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Chapter-5

Result and Discussion

Tecomella undulata

5. Tecomella undulata

The systematic studies were undertaken on leaves and stem bark of *Tecomella undulata* in order to evolve parameters for standardization and to access their hepatoprotective activity. The results of different studies are compiled and discussed under following headings:

- Pharmacognostical studies and proximate analysis
- Preliminary phytochemical evaluation
- Separation and identification of bioactive marker constituents
- Biological evaluation

5.1 Pharmacognostical Evaluation

Some important pharmacognostical features for various parts of *Tecomella undulata* are documented here:

5.1.1 *Macroscopic features*

Leaves of *Tecomella undulata* are elongated, alternate and rounded at the tips; lamina is elliptic-oblong to elliptic-lanceolate or linear-oblong; margin undulate and petiole is 6-18 mm long. Stem bark of *Tecomella undulata*, occur in flat or slightly curved pieces, about 6-8 mm in thickness. The outer surface of bark is greyish brown with occasional small dark patches. Longitudinal zigzag furrows and irregular transverse cracks are present on outer surface making it rough. A few vertically elongated lenticels are also present. The inner surface of bark is smooth and buff to brownish in colour. The photograph of leaves and stem bark are shown in Figure 5.1 and there characteristic features are reported in Table 5.1.

Figure 5.1 Photographs of leaves and stem bark of *Tecomella undulata*

Table 5.1 Characteristic macroscopic features of *Tecomella undulata* leaves and stem bark

Features	Observations	
	Leaves	Stem bark
Colour		
-Inner surface	Green	Dark Brownish
-Outer surface	Dark green	Dull Brown grey or grey in colour
Odour		Odourless
Taste	Bitter	Tasteless
Shape	Elongated	Curved
Fracture		Short
Size		
-Length	2.5 - 3 cm	20 cm
-Bridth		8 mm (thickness)

These features will assist in identifying the selected drug on preliminary basis.

5.1.2 Microscopic studies

In proper identification of medicinal herbs, results of morphological determination pose unacceptability; because the plants are either available in mutilated form or sold without definite structure of the organ. Thus, microscopy (Transverse sectioning) along with powder microscopy remains the indispensable and cost effective tools of conventional analytical pharmacognosy. In this regard, the important microscopic and powder microscopic features of *Tecomella undulata* leaves and stem bark have been documented herein.

5.1.2.1 Transverse section of Tecomella undulata leaf

Leaves are isobilateral with mesophyll tissue comprising of palisade layers on either side of the spongy tissue. Both epidermises are covered with thick cuticle. Below the upper epidermis there is a single layer of loosely arranged rod shaped palisade cells. Above the lower epidermis there are two layers of same sized palisade cells. Spongy tissue comprises of only 2-3 layers of cells sandwiched between the palisade layers.

Multicellular stalked glands are intermittently found deeply sunken in both epidermises. It is made up of 13-15 radiating small cells which form the head and the stalk is made up of a single cell. Midrib is uniconvex with abaxial side deeply cresceutic. Large cresceutic vascular bundles lies on the abaxial side with phloem towards the abaxial side and xylem elements arranged radially toward the adaxial

side. Opposite to the large cresceutic vascular bundle lays four small vascular bundles arranged in the form of an arc with phloem towards the adaxial side.

Multicellular glands are also present on the midrib with its density being more on the abaxial side. Below the upper epidermis and above the lower epidermis of the midrib 3-4 layer of distinct collenchyma cells are noticed. Palisade layer on the adaxial side extends in to the midrib. Next to the colenchyma cell layers, few layer of thin walled Parenchyma cells with starch grains are present in which the vascular bundles are embedded. The microscopic features of leaf T.S. are depicted in Figure 5.2.

5.1.2.2 Powder microscopic features of Tecomella undulata leaves

Powder analysis of leaf showed the presence of irregular shaped epidermal cells with undulating anticlinal walls. Anisocytic stomata with guard cells and surrounding 5-6 subsidiary cells with arched walls are seen embedded intermittently between epidermal cells. Fragments of the multicellular glands are present. The multicellular head made up of 13-15 radiating cells is prominently observed. Transverse view of leaf fragments showing multicellular glands embedded in epidermal layer with thick cuticle is also noticed. These features are depicted in Figure 5.3.

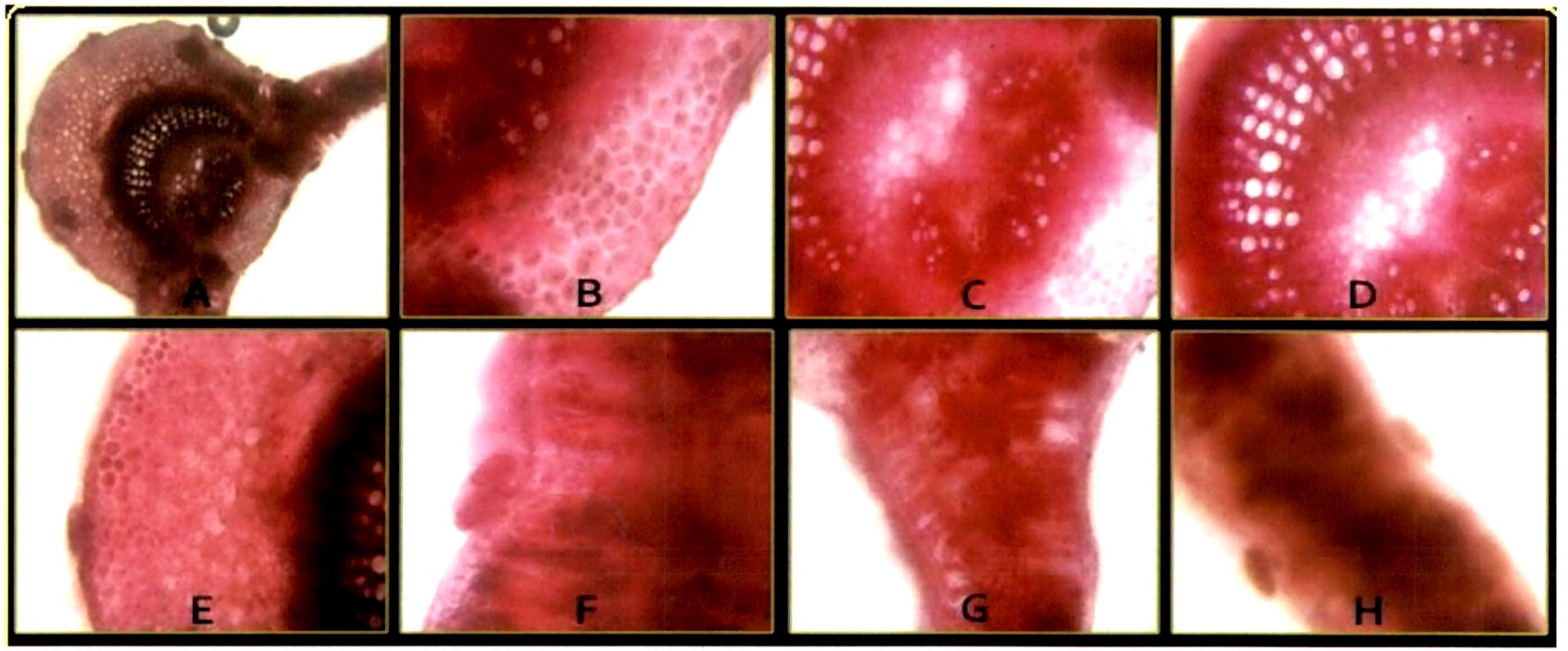
5.1.2.3 Transverse section of Tecomella undulata stem bark

Microscopically young stem shows a single layer thick walled epidermis, some of its cell forming non- glandular and glandular hairs, glandular hairs called stalk and 12-16 celled head, and multiseriate and branched non-glandular hairs. The microscopic features of stem bark T.S. are depicted in Figure 5.4

5.1.2.4 Powder microscopic features of Tecomella undulata stem bark

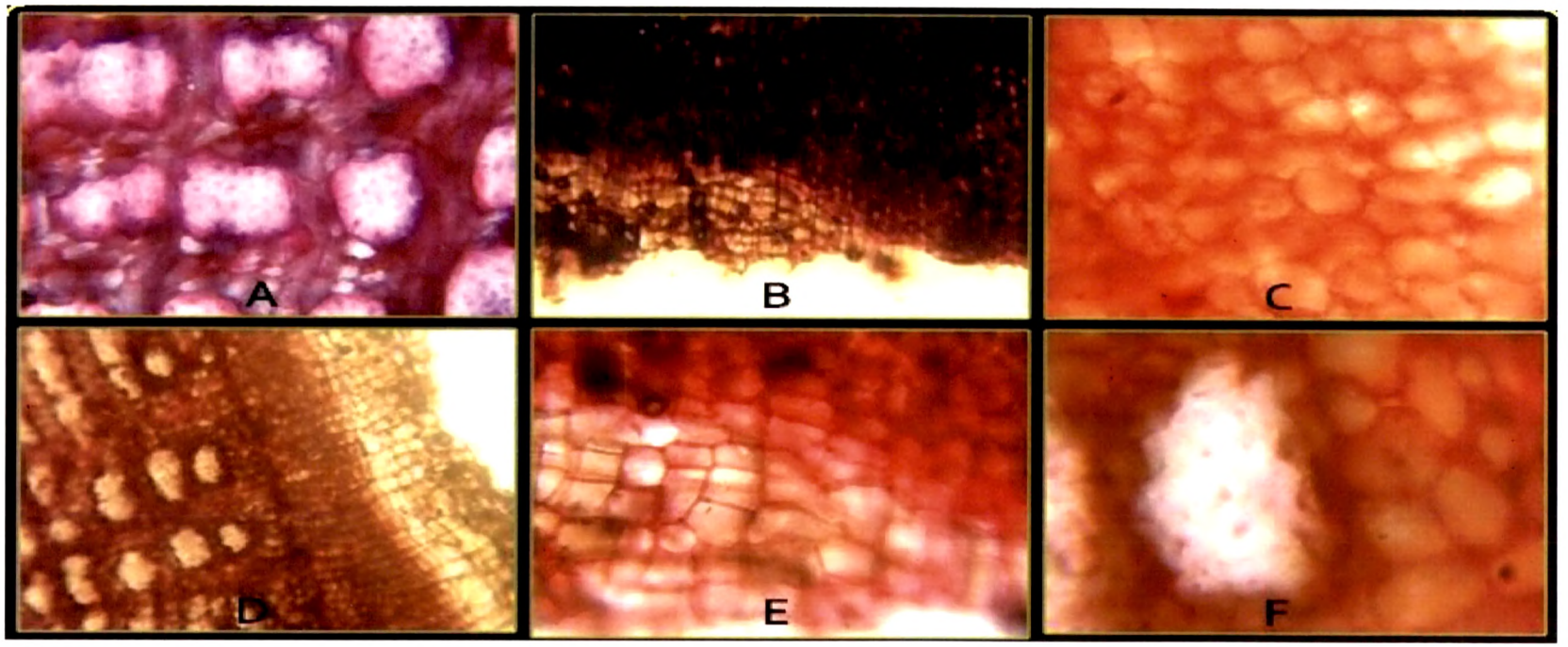
The stem bark powder is greenish brown in colour with a characteristic odour. The powder microscopy shows some important features like unicellular glandular trichomes, periderm cells and scleraids/ scleratic cells. These features are depicted in Figure 5.5.

Figure 5.2 Charestiristic features of transverse section of *Tecomella undulata* leaves



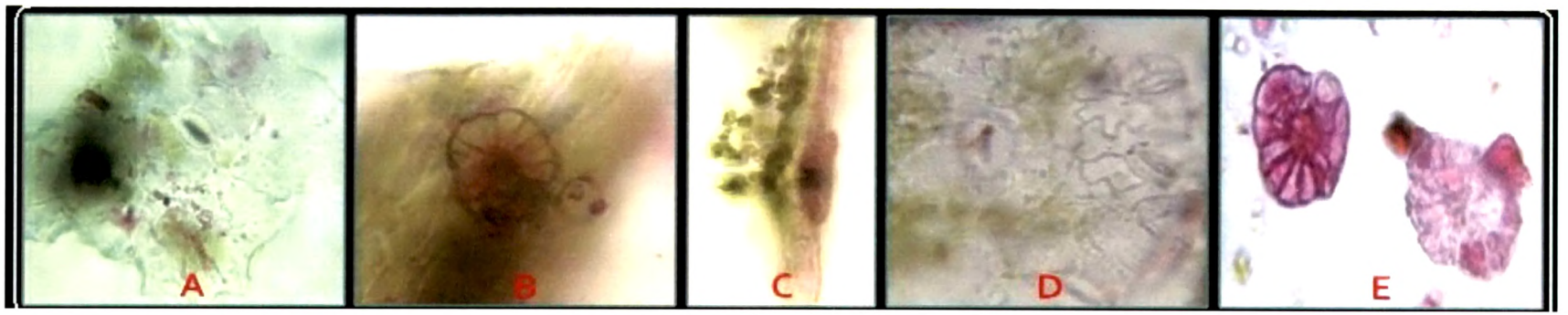
A)T.S. of leaves midrib showing cresceutic shaped large vascular bundles towards abaxial side, opposite to it four vascular bundles are arranged in omega form, B) Adaxial side of the midrib showing epidermis and 5-6 layers of collenchymas, C) 4 vascular bundles on abaxial side arranged in omega form, D) Large ascent shaped vascular bundle on abaxial side of midrib, E) Abaxial surface of midrib with the epidermis showing multicellular gland F) Multicellular gland embedded in epidermis, G) T.S. of lamina showing a single palisade layer on the adaxial surface and 2-3 layers of palisade on abaxial surface, H) Multicellular glands on upper and lower epidermis.

Figure 5.4 Charecteristic features of transverse section of *Tecomella undulata stem bark*



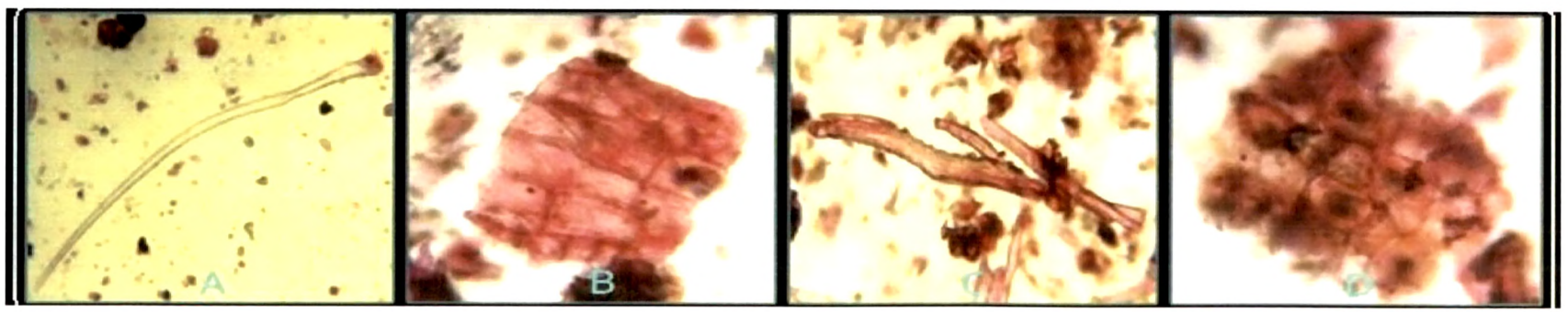
A) Layers of sclerotic cells alternating with phloem layers; B) Periderm with low magnification; C) Cells of phelloderm; D) T.S. of bark showing periderm and outer pheloem; E) Periderm 6 layers (Phellum); F) Phloem layer and scleratic cells patch.

Figure 5.3 Charestiristic features of powder microscopy of *Tecomella undulata* leaves



A) Surface view of epidermal layer ; B) Multicellular, glandular trichome ; Surface view of pellate hairs/trichomes; C) Fragments of leaves epidermis with pellate hairs/ multicellular hair; D) Stomata and undulating walls of epidermal cells; E) Multicellular trichomes

Figure 5.5 Characteristic features of powder microscopy of *Tecomella undulata stem bark*



A) Unicellular glandular trichome, B) Periderm cells, C) Sclererids/ scleratic cells, D) Periderm cells

5.1.3 Proximate analysis

Ash values are helpful in determining the quality and purity of crude drugs in powdered form. The total ash usually consists of inorganic radicals like carbonates, phosphates, silicates and silica of sodium, potassium, magnesium and calcium. Sometimes, inorganic variables like calcium oxalate, silica, carbonate content of crude drug effects "total ash" values, such variables are then removed by treating with acid (as they are soluble in hydrochloride acid) and then acid insoluble ash value is determined. The values vary within fairly wide limits and are therefore an important parameter for the purpose of evaluation of crude drugs. Water soluble ash is that part of the total ash content which is soluble in water. These values are determined for *Tecomella undulata* leaves and stem bark.

Ash values of *Tecomella undulata* leaves and stem bark shows relatively high total ash indicating high quantity of carbonates and oxide (Table 5.2). A low acid insoluble ash values are obtained in leaves and stem bark of *Tecomella undulata* indicates less silicious material like earth or sand.

Extractive values determining the amount of active constituents extracted with solvents from a given amount of meditional plant material. They are useful for evaluation of crude drugs and give in idea about the chemical nature of chemical constituents present in them. The water soluble extractive values of *Tecomella undulata* leaves and stem bark are high as compared to alcohol soluble extractive values, indicating that a high quantity of polar constituents are present in them as compared to non polar constituents (Table 5.2).

Table 5.2 Physicochemical analysis of *Tecomella undulata* leaves and stem bark

Parameters	Values% (w/w) * ±SEM	
	Leaves	Stem bark
Total Ash	9.32±0.48	7.92±0.33
Acid insoluble ash	4.54±0.02	1.49±0.08
Water soluble ash	2.49±0.22	4.02±0.21
Water soluble extractive values	21.49±0.65	9.31±0.54
Alcohol soluble extractive values	16.64±0.32	8.97±0.93

*** Values expressed as mean of three readings**

5.1.4 Elemental analysis

All the living organisms require inorganic elements for their growth and survival. Medicinal plant contain considerable amounts of mineral constituents, in particular, the presence of essential elements (Mg,Mn,Zn and many others) is a prerequisite for correct growth and development of plants. Inorganic elements in plants also plays role in the accumulation of secondary metabolites such as alkaloids, glycosides, terpenoids, phenolic compounds etc. (Andrijani, 1998) as their responsible for the activity of a number of enzymatic systems, which in turn regulate the metabolic pathways leading to the synthesis of these compounds. Results of elemental analysis of plant material under study viz., leaves and stem bark are given in table 5.3.

Table 5.3 Elemental analysis values of *Tecomella undulata* leaves and stem bark

Elements	Values in ppm/%	
	Leaves	Stem bark
Potassium	0.93 %	0.45 %
Sodium	0.11 %	0.05 %
Iron	5294.00 ppm	618.00 ppm
Zinc	58.00 ppm	10.00 ppm
Copper	727.00 ppm	5.8 ppm
Mangenese	3516.00 ppm	30.00 ppm

5.1.5 Total Phenolic, flavanoid and flavanol content

The total phenolic, flavanoid and flavanol contents of leaves and stem bark of *Tecomella undulata* in methanol and aqueous extracts are given in Table no 5.4 The phenolic content of methanolic extract of *Tecomella undulata* leaves and stem bark were found to be slightly higher than the aqueous extract and represent the various phenolic compounds like poly phenol, phenolic acid etc.

Table 5.4 Quantitative evaluation of total phenolic, flavanoid and flavanol content of leaves and stem bark of *Tecomella undulata*

Plant Parts	Extract	Total [a] Phenolics	Total flavanoids [b] ($AlCl_3$) method	Total [b] Flavanols
Leaves	Methanol	0.125±0.20	0.0139±0.66	0.0217±0.40
	Aqueous	0.0516±0.30	0.0058±0.36	0.0078±0.71
Stem bark	Methanol	0.173±0.18	0.0021±0.31	0.0066±0.91
	Aqueous	0.112±0.01	0.0048±0.81	0.0099±0.29

a= mg gallic acid/g; b= mg quercetin/g; * values expressed as mean of three readings; SEM

5.2 Preliminary phytochemical analysis of *Tecomella undulata*

5.2.1 Successive solvent extraction

The leaves and stem bark of *Tecomella undulata* were separately subjected to successive solvent extraction. Percentage yield and colour of the selected successive extracts were recorded in Table 5.5. 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.

Leaf and stem bark of Tecomella undulata gives maximum extractive value with methanol where as with ethyl acetate these extractive values were found to be very less.

Table 5.5 Successive extractive values of *Tecomella undulata* leaves

Solvent	Values%(w/w)*			
	Leaves	Appearance	Stem bark	Appearance
Petroleum ether	**2.75**	Yellowish green	**0.88**	Yellowish
Benzene	**1.4**	Dark brown	**1.78**	Greenish yellow
Chloroform	**1.05**	Yellowish brown	**2.53**	Brownish
Ethyl acetate	**0.25**	Yellowish green	**0.43**	Brownish
Methanol	**13.55**	Brownish	**7.05**	Brownish
Water/Aqueous	**7.23**	Brownish	**6.05**	Brown

5.2.2 Phytochemical analysis of successive extracts

Wide arrays of natural compounds like, alkaloids, glycosides, saponins, phytosterols, phenolics, terpenoids, flavonoids, coumarins, and tannins which exert physiological activity are synthesized in the plant, in addition to carbohydrates, protein and lipids utilized by man as food. A systematic and complete study of crude drug by different qualitative tests will provide detail information regarding the presence and absence of both primary and secondary metabolites derived as a result of plant metabolism. Establishing phyto-chemical profile of the extracts reveals the nature of chemical constituents and their composition. The extracts obtained in successive extraction

process were subjected to various qualitative chemical tests to determine the presence of various constituents. Results of phytochemical analysis for leaves and stem bark of all the extracts are summarized in Table 5.6

Table 5.6 Preliminary phytochemical investigations of *Tecomella undulata* leaves and stem bark

Phytochemical	Pet. Ether Extract		Chloroform Extract		Methanol Extract		Water Extract	
	L	SB	L	SB	L	SB	L	SB
Alkaloids	—	—	+	+	—	+	—	—
Phenols/ Tannins	—	+	+	+	+	+	+	+
Flavonoids	—	—	+	—	+	+	+	+
Saponins	—	—	+	+	+	+	+	+
Steroids	—	+	—	—	+	+	—	—
Terpenoids	—	—	+	-	+	-	—	—
Glycosides	—	—	—	—	—	+	+	+

'L' Leaves; 'SB' Stem bark; '+' presence; '-' absence

5.2.3 TLC of the extracts obtained From *Tecomella undulata* in successive extraction

TLC is very effective technique for the separation and identification of chemical constituents present in the extract. Components are separated by the differential migration of solute between two phases-a stationary phase and a mobile phase. Depending on the particular type of stationary phases and using different solvents, separation can be achieved on the basic of partition or a combination of partition and adsorption. The best advantage of TLC is the high sample throughput which results from the small amount of sample preparation required and the simultaneous quantification of several samples. Thus, TLC studies were performed on the extracts obtained after successive extraction of *Tecomella undulata* leaves and stem bark.

5.2.3.a TLC of the leaves extract obtained after successive extraction

The extracts obtained in the successive solvent extraction process were subjected to thin layer chromatography (TLC) studies in order to confirm the results obtained from qualitative tests. This assay provides qualitative insights into the bioactive constituents of the leaves.

For total terpenoids (triterpenes) TLC studies were done (toluene- chloroform-ethanol, 4: 4: 1; AS reagent) on the successive extract of leaves and results revealed that toluene, chloroform and ethyl acetate give positive results for the presence of terpenoids. Flavanoids were determined using ethyl acetate– formic acid– acetic acid–

water 100: 11: 11: 27 and Natural product reagent/PEG and results revealed1 and 5 light and dark blue florescent spots, in chloroform and methanol extracts, respectively. Alkaloids were detected in chloroform extract using toluene– ethyl acetate–diethyl amine 70:20:10; Dragendorff reagent and 3 orange colour spots were obtained on TLC . The results obtained from TLC of the successive extracts are recorded in Table 5.7

Table 5.7 TLC studies on successive extracts of leaves *Tecomella undulata*

Constituents	Pet-ether (Rf)	Toluene (Rf)	Chloroform (Rf)	Ethyl acetate (Rf)	Methanol (Rf)
Alkaloids	Not detected	Not detected	0.15, 0.26, 0.44	Not detected	Not detected
Flavonoids and Phenolics	Not detected	Not detected	0.87	Not detected	0.23, 0.53, 0.57, 0.70, 0.79
Terpenoids	Not detected	0.18,0.34, 0.48, 0.74	0.2, 0.33, 0.17, 0.37, 0.42, 0.54, 0.60, 0.62, 0.69.	0.6, 0.11, 0.64	Not detected

5.2.3.b TLC of the stem bark extract obtained after successive extraction

For total terpenoids (triterpenes) TLC studies were done (toluene- chloroform-ethanol, 4: 4: 1; AS reagent) on the successive extract of stem bark and results revealed that toluene and ethyl acetate, positive results for the presence of terpenoids. Detection of flavonoids using (ethyl acetate– formic acid– acetic acid– water; 100: 11: 11: 27; Natural product reagent/PEG) showed 2, 1 and 2 light and dark blue florescent spots in chloroform, ethyl acetate and methanol extracts respectively. Determination of alkaloids using toluene– ethyl acetate– diethyl amine; 70: 20: 10; Dragendorff reagent showed 1, 2 and 2 orange colour spots in chloroform, ethyl acetate and methanol extracts respectively. The results obtained from TLC of the successive extracts are recorded in Table 5.8

Table 5.8 TLC studies on successive extracts of stem bark *Tecomella undulata*

Constituents	Pet-ether (R_f)	Toluene (R_f)	Chloroform (R_f)	Ethyl acetate (R_f)	Methanol (R_f)
Alkaloids	Not detected	Not detected	0.33	0.35, 0.66	0.18, 0.63
Flavanoids and Phenolics	Not detected	Not detected	0.39, 0.84	0.82	0.76, 0.82
Terpenoids	Not detected	0.14, 0.17, 0.24, 0.41, 0.26, 0.53	Not detected	Not detected	Not detected

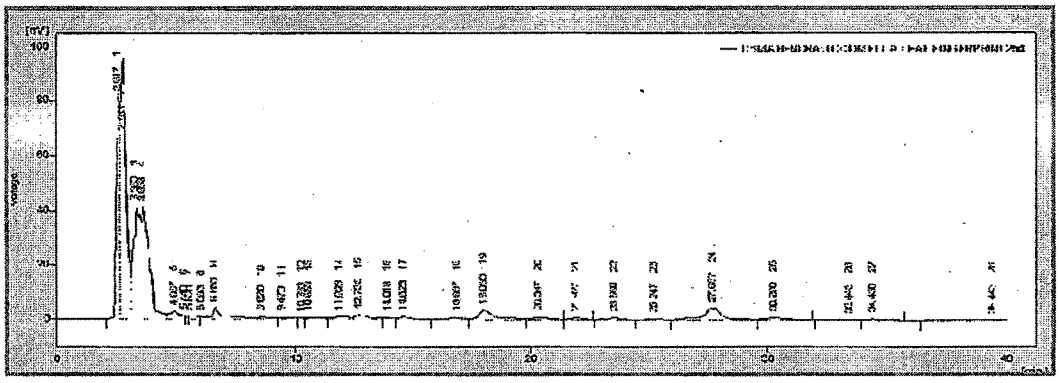
5.2.4 HPLC fingerprinting of Tecomella undulata leaves and stem bark methanolic extract

HPLC is a easy, rapid, convenient and cost effective methods for standardization of plant extract and its derived product/formulation due to its high sensitivity and accuracy. Thus standardization of *Tecomella undulata* leaves and stem bark methanolic extract is done by HPLC.

5.2.4.a Qualitative HPLC fingerprinting of Tecomella undulata leaves

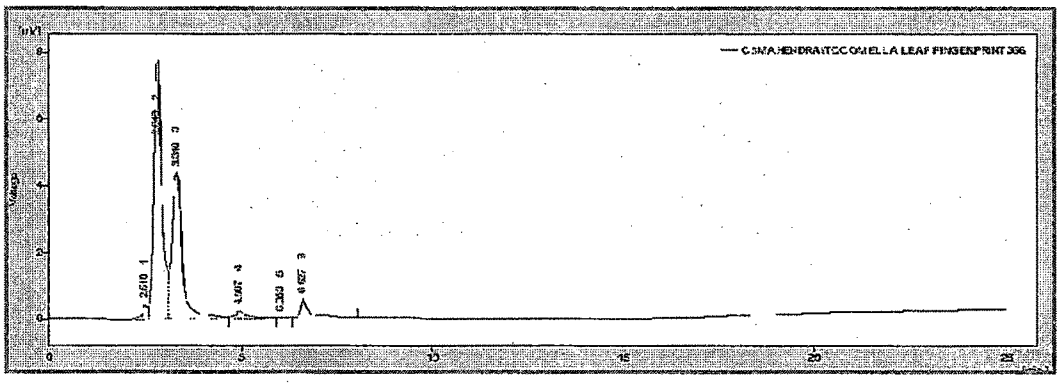
Results of HPLC analysis of leaves methanolic extract (mobile phase, Acetonitrile-water; 75: 25, flow rate; 1ml/min, detection; UV at 254 nm) shows that various constituents are present in it and their peaks are found in the chromatogram at different retention time (in mins) such as 2.617, 2.783, 3.360, 3.653, 4.967, 5.440, 5.620, 6.093, 6.653, 8.620, 9.470, 10.333, 10.653, 11.923, 12.733, 14.013, 14.623, 16.937, 18.030, 20.347, 21.927, 23.560, 25.247, 27.687, 30.290, 33.443, 34.430 and 39.443. Similarly, at 366 nm methanolic extract of leaves shows various peaks at retention time (in mins) 2.510, 2.840, 3.340, 4.967, 6.053 and 6.627 mins.

Figure 5.6 HPLC chromatogram of *Tecomella undulata* leaves methanolic extract at 254 nm



HPLC analysis of leaves methanolic extract (mobile phase, Acetonitrile-water; 75: 25, flow rate; 1ml/min, detection; UV at 254 nm)

Figure 5.7 HPLC chromatogram of *Tecomella undulata* leaves methanolic extract at 366 nm

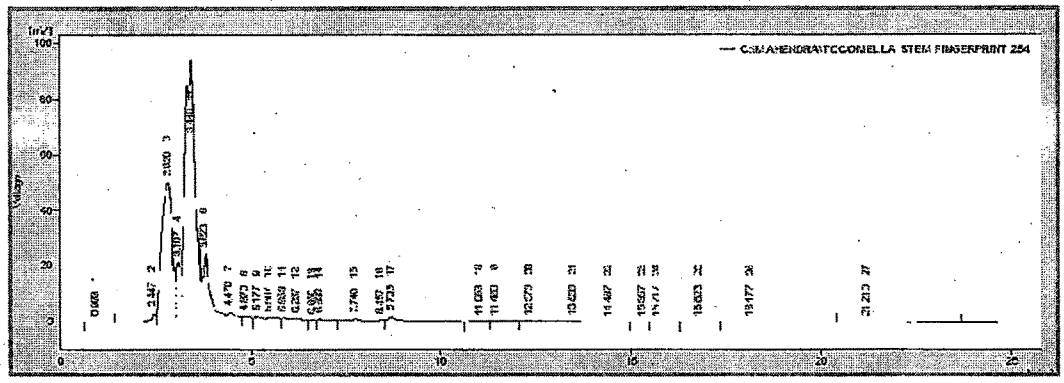


HPLC analysis of leaves methanolic extract (mobile phase, Acetonitrile-water; 75: 25, flow rate; 1ml/min, detection; UV at 366 nm)

5.2.4.b Qualitative HPLC fingerprinting of Tecomella undulata stem bark

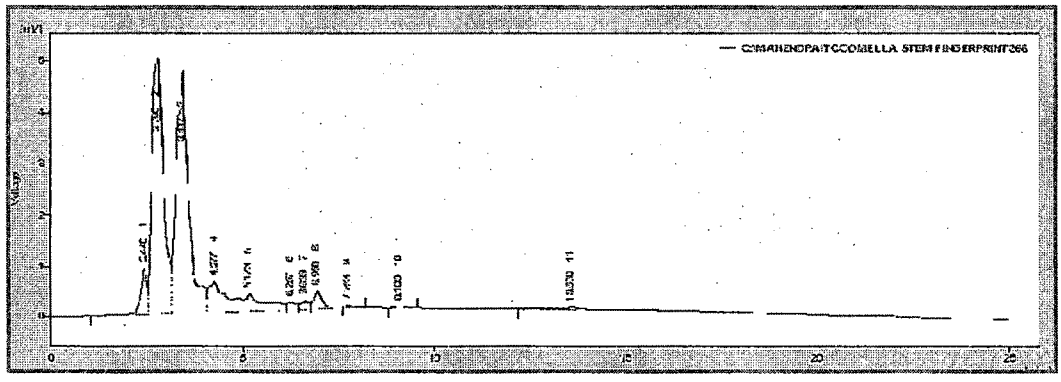
Results of HPLC analysis of *Tecomella undulata* stem bark methanolic extract (mobile phase, methanol-water; 1:1, flow rate; 2ml/min, detection; 254 nm) shows that various constituents are present in it and their peaks are found in the chromatogram at different retention time (in mins) such as such as 0.963, 2.447, 2.820, 3.107, 3.440, 3.823, 4.470, 4.870, 5.177, 5.507, 5.853, 6.237, 6.650, 6.833, 7.740, 8.457, 8.703, 11.053, 11.480, 12.373, 13.533, 14.487, 15.357, 15.717, 16.823, 18.177 and 21.210. Similarly, at 366 nm the stem bark methanolic extract shows various peaks at retention time (in mins) 2.440, 2.790, 3.447, 4.277, 5.173, 6.257, 6.633, 6.950, 7.753, 9.103 and 13.630 mins.

Figure 5.8 HPLC chromatogram of *Tecomella undulata* stem bark (methanolic extract) at 254 nm



HPLC analysis of leaves methanolic extract (mobile phase, Acetonitrile-water; 75: 25, flow rate; 1ml/min, detection; UV detector at 254 nm)

Figure 5.9 HPLC chromatogram of *Tecomella undulata* stem bark methanolic extract at 366 nm



HPLC analysis of leaves methanolic extract (mobile phase, Acetonitrile-water; 75: 25, flow rate; 1ml/min, detection; UV detector at 366 nm)

5.3. Phytochemistry of *Tecomella undulata*

5.3.1 Extraction, fractionation and identification of compound from leaves of Tecomella undulata

5.3.1.a Preparation of extracts

The leaves were shade dried, powdered (500 g) and extracted three times with petroleum ether (3X1.5 L) in a soxhlet apparatus. The filtrates were then combined and filtered and concentrated to dryness in a rotary evaporator (Buchi-R-215, Germany) to obtain a crude petroleum ether extract (TL-1; 12.8g; 2.5% w/w). The remaining marc was then dried and again exhaustively extracted at temperature (60-80°C) with methanol (3 × 1.5 L) in a soxhlet apparatus. The pooled extracts obtained

were then concentrated under vacuum to give methanolic extract (TL-7; 72g; 14.8% w/w).

5.3.1.b Unsaponified fraction

The petroleum ether extract (TL-1; 10g) was concentrated in vaccum by rotary evaporator and dried in desiccator. The dried petroleum ether extract was dissolved in 5% of methanolic KOH and refluxed for 3 hrs it is then partitioned with n-hexane and n-hexane layer was washed with water 4-5 times to get yellow coloured unsaponifiable matter (TL-2; 1.8g; 18% w/w).

5.3.1.c Chloroform fraction

Defatted methanolic extract (TL-7; 70g) was made hydroalcoholic by adding distilled water (1:3) and subjected to liquid-liquid partition by using chloroform, fraction was collected and concentrated in vaccum by rotary evaporator and dried in desiccator (TL-9; 13g; 18.77% w/w).

5.3.2 Identification of MS-2 in leaves of *Tecomella undulata* by HPLC

Reagents and Chemicals

All the chemicals, including solvents, were of analytical grade from E. Merck, India.

Preparation of Crude Extract

Accurately weighed 2 g coarse powder of *Tecomella undulata* leaves was extracted separately with methanol (3 X 50 ml) under reflux (30 min each time) on a water bath. The combined extracts were filtered and concentrated, and transferred to a 25 ml volumetric flask and the volume was made up with methanol.

Preparation of Standard Solution

A stock solution of MS-2 (500 µg mL-1) was prepared by dissolving 5 mg of accurately weighed betulinic acid in methanol and making up the volume of the solution to 10 ml with methanol.

Chromatography

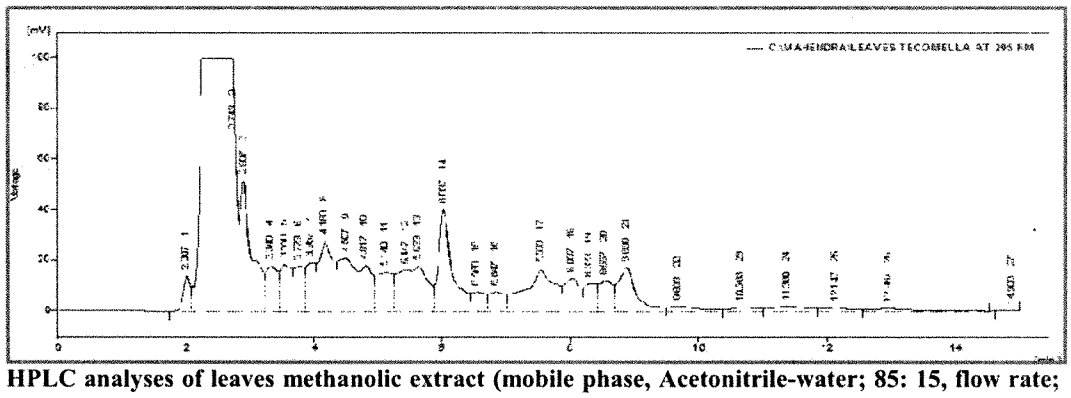
The HPLC analysis of methanolic extract of leaves *Tecomella undulata* was carried out at The chromatographic system (Shimadzu, Kyoto, Japan) consisted of a Shimadzu LC-20 AT Prominence solvent delivery module, a manual Rheodyne injector (PerkinElmer, Mumbai, India) with a 20 ml fixed loop, and an SPD-20A Prominence (Shimadzu) UV-Vis detector. The separation was performed on a

Hypersil C18 column (particle size 5 mm; 250 X 4.6 mm id; Thermoquest; Cheshire, UK) preceded by an ODS (Thermoquest) guard column (10 mm, 10 ´ 5 mm id) at an ambient temperature. Chromatographic data were recorded and processed using a Spinchrom Chromatographic Station® CFR Version 2.4.0.193 (Spinchrom Pvt. Ltd, Chennai, India). Peak purity analysis was carried out using an SPD M20A photo-diode array (PDA) detector from Shimadzu. The mobile phase consisted of acetonitrile: water (85:15 v/v) and the separation were performed by using isocratic elution at a flow rate of 1.0 mL min^{-1}. The samples were run for 25 minutes. Detection was done at 205 nm by UV detector.

The chromatogram of leaves methanolic extract (Figure 5.10) shows various peaks at different retention time(in mins) such as 2.007, 2.743, 2.907, 3.340, 3.550, 3.773, 3.957, 4.180, 4.507, 4.817, 5.140, 5.447, 5.633, 6.037, 6.560, 6.847, 7.653, 8.037, 8.323, 8.557, 8.880, 9.693, 10.663, 11.380, 12.147, 12.967, 14.903 and chromatogram of marker compound (MS-2) (Figure 5.11) showed single major peak at retention time 7.613. Retention time of marker constituents exactly matches with one of the constituent present in leaves methanolic extract, reconfirmation of identity of marker in leaves was done by adding standard solution in leaf methanolic extract sample. The chromatogram of overlap spectra of leaves methanolic extract and marker constituent are represented in figure 5.12.

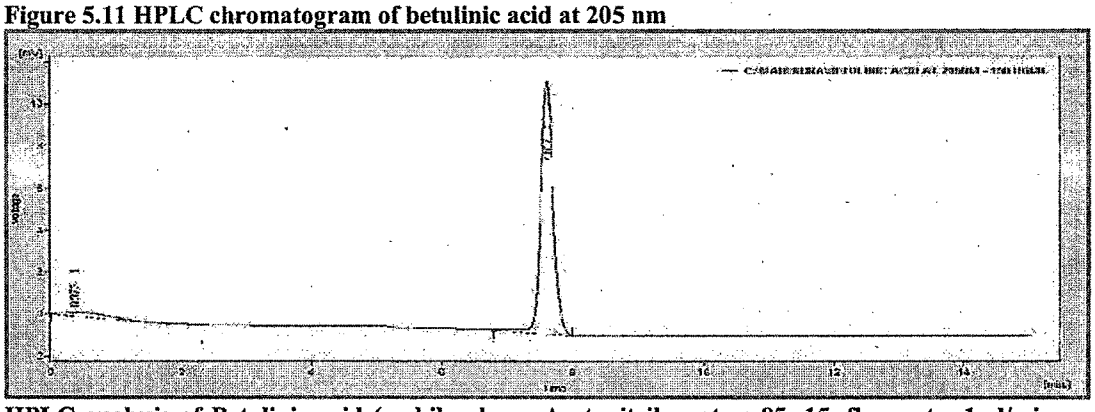
Based upon the fingerprinting results, it can be concluded that this analytical technique is a convenient method to qualitatively identify the bioactive constituent, present in the methanolic extract of *Tecomella undulata* leaves.

Figure 5.10 HPLC chromatogram of *T. undulata* leaves methanolic extract at 205 nm



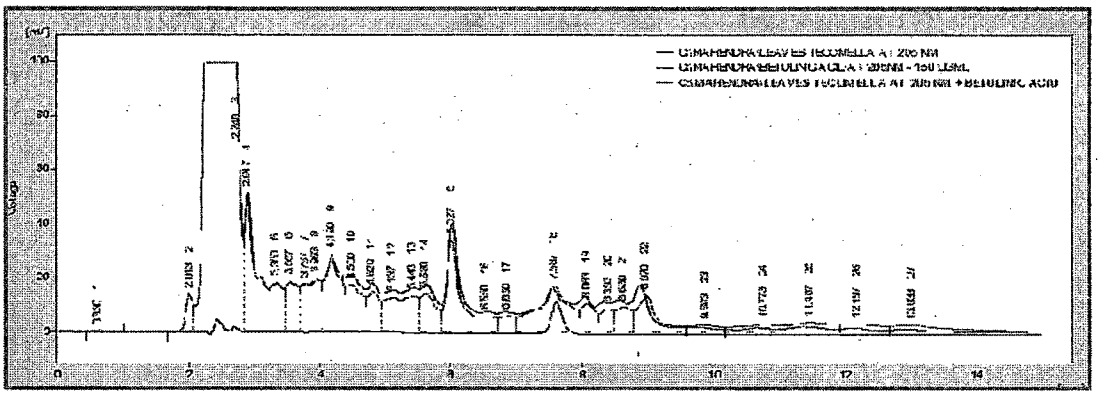
HPLC analyses of leaves methanolic extract (mobile phase, Acetonitrile-water; 85: 15, flow rate; 1ml/min, detection SPD-20A Prominence (Shimadzu) UV-Vis detector at 205 nm)

Figure 5.11 HPLC chromatogram of betulinic acid at 205 nm



HPLC analysis of Betulinic acid (mobile phase, Acetonitrile-water; 85: 15, flow rate; 1ml/min, detection SPD-20A Prominence (Shimadzu) UV-Vis detector at 205 nm)

Figure 5.12 HPLC chromatogram of *T. undulata* leaves sample, betulinic acid and Leaves sample after adding betulinc acid at 205 nm



HPLC analysis of all samples and standard (mobile phase, Acetonitrile-water; 85: 15, flow rate; 1ml/min, detection SPD-20A Prominence (Shimadzu) UV-Vis detector at 205 nm)

5.3.3 Extraction, fractionation and isolation of compounds from stem bark of *Tecomella undulata*

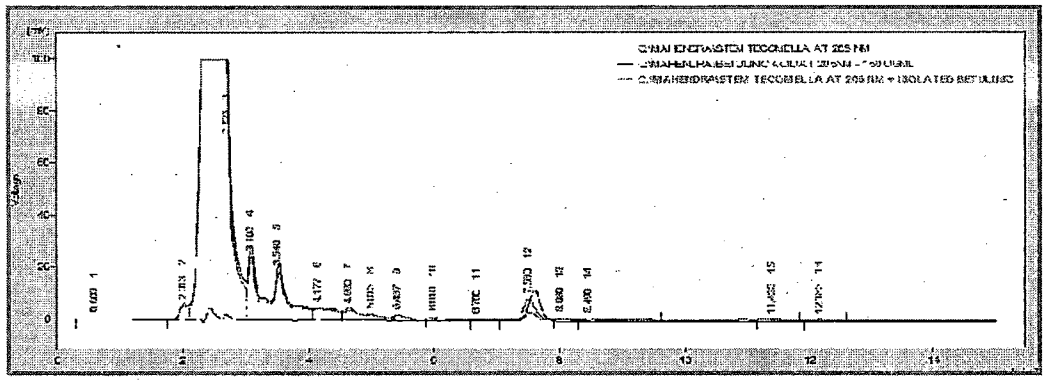
The stem barks were shade dried, powdered (2 kg) and extracted three times with petroleum ether (3X1.5 L) in a soxhlet apparatus. The filtrates were then combined and filtered and concentrated to dryness in a rotary evaporator (Buchi-R-215, Germany) to obtain a crude petroleum ether extract (TSB-1). The remaining marc was then dried and exhaustively extracted at 60-80°C with methanol (3 × 1.5 L) in a soxhlet apparatus. The pooled extracts were then concentrated under vacuum to obtain methanolic extract (TSB-7).

Dried petroleum ether extract (TSB-1) dissolved in diethyl ether and then extracted with 2N Na_2CO_3 aqueous solution and the sodium carbonate soluble fraction was acidified with 2N HCI giving yellow mass (TSB-2). This fraction was subjected for preparative TLC (benzene- ethyl acetate; 95:5) to obtain a pure compound (MS-1). Methanolic extract (TSB-7) was suspended in water and partitioned with EtOAc, From the EtOAc-soluble fraction (TSB-9), 13 sub fractions were obtained by silica gel chromatography eluting with a CH_2Cl_3-MeOH gradient by increasing the polarity. The resultant 6th fraction obtained from dichloromethane- methanol (50:50) yielded an amorphous brownish powder on drying (TSB -10). Fraction 6 (5.4 g) was loaded onto a silica gel column (70 × 6 cm) and eluted with a *n*-hexane- EtOAc gradient and yielded another 12 sub fractions. Fraction 4 and 5 (30 or 40 % hexane: ethyl acetate; TSB-11) showed the identical pattern on TLC plate. This fraction was subjected to preparative TLC (n-hexane- ethyl acetate; 70:30) to obtain a pure compound (MS-2). Which was characterised by using various spectroscopic techniques viz. Mass, NMR and overlaid IR spectra with using standard which is procured from sigma alderiched, Purity of MS-1 and MS-2 compound obtained herein was confirmed by analytical HPLC.

5.3.4 Identification of isolated marker in bioactive methanolic extract and flavanoidal fraction of stem barks *Tecomella undulata* by HPLC

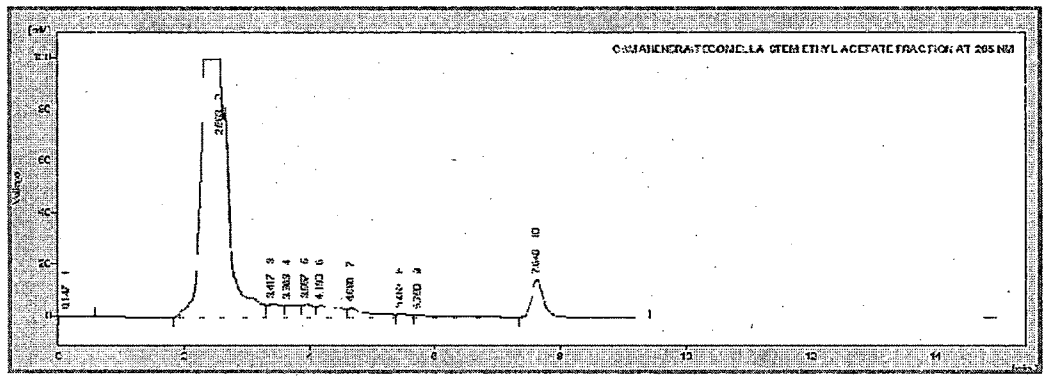
HPLC chromatography of isolated marker (MS-2), methanolic extract and total flavanoidal fraction of stem barks was carried out using HPLC (Shimadzu, Kyoto, Japan), column (Hypersil C18 column, particle size 5 mm; 250 x 4.6 mm id; Thermoquest, Cheshire, UK) and elution was carried out with acetonitrile: water (85:15) at a flow-rate of 1ml/min and the elute was monitored (SPD-20A Prominence (Shimadzu) UV-Vis detector) at 205 nm. The methanolic extract (TSB-7) showed 13 peaks with different retention time (2.020, 2.717, 3.107, 3.540, 4.170, 4.303, 4.667, 4.997, 5.420, 5.990, 7.543, 8.060 and 8.537). The overlaid spectra obtained for TSB-7+ isolated marker (Fig. 5.13) and flavanoidal fraction (TSB-9) was having 10 peaks at different retention time, (0.147, 2.593, 3.417, 3.663, 3.957, 4.190, 4.680, 5.463, 5.750 and 7.640) chromatogram was shown in figure 5.14. These results provide the qualitative identification as well as the standardization method for the TSB-7 and TSB-9.

Figure 5.13 HPLC chromatogram of *T .undulata* stem bark (TSB-7), betulinic acid and TSB-7 after adding betulinc acid at 205 nm



HPLC analysis of TSB-7 and standard (mobile phase, Acetonitrile-water; 85: 15, flow rate; 1ml/min, detection SPD-20A Prominence (Shimadzu) UV-Vis detector at 205 nm)

Figure 5.14 HPLC chromatogram of *T. undulata* ethyl acetate fraction (TSB-9) at 205 nm



HPLC analysis of TSB-9 (mobile phase, Acetonitrile-water; 85: 15, flow rate; 1ml/min, detection SPD-20A Prominence (Shimadzu) UV-Vis detector at 205 nm)

5.3.5 Identification and quantification of MS-1 from stem bark of *T. undulata* by HPLC

Instrumentation

The chromatographic system (Shimadzu, Kyoto, Japan) consisted of a Shimadzu LC-20 AT Prominence solvent delivery module, a manual Rheodyne injector (PerkinElmer, Mumbai, India) with a 20 mL fixed loop, and an SPD-20A Prominence (Shimadzu) UV-Vis detector. The separation was performed on a Hypersil C18 column (particle size 5 mm; 250 X 4.6 mm id; Thermoquest, Cheshire, UK) preceded by an ODS (Thermoquest) guard column (10 mm, 10 X 5 mm id) at an ambient temperature. Chromatographic data were recorded and processed using a Spinchrom

Chromatographic Station® CFR Version 2.4.0.193 (Spinchrom Pvt. Ltd, Chennai, India). Peak purity analysis was carried out using an SPD M20A photo-diode array (PDA) detector from Shimadzu. Degassing of the mobile phase was done by sonication in an Ultrasonics Selec (DTC 503, Vetra, Italy) ultrasonic bath. An analytical balance from the AW series from Shimadzu was used with an accuracy of 0.01 mg.

Materials

Standard substance MS-1 was isolated in our laboratory Acetonitrile, methanol, and water of HPLC grade were purchased from Qualigens (Mumbai, India). All the other solvents and reagents used were of analytical grade and were filtered through a 0.2 mm Ultipor® Nylon 66 membrane filter (Pall Life Sciences, East Hills, NY) prior to use.

Chromatographic Conditions

Chromatographic estimations were performed using an equilibrated Hypersil reversed-phase C18 column. Analysis of MS-1 was isocratic at a 2 mL/min flow rate with an Acetonitrile; 0.25% acetic acid in water 50 + 50, v/v as the mobile phase. The absorbance of MS-1 was good at 262 nm and it was free from any interference. The mobile phases were freshly prepared every day. The mobile phase was filtered through a 0.2 mm membrane filter to remove any particulate matter, mixed, and degassed by sonication before use. The sensitivity of the detector was set at 0.01 absorbance unit's full scale (AUFS). Prior to injecting the solutions, the column was equilibrated for at least 60 min with the mobile phase flowing through the system.

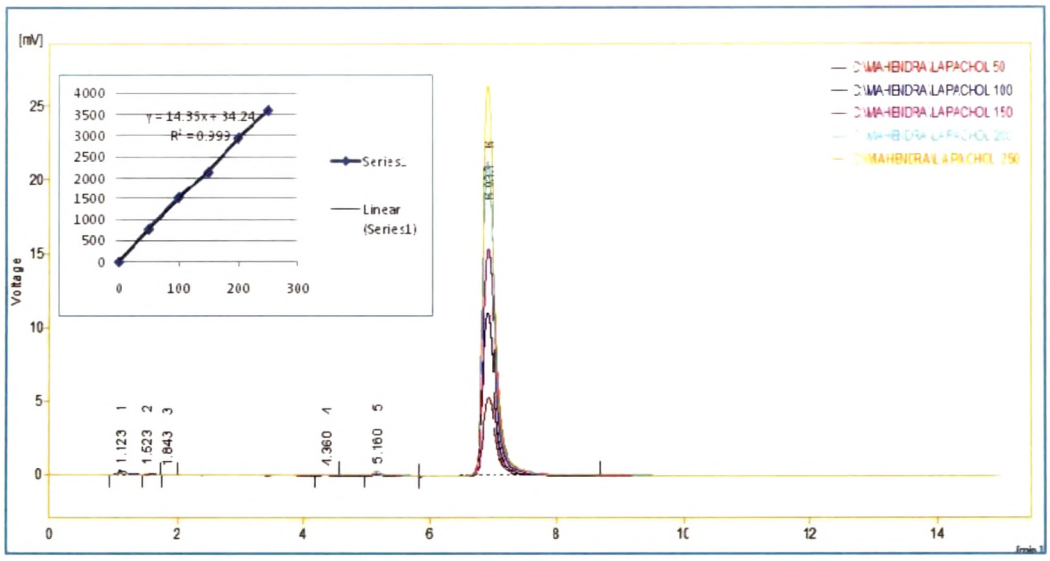
Preparation of Standard Solutions

Approximately 5 mg standard MS-1 was weighed precisely in separate volumetric flasks and dissolved in 5 mL acetonitrile to obtain stock concentrations of 1000 µg/mL. Aliquots of each standard were further diluted to obtain solutions in the range of 50–250 µg /mL in acetonitrile.

Method Validation

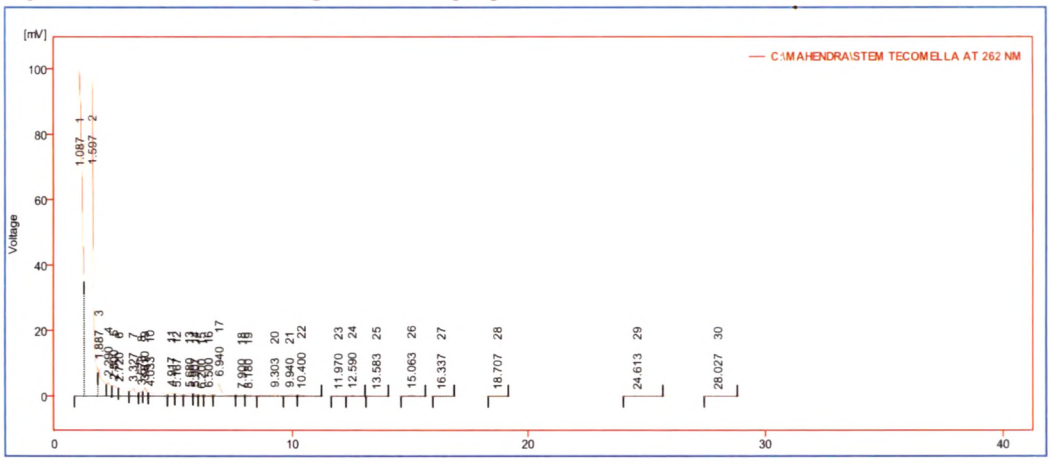
The methods developed for analyzing MS-1 individually were validated for linearity, accuracy, precision, specificity, and quantification limits as per ICH guidelines.

Figure 5.15 Representing chromatograms and calibration curve for isolated marker (MS-1; 50-250µg/ml)



HPLC process parameter of MS-1 (mobile phase, Acetonitrile- 0.25% acetic acid in water; 50: 50, flow rate; 2ml/min, detection; UV at 262 nm, Retention time 6.947 minutes

Figure 5.16 HPLC chromatogram showing separation of MS-1 in TSB-7 at UV 262 nm



HPLC process parameter of TSB-7 (mobile phase, Acetonitrile- 0.25% acetic acid in water; 50: 50, flow rate; 2ml/min, detection; UV at 262 nm, total running time 40 minutes

Calibration Curve (Linearity)

Linear regression analysis confirms that the *r*2 values for the drug was >0.999, confirming the linear relationship between the concentration of the drugs and area under the curve (Table 1). The calibration curves (n = 3) constructed for the markers were linear over the concentration range of 50–250 µg/mL for MS-1.

Precision and Stability

The precision results for the solution at the three concentrations are presented in Table 2. It was shown that the RSD values for retention time were < 2%, while the RSD values for peak area were <2% for both intra- and interday assay precision (intraday, 4 h, six injections; interday, 6 days). For the stability test, the same sample was analyzed within 24 h at room temperature, and the solution was found to be stable (RSD values for the retention time and peak area were both >3%).

Limit of Detection and Limit of Quantification

The LOD and LOQ were found to be 0.62 and 8.49 µg/ml for MS-1.

Specificity

Specificity evaluation was carried out by analyzing MS-1. It was observed that the peak of the drugs was well separated and was not interfered with by the degradation products and other components. Peak corresponding of the drugs showed positive values for the minimum peak purity index over the entire range of integrated chromatographic peaks, thus indicating the purity of the peaks (Table 5.9). Further, peak corresponding of the drug obtained by the proposed methods were seen to be pure (Figure 5.15). Thus, the methods were confirmed to be specific for the drug in the presence of the degradation products and other components.

Robustness

Table 4 shows the mean obtained (n = 6) for each factor studied, indicating that the selected factors remained unaffected by small variations of these parameters. The recovery obtained individually and the mean were between 97.2–102.3% for MS-1. Therefore, it can be concluded that the methods are consistent for detection wavelength, selected column, and solvent brand.

System Suitability

A system suitability test was performed to evaluate the chromatographic parameters [capacity factor, separation factor, column efficiency, number of theoretical plates, height equivalent to the theoretical plate (HETP) asymmetry of the peak, and resolution between two consecutive peaks] for each method before the validation runs. Three replicate injections of the standard solution and three injections of the solution prepared for the specificity procedure were used.

Accuracy

As shown in Table 6, the recovery of the investigated component ranged from 97.17–98.62%, and their %RSD values were all <3%. It was known from recovery tests that the developed methods manifested reliability and accuracy for the measurement of the MS-1.

Table 5.9 Method validation parameter for quantification of MS-1 using proposed HPTLC Densitometric method.

a) Overview of method development for the quantitation of MR-1 in *Tecomella undulata.*

Validation parameter	Results
HPLC Method	
Linearity range (µg mL^{-1})	50 – 250
Regression equation	Y=14.36x+34.238
Correlation coefficient	0.999
Limit of detection (LOD) (µg)	18.5
Limit of quantitation (LOQ) (µg)	22
System suitability	
Separation factor	1.60
Capacity factor	9.81
Tailing factor	1.21
Resolution factor	1.97

b) Intra- and inter-day precision of HPLC method

Marmesin (µg)	Intra-day		Inter-day	
	Mean (µg) (n=5)	RSD (%)	Mean (µg) (n=3)	RSD (%)
0.8	0.7034	1.01	0.7997	1.29
1.6	1.6014	0.56	1.6097	1.11
3.2	3.2010	0.40	3.2040	0.42

c) Recovery study (n=3)

Amount of marmesin added (µg)	Amount of MS-1 recovered (µg)	Recovery (%)
50.00	49.08	97.2
75.00	74.10	99.9
100.00	100.17	102.3

d) MS-1 content (on plant dry weight basis) in *Tecomella undulata* by HPLC method (n=3)

Samples	HPLC Method
	MS-1 (mg/gm) (Mean ± SD)
Stem barks	0.002 ± 0.02 % w/w

Applicability of the Developed Method in stem bark of Tecomella undulata

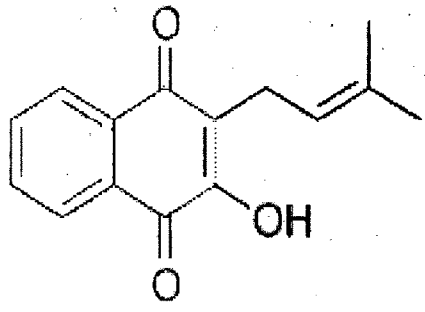
The developed methods were applied for the determination of MS-1 in the stem bark; the results are presented in Table 4.10. The chromatogram obtained for TSB-7 (Figure 5.16), showed a separated instinct peak for MS-1. The calibration curve (Fig. 5.15) was prepared with MS-1 and was found to be linear (R^2= 0.999) in the concentration range (50- 250µg/ml) used. The presence of MS-1 in TSB-7 was found to be 2.015 ± 0.08 mg per 100 gm powder.

5.3.6 Characterization of isolated compounds from *Tecomella undulata*

5.3.6.a Characterization of MS-1

The structure elucidation of MS-1 was performed with the help of overlaid IR spectroscopy and Co- TLC analysis that confirmed it as lapachol (**Figure 5.17**). Lapachol (MS-1): $C_{15}H_{14}O_3$, yellow needles m.p.139-140°; m/z (%) 242; IR spectra: ν_{max} (KBr) 3423, 3352, 2973, 2910, 2122, 1661, 1640, 1582, 1456, 1369, 1352, 1311, 1272, 1240, 1209, 1183, 1154, 1047, 1028, 936, 847, 790, 725 and 662 cm^{-1} confirmed it as lapachol after comparison with the authentic standard. Co-TLC (benzene- Glacial acetic acid; 95:5; violet red colour with ferric chloride and red with magnesium acetate) Chemical test; It dissolved in aqueous sodium hydroxide giving a deep red solution which turned light yellow on addition of sodium dithionite and the red colour was restored on shaking for some time in air.

Figure 5.17 Chemical structure of MS-1



Lapachol

Figure 5.18 IR spectrum of isolated compound (MS-1)

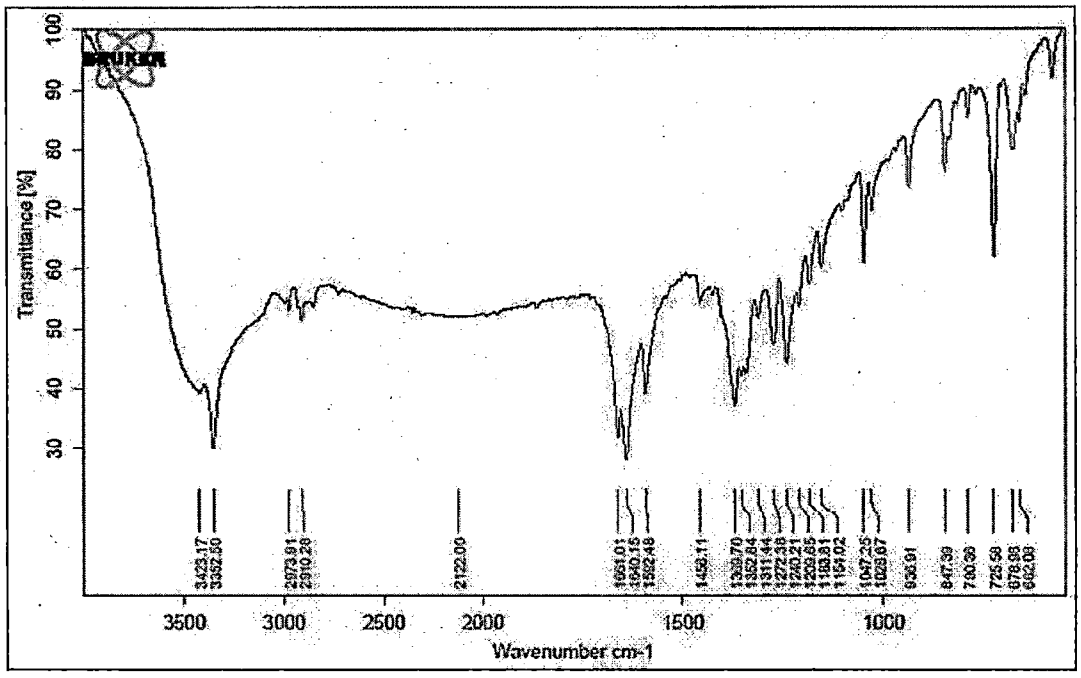
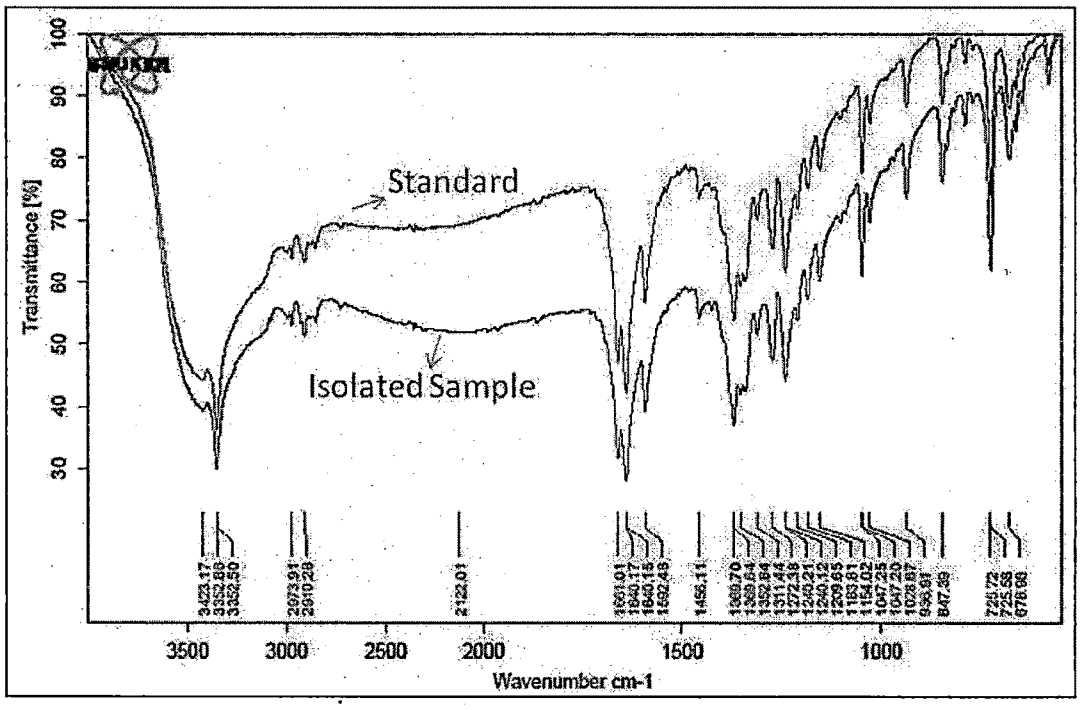


Figure 5.19 Overlaid IR spectrum of isolated compound (MS-1) and standard lapachol



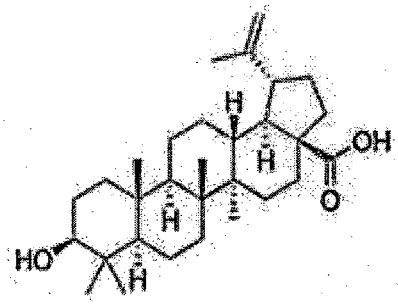
5.3.6.b Characterization of MS-2

The structure elucidation of MS-2 was performed with the help of overlaid IR spectroscopy and Co- TLC analysis that confirmed it as betulinic acid . Betulinic acid (MS-2) is 3β-Hydroxy-lup-20(29)-en-28-oic acid. Its molecular formula is $C_{30}H_{48}O_3$

and it have white needles shape crystal with melting point 316-318°. Its molecular weight is 456. IR spectra: ν_{max} (KBr) 3855, 3544, 3418, 3232, 2942, 2869, 2351, 2328, 1685, 1638, 1619, 1538, 1484, 1452, 1400, 1300, 1236, 1107, 1044, 984 and 887 cm-1confirmed it as betulinic acid comresion with an authentic standard Co-TLC (hexane- ethyl acetate- acetic acid; 7:3:0.03; derivatization was carried out with anisaldehyde and sulphuric acid followed by heating at 110° C for 3 min).

Betulinic acid and its derivatives have been discovered as a new class of compounds a potential anti-cancer and anti- HIV agents (Soler et al., 1996; Schmidt et al., 2010) Considering the wide therapeutic applications of betulinic acid it is also considered as one of the bioactive marker constituent.

Figure 5.20 Chemical structure of MS-2



Betulinic acid

Figure 5.21 IR spectrum of MS-2

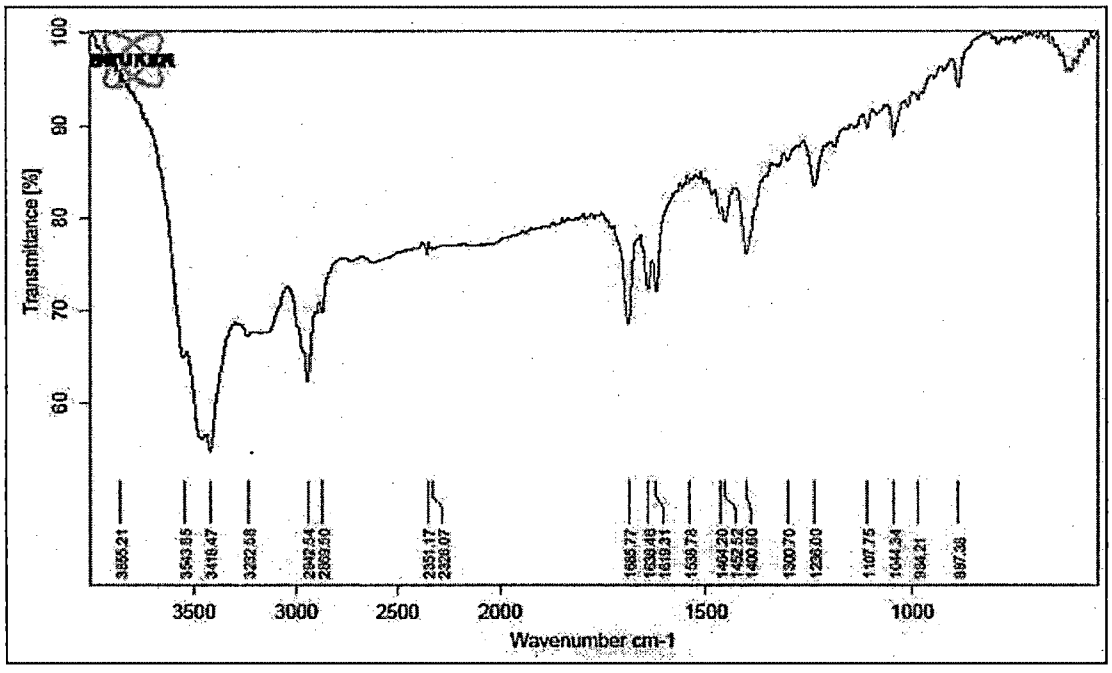


Figure 5.22 Overlaid IR spectra of MS-2 along with standard betulinic acid

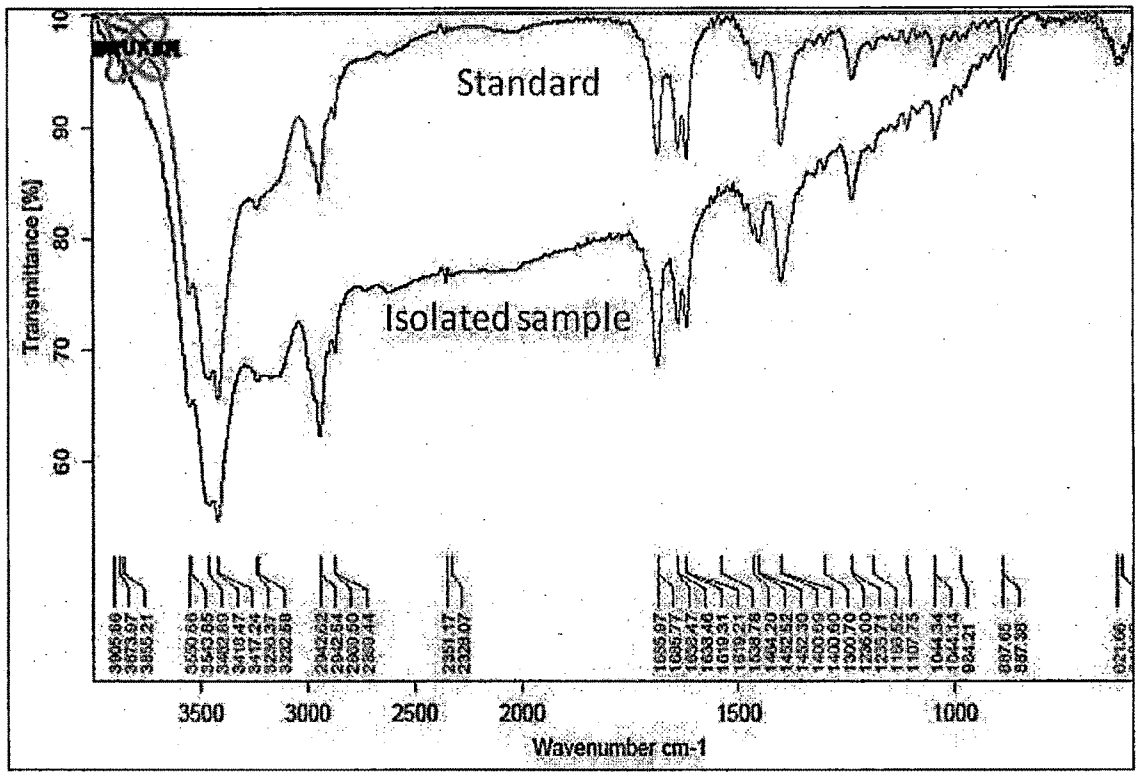
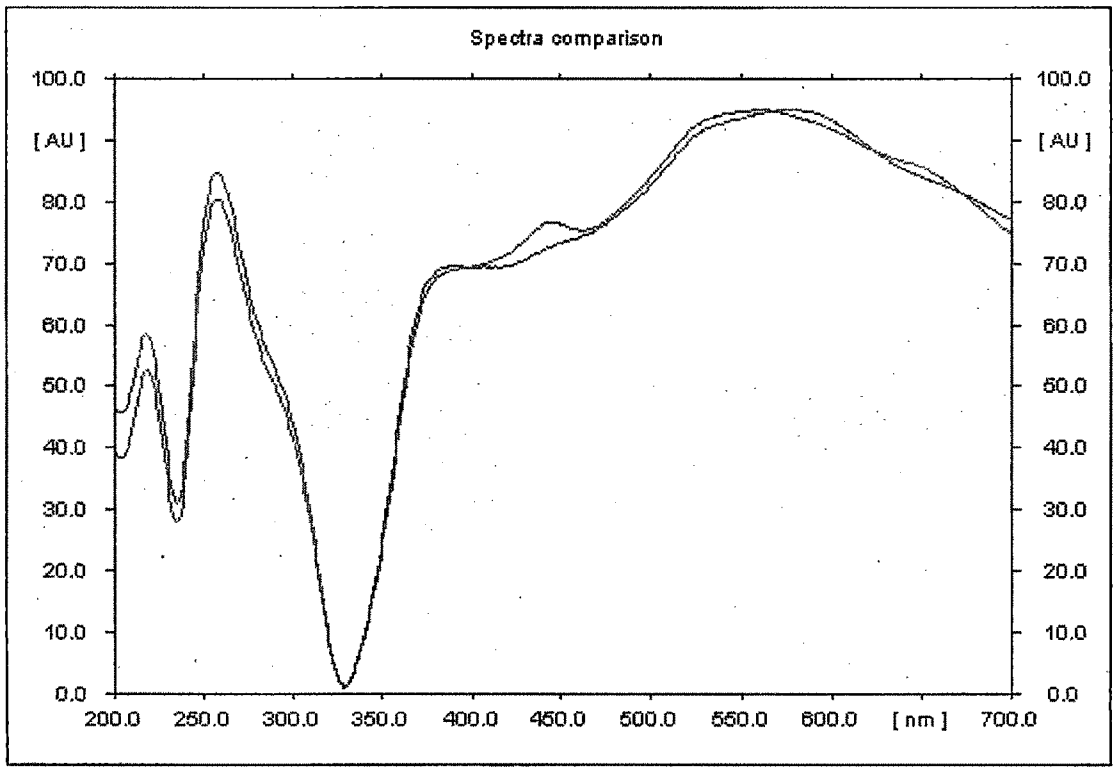


Figure 5.23 Overlaid UV spectrum of MS-2 along with standard betulinic acid



5.4 Biological studies

5.4.1 *In vitro* cytotoxicity studies of extracts, fractions and isolated compounds from *Tecomella undulata*

Prior to the therapeutic use of any herbal extract, it is imperative to perform a cytotoxicity assay. This is because crude extract of many herbs have been shown be non-toxic but some of its bioassay guided fraction or pure compound may show toxicity (Tshikalange and Hussein, 2010). The human hepatoma cell line (Hep G2) is a popular and an effective *in vitro* model for assessing hepatoprotective potential of phytoconstituents or bioassay guided fractions due to its functional similarity with an intact liver.

5.4.1.a In vitro cytotoxicity studies of TLs

Cytotoxicity assessment of *T. undulata* leaves chloroform fraction (TL-9) revealed less than 50% cell viability at 250 µg /ml dose. However petroleum ether extract and its fraction (TL-1 and TL-2) and methanolic extract (TL-7) showed a differential pattern of cytotoxicity in HepG2 cells. TL-2 exhibited superior percentage of cell viability (> 80%) at 250µg/ml. However TL-9 recorded significant cytotoxicity, which was characterized by less than 50% cell viability at all the doses studied herein. TL-2 recorded much improved cell viability as compared to its preceding extract (Figure 5.24).

5.4.1.b In vitro cytotoxicity studies of TSBs

Cytotoxicity assessment of *Tecomella undulata* stem bark petroleum ether fraction and its preceding phytoconstituents (TSB-2 and MS-1) revealed less than 50% cell viability at 250 µg /ml dose. However methanolic extract, fractions and it phytoconstituents (TSB-7, TSB-9, TSB-10 or MS-2) showed a differential pattern of cytotoxicity of Hep G2 cells. TSB-7, TSB-9 and MS-2 exhibited superior percentage of cell viability (> 65%) at 250µg/ml. MS-2 recorded much improved cell viability as compared to its preceding fractions and its extract (Figure 5.25).

Figure 5.24 Cytotoxicity assessment of *Tecomella undulata* leaves extracts and fractions

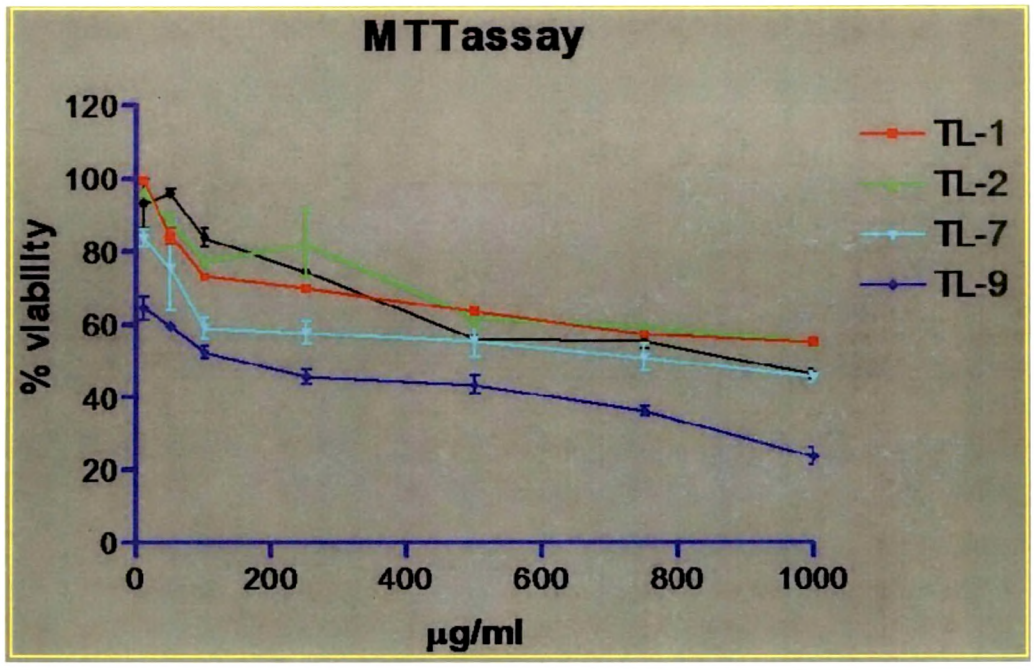
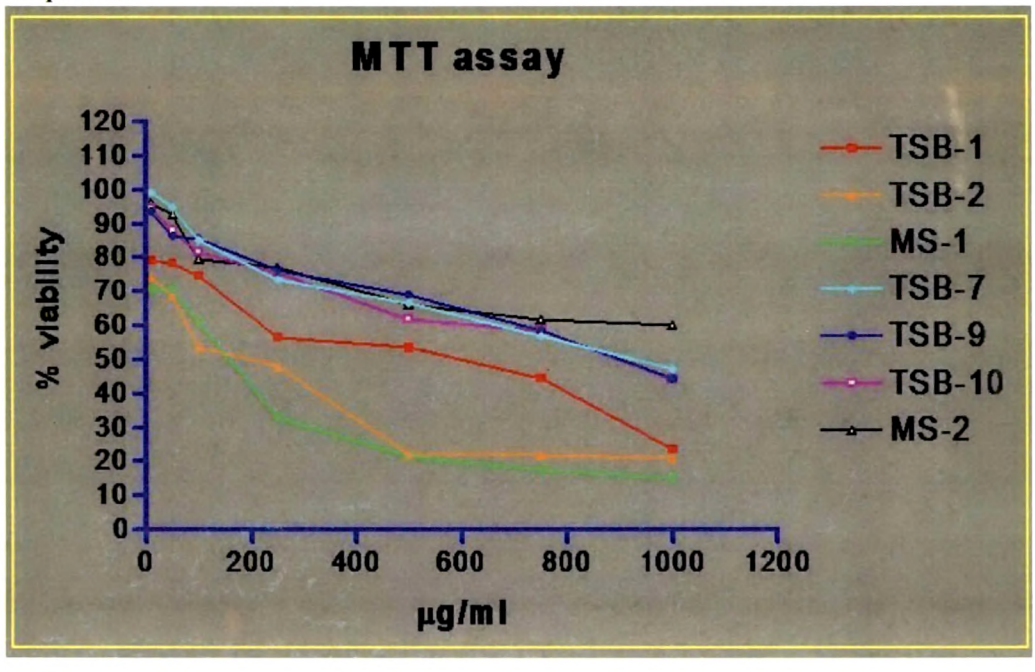


Figure 5.25 Cytotoxicity assessment of *Tecomella undulata* stem bark extracts, fractions and compounds



5.4.2 In vitro Hepatoprotective potential of *Tecomella undulata*

HepG2 cell line is a popular *in vitro* model where in, cells are subjected to CCl_4 induced toxicity and subsequent amelioration using a desired herbal extract or compound is question (Kritika et al., 2010).

Carbon tetrachloride (CCl_4) is a well-known hepatotoxicant that causes necrotic damage to cells and tissue, resulting in leakage of their enzymes (SGOT and SGPT) into the blood stream. High concentrations of CCl_4 *in vivo* can manifest extensive toxic damages to hepatocytes via generation of trichloromethyl free radicals (CCl_3* and/or CCl_3OO*) that induce biotransformation by hepatic microsomal Cytochrome P 450 (Jadeja et al., 2011).

In the present study, TLs and TSBs were evaluated for assessing their hepatoprotective potentials.

5.4.2.a In vitro hepatoprotective potential of Tecomella undulata leaves

In the present study, elevated activity levels of SGOT and SGPT were recorded in CCl_4 treated HepG2 cells which are in accordance with other reports (Krithika et al., 2009, Zeashan et al., 2009). However, co-supplementation of TL-1, TL-2 and TL-7 recorded a non-linear dose dependent decrease in activity levels of SGOT and SGPT. Results of the study indicate that TL-2 fraction of petroleum ether extract (TL-1) provides superior hepatoprotection as compared to TL-1 and TL-7. However, cytotoxicity obtained in TL-9 is inexplicable and warrants further study. It can be assumed that reappearance of hepatoprotection in TL-2 its non-toxic nature can be attributed to the possible removal of the unknown toxic substance in the insoluble portion (Table 5.10).

Table 5.10 Effect of *Tecomella undulata* leaf extracts, fractions and sylimarin on CCL_4 induced hepatotoxicity

TREATMENTS		SGOT (IU/l)	SGPT (IU/l)	CELL VIABILITY(%)
Control		4.39±0.57	3.33±0.33	100
1% CCL_4		10.33±1.73$^{###}$	9.66±0.88$^{###}$	20.81±1.21$^{###}$
1% CCL_4 + Sylimarin (µg/ml)	10	6.66±0.33***	5.66±0.66***	74.28±1.70***
	20	6.33±0.66***	3.66±0.33***	81.17±1.99***
	50	4±0.57***	3±0.57***	84.41±1.87***
	100	4±1.00***	2.66±0.33***	94.54±4.10***
	200	3.33±0.88***	2.33±0.88***	96.85±3.45***
1% CCL_4 + TL-1 (µg/ml)	10	9.33±0.88^{ns}	8.33±0.88*	45.67±2.00^{ns}
	20	7.66±0.33*	8.33±0.33*	84.43±4.99***
	50	8±0.57*	7.66±0.88**	78.48±5.49***
	100	7±0.57*	6±0.57***	76.79±6.29***
	200	7.66±0.88*	6.33±0.88***	79.60±8.00***
1% CCL_4 + TL-2 (µg/ml)	10	6.66±1.20**	7.25±1.52*	87.85±4.32***
	20	6.33±0.33**	5.66±0.33***	85.18±1.93***
	50	6±0.53**	6.33±0.33***	85.56±2.29***
	100	4.33±0.88***	4.66±1.29***	78.01±4.12***
	200	3.59±0.57***	3.43±0.66***	77.40±3.46***
1% CCL_4 + TL-7 (µg/ml)	10	9.33±0.33^{ns}	8.66±0.66*	42.02±2.08^{ns}
	20	8±0.15*	7±1.52**	58.82±2.78**
	50	5.33±0.33***	5.66±1.20***	85.23±3.23***
	100	6.66±0.20**	5.33±1.85***	87.57±1.44***
	200	10.66±0.45^{ns}	9.06±0.66^{ns}	39.09±2.81^{ns}

Data expressed as mean± S.E.M. for n=3. Where, $^{###}$p<0.001 compared to control and *p<0.05, **p<0,01 and ***p<0.001 compared to 1%CCL_4.

5.4.2.b In vitro hepatoprotective potential of Tecomella undulata stem bark

In the present study we have explored the potential hepatoprotective effects of *Tecomella undulata* stem bark extracts, fractions and phytoconstituents (TSBs). Results indicated that methanolic extract (TSB-7), fraction (TSB-9) and its preceding phytoconstituents (MS-2) reduces hepatocytes injury induced by model toxicants. However, different grades of protection were found. TSB-9 was the most potent *in vitro* hepatoprotective fraction. However, TSB-1 and TSB-10 does not show any hepatoprotective activity.

Activity levels of SGOT and SGPT in cell supernatants recorded a significant increment in cells treated with 1% CCl_4 (Table 5.11).

Table 5.11 Effect of *Tecomella undulata* stem bark extracts, fractions, MS-2 and sylimarin on CCL_4 induced hepatotoxicity

TREATMENTS		SGOT (IU/l)	SGPT (IU/l)	CELL VIABILITY (%)
Control		4.80±0.57	3.33±0.33	100
1% CCL_4		10.23±3.46$^{###}$	8.66±0.88$^{###}$	20.81±1.21$^{###}$
1% CCL_4 + Sylimarin (µg/ml)	10	6.66±0.33***	5.66±0.66***	74.28±1.70***
	20	6.33±0.66***	3.66±0.33***	81.17±1.99***
	50	4±0.57***	3±0.57***	84.41±1.87***
	100	4±1.00***	2.66±0.33***	94.54±4.10***
	200	3.33±0.88***	2.33±0.88***	96.85±3.45***
1% CCL_4 + TSB-1 (µg/ml)	10	7.33±0.88*	6.33±0.45*	60.71±2.53*
	20	7±0.57*	6.66±0.88*	75.40±0.61**
	50	5±0.57***	6.33±0.45*	99.34±0.72***
	100	8±1.52*	7±1.52^{ns}	66.02±1.34*
	200	8.66±0.37*	7.33±0.88^{ns}	66.26±1.25*
1% CCL_4 + TSB-7	10	7.66±0.88*	6.33±0.45^{ns}	75.82±2.86**
	20	6.33±0.31*	5.66±0.88*	76.39±1.00**

(µg/ml)	50	5.33±0.33***	5±0.57*	68.18±1.73**
	100	4.66±0.66***	4±0.00***	91.74±4.03***
	200	4.06±0.88***	3.94±0.33***	86.96±2.23***
1% CCL_4 + TSB-9 (µg/ml)	10	7.33±0.88*	6.33±1.45^{ns}	67.74±1.24**
	20	6±1.00**	5.66±0.33*	83.02±4.15***
	50	5±0.57***	5.03±0.66*	86.16±4.36***
	100	3.66±0.88***	4.66±0.88***	81.76±4.97***
	200	2.33±0.88***	3.33±0.66***	90.62±0.16***
1% CCL_4 + TSB-10 (µg/ml)	10	7.33±0.88*	8.03±0.66^{ns}	66.35±1.48**
	20	7.66±0.33*	8.33±1.33^{ns}	56.41±3.44*
	50	6±1.15**	7.66±0.88^{ns}	61.26±2.27**
	100	4.66±0.83***	7.33±1.85^{ns}	67.65±1.48**
	200	7.66±0.88*	8.52±0.57^{ns}	52.49±1.86**
1% CCL_4 + MS-2 (µg/ml)	10	6.33±1.20**	5.66±1.33*	54.40±0.70*
	20	5.66±0.33***	5.33±0.66*	61.52±0.99*
	50	5±0.57***	4.78±0.57***	67.96±0.89**
	100	4.02±0.57***	4.06±0.33***	75.64±0.39***
	200	3.66±0.66***	3.36±0.88***	92.88±0.29***

Data expressed as mean± S.E.M. for n=3. Where, $^{###}$p<0.001 compared to control and *p<0.05, **p<0,01 and ***p<0.001 compared to 1%CCL_4.

5.4.3 Acridine orange/ethidium bromide staining of *Tecomella undulata*

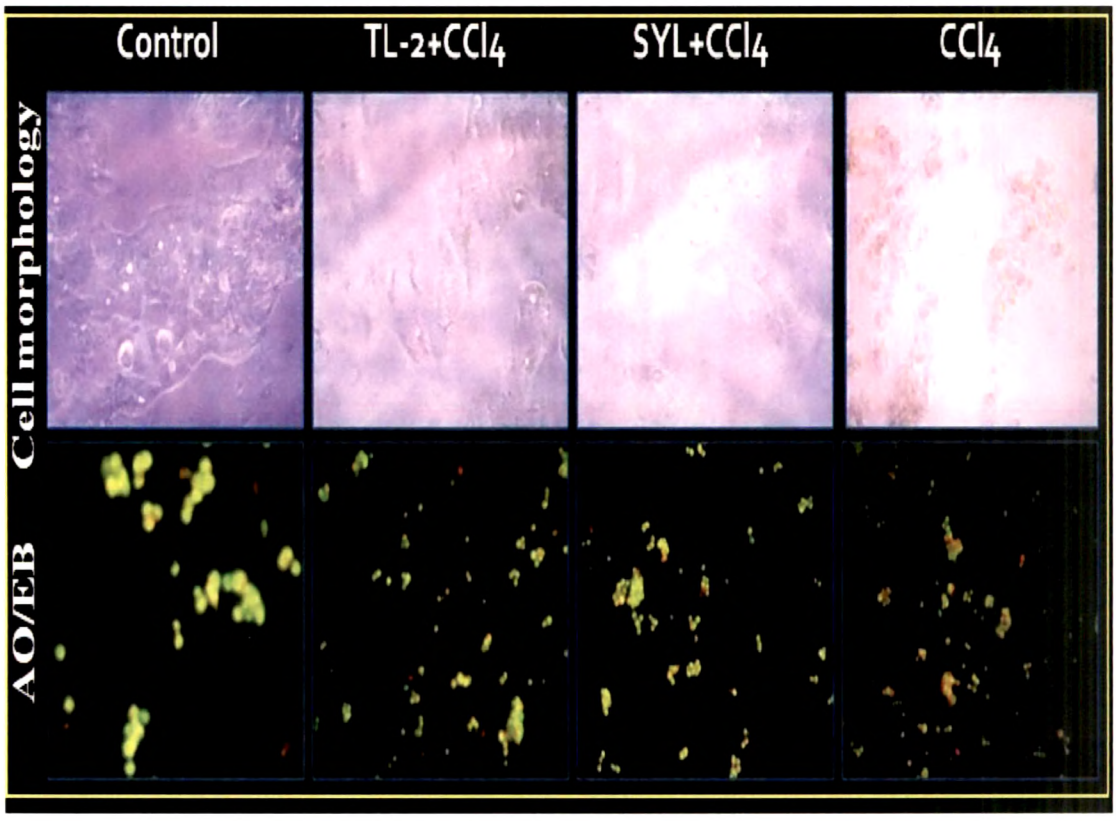
Two DNA intercalators, acridine orange (AO) and ethidium bromide (EB) were used to visualise condensed chromatin of apoptotic dead cells. The differential uptake of these two dyes allows the identification of live and dead cells. While the cationic dye acridine orange enters into live cells containing normal nuclear chromatin and exhibit

green colour, ethidium bromide is excluded by live cells and it stains fragmented nuclear chromatin in apoptotic cells in orange colour. These staining studies and photographic morphological evidence will further help in evaluating the hepatoprotective potential of various extracts, fractions and compounds of *Tecomella undulata.*

5.4.3.a Acridine orange/ethidium bromide staining of leaves of Tecomella undulata

A detailed scrutiny of AO and EB positive cells in the control and treated groups revealed that CCl_4 treatment accounted for maximum number of EB positive cells whereas the control group recorded AO positive cells. Also more number of AO positive cells were recorded in CCl_4 in TL-2 group as compared to CCl_4 treated group, CCl_4 +SYL group recorded maximum AO positive cells with result of this group comparable to that of control group. Similar set of results were observed in morphological changes of HepG2 cells (Figure 5.25).

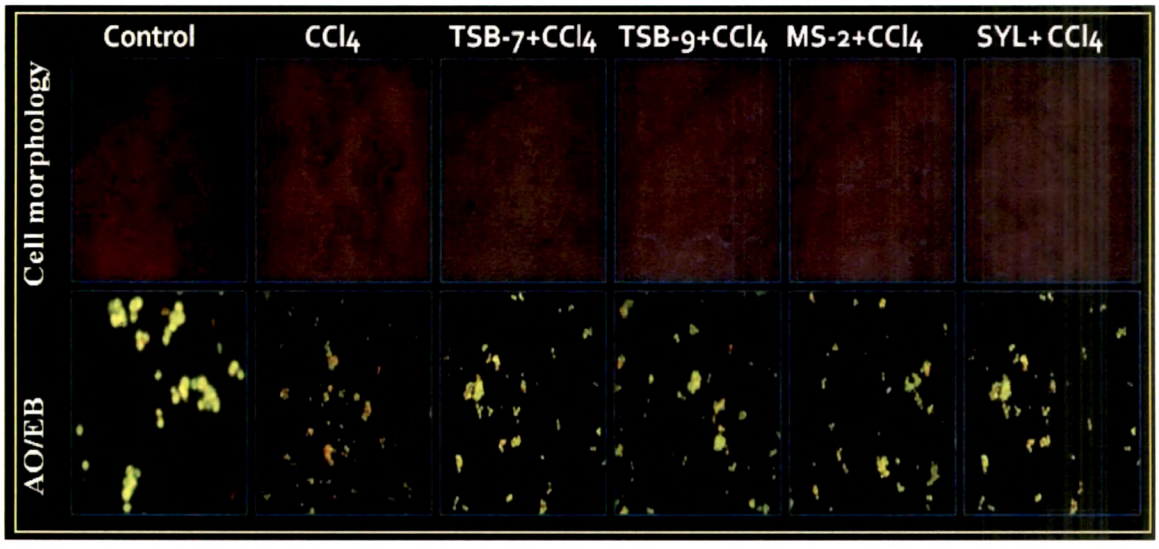
Figure 5.26 Phase contrast and AO/ EB stained photomicrographs of HepG2 cells exposed to 1% CCL4 alone or in presence of TL-2 or sylimarin.



5.4.3.b Acridine orange/ethidium bromide staining of stem bark of Tecomella undulata

Staining with AO and EB revealed that CCl_4 treatment accounted for maximum number of EB positive cells whereas; the control group recorded for more AO positive cells. Also, CCl_4+TSB-7, CCl_4+TSB-9 and MS-2 group recorded more number of AO positive cells respectively than CCl_4 treated group. But, CCl_4 +SYL group recorded maximum AO positive cells. Similar set of results were observed in morphological changes of HepG2 cells (Figure 5.27).

Figure 5.27 Phase contrast and AO/ EB stained photomicrographs of HepG2 cells exposed to 1% CCL_4 alone or in presence of TL-2 or sylimarin.



5.4.4 In vivo acute toxicity studies of *Tecomella undulata*

The acute toxicity studies in rats were performed only in those samples of TLs and TSBs which gave positive results for in vitro hepatoprotective activity.

5.4.4.a Acute toxicity studies of leaves Tecomella undulata

No mortality was recorded in animals that were orally administered up to 2000 mg/kg TL-2. There were also no adverse behavioral changes, diarrhoea, salivation or food aversion. There was no major change in the gross weight of animals.

5.4.4.b Acute toxicity studies of stem bark Tecomella undulata

No mortality was recorded in animals that were orally administered up to 5000 mg/kg of TSB-7; 2000 mg/kg TSB-9 and 1000 mg/kg MS-2. There were also no adverse behavioural changes, diarrhoea, salivation or food aversion.

5.4.5 In vivo* hepatic lipid peroxidation and antioxidant activity of *Tecomella undulata

5.4.5.a In vivo* hepatic lipid peroxidation and antioxidant activity of *TLs

To this end, the products' capacity to prevent or reduce hepatocytes damage induced by hepatotoxins (CCL_4), was examined. Although different biochemical alterations and mechanisms are responsible for the liver injury produced by these toxins, it is generally accepted that they all act as oxidants and involve ROS generation (Lumeng and Crabb, 2000; Balasubramaniyan et al., 2007; Wang et al., 2007).

One of the principal causes of CCl_4 induced liver injury is lipid peroxidation (LPO), induced and accelerated by free radical derivatives of CCl_4 (Maling et al., 1974). Free radicle scavenging enzymes, SOD and CAT and non-enzymatic antioxidants like GSH and AA are the first line of cellular defence against possible oxidative damage (Jayakumar et al., 2006; Lee et al., 2007; Gowri Shankar et al., 2008).SOD protects cells against free radicles by converting superoxide radicals into hydrogen peroxide, which is further metabolized by CAT to molecular oxygen and water, (Maheshwari et al., 2011) thus preventing oxidative damage. Changes in these parameters are hallmark of lipid peroxidation. Hence TLs and TSBs are evaluated for their antioxidant activity and for protection against CCl_4 induced lipid peroxidation.

CCl_4 treatment accounted for significant decrement in status of hepatic, enzymatic (SOD and CAT) and non-enzymatic (GSH and AA) antioxidants. TL-2 pre-treatment was able to prevent CCl_4 induced depletion of hepatic antioxidant with is higher dose (100 and 200 mg/kg body weight) being most efficient and comparable to SYL + CCl_4 group. TL-2 (200mg/kg body weight) recorded comparatively less significant level of mitigation as compared to SYL treated groups. Hepatic LPO levels were significantly high in CCl_4 treated groups. LPO level were also significant in SYL, TL-2 treated groups and comparable to control group (Table 5.12).

Table 5.12 Effect of TL-2 and sylimarin on hepatic antioxidant and lipid peroxidation during CCl4 induced hepatotoxicity

Treatments	Superoxide Dismutase U/min/ mg/protein	Catalase U/min/ mg/protein	Reduced glutathione (mg/g)	Ascorbic acid (mg/g)	Lipid peroxidation Nmol of MDA formed/ mg/protein
Control	60.00±1.52	34.33±1.36	6.64±0.30	4.2±0.04	2.76±0.04
CCl_4	25.33±0.88###	15.83±1.01###	2.36±0.04###	1.83±0.05###	5.82±0.05###
Silymarin	55.00±1.52###	32.33±0.33###	4.99±0.03##	3.77±0.03###	2.44±0.01###
TL-2 100	37.66±0.88*	20.33±0.88*	3.41±0.08*	2.68±0.01*	3.68±0.02**
TL-2 200	42.33±2.02**	23.33±1.20**	3.80±0.03*	2.88±0.02*	3.27±0.00**

Data expressed as mean± S.E.M. for n=6. Where, ###p<0.001 compared to control and *p<0.05, **p<0, 01 and ***p<0.001 compared to CCL4 control

5.4.5.b In vivo hepatic lipid peroxidation and antioxidant activity of *TSBs*

Significant decrement in status of hepatic enzymatic (SOD and CAT) and non-enzymatic (GSH and AA) antioxidants was recorded in CCl_4 treated rats. TSB-7, TSB-9 and MS-2 pre-treatment was able to prevent CCl_4 induced depletion of hepatic antioxidant. A dose of 400mg/kg, 200mg/kg and 150 mg/kg body weight of TSB-7, TSB-9 and MS-2 respectively were found to be most efficient and comparable to SYL + CCl_4 group. Hepatic LPO levels were significantly high in CCl_4 treated groups. But, LPO level were similar in SYL, TSB-9 and MS-2 treated groups and comparable to control group (Table. 5.13)

Table 5.13 Effect of TSB-7, TSB-9, MS-2 and sylimarin on hepatic antioxidants and lipid peroxidation during CCl4 induced hepatotoxicity

Treatments	Superoxide Dismutase U/min/ mg/protein	Catalase U/min/ mg/protein	Reduced glutathione (mg/g)	Ascorbic acid (mg/g)	Lipid peroxidation Nmol of MDA formed/ mg/protein
Control	60.00±1.52	34.33±1.36	6.64±0.30	4.2±0.04	2.76±0.04
CCl_4	25.33±0.88###	15.83±1.01###	2.36±0.04###	1.83±0.05###	5.82±0.05###
Silymarin	55.00±1.52***	32.33±0.33***	4.99±0.03***	3.77±0.03***	2.44±0.01***
TSB-7 200	45.00±1.52**	25.33±0.33**	4.01±0.04**	2.96±0.02**	3.74±0.02**
TSB-7 400	50.66±1.85***	30.00±0.57***	4.55±0.06**	3.32±0.04***	2.59±0.03***
TSB-9 100	47.66±0.66**	24.00±1.15**	4.23±0.03**	2.86±0.04**	3.12±0.02**
TSB-9 200	51.66±0.33***	29.33±1.33***	4.75±0.02**	3.26±0.06***	2.56±0.01***
MS-2 75	41.33±0.88***	23.33±0.33**	3.86±0.04**	2.91±0.01**	3.82±0.07**
MS-2 150	49.66±1.20***	27.66±0.66***	4.26±0.05**	3.15±0.04***	2.97±0.03***

Data expressed as mean± S.E.M. for n=6. Where, ###p<0.001 compared to control and *p<0.05, **p<0, 01 and ***p<0.001 compared to CCL4 control

5.4.6 *In vivo* studies

5.4.6.a In vivo: CCl_4 induced hepatotoxicity of leaves Tecomella undulata

Hepatotoxicity induced by CCl_4 is the most commonly used model system for the screening of hepatoprotective activity of plant extracts/ fractions. Administering CCl_4 to rats markedly increases serum AST, ALT, ALP, and bilirubin levels which reflect the severity of liver injury (Lin, Yao, Lin, & Lin, 1996). In this study, significant increase in AST, ALT, ALP, and bilirubin in the serum were observed after administration of CCl_4, as reported earlier. The leakage of large quantities of enzymes into the blood stream was associated with centrilobular necrosis and ballooning degeneration of the liver. However, the increased levels of these enzymes were significantly decreased by pretreatment with TL-2 (200mg/kg body weight) was the most efficient in mitigating CCl_4 induced hepatotoxicity (Table.5.14).

Table 5.14 Effects of TL-2 and sylimarin on plasma levels of hepatic injury markers during CCl4 induced hepatotoxicity.

Treat-ments	Alanine Transaminase (U/L)	Asparate Transaminase (U/I)	Alkaline Phosphatise (U/L)	Bilirubin (g%)	Total protein (g%)
Control	51.83±2.04	80.83±2.11	1.78±0.02	1.41±0.01	7.79±0.05
CCl4	152.33±2.41###	313.33±5.19###	3.53±0.03###	4.25±0.04###	4.39±0.12###
SYL	56.5±2.49***	86.83±4.28***	1.86±0.02***	1.56±0.01***	7.52±0.02***
TL-2 100	83.66±9.19***	172.50±4.81***	2.85±0.05*	2.87±0.02*	5.61±0.11*
TL-2 200	67.16±2.66***	121.00±8.78***	2.53±0.08*	2.59±0.03*	5.94±0.09*

Data expressed as mean± S.E.M. for n=6. Where, ###p<0.001 compared to control and *p<0.05, **p<0.01 and ***p<0.001 compared to CCL4.

5.4.6.b *In vivo CCl_4 induced hepatotoxicity of Tecomella undulata stem bark*

CCl_4 treated group recorded significantly elevated level of AST, ALT, ALP and total bilirubin whereas the total protein content in plasma significantly reduced. However, TSB-7, TSB-9 and MS-2 treated groups prevented CCl_4 induced elevation in plasma markers of hepatic damage and prevented CCl_4 induced decrement in plasma protein. Higher dose of TSB-7 (400mg/kg body weight) and TSB-9 (200mg/kg body weight) were the most efficient in mitigating CCl_4 induced hepatotoxicity. MS-2 (75 and 150 mg/kg body weight) also recorded similar set of changes (Table 5.15).

Table 5.15 Effects of TSB-7, TSB-9, MS-2 and sylimarin on plasma levels of hepatic injury markers during CCl4 induced hepatotoxicity.

Treat-ments (mg/kg)	Alanine Transaminase (U/L)	Asparate Transaminase (U/I)	Alkaline Phosphatise (U/L)	Bilirubin (g%)	Total protein (g%)
Control	51.83±2.04	80.83±2.11	1.78±0.02	1.41±0.01	7.79±0.05
CCl_4	152.33±2.41###	313.33±5.19###	3.53±0.03###	4.25±0.04###	4.39±0.12###
SYL 100	56.5±2.49***	86.83±4.28***	1.86±0.02***	1.56±0.01***	7.52±0.02***
TSB-7 200	76.33±4.94***	127.66±3.19***	2.91±0.03*	2.74±0.04*	6.07±0.21**
TSB-7 400	63.5±2.35***	99.66±2.29***	2.45±0.05*	1.99±0.03**	6.75±0.06**
TSB-9 100	67.16±2.01***	101.00±2.16***	2.63±0.05*	2.60±0.04*	6.30±0.06**

TSB-9 200	62.5±1.40***	90.16±3.39***	2.14±0.03**	1.94±0.01**	6.84±0.05**
MS-2 75	53.16±2.23***	98.33±3.09***	2.84±0.03*	2.75±0.03*	6.16±0.29**
MS-2 150	66.33±3.78***	82.66±2.59***	2.59±0.04*	2.14±0.03**	6.57±0.03***

Data expressed as mean± S.E.M. for n=6. Where, ###p<0.001 compared to control and *p<0.05, **p<0.01 and ***p<0.001 compared to CCL4.

5.4.7 In vivo: Histopathology of Liver

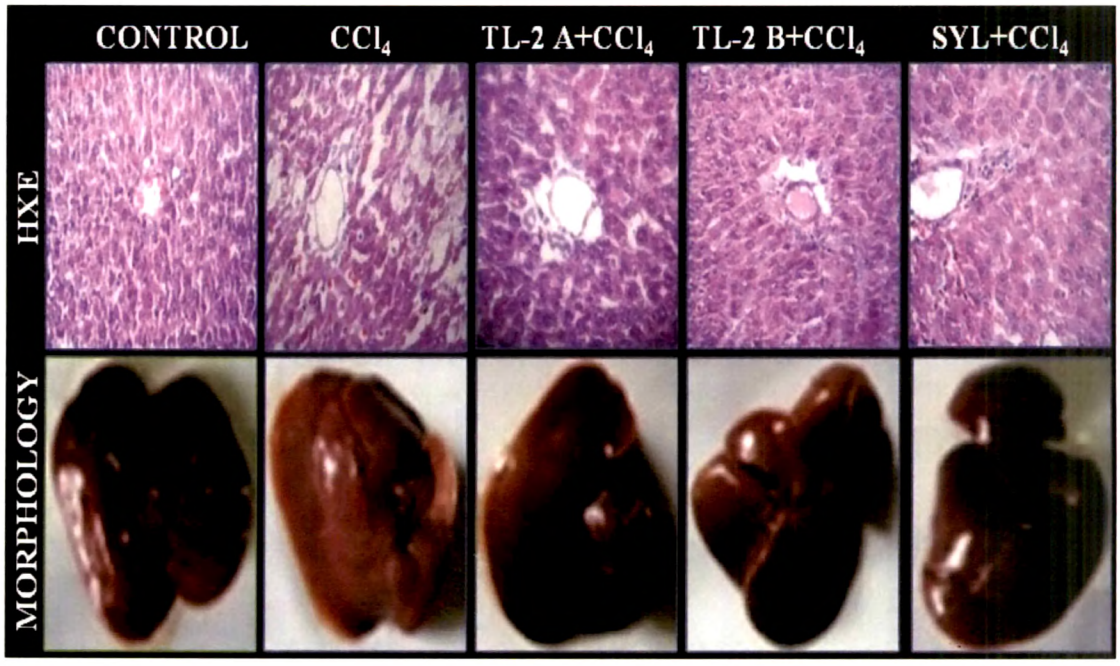
5.4.7.a Morphological and histopathological changes in liver after treatment with TLs

Microscopic evaluation of liver sections of control rats depicted unaltered cellular architecture with distinct hepatic cells, sinusoidal spaces, and a central vein.

Results of histopathology shows that the histo- architecture of liver was completely damaged in CCl_4 treated group. HXE stained section of CCl_4 treated rats, were characterized by loss of cellular boundries, infiltration of inflammatory cells, cytoplasmic vaculation, fatty change, centrilobular necrosis, and severe collagen deposition.

These cellular changes were greatly reduced in TL-2 treated group with higher dose (200 mg/kg body weight) recording mild necrosis and relatively healthy hepatocytes that were comparable to the control and SYL treated groups (Figure 5.28).

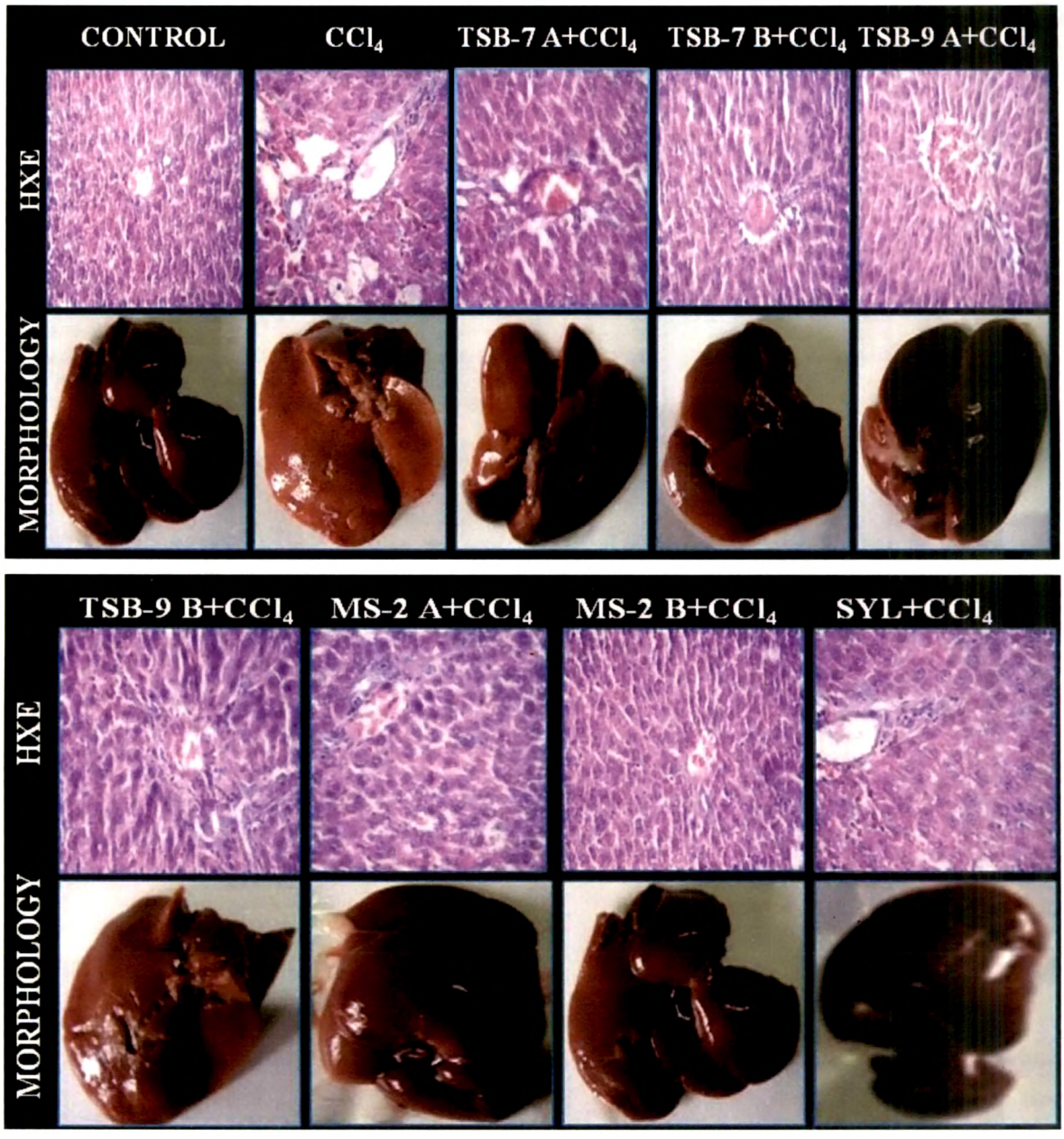
Figure 5.28 Morphological, haematoxyline-eosin photomicrographs of rat liver treated with CCL4 alone or in presence of TL-2 or sylimarin.



5.4.7.b Morphological and histopathological changes in liver after treatment with TSBs

The normal histoarchitecture of liver was completely in CCl_4 treated group. Hepatocytes vaculation, centrilobular necrosis and nuclear condensation were evident in HXE stand section of CCl_4 group. These cellular changes were greatly reduced in TSB-7, TSB-9 and MS-2 treated group with higher dose of TSB-7; TSB-9 and MS-2 (400; 200 and 150 mg/kg body weight) recording mild necrosis and healthy hepatocytes that were comparable to control and SYL treated group (Figure 5.29).

Figure 5.29 Morphological, haematoxyline-eosin photomicrographs of rat liver treated with CCL_4 alone or in presence of TSB-7, TSB-9, MS-2 or sylimarin.


CONTROL
CCl_4
TSB-7 A+CCl_4
TSB-7 B+CCl_4
TSB-9 A+CCl_4
HXE
MORPHOLOGY
TSB-9 B+CCl_4
MS-2 A+CCl_4
MS-2 B+CCl_4
SYL+CCl_4
HXE
MORPHOLOGY