKMFME at various dose levels against paracetamol-induced hepatotoxicity are shown in Table 43 and Graph 18. The maximum percentage restoration was obtained with MFMFME 150 mg/kg.

Graph 17: Percentage restoration of various parameters by AFEFEE and EFEFEE against paracetamol-induced hepatotoxicity



Graph 18: Percentage restoration of various parameters by EMKMFME and MFMFME against paracetamol-induced hepatotoxicity

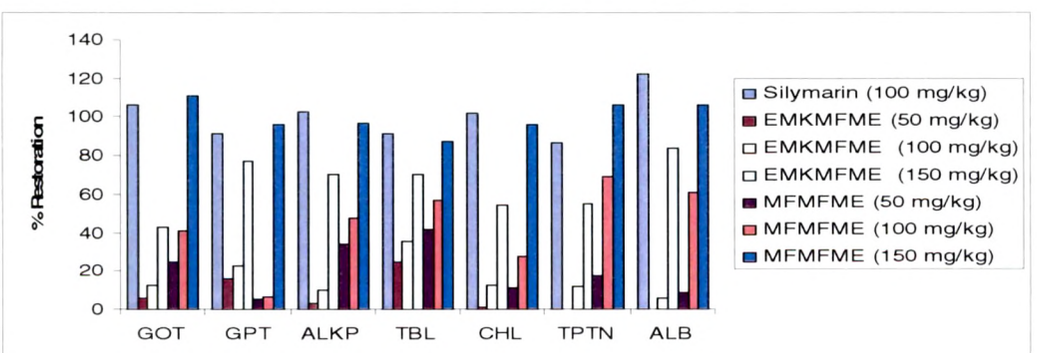


Table 42: Effect of EMKMFME and MFMFME of *B. montanum* on paracetamol-induced hepatotoxicity in rats

GROUP	GOT (IU/L)	GPT (IU/L)	ALKP (IU/L)	TBL (mg/dl)	CHL (mg/dl)	TPTN (g/dl)	ALB (g/dl)
Control	125.33 ± 02.98	79.50 ± 03.68	192.17 ± 05.59	1.17 ± 0.12	99.67 ± 03.45	5.65 ± 0.23	3.60 ± 0.25
Paracetamol	243.16 ± 16.31	157.83 ± 09.87	396.15 ± 14.71	3.05 ± 0.44	216.34 ± 25.93	2.38 ± 0.34	1.93 ± 0.43
Silymarin	116.83 ± 08.04*	86.00 ± 06.57*	186.67 ± 09.01*	1.33 ± 0.27*	96.33 ± 08.73*	5.21 ± 0.44**	3.98 ± 0.20**
EMKMFME 1	235.55 ± 20.20	145.33 ± 25.34	389.65 ± 39.40	2.58 ± 0.44	215.00 ± 20.98	2.05 ± 0.54	1.87 ± 0.43
EMKMFME 2	228.17 ± 18.93	139.83 ± 24.33	375.00 ± 36.18	2.38 ± 0.31	201.67 ± 14.39	2.78 ± 0.52	2.03 ± 0.47
EMKMFME 3	192.17 ± 08.96*	96.81 ± 06.95*	251.33 ± 32.81*	1.72 ± 0.18*	152.50 ± 07.28*	4.18 ± 0.54**	3.32 ± 0.22**
MFMFME 1	213.50 ± 09.46	153.50 ± 19.44	326.50 ± 32.39	2.27 ± 0.39	202.50 ± 20.21	2.95 ± 0.34	2.08 ± 0.49
MFMFME 2	194.17 ± 11.82	152.33 ± 18.30	298.00 ± 31.70	1.98 ± 0.32	183.50 ± 22.32	4.65 ± 0.45**	2.95 ± 0.35
MFMFME 3	110.67 ± 07.01*	82.17 ± 06.08*	198.50 ± 06.53*	1.40 ± 0.37*	103.00 ± 05.51*	5.86 ± 0.48**	3.71 ± 0.36**
F calculated	17.23	04.82	10.48	03.54	09.93	10.66	04.91
DV	49.82	60.86	104.57	01.32	63.74	01.75	01.39

Data represents the mean ± SEM of six animals. F theoretical = 2.18($p<0.05$). Silymarin: 100 mg/kg

EMKMFME 1, 2 and 3: EMKMFME 50, 100 and 150 mg/kg; MFMFME 1, 2 and 3: MFMFME 50, 100 and 150 mg/kg; DV: Dunnett value

*Significant reduction compared to paracetamol ($p<0.05$). ** Significant increase compared to paracetamol ($p<0.05$)

Table 43: Percentage restoration of various parameters showed by EMKMFME and MFMFME against paracetamol-induced hepatotoxicity

GROUP	GOT	GPT	ALKP	TBL	CHL	TPTN	ALB
Silymarin (100 mg/kg)	106.11	91.22	102.65	91.48	102.01	86.54	122.75
EMKMFME (50 mg/kg)	06.33	15.87	03.18	24.99	01.14	---	---
EMKMFME (100 mg/kg)	12.59	22.80	10.37	35.63	12.46	12.23	05.98
EMKMFME (150 mg/kg)	42.83	77.47	70.97	70.74	54.26	55.04	83.83
MFMFME (50 mg/kg)	24.91	05.49	34.13	41.48	11.76	17.43	08.98
MFMFME (100 mg/kg)	41.15	06.98	48.10	56.91	27.91	69.41	61.07
MFMFME (150 mg/kg)	111.29	96.08	96.85	87.76	96.34	106.41	106.58

EMKMFME: Ethyl methyl ketone sub-fraction of methanol extract; MFMFME: Methanol sub-fraction of methanol extract.

From Table 42, it was evident that MFMFME and EMKMFME at dose level of 150 mg/kg restored significantly the altered parameters induced by paracetamol. Histopathological studies revealed that the centrilobular necrosis extending to mid zone and bridged necrosis are well reversed by the fractions thus supplementing the results of biochemical studies.

Thioacetamide-induced hepatotoxicity

Effect of aerial parts of *P. daemia*

Administration of thioacetamide (100 mg/kg i.p.) induced a marked increase in the serum levels of GOT, GPT, ALKP, TBL, and CHL; decrease in the levels of TPTN and ALB, indicating parenchymal cell necrosis. A significant decrease ($p<0.05$) in the elevated levels of biochemical parameters and significant ($p<0.05$) increase in reduced TPTN and ALB levels was observed with the groups of rats which received AFEFEE (150 mg/kg) as observed with silymarin treated group. The rats treated with EFEFEE have not shown significant changes in the altered biochemical parameters except GPT and ALKP. Results of thioacetamide-induced hepatotoxicity are shown in Table 44 and Figure21.

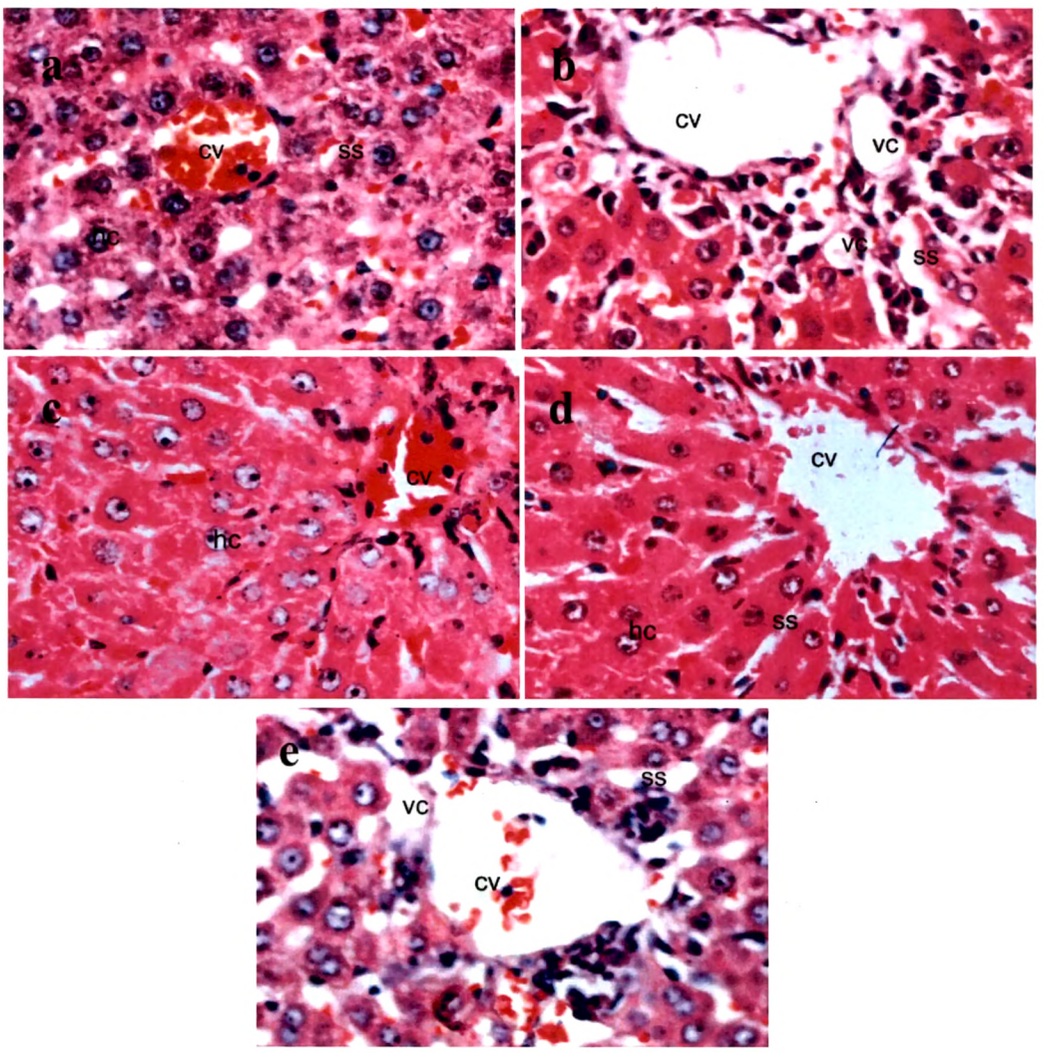
Histological examination of liver section of control group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces, and central vein (Figure 21a). Histopathological examination of liver sections of rats administered with TAA showed hepatic cells with severe toxicity characterised by centrilobular necrosis along with various gradation of fatty

changes comprising of tiny to large sized vacuoles, disarrangement of hepatic cells with blood pooling in sinusoidal spaces (Figure 21b). The liver sections of the rats administered with 150 mg/kg p.o. of AFEFEE (Figure 21d) followed by TAA intoxication, showed less visible changes as observed with silymarin treated rat liver sections (Figure 21c) there by suggesting the protective effect of the extracts. Restoration of altered changes was not seen in the rat liver sections treated with EFEFEE (Figure 21e) when compared to AFEFEE and silymarin treated rat liver sections.

The percentage restorations of various biochemical parameters showed by AFEFEE and EFEFEE at various dose levels against thioacetamide-induced hepatotoxicity are sown in Table 45 and Graph 19.

From Table 44 and Figure 21 it was evident that AFEFEE at the dose level of 150 mg/kg produced maximum effect by restoring the biochemical parameters altered by thioacetamide. Histopathological studies show that pre-treatment with the AFEFEE of *P. daemia* protects the liver against toxic effects of thioacetamide similar to silymarin.

Figure 21: Photomicrographs representing effect of AFEFEE and EFEFEE against thioacetamide-induced hepatotoxicity in rats



a: Normal rat liver section; b: Liver section of the rat intoxicated with thioacetamide; c: Liver section of the rat treated with silymarin and intoxicated with thioacetamide; d: Liver section of the rat treated with AFEFEE 150 mg/kg and intoxicated with thioacetamide; e: Liver sections of the rat treated with EFEFEE 150 mg/kg and intoxicated with thioacetamide. Eosin-Haematoxylin stain. 400X.

cv: central vein, vc: vacuole, ss: sinusoidal spaces, hc: hepatocytes.

Table 44: Effect of AFEFEE and EFEFEE of *P. daemia* on thioacetamide-induced hepatotoxicity in rats

GROUP	GOT (IU/L)	GPT (IU/L)	ALKP (IU/L)	TBL (mg/dl)	CHL (mg/dl)	TPTN (g/dl)	ALB (g/dl)
Control	115.17 ± 5.85	124.83 ± 9.52	185.33 ± 5.54	1.18 ± 0.11	110.33 ± 3.95	7.75 ± 0.42	4.25 ± 0.51
Thioacetamide	275.67 ± 28.89	297.17 ± 32.21	414.50 ± 32.99	2.83 ± 0.26	252.34 ± 20.26	1.98 ± 0.30	1.28 ± 0.25
Silymarin	120.67 ± 11.64*	118.33 ± 10.67*	191.67 ± 13.27*	1.52 ± 0.31*	106.83 ± 6.50*	8.08 ± 1.46**	3.98 ± 0.50**
AFEFEE 1	257.17 ± 30.51	246.17 ± 29.19	345.17 ± 26.64	2.41 ± 0.25	222.50 ± 24.78	2.45 ± 0.48	1.81 ± 0.34
AFEFEE 2	212.50 ± 33.45	213.50 ± 23.39	287.17 ± 29.20*	1.92 ± 0.31	169.17 ± 18.20*	3.06 ± 0.68	2.26 ± 0.27
AFEFEE 3	109.66 ± 8.30*	131.00 ± 14.91*	182.00 ± 11.42*	1.37 ± 0.20*	114.83 ± 8.05*	6.90 ± 0.53**	4.30 ± 0.84**
EFEFEE 1	253.67 ± 19.74	223.17 ± 30.31	335.16 ± 42.86	2.36 ± 0.30	233.00 ± 16.65	2.26 ± 0.35	1.50 ± 0.25
EFEFEE 2	264.00 ± 20.08	203.50 ± 21.06*	334.66 ± 20.77	2.16 ± 0.32	223.67 ± 13.44	2.93 ± 0.34	1.78 ± 0.24
EFEFEE 3	216.17 ± 20.67	232.67 ± 25.48	266.83 ± 21.51*	2.40 ± 0.25	207.50 ± 11.44	2.10 ± 0.31	2.10 ± 0.27
F calculated	9.83	7.02	10.79	4.32	14.69	16.09	8.02
DV	86.04	91.17	98.73	1.05	59.36	2.53	1.70

Data represents the mean ± SEM of six animals. F theoretical = 2.18($p<0.05$).

AFEFEE 1, 2 and 3: AFEFEE 50, 100 and 150 mg/kg; EFEFEE 1, 2 and 3: EFEFEE 50, 100 and 150 mg/kg; DV: Dunnett value

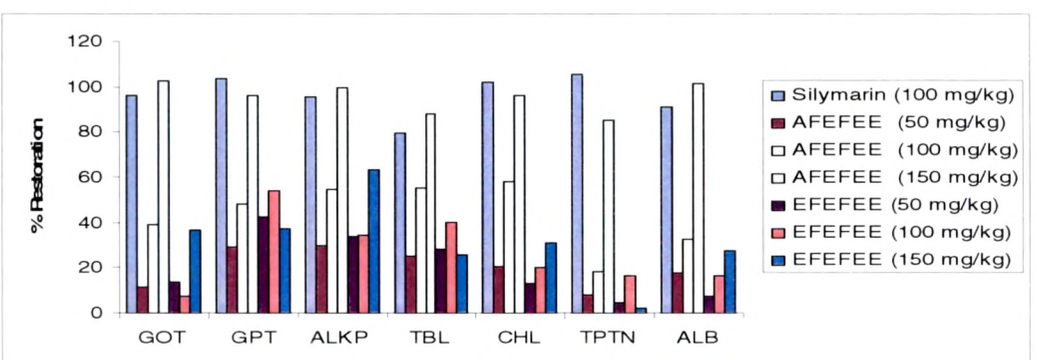
*Significant reduction compared to thioacetamide ($p<0.05$). ** Significant increase compared to thioacetamide ($p<0.05$)

Table 45: Percentage restoration of various parameters showed by AFEFEE and EFEFEE against thioacetamide-induced hepatotoxicity

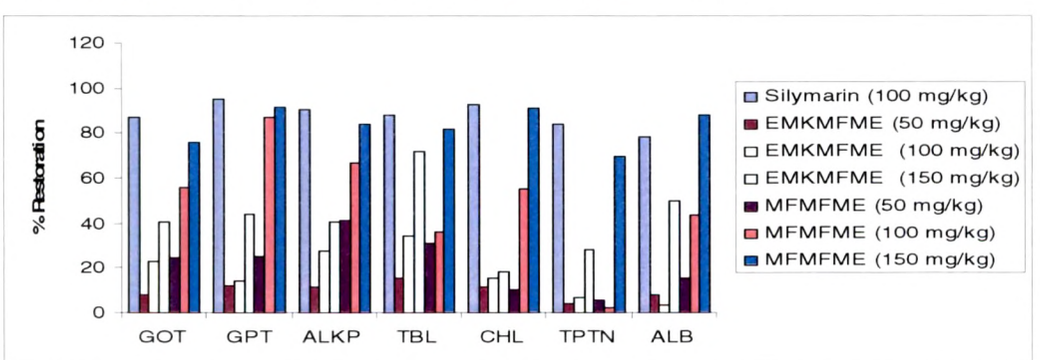
GROUP	GOT	GPT	ALKP	TBL	CHL	TPTN	ALB
Silymarin (100 mg/kg)	96.10	103.72	95.81	79.38	101.85	105.71	90.91
AFEFEE (50 mg/kg)	11.47	29.58	29.81	25.45	20.88	08.14	17.84
AFEFEE (100 mg/kg)	39.16	48.52	54.75	55.14	58.21	18.71	32.99
AFEFEE (150 mg/kg)	102.92	96.37	99.97	88.47	96.25	85.26	101.68
EFEFEE (50 mg/kg)	13.64	42.92	34.12	28.48	13.53	04.85	07.41
EFEFEE (100 mg/kg)	07.23	54.32	34.33	40.60	20.06	16.46	16.83
EFEFEE (150 mg/kg)	36.89	37.41	63.49	26.05	31.38	02.07	27.60

AFEFEE: Acetone sub-fraction of ethanol extract; EFEFEE: Ethanol sub-fraction of ethanol extract.

Graph 19: Percentage restoration of various parameters by AFEFEE and EFEFEE against thioacetamide-induced hepatotoxicity



Graph 20: Percentage restoration of various parameters by EMKMFME and MFMFME against thioacetamide-induced hepatotoxicity



Effect of roots of *B. montanum*

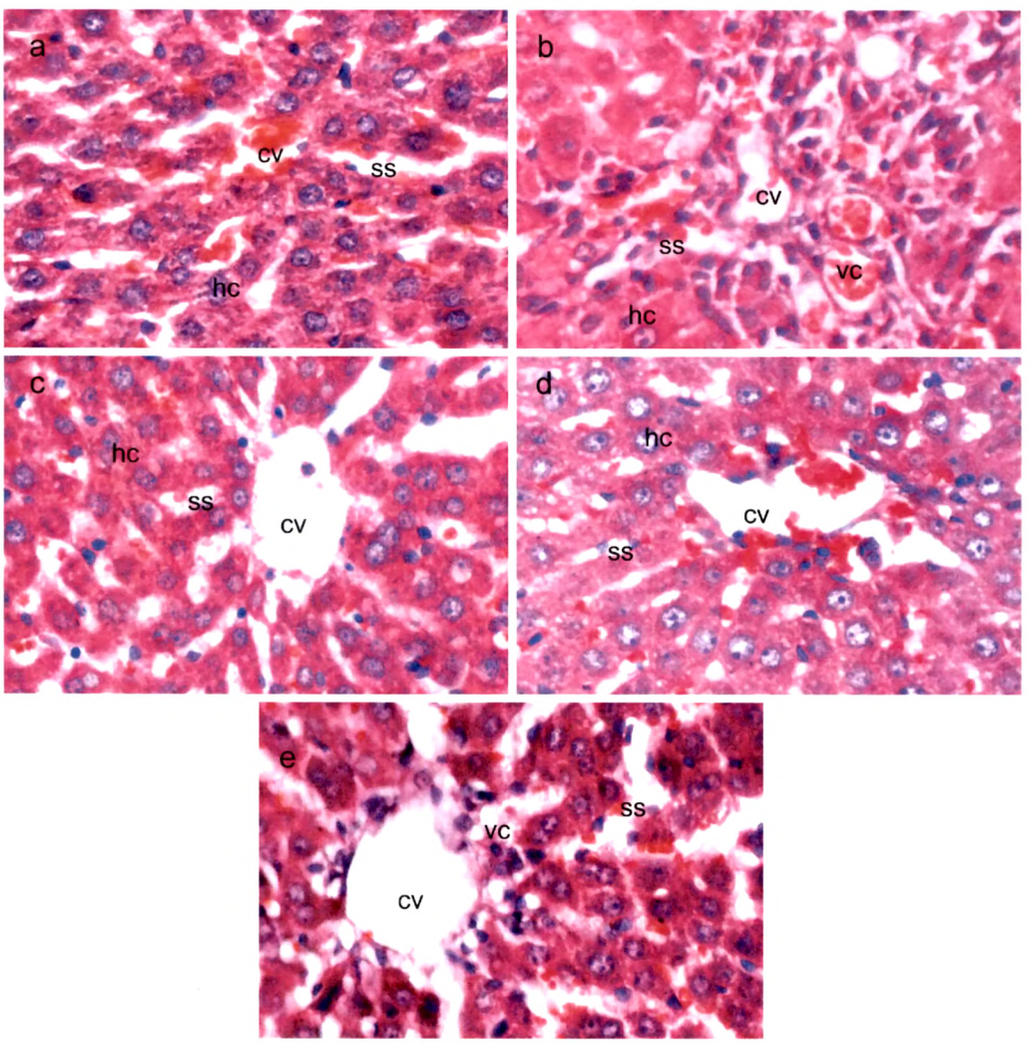
Significant ($p<0.05$) increase in the serum levels of GOT (100.50 ± 4.43 to 259.67 ± 19.58), GPT (101.67 ± 7.01 to 321.50 ± 21.74), ALKP (199.83 ± 8.35 to 412.17 ± 19.71), TBL (1.67 ± 0.27 to 4.73 ± 0.69) and CHL (94.67 ± 4.48 to 211.33 ± 21.13); decrease in the levels of TPTN (6.75 ± 0.26 to 2.71 ± 0.17) and ALB (4.02 ± 0.28 to 1.68 ± 0.13) occurred in normal rats upon intoxication. with thioacetamide. MFMFME at dose levels of 150 mg/kg, exhibited a significant decrease ($p<0.05$) in all the elevated biochemical levels and significant ($p<0.05$) increase in depleted TPTN and ALB levels similar to that observed in silymarin treated group. EMKMFME at a dose level of 150 mg/kg also restored the altered biochemical parameters except GOT and CHL, due to thioacetamide intoxication significantly ($p<0.05$) as observed with MFMFME treated group. There was significant ($p<0.05$) difference in the activities exhibited by EMKMFME and silymarin i.e. the activities exhibited by EMKMFME and silymarin are not statistically ($p<0.05$) similar. The results obtained are shown in Table 46 and Figure 22.

Histological examination of liver section of control group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces, and central vein (Figure 22a). Histopathological examination of liver sections of rats administered with thioacetamide showed hepatic cells with severe toxicity characterised by centrilobular necrosis along with various gradation of fatty changes comprising of tiny to large sized vacuoles, disarrangement of hepatic cells with blood pooling in sinusoidal spaces (Figure 22b). The liver sections of the rats administered with 150 mg/kg p.o. of MFMFME (Figure 22d) followed by thioacetamide intoxication, showed less visible changes similar to that observed in silymarin treated rat liver sections (Figure 22c) there by suggesting the protective effect of the extracts. Though less visible changes are seen in the liver sections of the rats treated with EMKMFME 150 mg/kg followed by thioacetamide intoxication (Figure 22e), the intensity was on lower side.

The percentage restoration of various biochemical parameters showed by MFMFME and EMKMFME at various dose levels against thioacetamide-

induced hepatotoxicity are shown in Table 47 and Graph 20. The maximum percentage restoration was obtained with MFMFME 150 mg/kg.

Figure 22: Photomicrographs representing effect of EMKMFME and MFMFME against thioacetamide-induced hepatotoxicity in rats



a: Normal rat liver section; b: Liver section of the rat intoxicated with thioacetamide; c: Liver section of the rat treated with silymarin and intoxicated with thioacetamide; d: Liver section of the rat treated with MFMFME 150 mg/kg and intoxicated with thioacetamide; e: Liver sections of the rat treated with EMKMFME 150 mg/kg and intoxicated with thioacetamide. Eosin-Haematoxylin stain. 400X.

cv: central vein, vc: vacuole, ss: sinusoidal spaces, hc: hepatocytes

Table 46: Effect of EMKMFME and MFMFME of *B. montanum* against thioacetamide-induced hepatotoxicity in rats

GROUP	GOT (IU/L)	GPT (IU/L)	ALKP (IU/L)	TBL (mg/dl)	CHL (mg/dl)	TPTN (g/dl)	ALB (g/dl)
Control	100.50 ± 04.43	101.67 ± 07.01	199.83 ± 08.35	1.67 ± 0.27	94.67 ± 04.48	6.75 ± 0.26	4.02 ± 0.28
Thioacetamide	259.67 ± 19.58	321.50 ± 21.74	412.17 ± 19.71	4.73 ± 0.69	211.33 ± 21.13	2.71 ± 0.17	1.68 ± 0.13
Silymarin	121.00 ± 06.51*	109.65 ± 07.76*	219.33 ± 13.79*	2.08 ± 0.39*	101.83 ± 06.32*	6.11 ± 0.31**	3.51 ± 0.27**
EMKMFME 1	247.17 ± 30.18	294.50 ± 26.35	387.00 ± 23.96	4.25 ± 0.66	198.00 ± 11.43	2.88 ± 0.21	1.87 ± 0.26
EMKMFME 2	223.33 ± 29.23	289.33 ± 17.97	353.17 ± 22.83	3.67 ± 0.64	192.67 ± 15.02	3.00 ± 0.26	1.76 ± 0.21
EMKMFME 3	194.50 ± 07.49	223.31 ± 24.81*	325.16 ± 29.54*	2.53 ± 0.33*	189.50 ± 10.05	3.85 ± 0.18**	2.85 ± 0.27**
MFMFME 1	220.17 ± 27.97	264.50 ± 29.04	321.33 ± 33.24	3.78 ± 0.63	198.83 ± 12.68	2.95 ± 0.17	2.05 ± 0.32
MFMFME 2	170.83 ± 22.04	128.50 ± 09.34*	269.17 ± 23.39*	3.61 ± 0.77	145.81 ± 13.29*	2.61 ± 0.43	2.70 ± 0.38
MFMFME 3	138.50 ± 06.89*	117.50 ± 08.16*	232.67 ± 12.03*	2.23 ± 0.29*	104.33 ± 05.78*	5.53 ± 0.27**	3.75 ± 0.36**
F calculated	08.11	22.97	36.94	03.70	15.51	36.67	09.85
DV	77.88	73.77	96.43	02.17	47.55	01.05	01.12

Data represents the mean ± SEM of six animals. F theoretical = 2.18($p<0.05$). Silymarin dose: 100 mg/kg

EMKMFME 1, 2 and 3: EMKMFME 50, 100 and 150 mg/kg; MFMFME 1, 2 and 3: MFMFME 50, 100 and 150 mg/kg; DV: Dunnett value

*Significant reduction compared to thioacetamide ($p<0.05$). ** Significant increase compared to thioacetamide ($p<0.05$)

Table 47: Percentage restoration of various parameters showed by EMKMFME and MFMFME against thioacetamide-induced hepatotoxicity

GROUP	GOT	GPT	ALKP	TBL	CHL	TPTN	ALB
Silymarin (100 mg/kg)	87.36	95.32	90.63	88.21	93.07	84.15	78.19
EMKMFME (50 mg/kg)	07.87	12.15	11.82	15.68	11.33	04.21	08.12
EMKMFME (100 mg/kg)	22.83	14.49	27.73	34.63	15.86	07.17	03.41
EMKMFME (150 mg/kg)	41.05	44.17	40.89	71.87	18.55	28.21	49.99
MFMFME (50 mg/kg)	24.88	25.65	41.75	31.03	10.62	05.94	15.81
MFMFME (100 mg/kg)	55.96	86.85	67.21	36.59	55.67	02.47	43.58
MFMFME (150 mg/kg)	76.34	91.80	84.36	81.67	90.95	69.79	88.45

EMKMFME: Ethyl methyl ketone sub-fraction of total methanol extract; MFMFME: Methanol sub-fraction of total methanol extract.

From Table 46 and Figure 22 it was evident that MFMFME at the dose level of 150 mg/kg produced significant ($p<0.05$) hepatoprotective effect by restoring the biochemical parameters altered by thioacetamide. Histopathological studies show that pre-treatment with the MFMFME of *B. montanum* protects the liver against toxic effects of thioacetamide, similar to silymarin. EMKMFME at the dose level of 150 mg/kg also produced significant ($p<0.05$) hepatoprotective effect by restoring the biochemical parameters altered by thioacetamide except GOT and CHL. Histopathological studies show that pre-treatment with the EMKMFME of *B. montanum* protects the liver against toxic effects of thioacetamide as observed with MFMFME, however with less intensity.

The hepatotoxicity of CCl_4 is due to metabolic formation of highly reactive trichloromethyl free radical which attacks the polyunsaturated fatty acids of the membrane of the endoplasmic reticulum. The initial damage produced is highly localised in the endoplasmic reticulum which results in loss of cytochrome P_{450} leading to its functional failure with a decrease in protein synthesis and accumulation of triglycerides, a characteristic of CCl_4 poisoning. If the damage is severe, it leads to disturbances in the water and electrolyte balance of hepatocytes leading to an abnormal increase in liver enzymes in plasma, there by impairing mitochondrial functions, followed by hepatocellular necrosis (Recknagel, 1967; Slater, 1966).This is evidenced by an elevation in

the serum marker enzymes and decreased protein synthesis. The ability of the extracts tested i.e. EE, EFEE and AFEFEE from aerial parts of *P. daemia* and ME, MFME, MFMFME and EMKMFME from roots of *B. montanum* to protect the liver against the CCl_4-induced hepatotoxicity may due to the stabilization of endoplasmic reticulum as well as repair of hepatic tissue damages caused by the CCl_4 leading to hepatic regeneration.

Paracetamol produces hepatic necrosis when ingested in large doses. It is metabolised in the liver primarily to glucuronide and sulphate conjugates. Paracetamol toxicity is due to formation of toxic metabolites when a part of it is metabolised by cytochrome P_{450}. Induction of cytochrome P_{450} or depletion of glutathione is a prerequisite for paracetamol induced hepatotoxicity (Savides et al., 1983; Rao and Mishra, 1998a). Therefore the hepato-protective activity of the EE, EFEE and AFEFEE from aerial parts of *P. daemia* and ME, MFME, MFMFME and EMKMFME from roots of *B. montanum* against paracetamol induced hepatotoxicity may be due to inhibition of cytochrome P_{450}; stimulation of hepatic regeneration or activation of the functions of reticuloendothelial systems.

Administration of a single dose of thioacetamide in rats produces centrilobular hepatic necrosis. It get metabolises to a toxic metabolite thioacetamide-S-oxide, a direct hepatotoxin, which is further metabolised, at least in part, by cytochrome P_{450} monooxygenases. The subsequent product formed exerts hepatotoxicity by binding to hepatocyte macromolecules and causes centrilobular necrosis by generation of reactive oxygen species. The chronic thioacetamide exposure leads to cirrhosis in rats. It induces toxicity by altering semipermeable character of the cell membrane of hepatocytes resulting in an increased influx of calcium ions and leading to necrosis and finally death (Gallagher et al., 1956; Neal and Halpert, 1982). Therefore extracts under study antagonise the effect of thioacetamide by inhibiting cytochrome P_{450} or by acting either as membrane stabiliser, thereby preventing the distortion of the cellular ionic environment associated with thioacetamide intoxication, or by preventing interaction of thioacetamide with

the transcriptional machinery of the cells resulting in regeneration of hepatic cells.

Thus the hepatoprotective activity of these extracts and fractions may be due to their ability to affect the cytochrome P_{450} mediated functions or stabilisation of endoplasmic reticulum resulting in hepatic regeneration.

In literature many authors reported the hepatoprotective activity of flavonoid compounds. Galisteo et al., (2006) reported the hepatoprotective activity of flavonoids of *Rosmarinus tomentosus*. The hepatoprotective effect of quercetin was reported by Janbaz et al., (2004). Protective effect of rutin was reported by Janbaz et al., (2002). Silymarin obtained from *Silybum marianum* is a good hepatoprotective agent (Hiroshi and Yoshinobu, 1988). In accordance with these results, it may be hypothesized that flavonoids with their anti-oxidant properties, which are present in EE, EFEE and AFEFEE of *P.daemia*; ME, MFME, EMKMFME and MFMFME of *B. montanum*, are responsible for the hepatoprotective activity.

3.4.2.2 Hepatoprotective activity in vitro

The sub-fractions AFEFEE and EFEFEE of TE of aerial parts of *P. daemia* and EMKMFME and MFMFME of ME of roots of *B. montanum* were subjected to in vitro hepatoprotective screening using carbon tetrachloride, paracetamol and thioacetamide models on primary cultured rat hepatocytes.

Isolation and culturing of hepatocytes:

Hepatocytes were isolated from rat liver according to the method of Sarkar and Sil with some modifications. The method adopted by Tingstrom and Obrink with slight modifications was used for the purpose of primary culture of hepatocytes. The percent viability of hepatocytes obtained after isolation and primary culturing was 97.25 in normal conditions.

Hepatic cytotoxicity testing:

The toxicity of the fractions to the hepatocytes was judged by incubating the isolated hepatocytes with the fractions individually with the compounds at the concentration levels of 500, 1000 and µg/ml. The results of hepatic cytotoxicity of AFEFEE and EFEFEE; EMKMFME and MFMFME are shown in Table 48 and 49 respectively.

Table 48: Effect of AFEFEE and EFEFEE on primary cultured rat hepatocytes

Group	Viable cells (%)	TPTN
Control	95.16 ± 1.12	4.01 ± 0.08
AFEFEE 500 µg/ml	92.17 ± 1.81*	3.93 ± 0.02*
AFEFEE 1000 µg/ml	90.33 ± 0.80*	3.90 ± 0.03*
AFEFEE 1500 µg/ml	89.62 ± 0.32*	3.85 ± 0.03 *
EFEFEE 500 µg/ml	93.68 ± 0.88*	4.04 ± 0.04*
EFEFEE 1000 µg/ml	93.94 ± 0.76*	3.82 ± 0.09*
EFEFEE 1500 µg/ml	92.02 ± 1.18*	3.86 ± 0.13 *
F calculated	2.00	1.31
Dunnett value	4.41	0.29

F theoretical: 4.32 ($p<0.01$); *not significant compared to control ($p<0.05$)

The values indicated in the Table 48 and 49 indicate the effect of these fractions on percent viability and TPTN content of the hepatocytes. The differences in the values of percent viability and protein content of hepatocytes incubated with the fractions were insignificant ($p<0.05$), compared to control indicating no toxic effect on the hepatocytes.

Table 49: Effect of EMKMFME and MFMFME on primary cultured rat hepatocytes

Group	Viable cells (%)	TPTN
Control	96.45 ± 0.54	3.95 ± 0.10
EMKMFME 500 µg/ml	94.25 ± 0.55*	3.91 ± 0.02*
EMKMFME 1000 µg/ml	93.27 ± 1.75*	3.86 ± 0.03*
EMKMFME 1500 µg/ml	92.55 ± 0.33*	3.82 ± 0.03*
MFMFME 500 µg/ml	93.19 ± 1.71*	4.01 ± 0.02*
MFMFME 1000 µg/ml	93.46 ± 0.39*	3.87 ± 0.05*
MFMFME 1500 µg/ml	92.50 ± 0.09*	3.75 ± 0.03*
F calculated	1.88	2.60
Dunnett value	5.67	0.21

F theoretical: 4.32 ($p<0.01$); *not significant compared to control ($p<0.05$)

Hepatoprotective activity:

CCl_4 -induced hepatotoxicity

Effect of aerial parts of *P. daemia*:

Incubation of hepatocytes with CCl_4 (10 µl/ml) resulted in the induction of sub maximal toxicity which was indicated by 64.78% and 54.59% depletion in viability and TPTN content of hepatocytes respectively. Similarly an elevation about 268.45% and 267.06% of GOT and GPT levels were observed respectively upon intoxication with CCl_4.

Hepatocytes treated with AFEFEE in the concentrations of 100-1000 µg/ml showed concentration dependant significant ($p<0.05$) protective effect by restoring the viability of hepatocytes (40.36-79.20%), TPTN content (24.08-63.63%), GOT (28.16-88.48%) and GPT (36.05-83.87%). EFEFEE at a concentration of 1000 µg/ml also showed significant ($p<0.05$) protective effect

Table 50: Effect of *P. daemia* against CCl_4-induced toxicity on rat hepatocytes

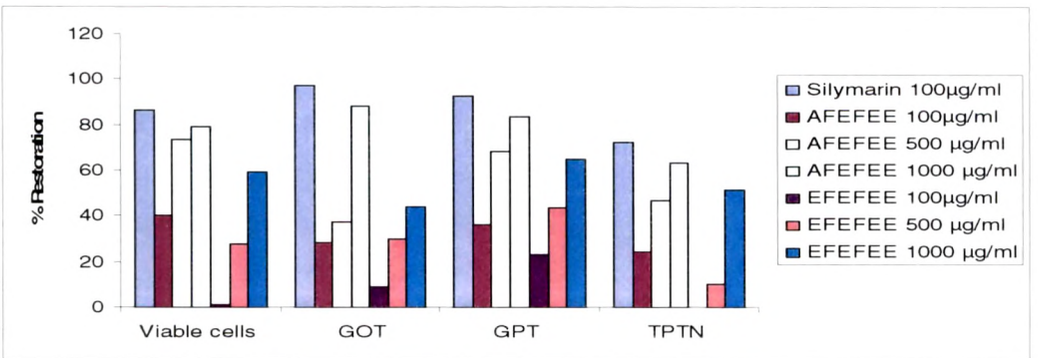
Group	Viable cells (%)	GOT (IU/L)	GPT (IU/L)	TPTN (g/dl)
Control	93.88 ± 0.81	18.70 ± 0.46	21.53 ± 0.63	4.03 ± 0.09
CCl_4 10 µl/ml	33.06 ± 1.14	50.20 ± 0.61	57.50 ± 0.94	1.83 ± 0.14
Silymarin 100µg/ml	85.84 ± 1.19 * (86.77)	19.46 ± 0.35* (97.59)	24.10 ± 0.37* (92.85)	3.43 ± 0.14** (72.72)
AFEFEE 100µg/ml	57.63 ± 1.58* (40.39)	41.33 ± 0.62* (28.16)	44.53 ± 1.25* (36.05)	2.36 ± 0.21** (24.08)
AFEFEE 500 µg/ml	77.60 ± 0.57* (73.72)	38.50 ± 0.45* (37.14)	32.80 ± 0.65* (68.66)	2.86 ± 0.12** (46.81)
AFEFEE 1000 µg/ml	81.24 ± 1.67* (79.20)	22.33 ± 0.50* (88.48)	27.33 ± 0.69* (83.87)	3.23 ± 0.18** (63.63)
EFEFEE 100µg/ml	33.67 ± 1.82 (01.00)	47.36 ± 0.75* (09.02)	49.20 ± 0.63* (23.07)	1.53 ± 0.08
EFEFEE 500 µg/ml	49.87 ± 2.46* (27.63)	40.70 ± 0.71* (30.16)	41.90 ± 0.81* (43.36)	2.06 ± 0.11 (10.45)
EFEFEE 1000 µg/ml	69.13 ± 0.44* (59.29)	36.26 ± 0.49 * (44.25)	34.06 ± 1.10* (65.16)	2.96 ± 0.20** (51.35)
F calculated	241.01	446.7	212.05	44.67
Dunnett value	05.95	02.34	03.44	0.47

Data represents the mean ± SEM of three values; Values in parenthesis indicate percentage protection against CCl_4. AFEFEE: Acetone sub-fraction of ethanol extract; EFEFEE: Ethanol sub-fraction of ethanol extract.

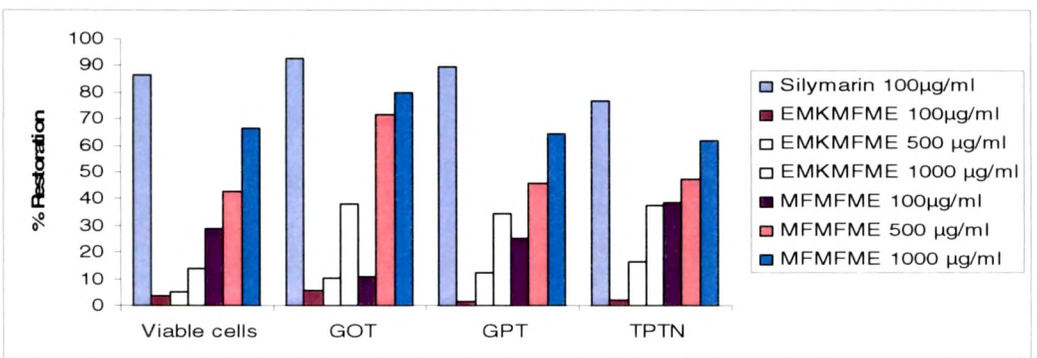
F theoretical: 5.64 ($p<0.01$). * Significant reduction compared to CCl_4 ($p<0.05$). ** Significant increase compared to CCl_4 ($p<0.05$).

by altering the viability of cells (59.29%), TPTN (51.35%), GOT 44.25%) and GPT (65.16%), while the positive control silymarin showed significant ($p<0.05$) protective effect by restoring viability (86.77), TPTN (72.72), GOT (97.59) and GPT (92.85). EFEFEE at dose level of 500 µg/ml also restored significantly ($p<0.05$) the viability (27.63), GOT (30.16) and GPT (43.36) levels. The maximum protection was seen with AFEFEE 1000 µg/ml and the activity was statistically ($p<0.05$) similar to the activity exhibited by silymarin. Results are shown in Table 50 and Graph 21.

Graph 21: Percentage restoration of various parameters by *P. daemia* against CCl_4-induced toxicity in vitro



Graph 22: Percentage restoration of various parameters by *B. montanum* against CCl_4-induced toxicity in vitro



Effect of roots of *B. montanum*:

Incubation of hepatocytes with 10 µl/ml solution of CCl_4 resulted in the induction of significant ($p<0.05$) sub maximal toxicity which was indicated by 62.32% and 56.60% depletion in viability and TPTN content of hepatocytes respectively. Similarly an elevation about 250.02% and 246.92% of GOT and GPT levels are observed respectively upon intoxication with CCl_4.

Hepatocytes treated with MFMFME in concentrations of 500-1000 µg/ml showed a concentration dependant significant ($p<0.05$) protective effect by restoring the viability of hepatocytes (43.03-66.69%), TPTN content (47.57-61.67%), GOT (71.82-79.65%) and GPT (45.63-64.18%). EMKMFME at a concentration of 1000 µg/ml also showed significant ($p<0.05$) protective effect by restoring the viability of cells (13.84%), TPTN (37.44%), GOT (38.03%)

and GPT (34.07%). The results obtained are shown in Table 51 and Graph 22.

MFMFME at dose level of 1000 µg/ml showed maximum protection though the activity was not similar ($p<0.05$) to that of silymarin.

Table 51: Effect of *B. montanum* against CCl_4-induced toxicity on rat hepatocytes

Group	Viable cells (%)	GOT (IU/L)	GPT (IU/L)	TPTN (g/dl)
Control	95.84 ± 0.34	19.11 ± 0.08	20.67 ± 0.29	4.01 ± 0.02
CCl_4 10 µl/ml	36.11 ± 0.61	47.78 ± 0.27	51.04 ± 0.33	1.74 ± 0.12
Silymarin 100µg/ml	87.82 ± 0.90* (86.35)	21.13 ± 0.52* (92.74)	23.83 ± 0.17* (89.52)	3.42 ± 0.08** (77.01)
EMKMFME 100µg/ml	38.22 ± 0.60 (03.52)	46.21 ± 0.63 (05.46)	50.62 ± 0.25 (01.38)	1.79 ± 0.06 (02.20)
EMKMFME 500 µg/ml	39.11 ± 1.14 (05.01)	44.79 ± 0.90 (10.40)	47.22 ± 0.03 (12.56)	2.12 ± 0.04 (16.73)
EMKMFME 1000 µg/ml	44.40 ± 0.56* (13.84)	36.85 ± 0.35* (38.03)	40.53 ± 0.36* (34.57)	2.59 ± 0.12** (37.44)
MFMFME 100µg/ml	53.46 ± 0.28* (28.97)	44.67 ± 0.39 (10.82)	43.42 ± 0.27* (25.06)	2.62 ± 0.18** (38.76)
MFMFME 500 µg/ml	61.88 ± 0.82* (43.03)	27.14 ± 0.57* (71.82)	37.17 ± 0.05* (45.63)	2.82 ± 0.06** (47.57)
MFMFME 1000 µg/ml	76.05 ± 0.80* (66.69)	24.89 ± 0.37* (79.65)	31.53 ± 0.31* (64.18)	3.14 ± 0.04** (61.67)
F calculated	970.99	239.07	846.9	62.80
Dunnett value	03.01	03.14	01.07	0.40

Data represents the mean ± SEM of three values; Values in parenthesis indicate percentage protection against CCl_4. EMKMFME: Ethyl methyl ketone sub-fraction of methanol extract; MFMFME: Methanol sub-fraction of methanol extract.

F theoretical: 5.64 ($p<0.01$). * Significant reduction compared to CCl_4 ($p<0.05$). ** Significant increase compared to CCl_4 ($p<0.05$).

Paracetamol-induced hepatotoxicity

Effect of aerial parts of *P. daemia*:

Incubation of hepatocytes with 100 µg/ml solution of paracetamol resulted in 69.63% and 49.75% depletion in viability and TPTN content of hepatocytes respectively. An elevation about 238.51% and 220.58% of GOT

and GPT levels were observed respectively upon intoxication with paracetamol.

Table 52: Effect of *P. daemia* against paracetamol-induced toxicity on rat hepatocytes

Group	Viable cells (%)	GOT (IU/L)	GPT (IU/L)	TPTN (g/dl)
Control	96.13 ± 0.45	19.37 ± 0.52	24.63 ± 0.21	4.10 ± 0.12
Paracetamol 100 µg/ml	29.19 ± 1.54	46.20 ± 0.51	54.33 ± 0.34	2.06 ± 0.20
Silymarin 100 µg/ml	84.11 ± 1.28* (82.04)	21.86 ± 0.81* (90.78)	28.06 ± 0.52* (88.53)	3.56 ± 0.26** (73.51)
AFEFEE 100 µg/ml	49.17 ± 1.70* (29.77)	33.36 ± 0.72* (47.89)	45.03 ± 0.49* (31.34)	2.73 ± 0.08** (32.84)
AFEFEE 500 µg/ml	63.84 ± 1.78* (51.62)	28.53 ± 0.26* (65.90)	39.62 ± 0.54* (49.53)	3.03 ± 0.09** (47.54)
AFEFEE 1000 µg/ml	79.83 ± 0.60* (75.45)	22.27 ± 0.58* (89.25)	30.82 ± 0.32* (79.19)	3.70 ± 0.05** (80.37)
EFEFETE 100 µg/ml	38.34 ± 1.98* (13.63)	43.40 ± 0.53* (10.44)	50.43 ± 0.43* (13.14)	2.30 ± 0.11 (11.76)
EFEFEE 500 µg/ml	50.97 ± 0.91* (32.45)	39.96 ± 0.55* (23.27)	47.20 ± 0.55* (24.02)	2.56 ± 0.09 (24.50)
EFEFEE 1000 µg/ml	68.93 ± 4.52* (59.21)	33.27 ± 0.35* (59.45)	40.60 ± 0.49* (46.27)	3.10 ± 0.12** (50.97)
F calculated	123.54	303.93	519.29	24.79
Dunnett value	08.29	02.32	01.88	0.58

Data represents the mean ± SEM of three values; Values in parenthesis indicate percentage protection against toxicant. AFEFEE: Acetone sub-fraction of ethanol extract; EFEFEE: Ethanol sub-fraction of ethanol extract.

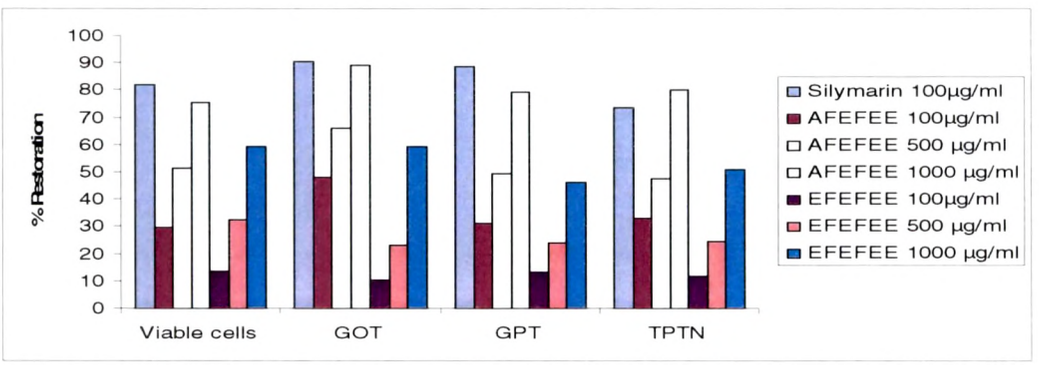
F theoretical: 5.64 ($p<0.01$)

* Significant reduction compared to paracetamol ($p<0.05$). ** Significant increase compared to paracetamol ($p<0.05$).

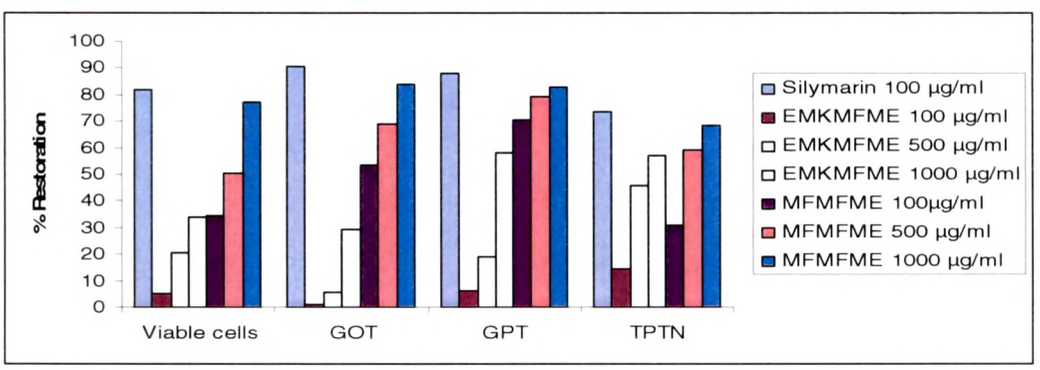
Hepatocytes incubated with AFEFEE in concentrations of 100-1000 µg/ml, afforded significant ($p<0.05$) concentration dependant protective effect by restoring the viability of hepatocytes (29.77-75.45%), TPTN content (32.84-82.04%), GOT (47.89-89.25%) and GPT (31.34-79.19). Hepatocytes incubated with EFEFEE at a concentration of 1000 µg/ml also exhibited significant ($p< 0.05$) protective effect by restoring the altered levels of cell viability (59.21%), TPTN content (50.97%), GOT (59.45%) and GPT (46.27%)

due to paracetamol intoxication. The Maximum protection was observed with the hepatocytes incubated with AFEFEE 1000 µg/ml and the activity was statistically ($p<0.05$) similar to silymarin. Results are shown in Table 52 and Graph 23.

Graph 23: Percentage restoration of various parameters by *P. daemia* against paracetamol-induced toxicity in vitro



Graph 24: Percentage restoration of various parameters by *B. montanum* against paracetamol-induced toxicity in vitro



Effect of roots of *B. montanum*:

Incubation of hepatocytes with 100µg/ml solution of paracetamol resulted in the induction of significant ($p<0.05$) sub maximal toxicity which was indicated by 70.43% and 49.51% depletion in viability and TPTN content of hepatocytes respectively. Similarly an elevation about 238.51% and 220.58% of GOT and GPT levels were observed respectively upon intoxication with paracetamol.

Hepatocytes treated with MFMFME in concentrations of 100-1000 µg/ml showed a concentration dependant significant ($p<0.05$) protective effect by restoring the viability of hepatocytes (34.58-77.35%), TPTN content (31.03-68.47%), GOT (53.67-83.81%) and GPT (70.45-83.19%). EMKMFME at concentrations of 500 and 1000 µg/ml also showed significant ($p<0.05$) protective effect by restoring the viability of cells (20.54-33.78%), TPTN (45.81-57.14%), GOT (05.84-19.16%) and GPT (19.02-58.12%). Results are shown in Table 53 and Graph 24.

Table 53: Effect of *B. montanum* against paracetamol-induced toxicity on rat hepatocytes

Group	Viable cells (%)	GOT (IU/L)	GPT (IU/L)	TPTN (g/dl)
Control	96.13 ± 0.46	19.37 ± 0.52	24.63 ± 0.27	4.10 ± 0.11
Paracetamol 100 µg/ml	28.82 ± 1.45	46.20 ± 0.51	54.33 ± 0.35	2.07 ± 0.17
Silymarin 100 µg/ml	84.11 ± 1.28* (81.82)	21.86 ± 0.81* (90.54)	28.06 ± 0.52* (88.26)	3.57 ± 0.27** (73.59)
EMKMFME 100 µg/ml	32.20 ± 1.28 (05.00)	45.87 ± 0.34 (01.23)	52.56 ± 1.17 (05.94)	2.36 ± 0.17 (14.28)
EMKMFME 500 µg/ml	42.70 ± 1.19* (20.54)	44.63 ± 0.26 (05.84)	48.67 ± 0.52* (19.02)	3.00 ± 0.05** (45.81)
EMKMFME 1000 µg/ml	51.65 ± 0.24* (33.78)	38.36 ± 0.17* (29.16)	37.03 ± 0.12* (58.12)	3.23 ± 0.08** (57.14)
MFMFME 100 µg/ml	52.37 ± 0.41* (34.58)	31.77 ± 0.72* (53.67)	33.36 ± 0.21* (70.45)	2.70 ± 0.05** (31.03)
MFMFME 500 µg/ml	63.04 ± 0.83* (50.64)	27.57 ± 0.43* (69.30)	30.76 ± 0.26* (79.19)	3.27 ± 0.04** (59.11)
MFMFME 1000 µg/ml	81.09 ± 1.27* (77.35)	23.67 ± 0.38* (83.81)	29.57 ± 0.25* (83.19)	3.46 ± 0.09** (68.47)
F calculated	612.32	459.64	493.23	21.29
Dunnett value	03.39	02.09	02.11	0.57

Data represents the mean ± SEM of three values; Values in parenthesis indicate percentage protection against paracetamol. EMKMFME: Ethyl methyl ketone sub-fraction of methanol extract; MFMFME: Methanol sub-fraction of methanol extract. * Significant reduction compared to paracetamol ($p<0.05$). ** Significant increase compared to paracetamol ($p<0.05$).

The maximum protection was obtained with MFMFME 1000 µg/ml and the activity was statistically similar ($p<0.05$) to the silymarin activity. Similarly

the activity obtained with EMKMFME 1000 mg/kg was not similar ($p<0.05$) to the activity of silymarin.

The hepatoprotective activity of the fractions tested may be due to inhibition of cytochrome P_{450}; stimulation of hepatic regeneration or activation of the functions of reticuloendothelial systems, as hepatotoxicity of paracetamol has been attributed to the formation of toxic metabolites when a part of paracetamol is activated by hepatic cytochrome P_{450} to a highly reactive metabolite N-acetyl-*p*-benzoquinoneimine.

Thioacetamide-induced hepatotoxicity

Effect of aerial parts of *P. daemia*:

Table 54: Effect of *P. daemia* against thioacetamide-induced toxicity on rat hepatocytes

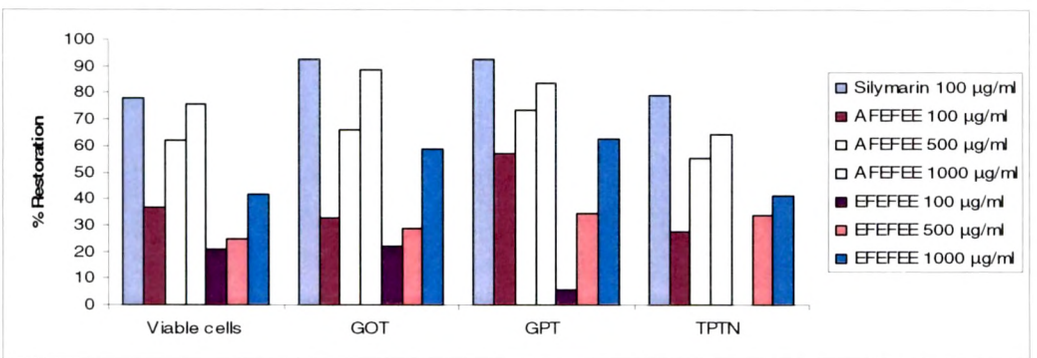
Group	Viable cells (%)	GOT (IU/L)	GPT (IU/L)	TPTN (g/dl)
Control	95.79 ± 0.63	23.67 ± 0.32	20.57 ± 0.44	4.26 ± 0.11
Thioacetamide 40 µg/ml	31.24 ± 0.85	46.93 ± 1.19	50.67 ± 0.83	1.97 ± 0.12
Silymarin 100 µg/ml	81.45 ± 1.47* (77.82)	25.33 ± 0.44* (92.6)	22.70 ± 0.71* (92.86)	3.76 ± 0.18** (79.04)
AFEFEE 100 µg/ml	54.87 ± 1.74* (36.62)	39.30 ± 0.69* (32.73)	33.40 ± 0.40* (57.34)	2.60 ± 0.15** (27.94)
AFEFEE 500 µg/ml	71.28 ± 3.15* (62.06)	31.53 ± 0.46* (66.06)	28.56 ± 0.43* (73.40)	3.23 ± 0.12** (55.46)
AFEFEE 1000 µg/ml	79.96 ± 1.03* (75.51)	26.23 ± 0.49* (88.80)	25.53 ± 1.16* (83.46)	3.43 ± 0.17** (64.19)
EFEFEE 100 µg/ml	44.74 ± 1.71* (20.93)	41.83 ± 0.87* (21.87)	49.03 ± 0.92 (5.44)	1.87 ± 0.12 (-)
EFEFEE 500 µg/ml	47.18 ± 1.80* (24.71)	40.20 ± 0.66* (28.86)	40.36 ± 0.43* (34.23)	2.73 ± 0.16** (33.62)
EFEFEE 1000 µg/ml	58.36 ± 3.89* (42.04)	33.17 ± 0.63* (59.03)	31.80 ± 0.57* (62.65)	2.90 ± 0.11** (41.04)
F calculated	100.23	143.29	239.04	30.08
Dunnett value	08.58	02.84	02.92	0.58

Data represents the mean ± SEM of three values; Values in parenthesis indicate percentage protection against thioacetamide. AFEFEE: Acetone sub-fraction of ethanol extract; EFEFEE: Ethanol sub- fraction of ethanol extract. F theoretical: 5.64 ($p<0.01$)

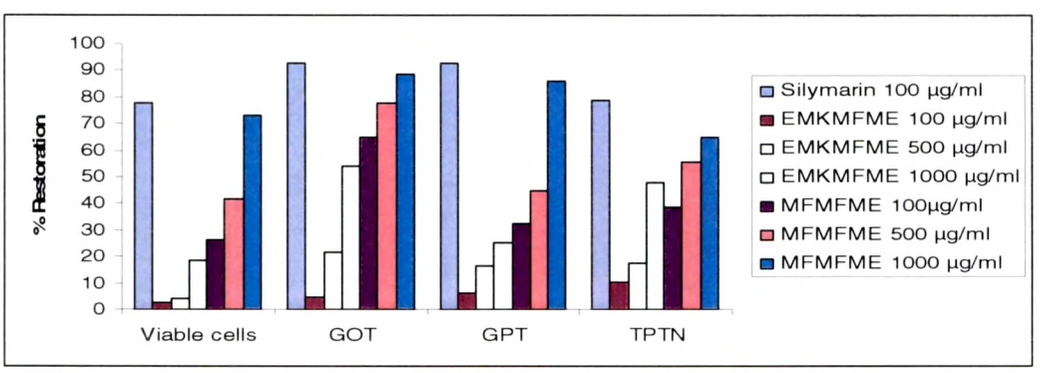
* Significant reduction compared to thioacetamide ($p<0.05$). ** Significant increase compared to thioacetamide ($p<0.05$).

Incubation of hepatocytes with 40 µg/ml solution of thioacetamide resulted in 67.38% and 53.75% depletion in viability and TPTN content of hepatocytes respectively. Thioacetamide intoxication was also resulted in an elevation of about 198% and 246% of GOT and GPT levels when compared to control.

Graph 25: Percentage restoration of various parameters by *P. daemia* against thioacetamide-induced toxicity in vitro



Graph 26: Percentage restoration of various parameters by *B. montanum* against thioacetamide-induced toxicity in vitro



Hepatocytes incubated with AFEFEE in concentrations of 100-1000 µg/ml showed significant ($P<0.05$) concentration dependant protective effect by restoring the altered levels of viability of hepatocytes (36.62-75.51%), TPTN content (27.94-64.19%), GOT (32.73-88.80%) and GPT (57.34-83.46%). Incubation with EFEFEE in concentrations of 500 and 1000 µg/ml also exhibited concentration dependant significant ($p<0.05$) protective effect by restoring the viability of cells (24.71-42.04%), TPTN content (33.62-

41.04%), GOT (28.86-59.03%) and GPT (34.23-62.65%). AFEFEE at concentration of 1000 µg/ml afforded maximum protection and the activity was similar ($P<0.05$) to silymarin. The results of the effect of AFEFEE and EFEFEE against thioacetamide-induced toxicity are shown in Table 54 and Graph 25.

Effect of roots of *B. montanum*:

Incubation of hepatocytes with 40 µg/ml solution of thioacetamide resulted in 67.38% and 53.75% depletion in viability and TPTN content of hepatocytes respectively. Thioacetamide intoxication was also resulted in an elevation of about 198% and 246% of GOT and GPT levels when compared to control.

Table 55: Effect of *B. montanum* against thioacetamide-induced toxicity on rat hepatocytes

Group	Viable cells (%)	GOT (IU/L)	GPT (IU/L)	TPTN (g/dl)
Control	95.79 ± 0.63	23.67 ± 0.32	20.57 ± 0.44	4.26 ± 0.11
Thioacetamide 40 µg/ml	31.24 ± 0.85	46.93 ± 1.19	50.67 ± 0.83	1.97 ± 0.12
Silymarin 100 µg/ml	81.45 ± 1.47* (77.82)	25.33 ± 0.44* (92.66)	22.70 ± 0.71* (92.86)	3.76 ± 0.18** (79.04)
EMKMFME 100 µg/ml	32.92 ± 0.93 (02.60)	45.86 ± 0.17 (04.59)	48.73 ± 0.27 (06.44)	2.20 ± 0.05 (10.47)
EMKMFME 500 µg/ml	33.87 ± 0.53 (04.07)	41.87 ± 0.42* (21.71)	45.70 ± 0.26* (16.50)	2.36 ± 0.06 (17.46)
EMKMFME 1000 µg/ml	43.20 ± 0.52* (18.53)	34.30 ± 0.41* (54.18)	43.06 ± 0.52* (25.26)	3.06 ± 0.08** (48.02)
MFMFME 100 µg/ml	48.23 ± 0.20* (26.33)	31.76 ± 0.38* (65.07)	40.90 ± 0.50* (32.43)	2.85 ± 0.03** (38.85)
MFMFME 500 µg/ml	58.06 ± 0.24* (41.57)	28.81 ± 0.26* (77.73)	37.10 ± 0.25* (45.05)	3.23 ± 0.12** (55.44)
MFMFME 1000 µg/ml	78.63 ± 0.32* (73.45)	26.30 ± 0.33* (88.50)	24.80 ± 0.37* (85.88)	3.45 ± 0.07** (65.05)
F calculated	1050.7	302.54	525.75	53.30
Dunnett value	03.08	02.14	02.10	0.43

Data represents the mean ± SEM of three values; Values in parenthesis indicate percentage protection against thioacetamide. EMKMFME: Ethyl methyl ketone sub-fraction of methanol extract; MFMFME: Methanol sub-fraction of methanol extract.

F theoretical: 5.64 ($p<0.01$). * Significant reduction compared to thioacetamide ($p<0.05$). ** Significant increase compared to thioacetamide ($p<0.05$).

Hepatocytes incubated with MFMFME in concentrations of 100-1000 µg/ml showed significant ($p<0.05$) concentration dependant protective effect by restoring the altered levels of viability of hepatocytes (26.33-73.45%), TPTN content (38.85-65.05%), GOT (65.07-88.50%) and GPT (32.43-85.88%) as observed with silymarin. Incubation of hepatocytes with 1000 µg/ml of EMKMFME resulted in restoration of viability (18.53%), TPTN (48.02%), GOT (54.18%) and GPT (25.26%) levels significantly ($p<0.05$) when compared to thioacetamide. The activity exhibited by EMKMFME was not similar ($p<0.05$) to silymarin. The maximum protection was obtained with MFMFME 1000 µg/ml and activity was similar ($p<0.05$) to silymarin. The results of the effect of EMKMFME and MFMFME against thioacetamide-induced toxicity are shown in Table 55 and Graph 26.

Thioacetamide intoxication leads to cell proliferation and production of cell necrosis by its metabolites. Extracts under study antagonize the effect of thioacetamide by acting as membrane stabiliser, there by preventing the distortion of the cellular ionic environment associated with reactive oxygen species generated by thioacetamide.

In the present study, the hepatotoxins employed reduced cell viability possibly due to injury of plasma membrane of hepatocytes resulting in the leakage of cellular enzymes. Incubation of hepatocytes with extracts significantly restored their viability as well as altered biochemical parameters induced by hepatotoxins.

3.5. Isolation and characterisation of active principles from bio active fractions

3.5.1 Isolation of active principles

Active sub-fractions, AFEFEE from *P. daemia* and MFMFME from *B. montanum* were then subjected to isolation of active constituents.

3.5.1.1 Isolation of PD1 from AFEFTE of *P. daemia*

Acetone sub-fraction (AFEFEE) of ethanol extract when subjected to thin layer chromatographic studies, revealed nine spots under UV 366 nm. Three were found to be major at R_f 0.41, 0.53 and 0.64 respectively. The

compounds at R_f 0.41 and 0.53 (PD1 and PD2) were separated by column chromatography using silica gel (200 mesh), followed by preparative TLC.

The compounds PD1 and PD2 are obtained in sufficient quantities using preparative TLC of the fractions 49-56 eluted in acetone: methanol (80:20) from column, which showed three compounds at R_f 0.30, 0.41 and 0.53 when detected by thin layer chromatography. PD2 was unstable at room temperature and it acquired brownish black colour from its original greenish yellow colour and lost its solubility in the solvents tried. Hence only PD1 was used for further studies.

3.5.1.2 Isolation of BM1 from MFMFME of *B. montanum*

Methanol sub-fraction (MFMFME) of methanol fraction from methanol extract (MFMFME) upon thin layer chromatographic studies, revealed ten spots under UV 366 nm with five as major at R_f 0.13, 0.23, 0.32, 0.43 and 0.51.The compound at R_f 0.43 (BM1) was obtained in sufficient quantity by preparative TLC, followed by purification using column chromatography with silica gel as an adsorbent.

3.5.2 Hepatoprotective activity of PD1 and BM1 in vitro

The compounds PD1 and BM1 were subjected to evaluation of hepatoprotective activity in vitro using CCl_4, paracetamol and thioacetamide as hepatotoxins.

3.5.2.1 Hepatic cytotoxicity testing

The effects of the PD1 and BM1 on primary cultured hepatocytes were judged by incubating the cultured hepatocytes with the compounds at the concentration levels of 500 and 1000 µg/ml. The results shown in Table 56 indicate the effect of these compounds on percent viability and TPTN content of hepatocytes.

The difference in the values of percent viability and protein content of hepatocytes incubated with the compounds were found to be insignificant ($p<0.05$) as compared to control group, indicating these compounds were not toxic to the hepatocytes at the selected dose levels.

Table 56: Effect of PD1 and BM1 on primary cultured rat hepatocytes

Group	Viable cells (%)	TPTN (g/dl)
Control	95.16 ± 1.13	4.01 ± 0.08
PD1 500 µg/ml	94.23 ± 0.11*	3.86 ± 0.03*
PD1 1000 µg/ml	93.17 ± 0.17*	3.79 ± 0.09*
BM1 500 µg/ml	95.27 ± 0.27	3.81 ± 0.02
BM1 1000 µg/ml	94.05 ± 0.04	3.77 ± 0.01
F calculated	2.69	2.70
Dunnett value	2.15	0.24

F theoretical: 3.48 ($p<0.05$); *not significant compared to control ($p<0.05$).

3.5.2.2 Hepatoprotective activity in vitro

CCl_4-induced hepatotoxicity

Incubation of hepatocytes with 10 µl/ml solution of CCl_4 resulted, induction of sub maximal toxicity which was indicated by significant ($p<0.05$) reduction in viability (95.34 ± 0.44 to 38.39 ± 0.87) and TPTN (3.69 ± 0.07 to 1.83 ± 0.05) levels as compared to normal hepatocytes. Similarly there was significant ($p<0.05$) increase in the GOT (17.62 ± 0.37 to 48.14 ± 1.07) and GPT (20.85 ± 0.25 to 52.37 ± 0.59) levels. (59.73% and 50.40% depletion in percent viability and TPTN respectively; while 273.21% and 251.17% elevation in GOT and GPT levels respectively).

The compounds PD1 and BM1 isolated form *P. daemia* and *B. montanum* respectively, protected the rat hepatocytes at concentrations of 100 and 500 µg/ml against CCl_4-induced toxicity. Hepatocytes incubated with PD1 at concentrations of 100-500 µg/ml showed significant ($p<0.05$) concentration dependant protective effect by restoring the altered levels of percent viability (75.66-93.17%), TPTN (60.71-90.30%), GOT (77.56-87.79%) and GPT (71.97-94.79%) as observed with silymarin treated hepatocytes. The Maximum protection was observed with 500 µg/ml.

Hepatocytes incubated with BM1 at concentrations of 100-500 µg/ml showed significant ($p<0.05$) concentration dependant protective effect by

restoring the percent viability (71.57-83.51%), TPTN content (84.69-88.79%), GOT (83.29-92.05%) and GPT (88.61-96.07%) levels as observed with silymarin treated hepatocytes. The Maximum protection was observed with 500 µg/ml. The positive control silymarin at a concentration 100 µg/ml showed significant ($p<0.05$) protective effect by restoring viability, TPTN, GOT and GPT. The results are shown in Table 57 and Graph 27.

Table 57: Effect of PD1 and BM1 against CCl_4-induced toxicity on rat hepatocytes

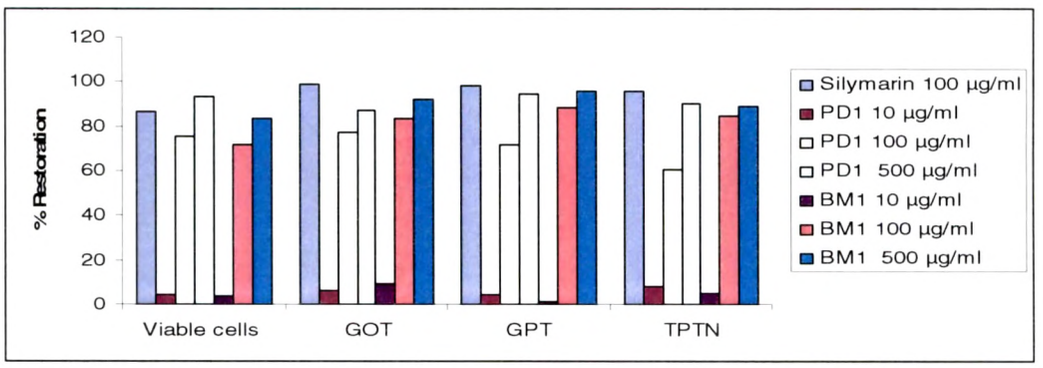
Group	Viable cells (%)	GOT (IU/L)	GPT (IU/L)	TPTN (g/dl)
Control	95.34 ± 0.44	17.62 ± 0.37	20.85 ± 0.25	3.69 ± 0.07
CCl_4 10 µl/ml	38.39 ± 0.87	48.14 ± 1.07	52.37 ± 0.59	1.83 ± 0.05
Silymarin 100µg/ml	87.44 ± 0.89* (86.82)	17.88 ± 0.20* (98.95)	21.40 ± 0.23* (98.17)	3.71 ± 0.04** (95.91)
PD1 10 µg/ml	40.82 ± 0.86 (04.27)	46.22 ± 0.62 (06.27)	51.01 ± 0.44 (04.45)	1.99 ± 0.02 (08.06)
PD1 100 µg/ml	81.38 ± 1.38* (75.66)	24.42 ± 0.29* (77.56)	30.36 ± 0.58* (71.97)	3.02 ± 0.06** (60.71)
PD1 500 µg/ml	91.83 ± 0.89* (93.17)	21.29 ± 0.64* (87.79)	23.38 ± 0.32* (94.79)	3.60 ± 0.03** (90.30)
BM1 10 µg/ml	40.48 ± 0.30 (03.67)	45.36 ± 0.34* (09.09)	52.08 ± 0.27 (0.94)	1.93 ± 0.03 (05.10)
BM1 100 µg/ml	79.06 ± 0.53* (71.57)	22.67 ± 0.34* (83.29)	25.27 ± 0.50* (88.61)	3.49 ± 0.02** (84.69)
BM1 500 µg/ml	85.84 ± 0.18*# (83.51)	19.99 ± 0.54*# (92.05)	22.99 ± 0.33*# (96.07)	3.57 ± 0.03*# (88.77)
F calculated	1029.10	568.15	1168.8	358.10
Dunnett value	03.07	02.29	01.72	0.18

Data represents the mean ± SEM of three values; Values in parenthesis indicate percentage protection against CCl_4. F theoretical: 5.64 ($p<0.01$)

* Significant reduction compared to CCl_4 ($p<0.05$). ** Significant increase compared to CCl_4 ($p<0.05$).

not significant ($p<0.05$) compared to silymarin.

Graph 27: Percentage restoration of various parameters by PD1 and BM1 against CCl_4-induced toxicity in vitro.



PD1 at concentration level of 500 µg/ml showed maximum protection. Similarly BM1 at 500 µg/ml exhibited maximum protection and activity was statistically ($p<0.05$) similar to silymarin.

Paracetamol-induced hepatotoxicity

Incubation of hepatocytes with 100 µg/ml solution of paracetamol resulted the induction of sub maximal toxicity which was indicated by significant reduction ($p<0.05$) in viability (96.41 ± 0.41 to 31.20 ± 0.56) and TPTN (4.11 ± 0.02 to 2.13 ± 0.04) levels as compared to normal hepatocytes. Similarly there was significant increase in the GOT (18.58 ± 0.35 to 47.32 ± 0.58) and GPT (23.69 ± 0.11 to 51.95 ± 0.459) levels (67.68% and 48.17% depletion in percent viability and TPTN respectively; 254.81% and 229.89% elevation in GOT and GPT levels respectively).

Hepatocytes incubated with PD1 100-500 µg/ml afforded significant ($P<0.05$) concentration dependant protective effect by restoring the viability of hepatocytes (67.76-78.56%), TPTN content (41.41-80.29%), GOT (64.57-79.63%) and GPT (59.51-90.01%) as observed with silymarin treated hepatocytes. The Maximum protection was observed with 500 µg/ml. The positive control silymarin showed significant ($p<0.05$) protective effect by restoring viability, TPTN, GOT and GPT levels.

Hepatocytes incubated with BM1 at concentrations of 100-500 µg/ml showed significant ($p<0.05$) concentration dependant protective effect by

restoring the viability of hepatocytes (63.03-81.15%), TPTN content (65.14-83.83%), GOT (48.54-89.04%) and GPT (48.43-95.55%) as observed with silymarin treated hepatocytes. The Maximum protection was observed with 500 µg/ml. The results are shown in Table 58 and Graph 28.

Table 58: Effect of PD1 and BM1 against paracetamol-induced toxicity on rat hepatocytes

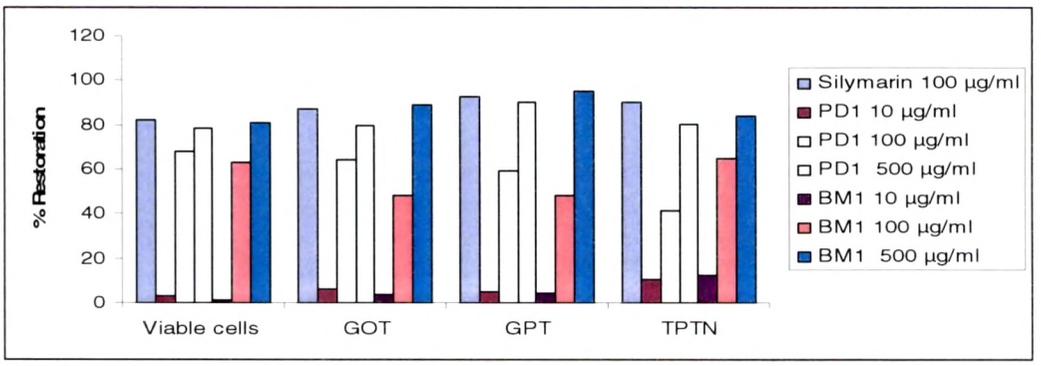
Group	Viable cells (%)	GOT (IU/L)	GPT (IU/L)	TPTN (g/dl)
Control	96.41 ± 0.41	18.57 ± 0.35	23.69 ± 0.11	4.11 ± 0.02
Paracetamol 100 µg/ml	31.20 ± 0.56	47.32 ± 0.58	51.95 ± 0.45	2.13 ± 0.03
Silymarin 100 µg/ml	85.01 ± 0.69* (82.32)	23.16 ± 0.62* (87.30)	25.71 ± 0.36* (92.62)	3.92± 0.05** (90.39)
PD1 10 µg/ml	33.19 ± 0.62 (03.04)	45.62 ± 0.99 (05.89)	50.46 ± 0.87 (05.25)	2.34 ± 0.11 (10.60)
PD1 100 µg/ml	75.49 ± 0.91* (67.76)	28.71 ± 0.31* (64.57)	35.09 ± 0.46* (59.51)	2.95 ± 0.07** (41.41)
PD1 500 µg/ml	82.55 ± 0.46*# (78.56)	24.37 ± 0.26*# (79.63)	26.45 ± 0.31*# (90.01)	3.72 ± 0.04**# (80.29)
BM1 10 µg/ml	32.12 ± 0.25 (01.41)	46.21 ± 0.26 (03.85)	50.75 ± 0.33 (04.23)	2.37 ± 0.08 (12.12)
BM1 100 µg/ml	72.40 ± 1.98* (63.03)	33.32 ± 0.64 (48.54)	38.23 ± 0.18* (48.43)	3.42 ± 0.14** (65.14)
BM1 500 µg/ml	84.24 ± 0.80*# (81.15)	21.66 ± 0.44*# (89.04)	24.88 ± 0.31*# (95.55)	3.79 ± 0.04**# (83.83)
F calculated	869.53	450.49	779.96	96.34
Dunnett value	03.68	02.27	01.79	0.32

Data represents the mean ± SEM of three values; Values in parenthesis indicate percentage protection against paracetamol.

F theoretical: 5.64 ($p<0.01$). # not significant compared to silymarin

* Significant reduction compared to paracetamol ($p<0.05$). ** Significant increase compared to paracetamol ($p<0.05$).

Graph 28: Percentage restoration of various parameters by PD1 and BM1 against paracetamol-induced toxicity in vitro



The activities exhibited by PD1 and BM1 at concentration of 500µg/ml, were statistically ($p<0.05$) similar to silymarin activity.

Thioacetamide-induced toxicity

Incubation of hepatocytes with 40 µg/ml solution of thioacetamide resulted, induction of toxicity which was indicated by significant ($p<0.05$) reduction in viability (96.53 ± 0.24 to 34.40 ± 0.59) and TPTN (4.04 ± 0.04 to 1.82 ± 0.01) levels when compared to normal hepatocytes. Similarly there was significant increase in the GOT (22.54 ± 0.10 to 45.36 ± 0.13) and GPT (19.60 ± 0.17 to 48.91 ± 0.47) levels (64.36% and 54.95% depletion in percent viability and TPTN levels respectively; 201.33% and 249.41% elevation in GOT and GPT levels respectively).

Hepatocytes incubated with PD1 at concentrations of 100-500 µg/ml showed significant ($p<0.05$) concentration dependant protective effect as evidenced by restoration of altered levels of percent viability (54.69-85.12%), TPTN content (50.89-82.42%), GOT (49.36-82.78%) and GPT (38.19-87.70%) as observed with silymarin treated hepatocytes. PD1 at concentration of 500 µg/ml afforded maximum protection and the activity was statistically ($p<0.05$) similar to silymarin.

Hepatocytes incubated with BM1 at concentrations of 100-500 µg/ml showed significant ($p<0.05$) protective effect by restoring the viability of hepatocytes (65.34-82.56%), TPTN content (63.46-80.56%), GOT (63.64-73.58%) and GPT (62.06-55.41%) as observed with silymarin treated

hepatocytes. The maximum restoration of all the parameters was observed with BM1 at concentration of 500 µg/ml, except in levels of GPT which was obtained with 100 µg/ml concentration. The activity was statistically similar to silymarin. The results of the effect of PD1 and BM1 against thioacetamide induced toxicity are shown in Table 59 and Graph 29.

Table 59: Effect of PD1 and BM1 against thioacetamide-induced toxicity on rat hepatocytes

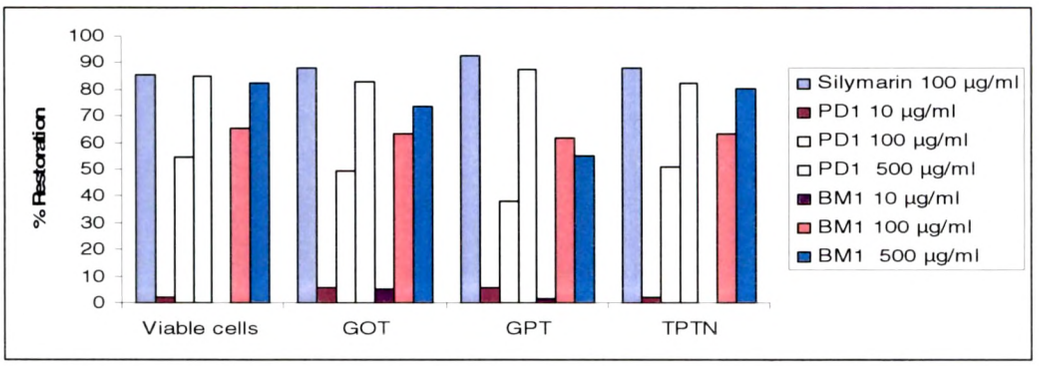
Group	Viable cells (%)	GOT (IU/L)	GPT (IU/L)	TPTN (g/dl)
Control	96.53 ± 0.24	22.54 ± 0.10	19.61 ± 0.17	4.04 ± 0.04
Thioacetamide 40 µg/ml	34.40 ± 0.59	45.38 ± 0.15	48.91 ± 0.47	1.82 ± 0.01
Silymarin 100 µg/ml	87.42 ± 0.59* (85.36)	25.26 ± 0.05* (88.03)	21.76 ± 0.29* (92.58)	3.78 ± 0.04** (88.27)
PD1 10 µg/ml	35.83 ± 0.34 (02.30)	44.02 ± 0.46 (05.86)	47.18 ± 0.56* (05.89)	1.87 ± 0.14 (02.25)
PD1 100 µg/ml	68.37 ± 0.87* (54.69)	34.09 ± 0.49* (49.36)	37.71 ± 0.27* (38.19)	2.95 ± 0.03** (50.89)
PD1 500 µg/ml	87.27 ± 0.87*# (85.12)	26.46 ± 0.65*# (82.78)	23.19 ± 0.57*# (87.70)	3.65 ± 0.03** (82.42)
BM1 10 µg/ml	34.46 ± 0.22 (0.09)	44.19 ± 0.29 (05.12)	48.46 ± 0.27 (01.53)	1.76 ± 0.02 (-)
BM1 100 µg/ml	74.49 ± 0.87* (65.34)	30.83 ± 0.29* (63.64)	30.71 ± 0.52* (62.06)	3.23 ± 0.07** (63.46)
BM1 500 µg/ml	85.68 ± 0.18*# (82.56)	28.56 ± 0.32*# (73.58)	32.66 ± 0.49*# (55.41)	3.61 ± 0.05*# (80.56)
F calculated	216.8	590.79	743.78	222.56
Dunnett's value	02.27	01.52	01.79	0.27

Data represents the mean ± SEM of three values; Values in parenthesis indicate percentage protection against paracetamol.

F theoretical: 5.64 ($p<0.01$). # not significant compared to silymarin.

* Significant reduction compared to thioacetamide ($p<0.05$). ** Significant increase compared to thioacetamide ($p<0.05$).

Graph 29: Percentage restoration of various parameters by PD1 and BM1 against thioacetamide-induced toxicity in vitro



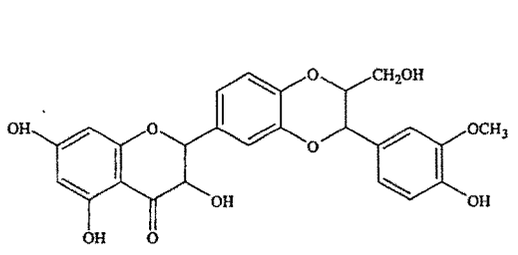
The ability of the PD1 and BM1 against to protect the hepatocytes against the CCl_4-induced hepatotoxicity may due to the stabilization of endoplasmic reticulum as well as repair of hepatic tissue damages caused by the CCl_4 leading to hepatic regeneration, as the intoxication with CCl_4 results in the metabolic formation of the highly reactive trichloromethyl free radical which attacks the membrane of the endoplasmic reticulum resulting in loss of cytochrome P_{450} leading to its functional failure with a decrease in protein synthesis.

The hepatoprotective activity of the PD1 and BM1 against paracetamol-induced hepatotoxicity may be due to inhibition of cytochrome P_{450} thereby altering its metabolism or stimulation of hepatic regeneration or activation of the functions of reticuloendothelial systems or all of these mechanisms.

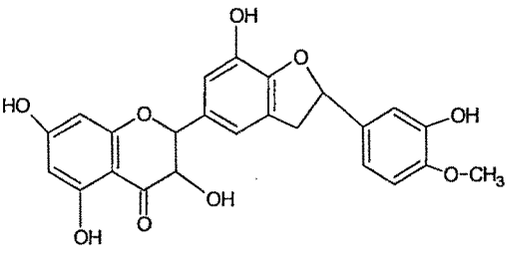
Thioacetamide gets metabolises to its toxic metabolite and induces toxicity by altering the semipermeable character of the cell wall, which leads to imbalance of ions, a rise in calcium levels and inhibition of mitochondrial activity, thus leading to the death of the cells. Both the compounds PD1 and BM1 were found to protect the isolated hepatocytes against the thioacetamide toxicity. The compounds may be thus protecting the hepatocytes by altering the metabolism of the toxicant, thus preventing the toxicant from altering the semipermeable character of the cell wall.

Thus incubation of hepatocytes with PD1 and BM1 significantly ($p<0.05$) restored the viability as well as altered biochemical parameters induced by all the three selected hepatotoxins. The activities exhibited by both the compounds were significantly comparable to silymarin and at concentrations of 500 µg/ml both the compound exhibited same spectrum of activity as silymarin (100 µg/ml).

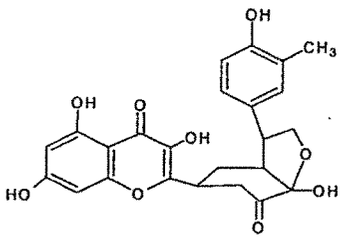
A number of natural products that are currently used as hepatoprotective agents or those that possess antioxidative activity are believed to exert their effect as a result of interaction with cytochrome P_{450} dependent monooxygenase system leading either to reduced formation of the ultimate active metabolite or scavenging of the reactive molecular species to prevent their reaching the target site. In present study the hepatoprotective activity of the PD1 (later identified as chlorogenic acid-section 3.5.3)) may be attributed to its protective action on lipid peroxidation, drug metabolizing enzymes to recover their levels during toxicity or to the enhancing effect on cellular antioxidant defence contributing to the protection of liver against oxidative injury by toxicants. The activity of BM1 (later identified as quercetin-3-O-galactosyl-7-O-rhamnoside) may be attributed to its ability to stabilize the endoplasmic reticulum like that of silymarin involving cytochrome P_{450} mediated functions. Due to the structural similarity, the compound BM1 proposed later (section 3.5.3) as quercetin-3-O-galactosyl-7-O-rhamnoside may be acting in a similar manner to protect the liver as silymarin, which stabilizes the cell membrane, stimulates protein synthesis and accelerates the process of regeneration, all of which play an important role in the hepatoprotective activity (Handa and Sharma, 1986).



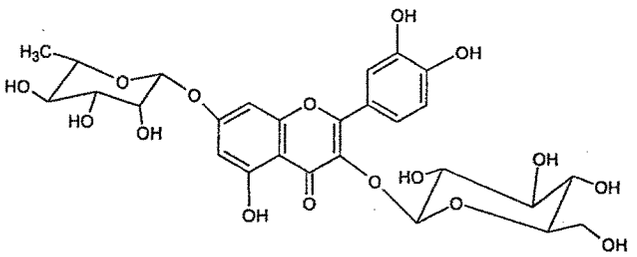
Silybin



Silychristin



Silydianin



Quercetin-3-O-galactosyl-7-O-rhamnoside

3.5.3 Characterisation of isolated compounds

The isolated compounds from *P. daemia* and *B. montanum* were identified using physicochemical and spectral data.

3.5.3.1 Characterisation of PD1

Description: White amorphous powder, melting point 207-208°C and λ max was found to be 256.5 nm (in acetone).

Solubility: Soluble freely in acetone, ethanol and methanol. It is very slightly soluble in chloroform and insoluble in benzene.

Chromatographic studies: Thin layer chromatography of the compound using ethyl acetate: formic acid: acetic acid: water (100: 11: 11: 27) as mobile phase, silica gel 60 F_{254} pre-coated plates as stationary phase and NP-PEG as a detecting agent revealed single spot (R_f 0.41) with blue colour fluorescence under UV 366 nm indicating phenolic/flavonoidal nature of the compound.

Spectral studies:

FT-IR spectra

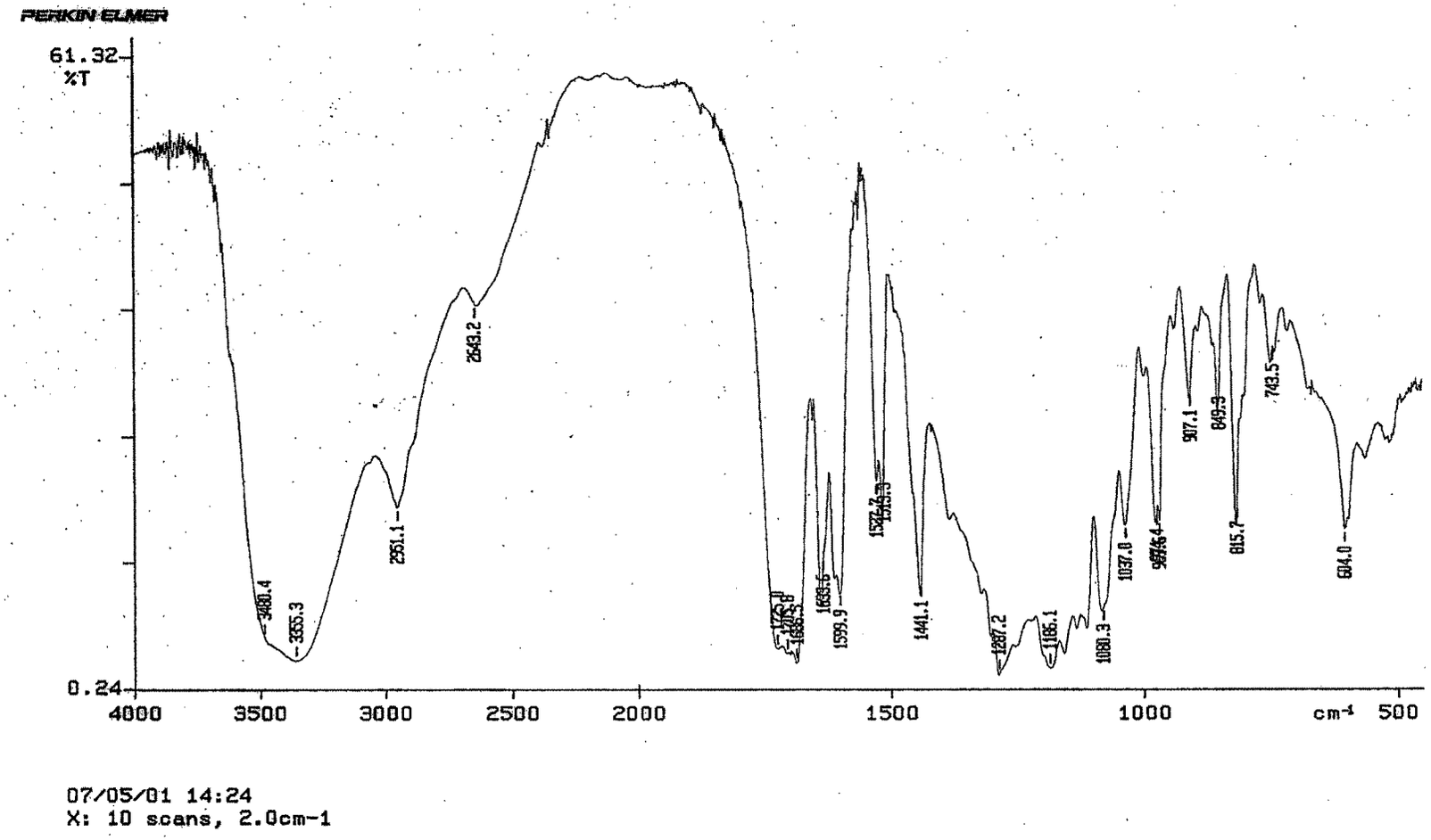
The IR spectra of the sample show peaks from 3470.7 to 604.0 cm^{-1}.

1. The peaks in the IR spectra from 3600-3300 cm-1 generally represent alcoholic O-H, amine or amide N-H and alkyne C-H. The spectra of the sample show a strong peak near 3400(3470.7 and 3360.7). The alcohol OH stretch is usually a broad and strong near 3400cm^{-1}which indicated the presence of alcoholic OH.

2. Peaks at 3000-2800cm^{-1} generally indicate the presence of alkyl (sp3) C-H. The spectra of the sample show a peak at 2951.1 confirming the presence of one alkyl CH.

3. Peaks between 1700 -1750cm^{-1} represent the presence of aldehyde C=O, ketone C=O, ester C=O, acid C=O and peaks at 1700-1640cm^{-1} represent amide C=O and conjugated C=O. As the conjugation lowers the absorption by 20-50 cm^{-1} the conjugated carbonyl's come slightly lower than normal C=O. The IR spectra of the sample show a peak at 1725.0cm^{-1} indicating the

Figure 23: IR spectrum of PD1



presence of carbonyl C=O, and the peaks at 1686.5cm^{-1} confirming the presence of conjugated carbonyl C=O.

4. Peaks between 1680-1620 cm^{-1} represents the presence of alkene C=C. The IR spectra of the sample show a peak a 1633.6cm^{-1} indicating the presence of alkene C=C. Generally the peaks at 1670-1600cm^{-1} indicate unsaturation.

5. Peaks between 1600-1400cm^{-1} indicate the presence of aromatic C=C. The IR spectra of the sample show peaks at 1599.9cm^{-1}, 1527.7cm^{-1}, 1513.3cm^{-1}, 1441.1cm^{-1} indicating the presence of aromatic C=C. more over peaks at 2000-1700cm^{-1},and peaks over 3000cm^{-1} (for CH) confirms the presence of simple aromatic C=C compounds.

6. The region at 1500-400cm^{-1} is generally called as finger print region. Peaks at 1300-1000cm^{-1} indicated the presence of C-O. The IR spectra of the sample show peaks at 1287.2cm^{-1}, 1190.9cm^{-1}, confirming the presence of C-O.

7. Peaks at 1000-675cm^{-1} indicate the presence of C-H alkene bending vibrations and the peaks at 870-625cm^{-1} indicate the presence of phenyl rings substitution bands. The IR spectra of the sample show peaks 1109.1cm^{-1}, 1080.3cm^{-1}, 1032.2cm^{-1}, 969.6cm^{-1}, 907.1cm^{-1} confirming CH bending vibrations and the peaks at 849.3cm^{-1}, 875.7cm^{-1} further indicates the presence CH bending vibrations. The FT-IR spectrum of the compound was shown in Figure 23.

The information obtained was confirmed and the structure was determined by interpreting the rest of spectra.

Mass spectra

From the mass spectrum of the sample the following conclusions were made.

1. In the mass spectrum of the sample, the line produced by the heaviest ion passing through the machine, *m/z* 353.8 is due to molecular ion.

2. The tallest line in the stick diagram, in this case at *m/z* = 162.9 is called the base peak. This is usually given in arbitrary height of 100, and the height of everything else is measured relative to this.

3. The spectrum also shows a peak at 179.9 with a relative abundance of around 68%. From the data obtained from the IR spectra and from the molecular weight (*m/z* 179.9) from mass spectra, the molecular formula of this fragment can be taken as $\mathbf{C_7H_{11}O_5}$ (12 X 7+ 11 X 1+16 X 5).

4. The molecular weight of the sample was found to be 353.8 from the spectra. The molecular weight of one of the fragment was found to be 179.9 and the molecular weight of the rest of the fragment can be expected as 173.9 and the molecular formula for this fragment can be taken as $\mathbf{C_9H_7O_4}$ (12 X 9+ 7 X 1+16 X4).

Thus from IR and Mass spectra the molecular formula of the sample was found as $\mathbf{C_{16}H_{18}O_9}$. The mass spectrum was shown in Figure 24.

NMR spectra

A careful interpretation of all the NMR spectra (^{1}H NMR, ^{13}C NMR, ^{13}C DEPT, COSY, NOESY, HMBC and HSQC) was done to determine the final chemical structure of the compound.

^{1}H NMR spectrum: From the ^{1}H NMR spectra the following different types of protons are observed.

1. A peak at 7.42 indicated the presence of a doublet at 15.9 Hz.
2. A peak at 7.02 indicated the presence of a doublet at 1.75 Hz.
3. A peak at 6.96 (showing two splitted peaks in the spectra) is indicating a double doublet at 8 and 1.9 Hz.
4. A peak at 6.13 is also indicating a doublet at 15.9 Hz.
5. A peak at 5.05 (showing a peak that splitted into four in the spectra) indicated the presence of quadruplet at 4.2 Hz.
6. Peaks at 4.90, 4.75, 3.92 and 3.55 are very broad indicating the presence of singlets at all the respective peaks.
7. Peaks at 2.02, 2.01, 1.97 and 1.78 (showing multiple splittings in the spectrum) are indicating the presence of multiplets.

The results of ^{1}H NMR spectrum are tabulated in Table 60. The ^{1}H NMR spectra of the PD1 are shown in Figure 25.

Figure 24: The mass spectrum of PD1

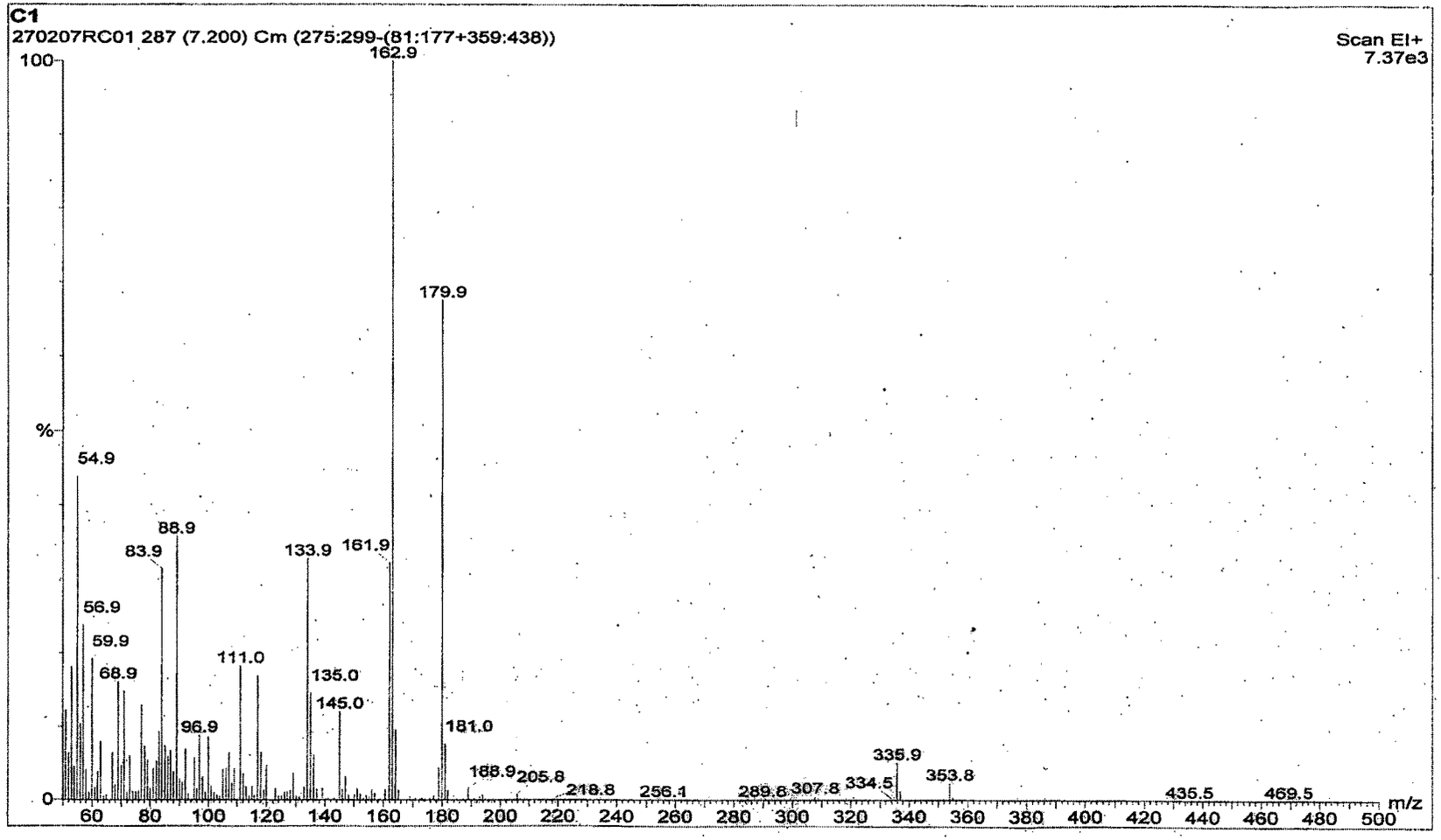


Figure 25: ^{1}H NMR spectrum of PD1

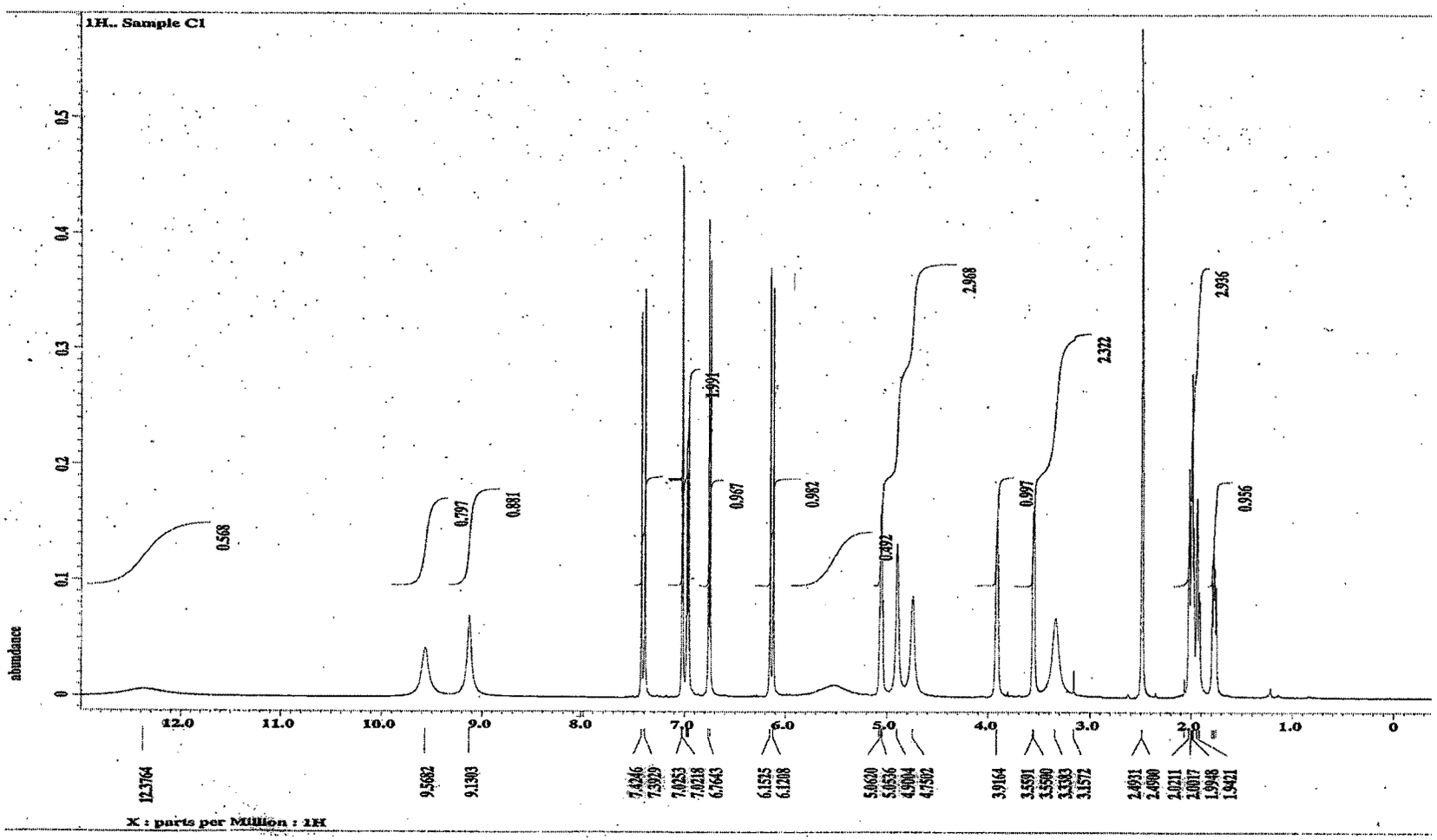


Table 60: Proton identification from ^{1}H NMR spectrum		
Sl no	**ppm**	**Peak**
1	9.67	Broad singlet
2	9.56	Singlet
3	7.40	Doublet
4	7.02	Doublet
5	6.96	Double Doublet
6	6.75	Doublet
7	6.13	Doublet
8	5.05	Quadruplet
9	4.90	Broad singlet
10	4.75	Broad singlet
11	3.92	Broad singlet
12	3.55	Broad singlet
13	3.33	Broad singlet
14	3.15	Singlet
15	2.02	Multiplet
16	2.01	Multiplet
17	1.97	Multiplet
18	1.78	Multiplet

Table 61: Various peaks from ^{13}C NMR	
Sl no	**ppm**
1	177.2
2	168.0
3	150.6
4	147.9
5	147.2
6	127.9
7	123.6
8	118.0
9	117.1
10	116.6
11	75.8
12	73.2
13	72.7
14	70.4
15	39.5
16	38.5

^{13}C NMR spectra: The number of signals in the ^{13}C NMR spectrum indicates the number of different types of carbon atoms. Thus from ^{13}C NMR spectra the following carbon atoms were identified (Table 61). The ^{13}C NMR spectrum was shown in Figures 26-28.

Figure 26: ^{13}C NMR spectrum of PD1

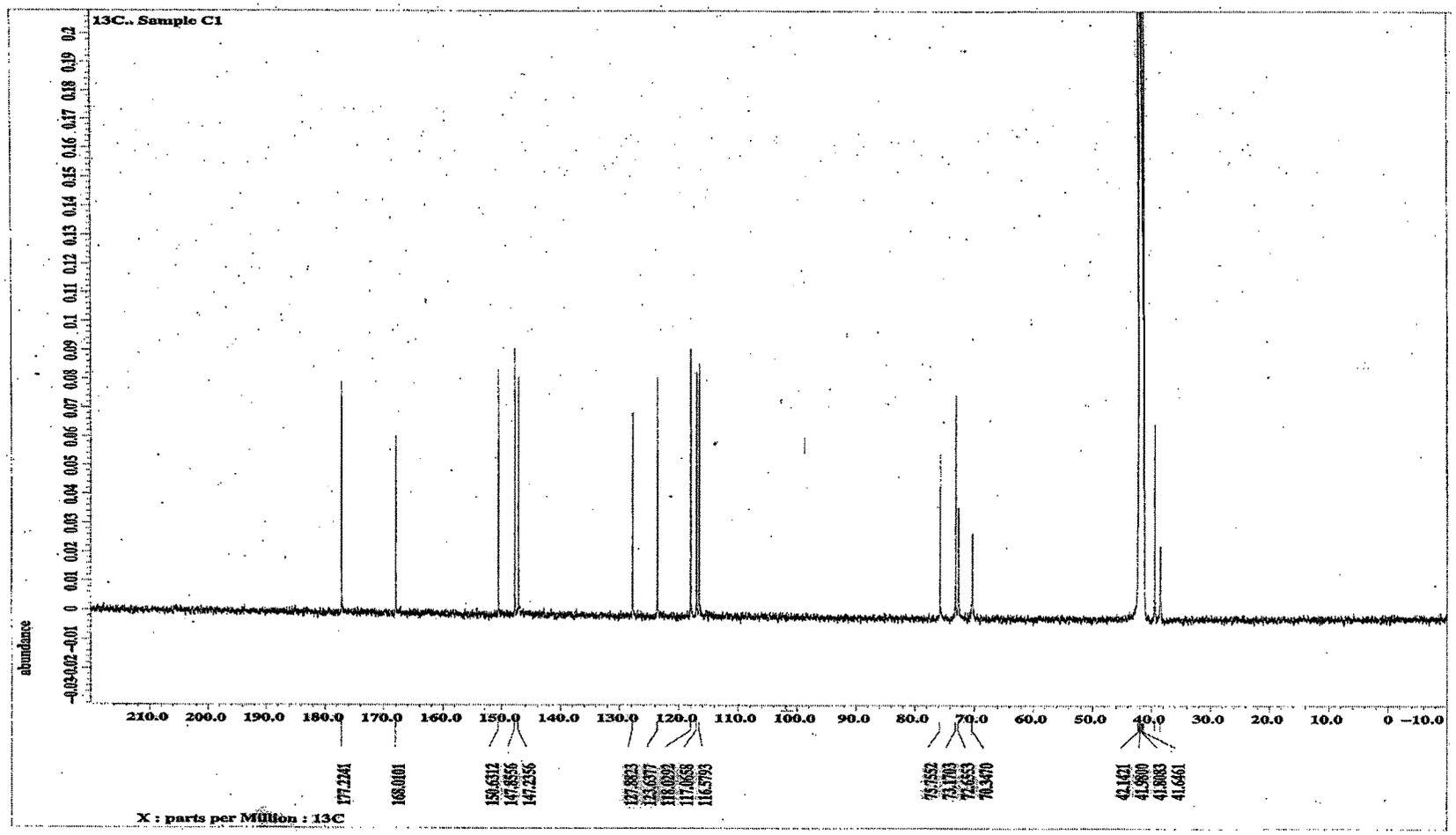


Figure 27: ^{13}C NMR spectrum of PD1 (resolved 1)

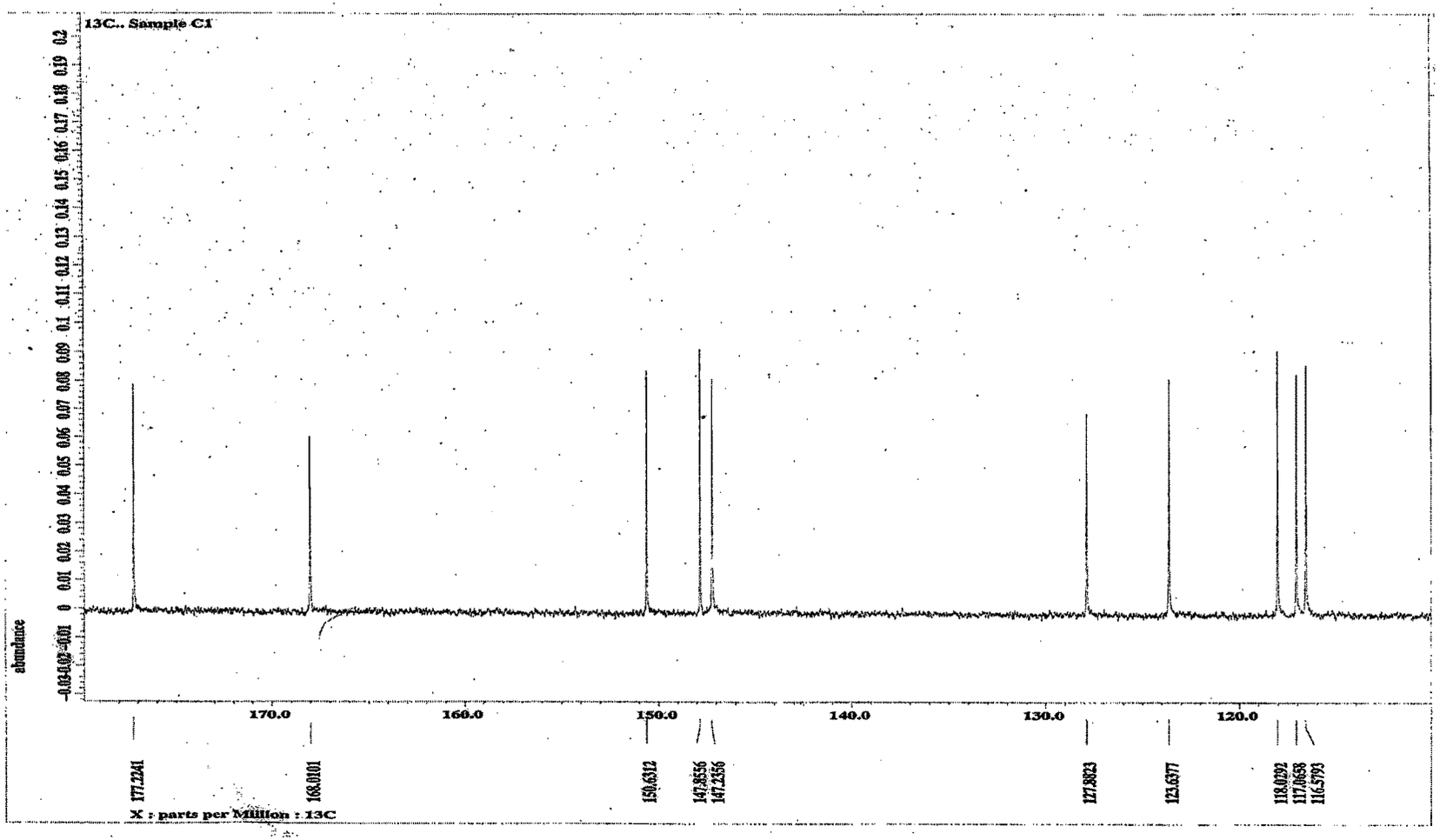
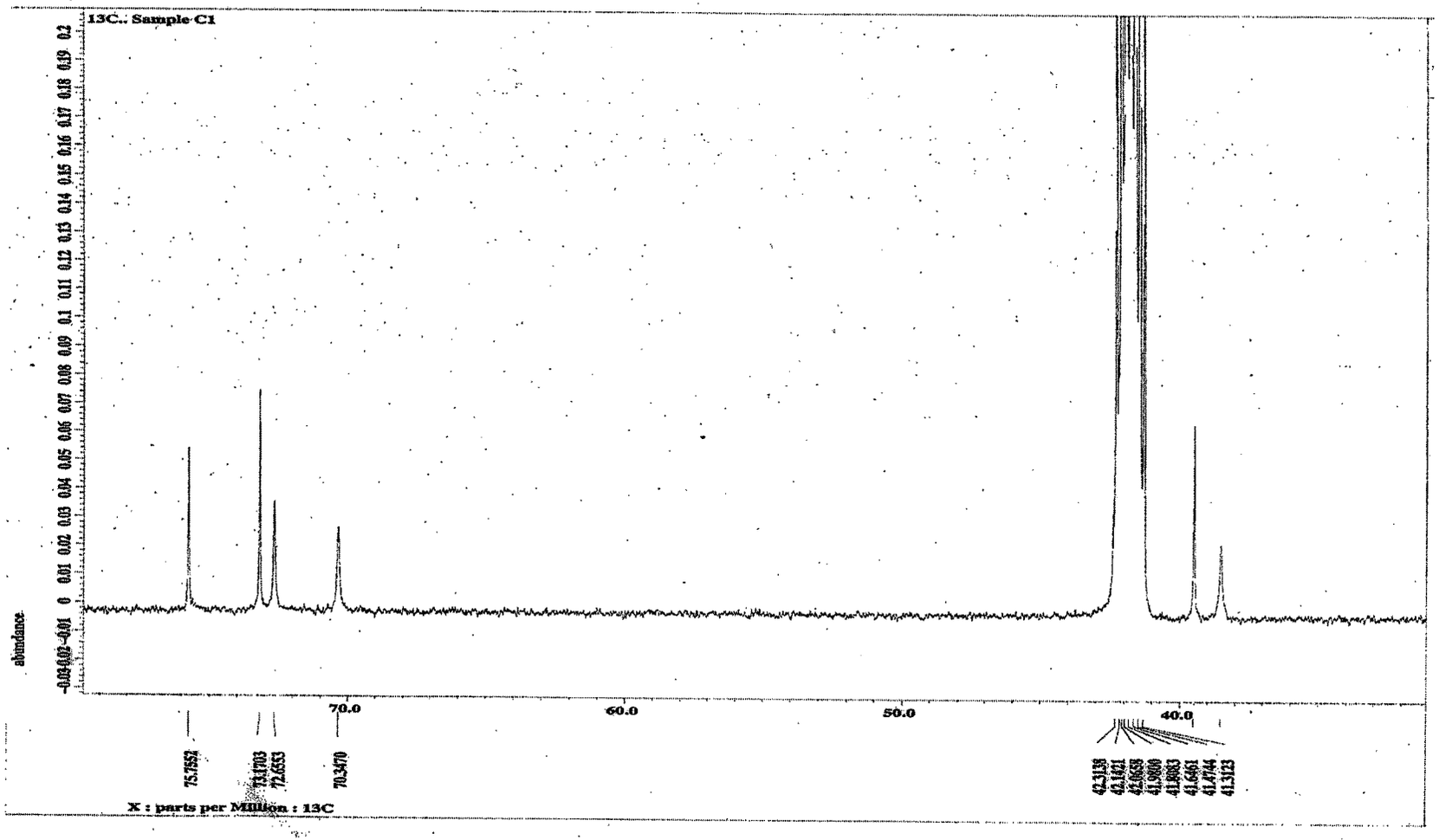


Figure 28: ^{13}C NMR spectrum of PD1 (resolved 2)



^{13}C DEPT NMR spectra: DEPT ^{13}C NMR changes the intensities of the ^{13}C signals based on the number of attached protons. Quaternary carbons are not observed and others are positive, negative and null depending on the angle used in the experiment. This helps to identify C, CH, CH_2 and CH_3. Thus from the data from ^{13}C NMR spectrum and ^{13}C DEPT NMR spectrum the following observations were made (Table 62). The ^{13}C DEPT NMR spectrum was shown in Figure 29.

Table 62: Various peaks and nature of the carbons from ^{13}C DEPT NMR

Carbon	ppm	Nature of carbon
1	177.2	Quadruplet
2	168.0	Quadruplet
3	150.6	Quadruplet
4	147.9	Quadruplet
5	147.2	CH
6	127.9	Quadruplet
7	123.6	CH
8	118.0	CH
9	117.1	CH
10	116.6	CH
11	75.8	Quadruplet
12	73.2	CH
13	72.7	CH
14	70.4	CH
15	39.5	CH_2
16	38.5	CH_2

Table 63: HSQC NMR data representing C-H bond couplings.

Sl no	Proton peak	Bonded carbon peak
1	7.40	147.9
2	7.02	117.1
3	6.96	123.6
4	6.75	118.0
5	6.13	116.6
6	5.05	73.2
7	3.92	70.4
8	3.55	72.7
9	2.02	39.5
10	2.01	38.5
11	1.97	38.5
12	1.78	39.5

HSQC NMR spectrum: The HSQC NMR spectrum was used to correlate directly bonded carbon to proton nuclei. The experiment utilizes proton and has high sensitivity. The one bond correlation between H-C, is facilitated by the pulse sequence. By interpreting the HSQC NMR spectra the C-H

Figure 29: ^{13}C DEPT NMR spectrum of PD1

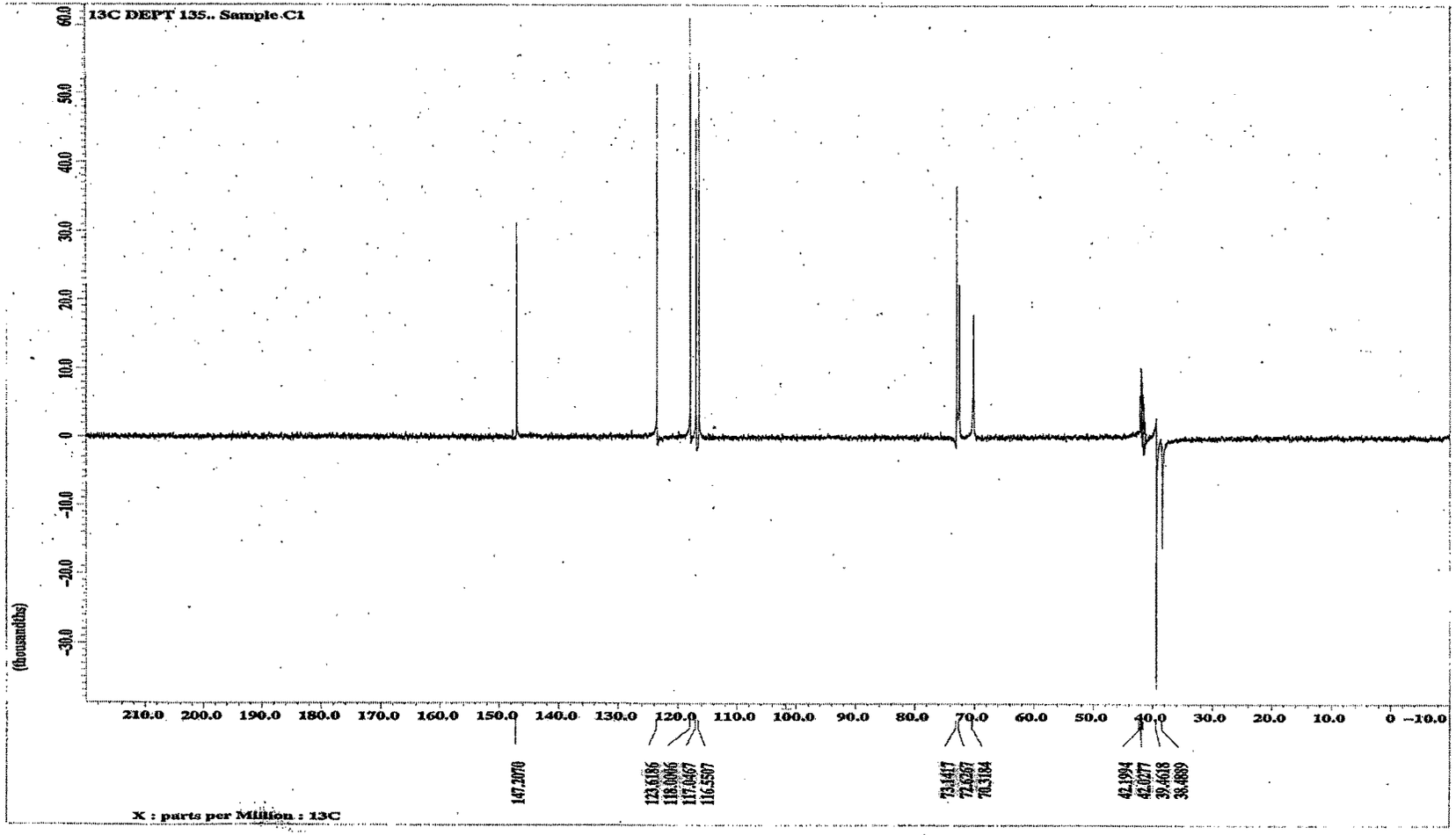
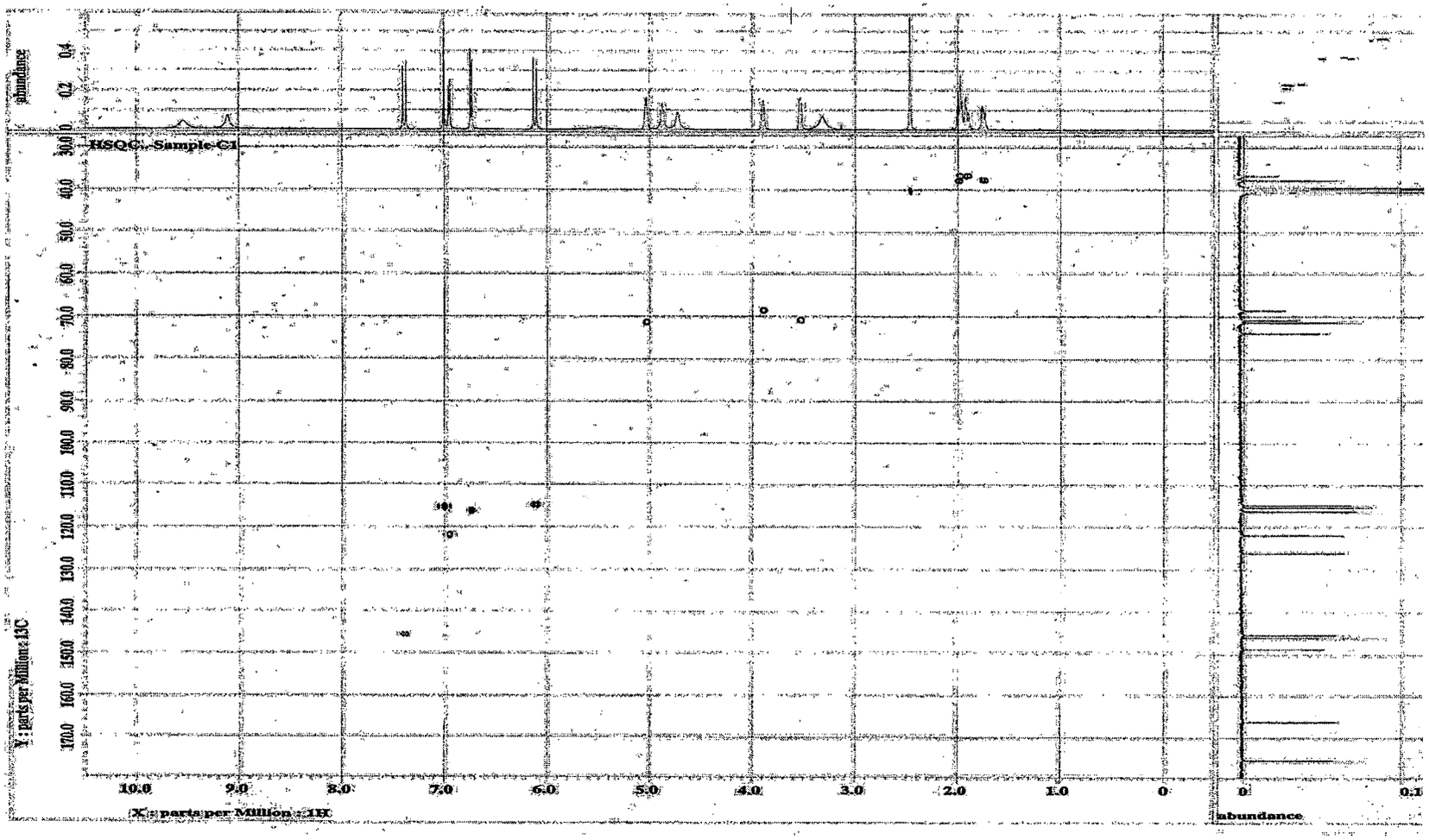


Figure 30: HSQC NMR spectrum of PD1



bond couplings were found (Table 63). The spectrum was shown in Figure 30. Thus the protons identified from the ^{1}H NMR spectrum are correlated with the carbon atoms from ^{13}C NMR spectrum with the help of HSQC spectrum. The rest of the protons at 9.56, 9.13 (carbonyl protons), 3.33 and 3.15 are found to be correlates with 'Ó' (OH).

DQF COSY NMR spectra: This is a variation to the standard HH COSY experiment, used for determining *scalar (J) couplings between protons*. The DQF-COSY sequence has two significant advantages. First, partial cancellation of the diagonal peaks in a DQF-COSY spectrum means that the diagonal ridge is much less pronounced in a DQF-COSY spectrum than it is in a normal COSY spectrum. This makes it easier to observe cross peaks between signals that are close together in chemical shift. Second, the double quantum filter eliminates the strong signals from, e.g., solvent.

The following proton couplings were confirmed from the DQF-COSY spectra.

1. Protons at 7.40 is coupled with proton at 6.13.
2. Protons at 7.02, 6.96 and 6.75 are found to be in one system.
3. Proton at 5.05 is coupled with proton at 2.01.
4. Proton at 3.55 is coupled with proton at 5.05.
5. Proton at 3.92 is coupled with proton at 1.78.
6. Proton at 3.55 is in the middle of 5.05 and 3.92.
7. The cosy spectrum shows a weak bond between 3.55 and 3.92 because of the stereochemistry as there is an axial and equatorial confirmation.
8. Proton at 1.78 is also coupled with proton at 2.02.

Thus from the DQF-COSY spectrum the possible scalar proton couplings are confirmed. The spectrum was shown in Figure 31.

NOESY spectrum: The NMR NOESY spectrum helps to find out the protons that are close in space. From the NOESY spectra the following confirmations were made.

1. Protons at 6.96 and 6.95 are closely spaced.
2. Protons at 1.97 and 2.01 are closely spaced.

Figure 31: DQF COSY NMR spectrum of PD1

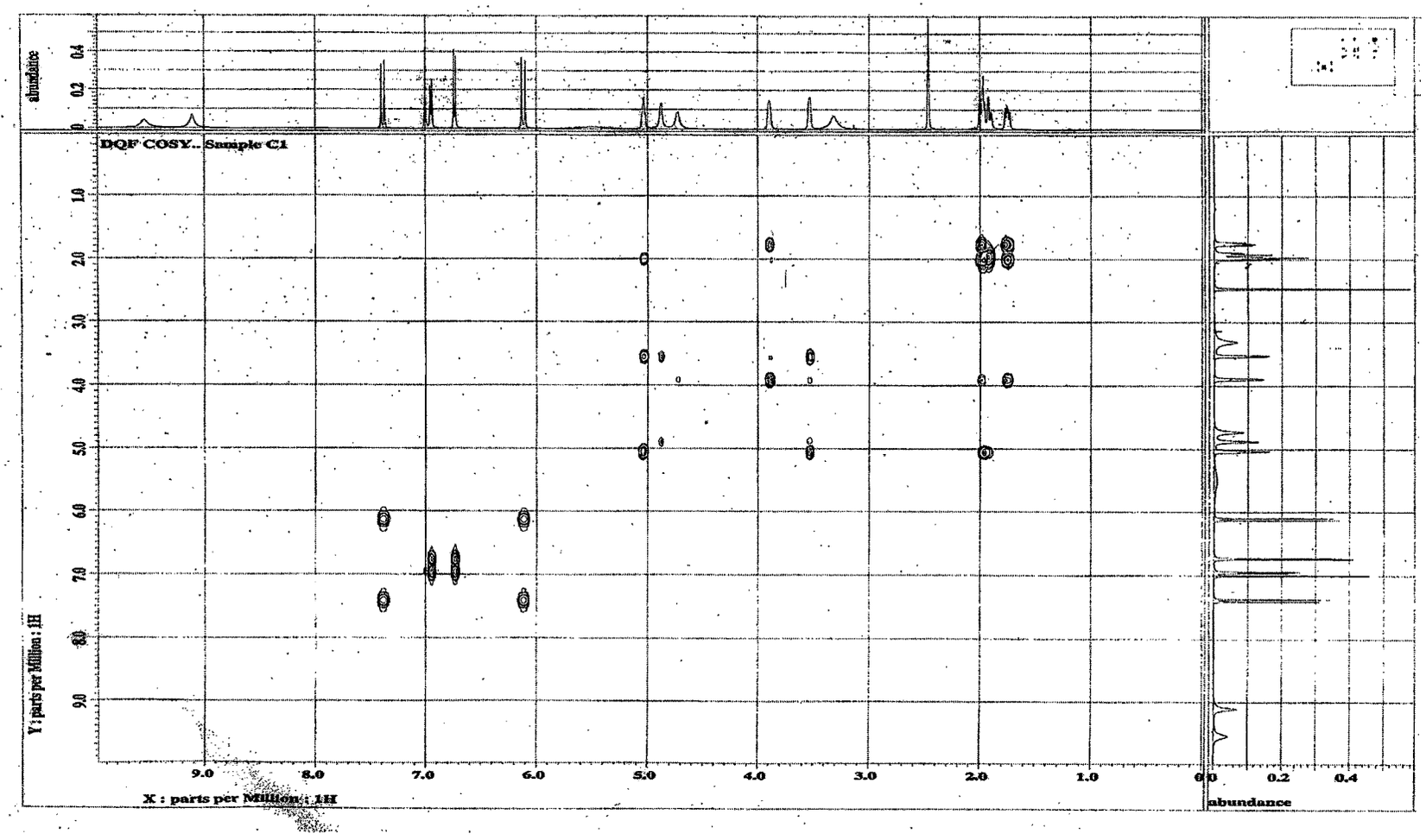
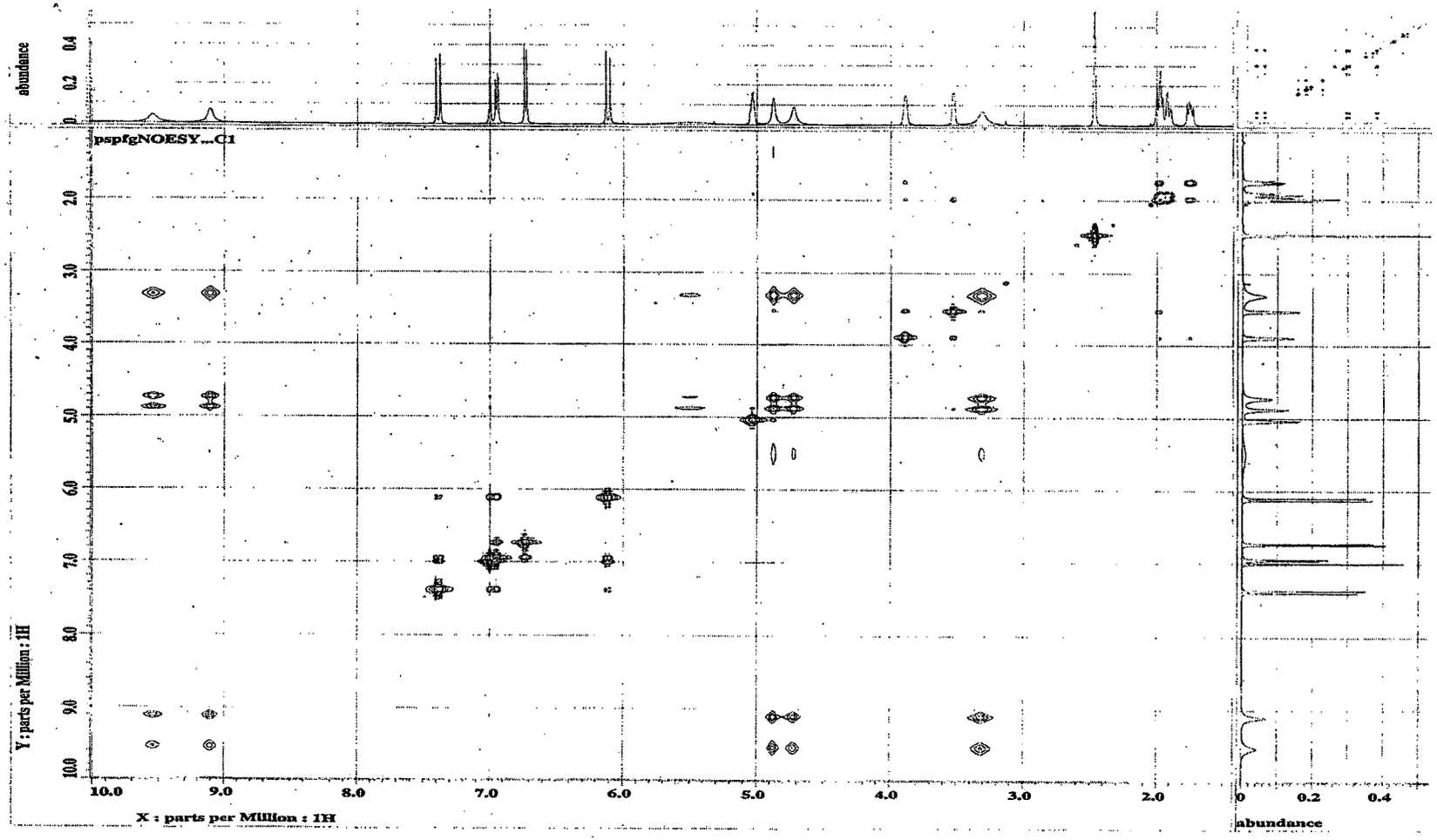


Figure 32: NOESY NMR spectrum of PD1



3. Protons at 7.02, 6.96, 5.05, 4.90, 3.92, 3.55, 3.33, 2.02, 2.01, 1.97 and 1.78 are found on the diagonal of the NOESY spectra.

The NOESY spectrum was shown in Figure 32.

Table 64: Assigning of carbon number to the carbons from HMBC spectra.

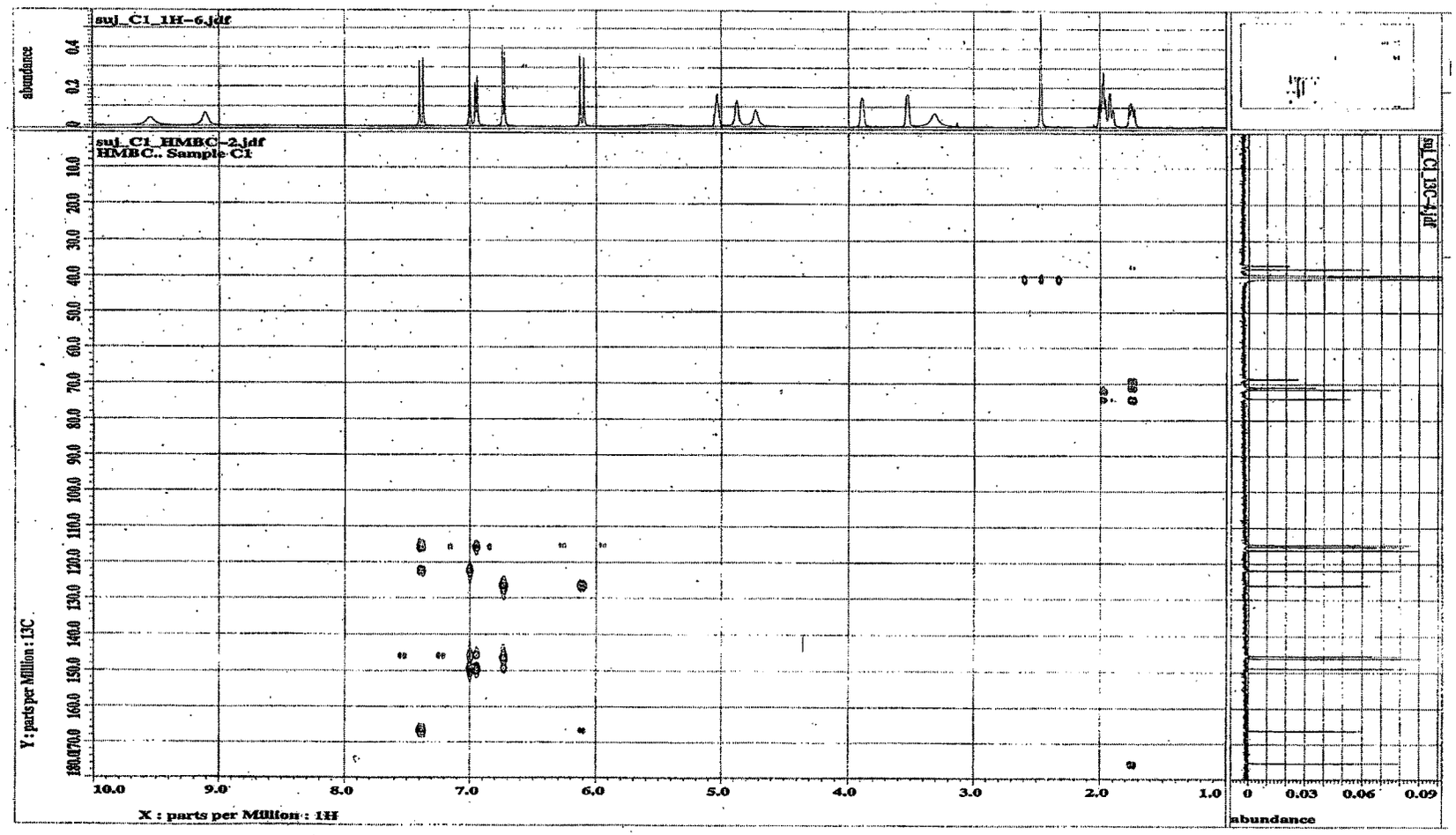
Carbon no	ppm
1	150.0
2	147.2
3	117.1
4	127.9
5	123.6
6	118.0
7	147.9
8	116.6
9	168.0
1^{I}	75.8
2^{I}	39.5
3^{I}	70.3
4^{I}	72.7
5^{I}	73.2
6^{I}	38.5

HMBC spectrum: This long-range 1H-13C correlation experiments also gives a 2d spectrum where ^{13}C chemical shifts are plotted on one-axis and ^{1}H chemical shifts plotted on another axis. Long range refers to 2 or 3 bonds since coupling over more bonds are extremely small. This experiment utilizes proton detection and has high sensitivity.

The final structure of the compound was confirmed by interpreting the HMBC spectrum. The following conclusions were made from the spectrum (Figure 33).

1. The carbon at 150.0 is correlated with carbons at 118.0 as well as 123.6.
2. Carbon at 147.7 is correlated with 150.6 and 117.1.
3. Carbon at 117.1 is correlated with carbons at 147.2, 150.6, 127.9 and 147.9.

Figure 33: HMBC spectrum of PD1



4. Carbon at 147.9 is correlated with carbons at 127.9, 123.6 and also with carbons at 117.1 and 116.6.

5. A carbon at 116.6 is correlated with carbons at 168.0, 147.9 and 127.9.

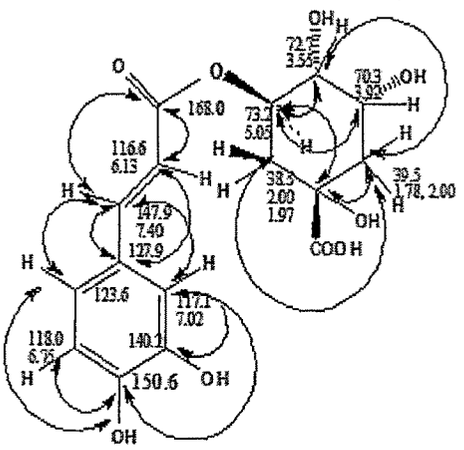
6. Carbon at 73.2 is correlating with carbon at 168.0 and also with 70.3, 72.7 and proton at 2.02.

7. Carbon at 39.5 is correlating with carbons at 70.3, 72.7 and 73.2 and with proton at 2.02.

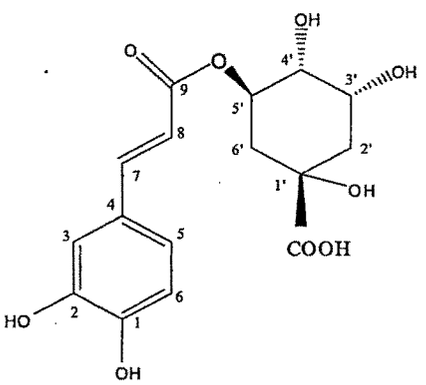
8. Carbon at 38.5 is correlating with 73.2, 39.5 and proton at 2.02.

Thus the following numbers were assigned for the carbons (Table 65)

This was showed below in the form of structure.



Thus from the HMBC spectra with the help of all other NMR spectra the structure of the compound was confirmed as **chlorogenic acid** which is as below.



IUPAC name: 3-[(E)-3-3(3,4-Dihydroxy phenyl)prop-2-enyl]oxy-1,4,5-trihydroxy-cyclohexane.

3.5.3.2 Characterisation of BM1:

The isolated BM1 was identified using physical and spectral data.

Description: White amorphous powder, and λ max was found to be 252 nm (in methanol).

Solubility: Soluble freely in ethanol and methanol. It is slightly soluble in acetone and insoluble in benzene.

Chromatographic studies: Thin layer chromatography of the compound using ethyl acetate: formic acid: acetic acid: water (100: 11: 11: 27) as mobile phase, silica gel 60 F_{254} pre coated plates as stationary phase and NP-PEG as a detecting agent revealed single spot (R_f 0.41) with orange colour fluorescence under UV 366 nm indicating flavonoidal nature of the compound.

Spectral studies:

FT-IR spectra

The IR spectra of the sample show peaks from 3413.7 to 791.0 cm^{-1}.

From the IR spectra the following deductions were made:

1. The peaks in the IR spectra from 3600-3300 cm^{-1} generally represent alcoholic O-H, amine or amide N-H and alkyne C-H. The spectra of the sample show a strong peak near 3413.0(3470.7 and 3360.7). The alcohol OH stretch is usually a broad and strong near 3400cm^{-1} which indicated the presence of alcoholic OH.

2. Peaks at 3000-2800cm-1 generally indicate the presence of alkyl (sp3) C-H. The spectra of the sample show peak at 2816.4 cm^{-1}confirming the presence of one alkyl CH. Peaks between 2720 and 2750 indicate the presence of carbonyl functional groups. The spectra of the sample shows a peak at 2729.8 cm^{-1} confirming the presence of =C-H aldehyde group.

3. Peaks between 1700-1640cm^{-1} represent amide C=O and conjugated C=O. As the conjugation lowers the absorption by 20-50 cm^{-1} the conjugated carbonyl's come slightly lower than normal C=O. The IR spectra of the sample shows a peak at 1648.0cm^{-1} indicating the presence of carbonyl C=O.

4. Peaks between 1600-1400cm^{-1} indicated the presence of aromatic C=C. The IR spectra of the sample show peaks at 1604.7cm^{-1}, 1503.7cm^{-1},

1455.6cm^{-1}, indicate the presence of aromatic C=C. more over peaks above 3000cm-1(for CH) confirms the presence of simple aromatic C=C compounds.

5. The region at 1500-400cm^{-1} is generally called as finger print region. Peaks at 1300-1000cm^{-1} indicated the presence of C-O. The IR spectra of the sample show peaks at1383.4cm^{-1}, 1359.3cm^{-1}, 1292.0cm^{-1}, 1200.6cm^{-1}, confirm the presence of C-O.

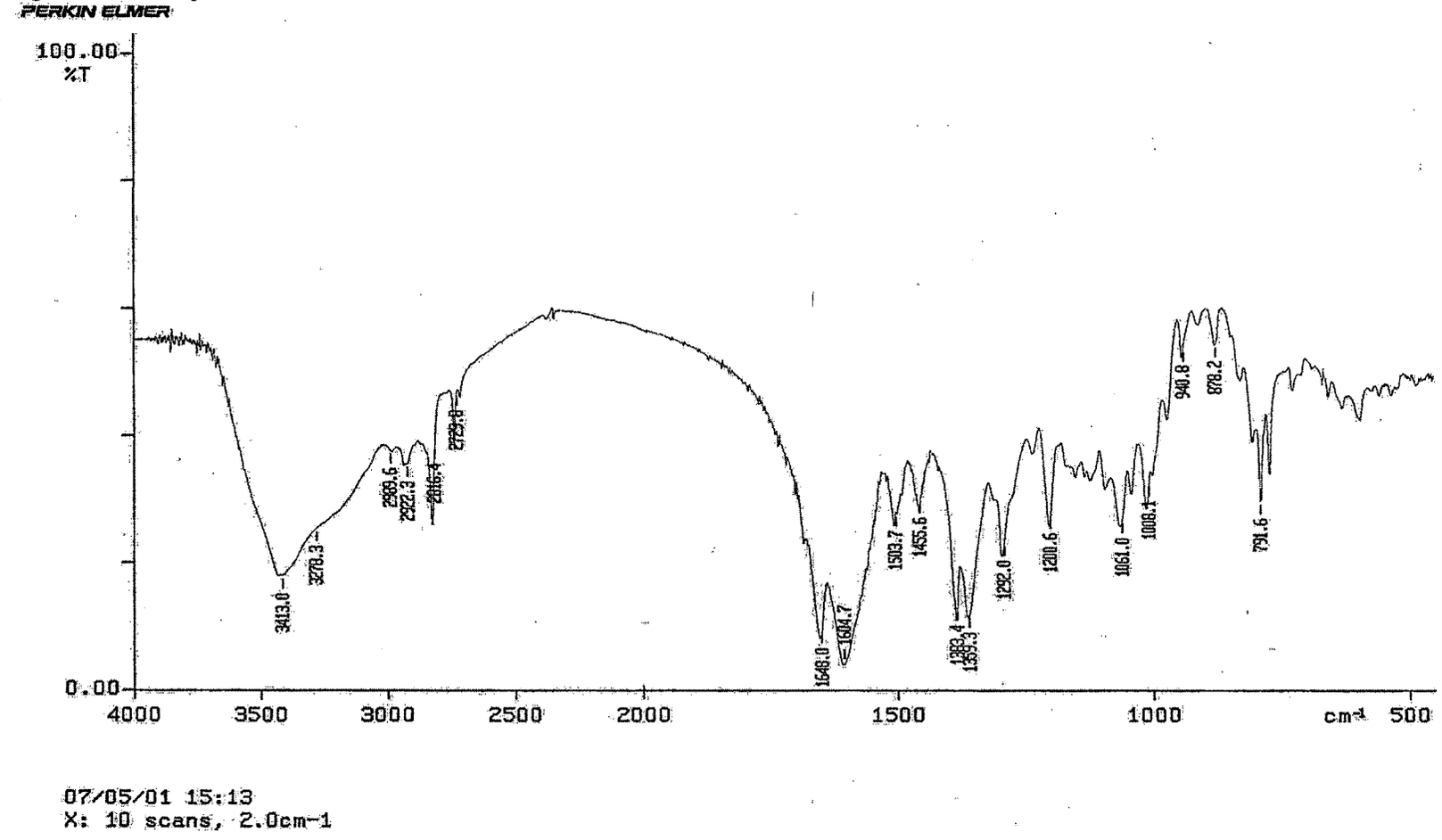
6. Peaks at 1000-675cm^{-1} indicated the presence of C-H alkene bending vibrations and the peaks at 870-625cm^{-1} indicate the presence of phenyl rings substitution bands. The IR spectra of the sample shows peaks 1061.0cm^{-1}, 1008.1cm^{-1}, 940.8cm-1,878.2cm^{-1},791.6cm^{-1} confirms CH bending vibrations and the peaks 878.2cm^{-1} further indicated the presence CH bending vibrations.

The FT-IR spectrum of the compound was represented in Figure 34.

The information obtained was confirmed and the structure was determined by interpreting the rest of spectra.

From the mass spectra the molecular formula was found to be C_{27} H_{30} O_{16}

Figure 34: IR spectrum of BM1



NMR spectra

Interpretation of all the NMR spectra (^{1}H NMR, ^{13}C NMR) was done to determine the final chemical structure of the compound.

^{1}H NMR spectrum: From the ^{1}H NMR spectra the following different types of protons are observed.

1. A peak at 8.45 ppm indicated the presence of a *singlet.*
2. A peak at 7.54 ppm also indicated the presence of a *quadruplet*
3. A peak at 6.81 ppm also indicated the presence of a *doublet.*
4. A peak at 6.31 ppm indicated the presence of a *singlet.*
5. A peak 6.10 ppm indicated the presence of a *singlet.*
6. A peak at 5.28 ppm indicated the presence of a *doublet.*
7. A peak at 4.38 ppm indicated the presence of a *singlet.*
8. A peak at 3.70 ppm indicated the presence of a *doublet.*
9. A peak at 3.41 ppm indicated the presence of a *singlet.*
10. Peaks at 3.28, 3.27, 3.25, 3.20, and 3.06 ppm indicated the presence of multiplets (showing multiple splittings in the spectrum) is indicating the presence of multiplets).
11. Peaks at, 2.49 and 1.21 ppm indicated the presence of a *singlets.*
13. Peaks at 1.12 and 0.99 ppm indicated the presence of *doublets.*

The results of ^{1}H NMR spectrum are tabulated in Table 65. The ^{1}H NMR spectrum of the BM1 was shown in Figure 35.

^{13}C NMR spectra: The number of signals in the ^{13} C NMR indicates the number of different types of carbon atoms.

Thus from ^{13}C NMR spectra the following carbon atoms were identified.

ppm: 176.7, 167.0, 161.2, 156.6, 149.5, 145.0, 133.0, 121.5, 120.8, 115.4, 115.0, 104.2, 101.2, 98.5, 93.5. 100.8, 71.9, 70.5, 70.3, 69.9, 17.7. 101.7, 74.12, 76.54, 66.9.

The ^{13}C NMR spectrum was shown in Figures 36.

Figure 35: ^{1}H NMR spectrum of BM1

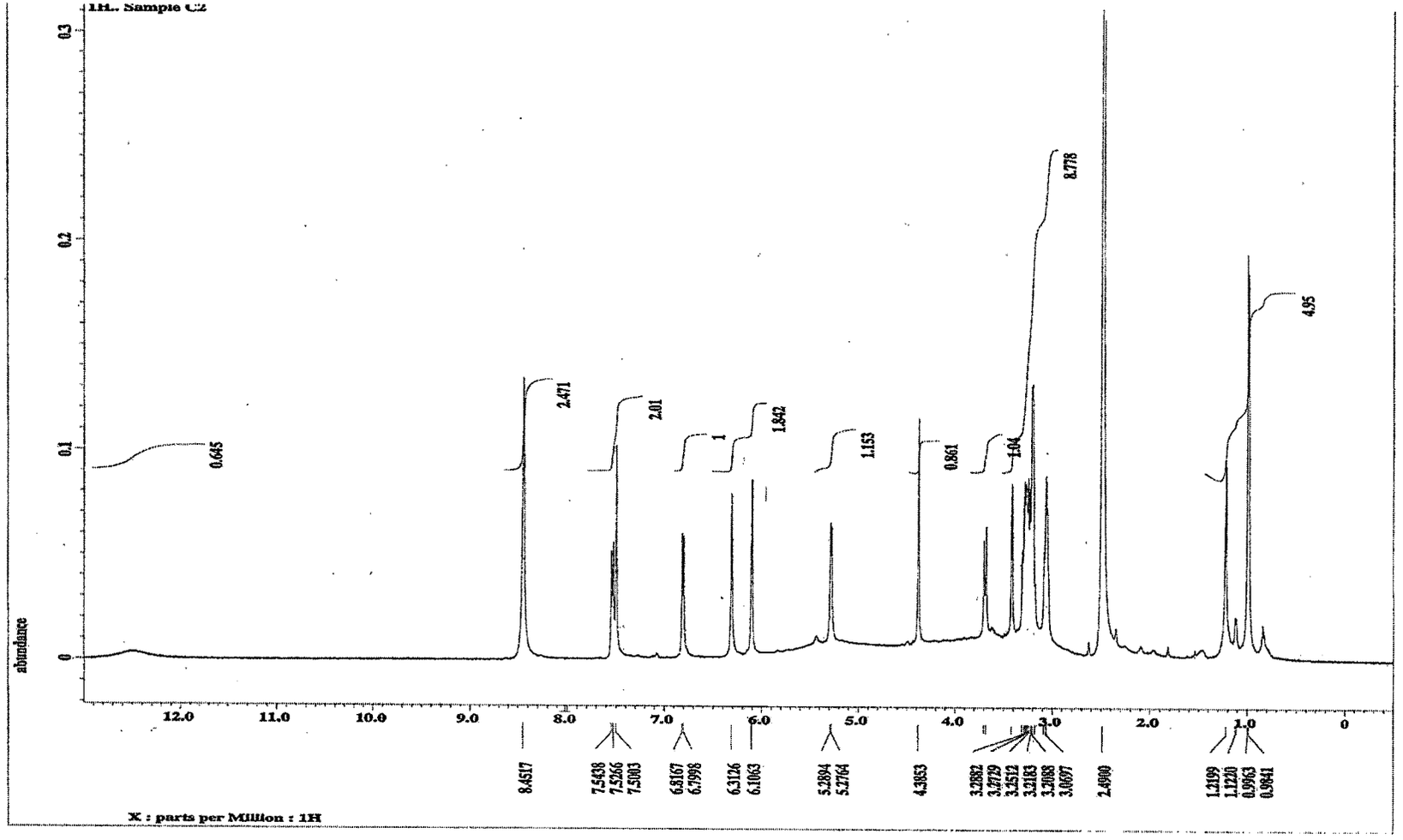
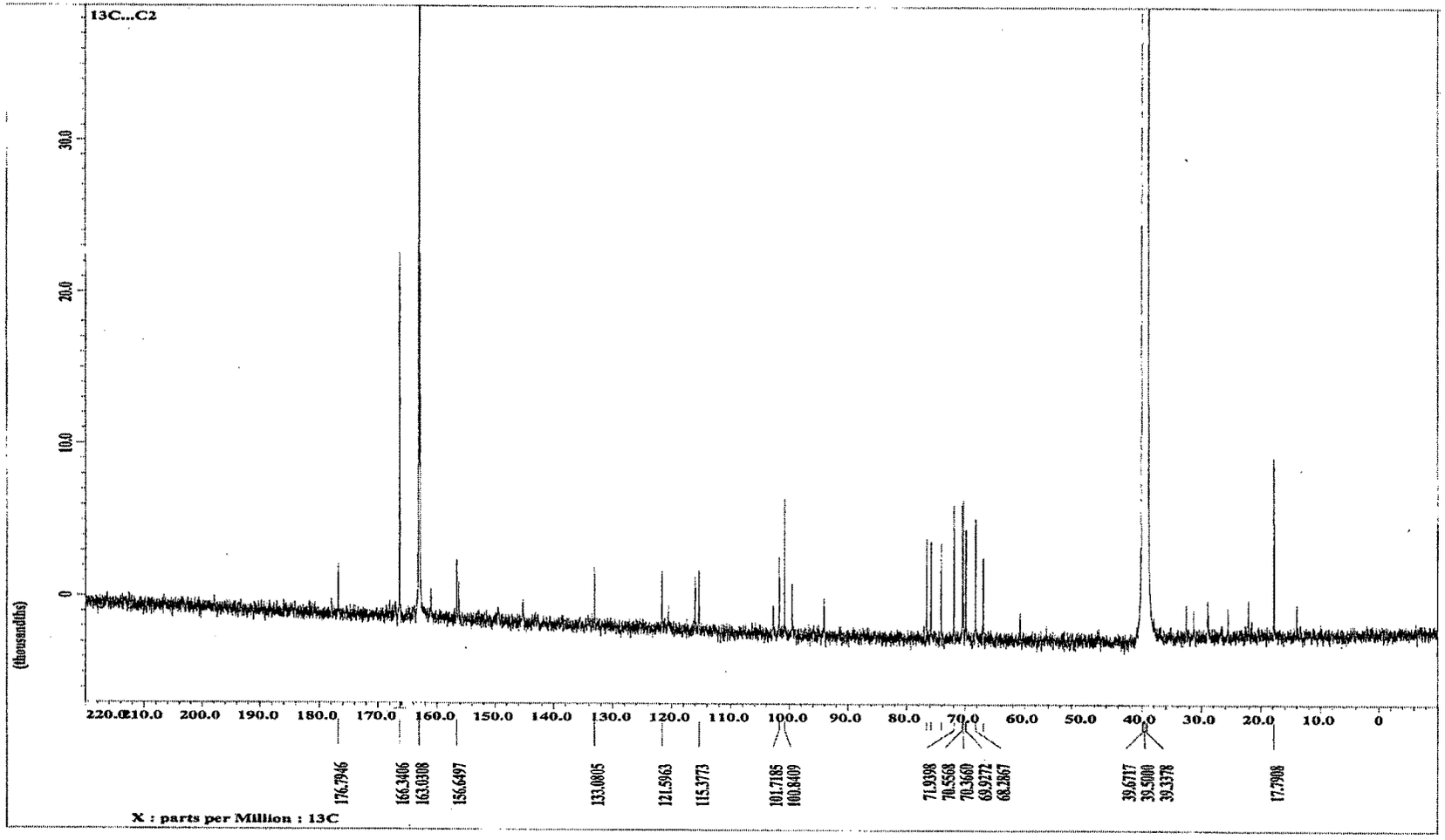
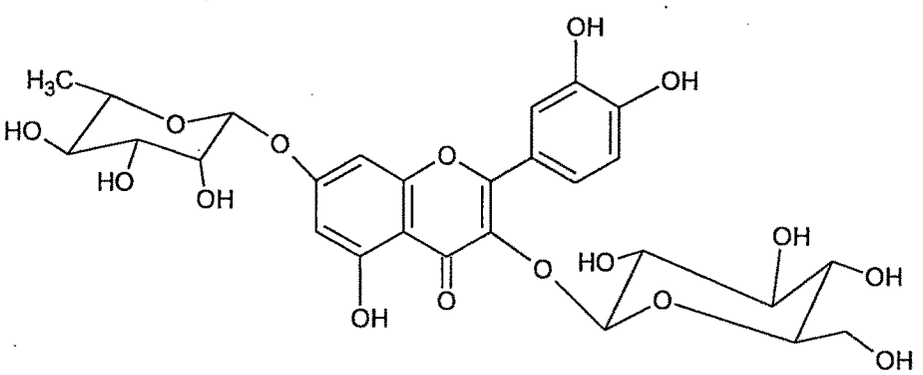


Figure 36: ^{13}C NMR spectrum of BM1



Based on these findings and literature (Markham et al., 1977) the following structure was proposed for the BM1 i.e. Quercetin-3-O-galactosyl-7-O-rhamnoside.



Quercetin-3-O-galactosyl-7-O-rhamnoside.

3.6 High performance thin-layer chromatographic analysis

3.6.1 Aerial parts of *P. daemia*

3.6.1.1 Fingerprint profile of EE, EFEE and AFEFEE

The HPTLC of EE resulted 8 bands at R_f 0.16, 0.29, 0.40, 0.54, 0.64, 0.81, 0.86 and 0.97, with the percentage areas of 8.39, 7.64, 33.32, 18.98, 17.94, 1.95, 1.95, 1.52 and 10.25 respectively. The EFEE exhibited 10 bands at R_f 0.06, 0.16, 0.30, 0.40, 0.52, 0.63, 0.75, 0.85, 0.92 and 0.98 with the percentage areas of these compounds are found to be 1.36, 11.21, 17.57, 43.91, 11.93, 9.98, 0.28, 0.94, 0.98 and 1.83 respectively. The AFEFEE exhibited 5 bands at R_f 0.28, 0.40, 0.53, 0.63 and 0.86 with percentage areas of 18.34, 59.77, 10.55 and 10.66 respectively. The standard quercetin-3-glucoside and PD1 exhibited a band at R_f 0.53 (orange) and 0.40 (blue) respectively.

The EE, EFEE and AFEFEE showed the presence of quercetin-3-glucoside and chlorogenic acid in the respective regions which matched with that of standard quercetin-3-glucoside and chlorogenic acid. The color, R_f value and spectral comparison was taken into consideration to detect the presence of quercetin-3-glucoside and PD1 in extracts. The results are represented in Figure 37.

These fingerprint profiles of the extracts developed will help as parameters in development of standardised bioactive extracts and fractions and plant material of *P. daemia*.

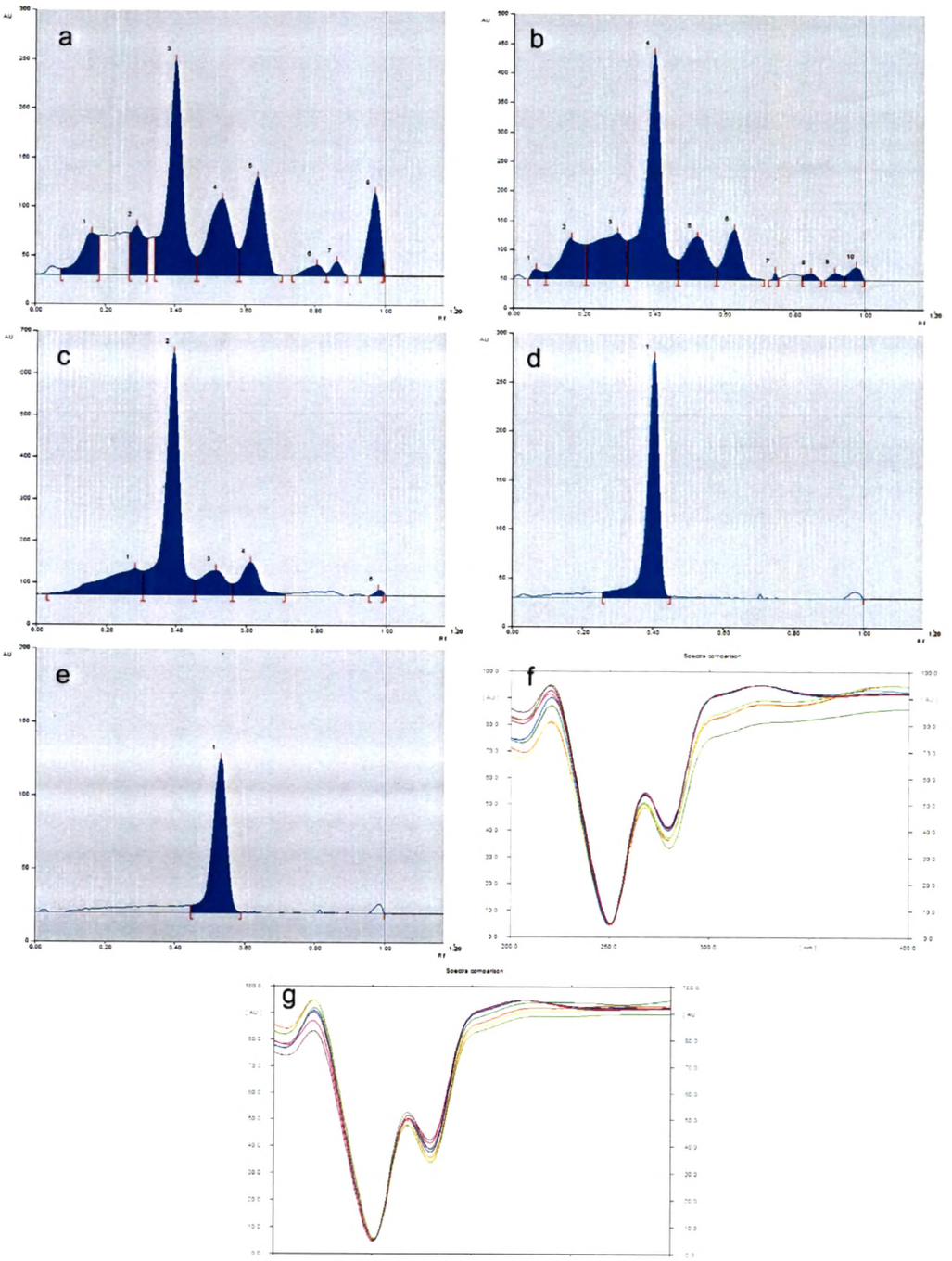
3.6.1.2 Estimation of PD1 in ethanol extract

The PD1 isolated from the ethanol extract (EE) was quantified. The calibration curve for the same was obtained in terms of peak area. The calibration curve was linear with the concentrations between 32 and 64 ng. The concentration of the PD1 in the extract was calculated form the calibration curve and was found to be 0.174 % w/w.

3.6.1.3 Estimation of quercetin-3-glucoside in ethanol extract

The estimation of quercetin-3-glucoside in the ethanol extract (EE) was carried out by constructing the linear calibration curve for the compound

Figure 37: HPTLC analysis of *P. daemia*



a: Chromatograph of EE 16 µl; b: Chromatograph of EFEE; c: Chromatograph of AFEFEE; d: Chromatograph of PD1; e: Chromatograph of quercetin-3-glucoside 8; f: Spectra comparison of PD1 in EE, EFEE and AFEFEE; g: Spectra comparison of quercetin-3-glucoside in EE, EFEE and AFEFEE

and then interpolating the test data from the curve. The calibration curve was linear with the concentration range between 16-32 ng. Calibration curve for the quercetin-3-glucoside was obtained in terms of peak area. The concentration of quercetin3-glucoside in ethanolic extract was found to 0.086% w/w.

Quantification of marker may reflect the quality of the raw material in general and quantitative composition of other phytoconstituents in particular.

3.6.2 Roots of *B. montanum*

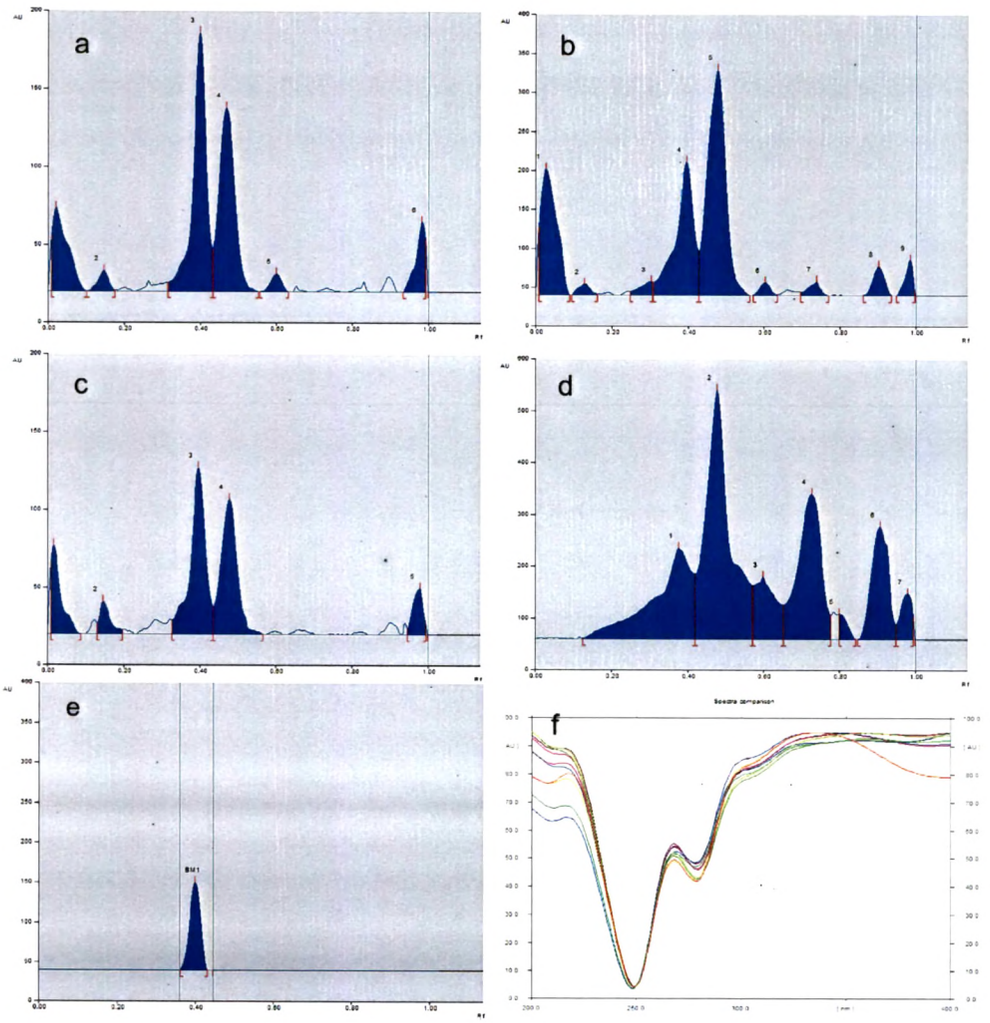
3.6.2.1 Fingerprint profile of ME, MFME, EMKMFME and MFMFME

The HPTLC of ME resulted 6 bands at R_f 0.02, 0.15, 0.40, 0.47, 0.60 and 0.98 having the peak areas of 14.60, 2.60, 39.28, 33.52, 2.32 and 7.67 respectively. The MFMFME resulted 9 bands at R_f 0.03, 0.13, 0.30, 0.40, 0.48, 0.60, 0.74, 0.90 and 0.98 having peak areas of 20.61, 1.45, 1.87, 24.32, 42.13, 1.45, 1.71, 3.49 and 2.98 respectively. MFFMFME showed 5 bands at R_f 0.02, 0.14, 0.40, 0.48, 0.98 having peak areas of 13.69, 5.47, 39.27, 34.00 and 7.57 respectively, while the EMKMFME showed 8 bands at R_f 0.40, 0.50, 0.56, 0.60, 0.61, 0.75, 0.82 and 0.93 with corresponding peak areas of 23.47, 35.30, 6.23, 2.19, 8.30, 16.06, 1.57 and 6.88 respectively. The BM1 exhibited a band at R_f 0.41. The results are shown in Figure 38.

3.6.2.2 Estimation of BM1 in methanol extract

The BM1 isolated from the methanol extract (ME) was quantified. The calibration curve for the same was obtained in terms of peak area and concentration. The calibration curve was linear with the concentrations between 400 and 1400 ng. The concentration of the quercitrin in the extract was calculated form the calibration curve was found to be 1.13% w/w.

Figure 38: HPTLC analysis of *B. montanum*



a: Chromatograph of ME; b: Chromatograph of MFME; c: Chromatograph of MFMFME; d: Chromatograph of EMKMFME e: Chromatograph of BM1; f: Spectra comparison of BM1 in ME, MFME, MFMFME, EMKMFME.

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