MATERIALS AND METHODS

3.1 General

3.1.1 Chemicals and Solvents

All solvents used for extraction and isolation were purified by distillation (**Vogel, 2004**). Silica gel of 60-120 mesh activated at 120 °C and activated charcoal were used as adsorbent in column chromatography. Thin Layer Chromatography was performed using Aluminium sheets (TLC Silica gel 60 F_{254}) and with glass plates. Silica gel (100 mesh) with 13% $CaSO_4.½H_2O$ binder was used as solid stationary phase in Thin Layer Chromatography. The spots were visualized under UV light at wavelength 254 nm and 365 nm and in iodine vapours.

3.1.2 Instruments

The instruments/equipments used for different analyses during the research are given in Table 1.

Table 1. Instruments/equipments used during the research

Instrument	Company
Melting point apparatus	Joshiba
UV Doublebeam spectrophotometer	Systronics 2202
FT-IR (4000-400) spectrophotometer	Shimadzu
NMR 500MHz spectrophotometer	Bruker Avance III
GC-MS	Thermo GC-TRACE ULTRA VER: 5.0,Thermo MS DSQ II and JEOL GCMATE II Schimadzu GC-2010; MS Schimadzu QT 2010 PLUS JEOL GCmate II GC-MS
HPLC	Shimadzu
HPTLC	Camag
Rotary vacuum evaporator	Equitron
Sonicator	PCi
Ultrasonic Homogeniser	BIOLOGICS, Inc
Tensiometer	Instron
Light Microscope	Labomed

3.2 Plant Material

E. crassipes (1050 kg) was collected from Singanallur boat house, Coimbatore. The plant was authenticated by Dr.G.V.S.Murthy, Scientist F & Head of Office, Botanical Survey of India, Southern Regional Centre, Coimbatore - 641 002 with the number BSI/SRC/5/23/2011-12/Tech (Certificate 1). The shoots and leaves of *E. crassipes* were used in the present study.

The methods adopted in the present study are

- Extraction of *E. crassipes*
- Fractionation of the aqueous extract of *E. crassipes*
- Secondary metabolite profiling - sterols, alkaloids and terpenoids
- Phytochemical screening of the extracts
- Open column chromatographic isolation of compounds from extracts of *E. crassipes*
- Characterization of isolated compounds
- High performance chromatographic studies
- Antimicrobial Assay
- Antioxidant assay
- *In vivo* Acute Toxicity Studies
- Preparation of ointments for *in vivo* wound healing testing
- Preparation of skin creams for skin whitening and antiageing assay
- Larvicidal, pupicidal and repellent studies of extracts and fractionates of *E. crassipes*
- *In silico* studies

3.3 Extraction of plant material

3.3.1 Extraction of *E. crassipes*

The plant material was washed several times to remove soil and other debris. The roots were cut off and shade dried for 20 days, chopped and pulverized.

3.3.1.1 Alkaline ethanolic extraction

The plant (1.5 kg) was extracted twice with petroleum ether (60-80 °C) for 6 h. The residual plant material was extracted twice with 2 N ethanolic potassium hydroxide for 6 h. The extract was desolvetised under reduced pressure and the residue was extracted thrice with acetone for 1 h. The acetone extracts were pooled and concentrated to a semi-solid mass using rotary flash evaporator to yield 280 g.

3.3.1.2 Sequential extraction

The bulk extraction of *E. crassipes* was carried out in Cymbio Pharma Pvt. Ltd., Bengaluru. *E. crassipes* (28 kg) was extracted by percolation twice with ethyl acetate for 6 h and then twice with water for 6 h yielding a black and brown extract respectively. A small

portion (1.5 kg) of the plant residue was extracted with 1% hydrochloric acid (3 L) for 6 h to get hydrolyzed extract.

3.3.1.3 Fractionation of aqueous extract

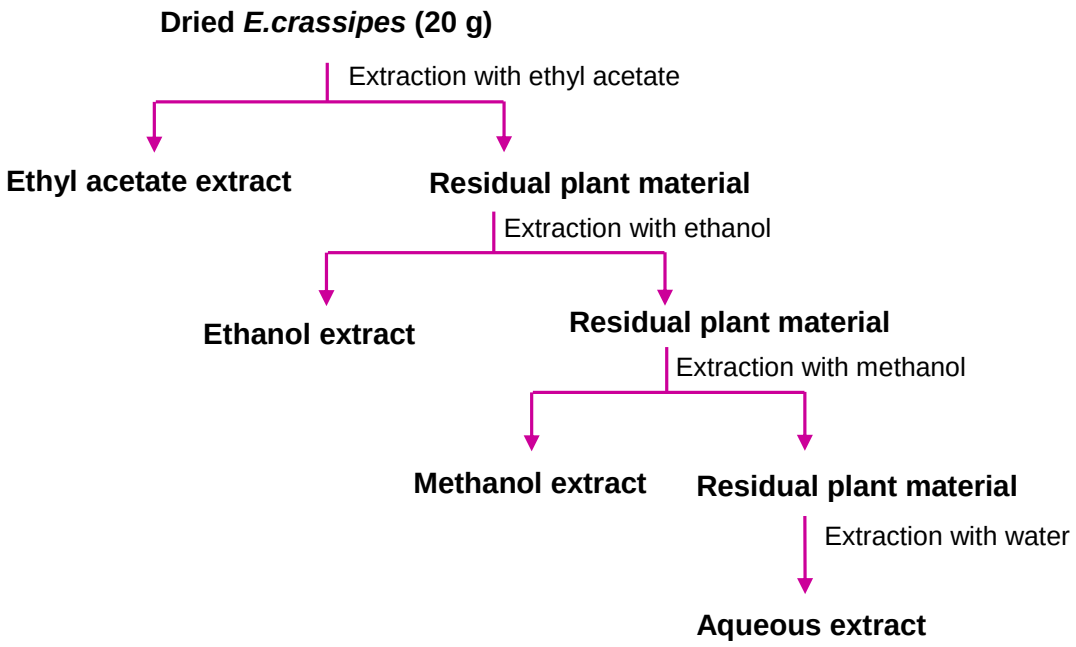
The aqueous extract was fractionated with ethanol and methanol respectively yielding ethanol fractionate (EFA), aqueous fractionate (AFE), methanol fractionate (MFA) and aqueous fractionate (AFM) of aqueous extract.

3.3.1.4 Extraction of *E. crassipes* by different methods

The suitability of the extraction method for extraction of dried and fresh *E. crassipes* was analysed using three different methods *viz.,* reflux and sonic assisted techniques *viz.,* ultrasonic bath and ultrasonic homogeniser. The extraction of the dried and fresh plant (20 g and 300 g respectively) was carried out for a uniform period of 3 h and 1 h respectively. Scheme 1 and 2 represents the procedure adopted for the extraction of dried and fresh *E. crassipes*.

The fresh plant was extracted with ethyl acetate by **ten** different methods to find the suitability of extraction with ethyl acetate as solvent *viz.,* reflux, ultrasonic bath, ultrasonic homogeniser, hot continuous extraction (soxhlet extraction), microwave, percolation, maceration, infusion, digestion and decoction (**Handa *et al,* 2008**). The volume of the solvent used and time of extraction are given in Table 2. The extraction was carried out based on the visual observation till the solvent became colourless indicating the completion of extraction.

Scheme 1. Extraction of dried *E. crassipes* by different methods



Scheme 2. Extraction of fresh *E. crassipes* by three different techniques

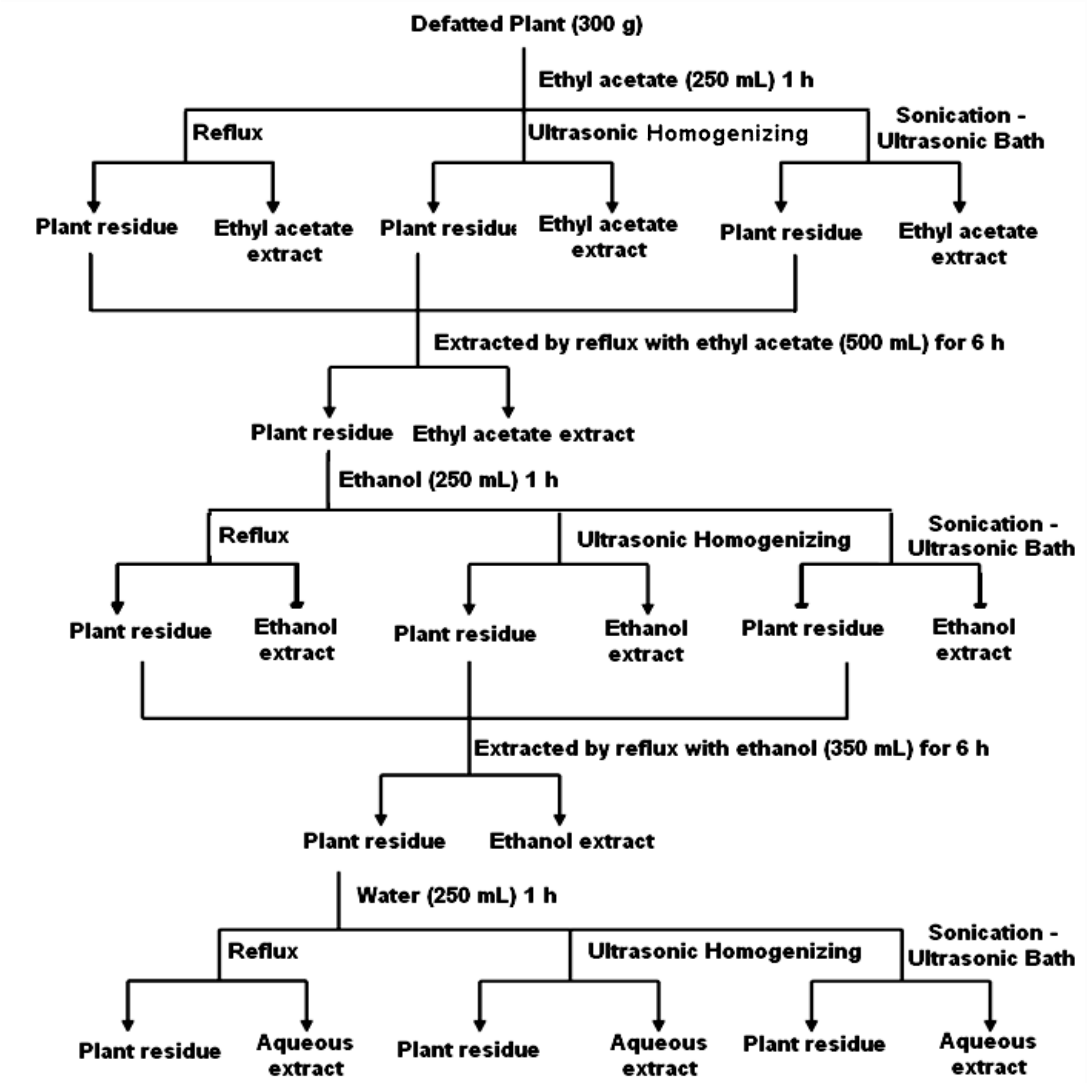


Table 2. Comparison of different extraction methods for fresh *E. crassipes* with ethyl acetate

S.No	Method	Time (h)	Volume of Solvent used for Extraction (mL)
1	Reflux	6	1000
2	Sonication using Ultrasonic bath	5	520
3	Ultrasonic Homogenizing	2	400
4	Hot Continuous extraction (Soxhlet)	1	100

5	Microwave assisted extraction	1¾	890
6	Percolation	600	540 (hot condition)
		600	500 (cold condition)
7	Maceration	72	280
8	Infusion	½	100
9	Digestion	1	150
10	Hot aqueous extraction (Decoction)	1	140

3.4 Seconday Metabolite Profiling

3.4.1 Isolation and profiling of sterols (Wang *et al,* 2002)

Powdered *Eichhornia crassipes* (Mart.) Solms (700 g) was extracted with a binary mixture of alcohol and water and KOH solution for 4 h. The reaction mixture was extracted with toluene (5.5 L) until the organic layer became colourless. The organic phase was washed with distilled water until neutrality was obtained. The organic layer was dried and the solvent evaporated to obtain the raw unsaponifiables. The raw unsaponifiables were treated with acetone and butanone (1:1) to remove the residual soap and to obtain the refined unsaponifiables. The refined unsaponifiables were recrystallized using ethyl acetate and the phytosterols thus obtained was chromatographed on TLC in 1:1 petroleum ether:ethyl acetate and detected in anisaldehyde reagent and 10% sulphuric acid. The sterols were subjected to Salkowski's test and Liebermann-Burchard test. The sterols were profiled by GC-MS with the gas chromatographic column at an oven temperature set initially at 50 °C (held for 1 min) and increased by 10 °C min^{-1} until it reached 300 °C (held for 15 min). Other gas chromatographic and mass spectrometric conditions were kept standard for all the measurements.

3.4.2 Isolation and profiling of alkaloids from the aqueous extract of *E. crassipes* (Shekar, 2008)

The methanol fractionate of aqueous extract (100 g) of *E. crassipes* was dissolved in millipore water (500 mL) and acidified with 20 mL of 1:1 HCl. The extract was then steam distilled and the filtrate was shaken with ether (180 mL). The ether layer was concentrated under reduced pressure to yield neutral alkaloids. The aqueous layer was made basic with sodium hydroxide and then shaken with ether (250 mL). The ether layer was concentrated in a rotary evaporator to yield basic alkaloids. The alkaloids obtained were subjected to Dragendorff's test, Mayer's test and Wagner's test. The alkaloids were analysed by TLC in Methanol: NH_4OH (200:3) and detected by spraying Dragendorff's reagent. The alkaloids were also profiled by GC-MS where the oven temperature was programmed from 150 °C to 270 °C at a rate of 5 °C min^{-1} with a 10 minutes hold.

3.4.3 Isolation and profiling of terpenoids

Isolation of terpenoids was carried out using flash column chromatography over silica. Ethyl acetate extract (5 g) of *E. crassipes* was made into slurry with silica and loaded onto a silica column and eluted with solvents of varying polarity. The column was eluted with chloroform:methanol (10:1), n-butanol:2M NH_4OH (1:1) and petroleum ether:ethyl acetate:formic acid (93:7:0.7). The homogeneity of the fractions was detected by TLC. The terpenoids were analysed by Liebermann-Burchard test and by TLC in petroleum ether:ethyl acetate (8.5:1.5) and detecting in 10% sulphuric acid. The concentrated fractions were analysed by GC-MS with an initial oven temperature of 60 °C and final temperature of 240 °C. The rate of increase in temperature of the column was set as 3 °C min^{-1}.

3.5 Phytochemical screening of the extracts of *E. crassipes*

The phytochemical screening tests for the extracts and fractionates of *E. crassipes* were carried out adopting standard procedures.

Metabolite	Phytochemical test	Reference
Alkaloids	Mayer's test	Raaman, 2006
	Wagner's test	Raaman, 2006
	Hager's test	Raaman, 2006
Flavonoids	NaOH Test	Raaman, 2006
	H_2SO_4 test	Raaman, 2006
Sterols	Liebermann-Burchard test	Harborne, 1973
Terpenoids	Liebermann-Burchard test	Harborne, 1973
Anthraquinones	Borntrager's test	Raaman, 2006
Anthocyanins	NaOH test	Raaman, 2006
Phenols	Ferric chloride test	Raaman, 2006
	Liebermann's test	Harborne, 1973
Proteins	Ninhydrin test	Harborne, 1973
Quinones	HCl test	Harborne, 1973
Carbohydrates	Molisch test	Harborne, 1973

3.6 Isolation of compounds from *E. crassipes*

3.6.1 Isolation of compounds from acetone extract of *E. crassipes*

Silica gel (60-120 mesh for column chromatography) (1300 g) was mixed with petroleum ether to form a slurry and packed to a height of 100 cm in a 130 cm x 6 cm glass column. Acetone extract (280 g) was dissolved in a small volume of acetone, mixed with silica gel (1100 g), allowed to dry and a portion (300 g) of this was loaded on top of the packed column. A step gradient of solvent from 100% petroleum ether

through ethyl acetate and ethanol to 100% methanol was used for elution. A total of 733 fractions of 400 mL were collected and concentrated by distillation. The concentrated fractions were loaded onto 10 x 20 cm TLC plates, developed in 1:1 petroleum ether - ethyl acetate and 1:1 ethyl acetate - ethanol, examined under UV lamp and monitored in iodine chamber. Fractions showing similar spots were combined giving 14 fractions (AC1-AC14). The nature of the fractions and the polarity of the solvent used for elution are given in Table 3 and the procedure involved is shown in Scheme 3.

Scheme 3. Isolation of compounds from the acetone extract of *E. crassipes*

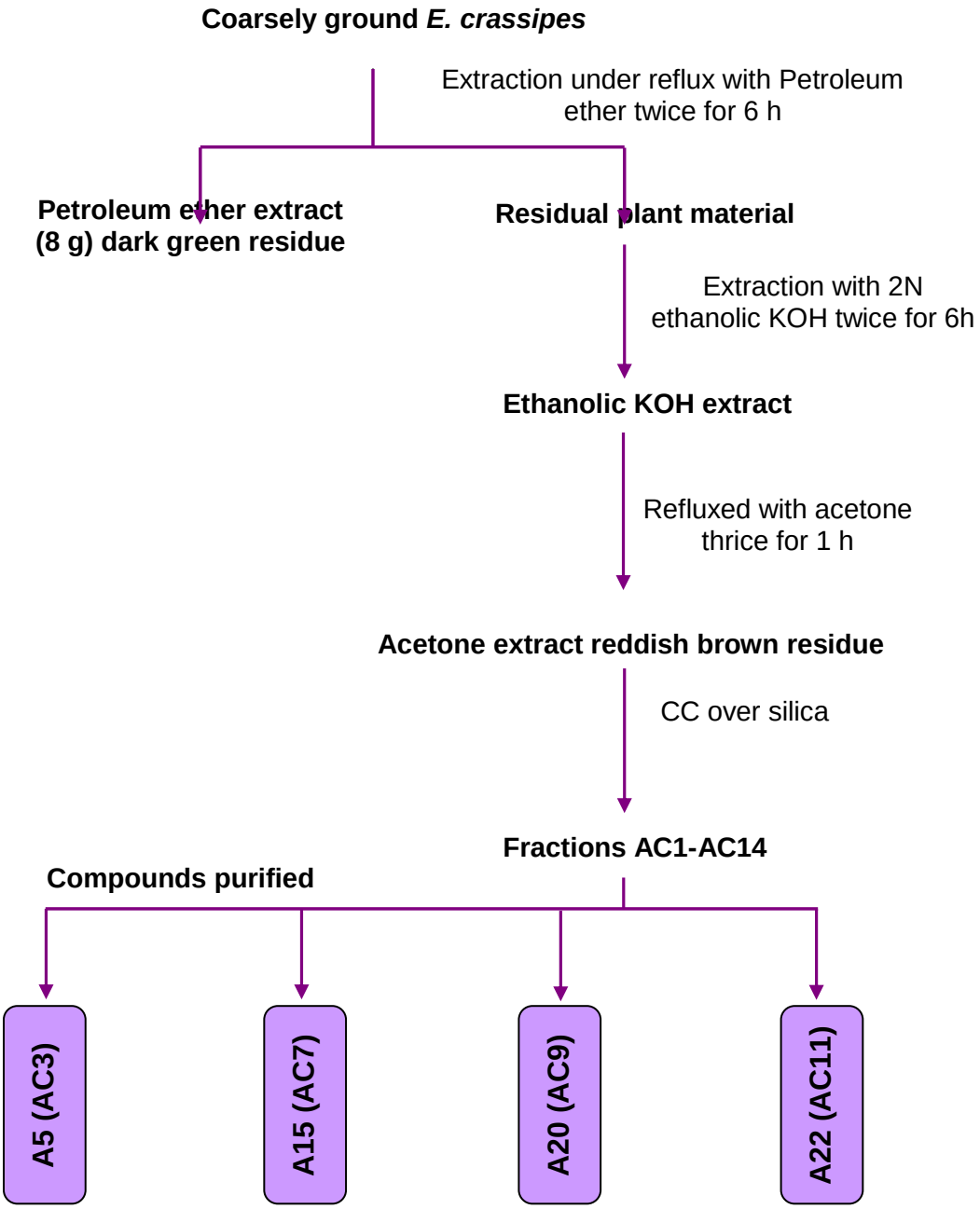


Table 3. Nature of the fractions and polarity of the solvent used in acetone column of *E. crassipes*

Fraction Number	Fractions combined	Nature of the fraction	Polarity of eluting solvent
AC1	1-20	Yellow wax	100% Petroleum ether
AC2	21-23	Reddish Orange Liquid	100% Petroleum ether
AC3	24-39	Reddish Orange Liquid	1% Ethyl acetate/ Petroleum Ether
AC4	40-53	Orange	1,5% Ethyl acetate/ Petroleum Ether
AC5	54-58	Orange Liquid	5% Ethyl acetate/ Petroleum Ether
AC6	59-87	Orange Liquid	5, 10% Ethyl acetate/ Petroleum Ether
AC7	88-97	Reddish Brown Liquid	20, 30, 40% Ethyl acetate/ Petroleum Ether
AC8	98-114	Reddish Brown Liquid	50, 70% Ethyl acetate/ Petroleum Ether
AC9	115-191	Reddish Brown Liquid	80% Ethyl acetate/ Petroleum Ether; 100% Ethyl acetate; 1% Ethanol/ Ethyl acetate; 5% Ethanol/ Ethyl acetate; 10% Ethanol/ Ethyl acetate; 15% Ethanol/ Ethyl acetate
AC10	192-279	Reddish Brown Liquid	25% Ethanol/ Ethyl acetate; 30% Ethanol/ Ethyl acetate; 40-80% Ethanol/ Ethyl acetate
AC11	280-440	Reddish Brown Liquid	80% Ethanol/ Ethyl acetate; 100% Ethanol
AC12	441-590	Reddish Brown Liquid	100% Ethanol
AC13	591-691	Reddish Brown Solid	100% Ethanol
AC14	692-733	Reddish Brown Solid & Liquid	100% Ethanol; 100% Methanol

3.6.2 Isolation of compounds from ethyl acetate extract of *E. crassipes*

The ethyl acetate extract (300 g) was made into slurry with 400 g of silica and was packed in a chromatographic column (150 cm x 7 cm) filled with 1600 g of silica gel (60-120 mesh). The elution of the components of the sample incorporated to the column was initially performed with petroleum ether and successively with gradient mixtures of petroleum ether-ethyl acetate, ethyl acetate-ethanol and ethanol-methanol. Fractions of 400 mL were collected, analysed by TLC and similar fractions were combined. A total of 1120 fractions yielded forty fractions (E1-E40). The nature of the combined fractions together with the polarity of the solvent used for elution is given in Table 4.

Table 4. Nature of the fractions and polarity of the solvent used in ethyl acetate column of *E. crassipes*

Fraction Number	Fractions combined	Nature of the fraction	Polarity of eluting solvent
E1	1-3	Powder	100% Petroleum ether
E2	5-10	Yellow wax	100% Petroleum ether
E3	11-19	Yellow wax	100% Petroleum ether
E4	20-24	Orange wax	5% Ethyl acetate/ Petroleum ether
E5	25-37	Orange wax	5% Ethyl acetate/ Petroleum ether
E6	38-53	White powder & Reddish Brown wax	5% Ethyl acetate/ Petroleum ether
E7	54-56	Buff Colour Wax	5% Ethyl acetate/ Petroleum ether
E8	57-60	Reddish Brown Wax & White powder	5% Ethyl acetate/ Petroleum ether
E9	61-63	Brown Wax	10%Ethyl acetate/ Petroleum ether
E10	64-67	Dark Brown Fluid	10%Ethyl acetate/ Petroleum ether
E11	68-71	Reddish Brown Solid	10%Ethyl acetate/ Petroleum ether
E12	72-75	Reddish Brown Solid	10%Ethyl acetate/ Petroleum ether
E13	76-79	Reddish Brown Solid	10%Ethyl acetate/ Petroleum ether
E14	80-87	Greenish Brown Solid	10%Ethyl acetate/ Petroleum ether
E15	88-95	Dark Brown Solid	10%Ethyl acetate/ Petroleum ether
E16	96-124	Green Solid	10%Ethyl acetate/ Petroleum ether
E17	125-154	Solid (Black)	10%Ethyl acetate/ Petroleum ether
E18	155-356	Solid (Black)	10,15,20,30,40% Ethyl acetate/ Petroleum ether
E19	357-394	Solid (Black)	40% Ethyl acetate/ Petroleum ether
E20	395-586	Solid (Black)	50-80% Ethyl acetate/ Petroleum ether
E21	587-650	Solid (Black)	80, 100% Ethyl acetate/ Petroleum ether
E22	651-668	Solid (Black)	100%Ethyl acetate/ Petroleum ether
E23	669	Powder (Black)	100%Ethyl acetate/ Petroleum ether
E24	670-671	Powder (Black)	1% Ethanol/ Ethyl acetate
E25	672-679	Powder (Black)	1% Ethanol/ Ethyl acetate
E26	680-685	Solid (Black)	1% Ethanol/ Ethyl acetate

E27	686-737	Solid (Black)	5, 10, 15% Ethanol/ Ethyl acetate
E28	738-750	Powder (Black)	15% Ethanol/ Ethyl acetate
E29	751-757	Powder (Black)	15% Ethanol/ Ethyl acetate
E30	758-770	Powder (Black)	20% Ethanol/ Ethyl acetate
E31	771-786	Solid (Black)	20, 25% Ethanol/ Ethyl acetate
E32	787-800	Solid (Black)	25% Ethanol/ Ethyl acetate
E33	801-911	Solid (Black)	30-70% Ethanol/ Ethyl acetate
E34	912-921	Solid (Black)	70% Ethanol/ Ethyl acetate
E35	922-939	Reddish Brown Solid	70, 80% Ethanol/ Ethyl acetate
E36	940-962	Solid (Brown)	80% Ethanol/ Ethyl acetate; 100 % Ethanol
E37	963-1044	Solid (Brown)	100% Ethanol; 10% Methanol
E38	1045-1109	Solid (Brown)	100% Methanol
E39	1110-1111	Solid (Brown)	100% Methanol
E40	1112-1120	Solid (Brown)	100% Methanol

The solvents in the combined fractions were evaporated, added ethanol and refrigerated. The fractions in which solid was seen on refrigeration were further processed and compounds isolated from those fractions. The purified compounds were characterized by employing spectral techniques (Scheme 4).

3.6.3 Isolation of compounds from aqueous extract of *E. crassipes*

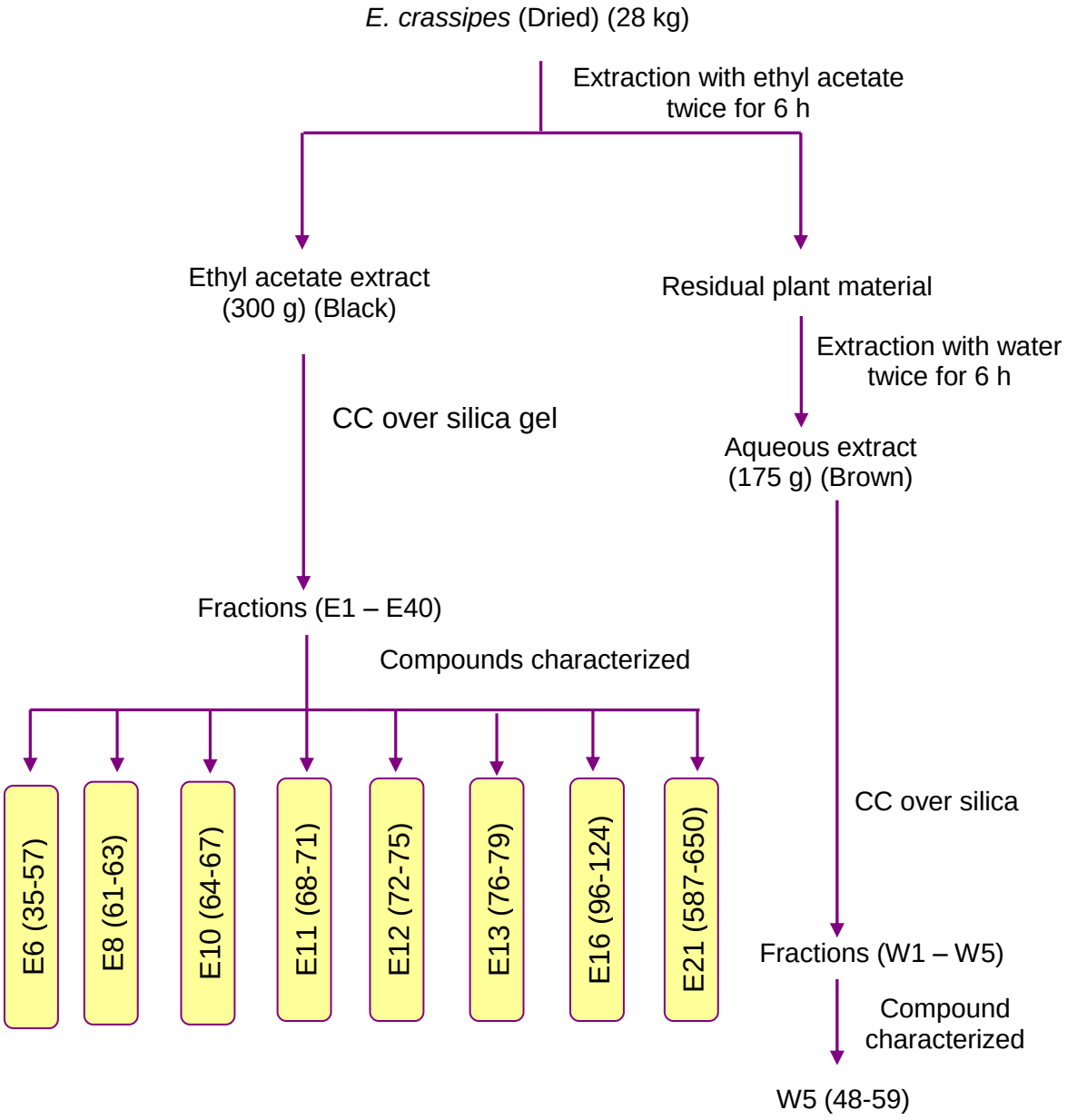
The isolation of compounds from crude aqueous extract (175 g) was done in a chromatographic column (150 cm x 7 cm) filled with 1500 g of silica gel (60-120 mesh). The silica gel was packed in to the column with 75:25 ethyl acetate: ethanol. The elution of the components of the sample incorporated to the column was performed with step gradient of ethyl acetate-ethanol. A total of 110 fractions of 500 mL each was collected and similar fractions based on TLC were combined yielding 6 fractions (W1-W6). The nature of the fractions and the polarity of the solvent used for elution are given in Table 5. The combined fractions were evaporated, alcohol added and refrigerated. The TLC of the fractions showed it to be a mixture of compounds. The fractions were treated with alcohol and refrigerated. A white solid (W5) was obtained which was filered off and recrystallised.

Table 5. Nature of the fractions and polarity of the solvent used in aqueous column of *E. crassipes*

Fraction Number	Fractions combined	Nature of the fraction	Polarity of eluting solvent
W1	1-2	Green wax	75% Ethylacetate: Ethanol

W2	3-12	Brown liquid	75% Ethylacetate: Ethanol
W3	13-26	Brown liquid	75% Ethylacetate: Ethanol
W4	27-47	Brown liquid	75% Ethylacetate: Ethanol
W5	48-59	White powder	75% Ethylacetate: Ethanol
W6	60-110	White crystals	50% Ethylacetate: Ethanol

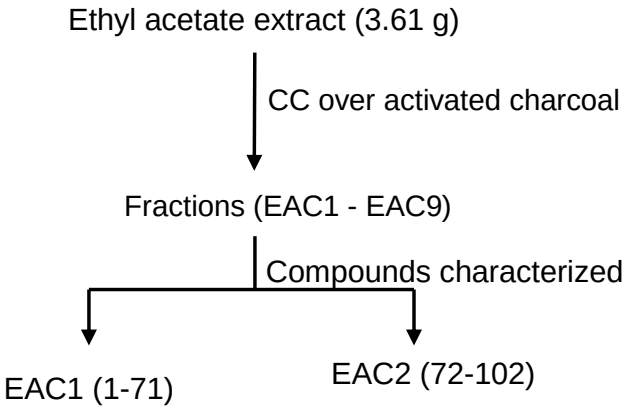
Scheme 4. Isolation of compounds from ethyl acetate and aqueous extract column over silica



3.6.4 Isolation of compounds from ethyl acetate extract of *E. crassipes* using activated charcoal

Fine activated charcoal (7.5 g) which has high binding interaction towards polar compounds was made into slurry with petroleum ether (60-80 °C) and was packed into the column. The ethyl acetate extract (3.61 g) was packed onto the column as slurry and eluted with solvents starting with petroleum ether through ethyl acetate and ethanol to methanol. A total of 146 fractions of 50 mL each were collected and similar fractions from TLC were combined giving fractions EAC1- EAC9.

Scheme 5. Isolation of compounds from the ethyl acetate extract of *E. crassipes* by column chromatography over activated charcoal



3.6.5 Isolation of compounds from the petroleum ether fractionate of ethyl acetate extract of *E. crassipes*

Ethyl acetate extract (60 g) was fractionated with petroleum ether (1000 mL) to give petroleum ether fractionate. Petroleum ether fractionate (20 g) thus obtained from ethyl acetate extract of *E. crassipes* was made in to a slurry with petroleum ether. This was then column chromatographed over silica (300 g) with the eluting solvent as 100 % petroleum ether. A total of 537 fractions of 100 mL volume each was collected and combined based on TLC of the fractions giving HP1-HP7 (Table 6).

Table 6. Nature of the fractions and the polarity of the solvent used in petroleum ether fractionate column

Fraction Number	Fractions combined	Nature of the fraction	Polarity of eluting solvent
HP1	1-8	Pale white solid	100% Petroleum ether
HP2	9-180	White wax	100% Petroleum ether

HP3	181-336	Yellow solid	100% Petroleum ether
HP4	181-336	White solid	100% Petroleum ether
HP5	337-429	White solid	100% Petroleum ether; 1%Ethyl acetate/ Petroleum ether
HP6	430-457	White solid	1%Ethyl acetate/ Petroleum ether
HP6A	458-515	White solid	10%Ethyl acetate/ Petroleum ether
HP7	516-521	Brown liquid	10%Ethyl acetate/ Petroleum ether

3.7 Characterization of compounds

Preliminary colour tests were carried out for the isolated compounds. Melting points were determined in the digital melting point apparatus. The isolated compounds were characterized by various spectral techniques namely UV, IR, ^{1}H NMR, ^{13}C NMR, DEPT, ^{1}H - ^{1}H COSY, HSQC, HMBC and Mass spectral techniques.

3.8 High performance chromatographic studies of *E. crassipes*

3.8.1 Qualitative estimation of glutathione in the ethyl acetate and aqueous extracts of *E. crassipes* extracts by HPLC

3.8.1.1 Instrument

A Shimadzu HPLC system (Class VP series) with photo diode array detector (SPD M10A VP), CTO-10AS VP column oven, CBM- 10A VP system controller and reverse phase C18 phenomenex column (250 mm × 4.6 mm) was used for the analysis. The HPLC system was equipped with Class VP series version 6.1 software.

3.8.1.2 Sample preparation

The ethyl acetate extract (10 mg) and aqueous extract (200 mg) were dissolved in HPLC grade methanol and water respectively (10 and 8 mL). The standard was prepared by dissolving reduced glutathione (1 mg) in 10 mL HPLC grade water. This was then filtered using SRP 15 syringe with 0.20 µm diameter filter that has a filter area of 1.7 cm^2 for rapid ultra-cleaning of the sample. A volume of 1 mL was injected into the column for each analysis.

3.8.1.3 Chromatographic conditions

The mobile phase consisted of two solvent systems (A: 0.06% trifluoroacetic acid (TFA) in water (v/v) and B: 100% acetonitrile) in a gradient (50% A and 50% B). The column temperature was maintained at 0 °C and the flow rate was set at 1.0 mL/min and the run

was continued for 40 minutes. The detection of the glutathione was carried out at 225 nm using a PDA detector.

3.8.2 HPTLC studies of compounds of *E. crassipes*

3.8.2.1 Equipment, Instrument and Materials

The analysis was done by using computerized Camag HPTLC system (Camag, Muttenz, Switzerland) consisting of a semiautomatic spotting device connected to a nitrogen tank and WinCATS 4 software (version 4.05, Camag), a TLC scanner III densitometer equipped with mercury, tungsten and deuterium lamp, a 100 µL HPTLC sample syringe (Hamilton, Bonaduz, Switzerland) and a glass twin-trough (10 cm x 10 cm) development chamber, volumetric flasks, measuring cylinders, micro-syringes and ruler. A percolated silica gel $60F_{254}$, (Mean pore size of 60 A^0 fluorescent excitation wavelength 254 nm) was used for spotting the samples for analysis.

3.8.2.2 Sample preparation

The compounds E11, E12, E13 and ethyl acetate extract (1 mg) were dissolved in 10 mL of chloroform in 10 mL volumetric flask and appropriate working concentrations of the sample (0.1 mg/µL) were used in the study.

3.8.2.3 Chromatographic conditions

The samples were spotted as bands of width 6 mm with a micro litre syringe on precoated silica gel $60F_{254}$ plate (10 cm x 10 cm with 200 µm thickness), using Linomat V applicator. A constant application rate of 0.1 µL/s was maintained and space between two bands was 15 mm. The slit dimension was kept at 5 mm x 0.45 mm and 20 mm/s scanning speed was employed. The mobile phase used in the analysis was 7:3 petroleum ether:ethyl acetate. Linear ascending development was carried out in twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 40 minutes at room temperature. The length of chromatogram run was 8 cm. Subsequent to the development, TLC plates were dried in a current of air with the help of an air-dryer.

Densitometric scanning was performed on TLC scanner III in the absorbance mode at 255 and 361nm. The source of radiation utilized was deuterium lamp that

emits a continuous UV spectrum between 190 and 400 nm. Concentrations of chromatographed compound were determined from the intensity of diffusely reflected light.

3.8.3 Gas chromatographic analysis

3.8.3.1 Instrument and chromatographic conditions

Metabolite profiling by GC-MS was carried out in a Schimadzu GC-2010 linked to Schimadzu MS QT 2010 PLUS equipped with 30 m long, 0.25 mm id and 0.25 µm film thickness HP-5-MS capillary column. The column temperature program was varied with the analysis. GC-MS analysis of the compounds was performed using Thermo GC - Trace Ultra VER: 5.0, equipped with a Thermo MS DSQ II. Compounds were separated on TR 5 - MS capillary standard non-polar column (30 m x 0.25 mm, film thickness 0.25 µm). Column oven temperature was programmed from 40 to 250 °C at the rate of 8 °C min^{-1}; initial and final temperatures were held for 3 and 10 minutes respectively for the compounds.

3.8.3.2 Sample preparation

The sterols, terpenoids, alkaloids, aqueous extract and the pure compounds A15, E10, E11, E21, W5 (1 mg) were dissolved in 1 mL of methanol and the injection volume of the samples was 1.0 µL. The samples were analysed using the split mode (split ratio 30:70). For GC-MS detection, an electron ionization system, with ionization energy of 70 eV was used. Helium was used as a carrier gas at a flow rate of 1.0 mL min^{-1}. The identification of the components was based on comparison of their mass spectra with NIST library 08 and Wiley Spectral Library 8.

3.9 Biological and pharmacological activity screening

3.9.1 Antimicrobial Assay

The plant extract and fractionates of *E. crassipes* were tested for their antibacterial and antifungal activity. Two bacteria *Staphylococcus albus*, *Pseudomonas aerugniosa* and two fungi *Aspergillus niger*, *Mucor* sp used for the studies were procured from Jebi Laboratory, Pollachi.

3.9.1.1 Disc Method for Determination of Zone of Inhibition for Antibacterial activity

Antimicrobial assay was conducted adopting standard procedure (**Arulpriya *et al,* 2010**). Paper discs of 4 mm diameter and glass Petri plates of 90 mm diameter were used throughout the experiment. Paper discs were sterilized in an autoclave and dried at

100 °C in an oven. The discs were soaked with test chemicals at the rate of 50 μg (dry weight) per disc for antibacterial analysis. One drop of bacterial suspension was taken in a sterile petri dish. Approximately 20 mL of sterilized and melted nutrient agar (~45 °C) was poured into the plate, and then mixed thoroughly.

The paper discs after soaking with test chemicals were placed at the center of the inoculated pour plate. A control plate was also maintained in each case with alcohol. The plates were initially maintained at low temperature (4 °C) for 4 h, then incubated at (35 ± 2) °C for growth of test organisms and observed at 24 h intervals for two days. The activity was expressed in terms of zone of inhibition in mm. Each experiment was repeated three times. The standard antibiotic gentamycin was used as a positive control and compared with test chemicals under identical conditions.

3.9.1.2 Streak Plate Isolation Method for Determination of Zone of Inhibition for Antifungal Activity

The required amount of SDA medium was taken in a conical flask separately and was sterilized in autoclave (at 121 °C and 15 Psi) for 15 minutes. A tube of SDA was liquefied and poured into the Petri dish. The plate was rotated gently for uniform distribution of the medium. The inoculating loop was held at a 60 °C angle in the hottest part of the Bunsen burner flame. The entire tube was heated to redness. The loop was allowed to cool for 15 to 20 seconds before it touches the culture. A small amount of the culture was taken from the tube with a sterilized inoculating loop and the microorganisms were streaked in a plate following quadrant. The stock solutions were prepared by dissolving the compounds in ethanol. The standard antibiotic flucanozole was used as positive control and was compared with test chemicals under identical conditions.

The process of inoculation was done under aseptic condition and the spores were inoculated in the medium and incubated for 5 days. A clear zone or ring on the SDA plate develops, the diameter of which is measured as the zone of inhibition. The antimicrobial activities of the compounds were recorded by photographing the Petri dishes.

3.9.2 Antioxidant assay

3.9.2.1 DPPH radical scavenging assay

The determination of DPPH radical scavenging activity of extracts and fractionates of *E. crassipes* was carried out by the method of **Nikhat *et al* (2009)**. 0.3 mM solution of DPPH radical in methanol was prepared and 1.0 mL of this solution was added

to 3.0 mL of extract solution in methanol at different concentrations. Thirty minutes later, the absorbance was measured at 517 nm. A control was prepared without adding extract. L-Ascorbic acid was used as the standard. The capability of the extract to scavenge the $DPPH^{\cdot}$ was calculated using the following equation:

$$\text{DPPH Scavenged } (\%) = \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \times 100$$

where $A_{control}$ is the absorbance of the control reaction and A_{test} is the absorbance in the presence of the sample of the extracts.

3.9.2.2 Reducing Power Assay

The reducing power of the extracts and fractionates of *E. crassipes* was determined by the slight modification of the method of **Oyaizu** (**1986**). Various concentrations of the plant extracts in corresponding solvents were mixed with phosphate buffer (2.5 mL) and potassium ferricyanide (2.5 mL). This mixture was kept at 50 °C in water bath for 20 min. After cooling, 2.5 mL of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 min whenever necessary. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and a freshly prepared ferric chloride solution (0.5 mL). The absorbance was measured at 700 nm. Control was prepared in similar manner excluding sample. Ascorbic acid at various concentrations was used as standard. Reducing power was measured at different concentrations of the extract and contact time (10 to 30 minutes).

3.9.3 *In vivo* Acute Toxicity Studies and Wound healing activity

The institutional ethical committee of KMCH College of Pharmacy approved the protocol for acute toxicity studies and incision wound healing studies under number KMCHRET/PhD23/2009-10 (Certificate 2).

3.9.4 *In vivo* Acute Toxicity Studies

Acute oral toxicity test was performed as per Organization for Economic Co-operation and Development (OECD) guidelines 423.

Experiments were performed using healthy young adult female Swiss albino mice, nulliparous, non-pregnant and weighing 25-30 g. The animals were randomly divided into six groups each containing six mice. They were identified by the markings using a yellow stain. One mouse was unmarked and the others were marked on head, body, tail, head and body, body and tail, to ease the observation.

The animals were housed in polypropylene cages (55 x 32.7 x 19 cm), with sawdust litter in a temperature controlled environment (23±2 °C). Lighting was controlled to supply 12 h of light and 12 h of dark for each 24 h period. Each cage was identified by a card. This card stated the cage number, number and weight of the animals it contained, test substance code, administration route and dose level. The animals were fed with standard laboratory animal food pellets with water *ad libitum*.

The test substance was administered in a single dose by gavage using specially designed mice oral needle. Animals were fasted 3 h prior to dosing (only food was withheld for 3 h but not water). Following the period of fasting, animals were weighed and test substance was administered orally at a dose of 100, 250, 500, 750, 1000 and 2000 mg/kg. After the administration of test substance, food for the mice was withheld for 2 h.

Animals were observed individually during the first 30 minutes after injection of the test substance, periodically during the first 24 h, with special attention given during the first 4 h, and daily thereafter, for a total of 14 days. All the rats were observed at least twice daily with the purpose of recording any symptoms of ill-health or behavioural changes.

Direct observation parameters include tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Skin, fur, eyes, mucous membrane, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern are the other parameters observed. The time of death, if any, was recorded. The number of survivors was noted after 24 h and then these were maintained for a further 14 days under observation.

3.9.5 Preparation of polyherbal formulations

The preparation of the polyherbal formulations were carried out by the procedure of **Akhtar *et al*** (**2011**) with slight modifications. Two polyherbal formulations were prepared. Fresh aloevera gel (180 mg), rhizome powder of turmeric (40 mg) and sandal wood (180 mg) were mixed thoroughly. The prepared herbal formulation was mixed with ethyl acetate extract (1.6 g) yielding ethyl acetate extract polyherbal formulation (PHF1) and aqueous extract (1.6 g) yielding aqueous extract polyherbal formulation (PHF2).

PHF1 : Fresh aloevera gel + rhizome powder of turmeric + sandal wood + ethyl acetate extract

PHF2 : Fresh aloevera gel + rhizome powder of turmeric + sandal wood + aqueous extract

3.9.6 *In vivo* Wound Healing Activity

Incision wound healing activity was performed according to the protocol of **Akkol *et al*** (**2012**). Albino Wistar rats of either sex (200 and 220 g) was used for the studies. The animals were left for 3 days at room conditions for acclimatization. They were housed in clean polypropylene cages under standard conditions of humidity (50±5%), temperature (25±2 °C) and light (12 h light/12 h dark cycle). The animals were fed with standard pellet diet and water *ad libitum* throughout the experiment.

3.9.6.1 Preparation of ointments for wound healing activity

The test samples for topical application were prepared in an ointment base (vehicle) consisting of yellow soft paraffin (17 g), hard paraffin (1 g), cetostearyl alcohol (1 g) and wool fat (1 g) in 2% concentration. Hard paraffin and cetosteryl alcohol were taken and melted on a water bath. Wool fat and white soft paraffin was added to the melted mixture with stirring. The stirring was continued until all the ingredients melted giving a homogenous mixture. The mixture was allowed to cool to room temperature. The polyherbal formulation PHF1 (20 mg) and ethyl acetate extract (20 mg) was added to the vehicle (1 g) yielding ointment F4 and F5 respectively. The polyherbal formulation PHF2 (20 mg) and aqueous extract (20 mg) was added to the vehicle (1 g) yielding ointment F6 and F7 respectively.

Sample Code	Test substance
F1	Negative control
F2	Betadine
F3	Vehicle
F4	Vehicle + PHF1
F5	Vehicle + Ethyl acetate extract
F6	Vehicle + PHF2
F7	Vehicle + Aqueous extract

3.9.6.2 Wound healing activity- Linear incision wound model

The animals were anesthetized with 0.2 cc Ketamine chloride prior to and during the creation of the wounds. The dorsal fur of the animals was shaved by using a razor. A longitudinal paravertebral incision of 5 cm long was made through the skin and cutaneous tissue on the back (**Ehrlich and Hunt, 1968**). The parted skin was sutured 1 cm apart using a surgical thread and curved needle. The sutures were non-absorbable, non-capillary and siliconised. The wounds were left undressed (**Hukkeri *et al,* 2006**). The wounded animals were divided into 7 groups containing four animals each. The negative control group (F1) was left untreated. The prepared ointment, reference drug (Betadine) and the vehicle was topically applied to the wound once a day for the remaining groups. The sutures were removed on the 11th post wound day and continued the application of the formulation till 14th day. On the 14th day, all the animals were sacrificed under anesthesia. The linear paravertebral incised skin was examined for its tensile strength and histopathology.

3.9.6.3 Wound area of the incised skin

The changes in wound area were monitored by a digital camera (Kodak) daily till 11th day. The wound area was measured by tracing the wound length on a transparent paper and matching it with a graph paper (**Jarrahi and Vafaei, 2004**).

3.9.6.4 Percentage of wound healing

Wound contraction was calculated by measuring the wound area (**Jarrahi and Vafaei, 2004**), and substituting in the formula :

$$\%\,\text{Wound contraction} = \frac{\text{Wound area in the day of X}}{\text{Wound area in the first day}} \times 100$$

$$\%\,\text{Wound healing} = 100 - \text{percentage wound area}$$

3.9.6.5 Tensile strength of the incised skin

Tensile strength of wound represents the effectiveness of wound healing (**Hayouni *et al,* 2011**). Tensile strength has commonly been associated with the organization, content and physical properties of the collagen fibril network. Tensile strength is the resistance to breaking under tension. It indicates how much the repaired tissue resists breaking under tension and may indicate in part the quality of the repaired tissue. The tensile strength of the skin on the 14th day was measured using a tensiometer.

3.9.6.6 Histopathological examination

The incised skin specimens collected on the post 14th day of the treatment were examined for the histopathalogical changes. The incised skin was collected in 10% formalin and stored for 24 h, dehydrated with a sequence of ethanol-xylene. The skin was processed and blocked with parasin mixed paraffin wax (55-57 °C) and then sectioned into 5 µm sections and stained with hematoxylin and eosin (HE) stains. The tissues were observed under light microscope and examined for epithelialization, inflammation, collagen and fibroblasts.

3.9.6.7 Statistical analysis of the data

Results of wound area and rate of wound contraction were expressed as mean ± S.E.M. Analysis of data was carried out using the statistical software Sigmastat version 3.1. Statistical comparisons of the wound area, rate of wound contraction and tensile strength were made between F1 *vs* F2, F3, F4, F5, F6, F7 and F2 *vs* F3, F4, F5, F6 and F7. The data on percentage wound healing was statistically evaluated using two-way ANalysis Of VAriance (ANOVA). The values of p≤ 0.05 were considered statistically significant. Tensile strength was statistically analysed using one-way ANOVA. Histopathological data were considered to be nonparametric and hence, no statistical tests were performed.

3.9.7 Preparation of skin cream for skin whitening and antiageing assay

Two skin creams LPR1 and LP3 were prepared for analyzing the skin whitening and antiageing properties of the extract of *E. crassipes*. The weight of the constituents in the skin creams is given in Table 7.

Table 7. Constituents of the skin cream

Sample	Additives/ Extracts	LPR1	LP3
Oil phase	Bees wax	9.71 g	11.49 g
	Emulsifying wax	35.12 g	38.48 g
Aqueous phase	Glycerol	30 mL	30 mL
	Water	97 mL	30 mL
	Ethyl acetate Extract	3.29 g	0.192 g
	Lemon extract	-	1.056 g
	Musk	-	0.057 g

The skin cream was prepared by the addition of aqueous phase to the oily phase with continuous stirring. Paraffin wax and emulsifying wax was heated upto 70±5 °C.

Aqueous phase consisting of glycerol and water was heated upto 80±1 °C and was added to the oil phase drop wise with continuous stirring. The ethyl acetate extract (3.29 g) and ethyl acetate extract (0.192 g) together with lemon (1.056 g) and musk (0.057 g) was added to this mixture respectively yielding LPR1 and LP3. Stirring of the mixture was continued in a magnetic stirrer until homogeneity.

3.9.7.1 *In vitro* skin whitening property of the skin cream by Tyrosinase assay (Narayasamy *et al,* 2011)

Tyrosinase catalyses the transformation of L-tyrosine into L-DOPA by hydroxylation and into *O*-dopaquinone by oxidation. Then, through a series of non-enzymatic reactions, *O*-dopaquinone is rapidly transformed into melanin, which is measured at 492 nm in a spectrophotometer. The skin cream LPR1 and LP3 was assayed for tyrosinase inhibition by measuring its effect on tyrosinase activity using a 96-well reader. The reaction was carried out in a 50 mM potassium phosphate buffer (pH 6.8) containing 20 mM L-tyrosine and 312.5 U/mL mushroom tyrosinase at 30 °C. The reaction mixture was pre-incubated for 10 min before adding the enzyme. The reaction mixture without the enzyme serves as blank. The reaction mixture with the corresponding solvents (without plant material) serves as control. The change of the absorbance at 492 nm was measured. The percent inhibition of tyrosinase was calculated as follows:

$$\text{Tyrosinase inhibition (\%)} = \frac{\text{OD of Control} - \text{OD of Test}}{\text{OD of Control} - \text{OD of Test}} \times 100$$

3.9.7.2 *In vitro* anti-ageing property of the skin cream

3.9.7.2.1 Inhibition of DNA damage (Guha *et al,* 2011)

The potential of LPR1 and LP3 to prevent DNA damage was carried out by the method of **Halliwell and Gutteridge** (**1981**). The skin cream in varying quantity (1.0, 2.0, 5.0, 10.0 and 20.0 mg) was added to the reaction mixture containing 0.5 mL deoxyribose (1 mg/mL in 0.15 M NaCl), 0.5 mL phosphate buffer (0.1 M NaCl) and 0.2 mL ammonium ferrous sulphate (4.8 mM) to make a final volume of 1 mL. The reaction mixture was then incubated at 37 °C for 1 h and the reaction was stopped by the addition of I mL TBA (1%) followed by the addition of 1 mL TCA (2.8%). The tubes containing the reaction mixture were incubated in boiling water for 20 minutes. This was then extracted with butanol and the absorbance was measured at 532 nm in a spectrophotometer.

3.9.7.2.2 DPPH radical scavenging activity

The skin creams LPR1 and LP3 were screened for their DPPH radical scavenging activity by the method of **Nikhat *et al* (2009)** with 0.25 mM DPPH at different concentrations (2, 10, 20 and 50 mg)**.**

3.9.7.3 Stability and chemical tests for the skin cream

The prepared skin creams LPR1 and LP3 were submitted to T.Stanes, Herbal Division & Phytopharma Testing lab, Coimbatore for certification.

3.9.8 Larvicidal, pupicidal and repellent studies of extracts and fractionates of *E. crassipes* against *Culex quinquefasciatus*

3.9.8.1 Mosquito culture

The eggs of *C.quinquefasciatus* were collected from in and around Coimbatore districts (Sewage water bodies) with the help of 'O' type brush. The eggs were transferred to 18 x 13 x 4 cm size enamel trays containing 500 mL of water for larval hatching. The mosquito larval and pupal culture was inoculated in the laboratory. The pupae were transferred to the plastic jars containing tap water which was placed in a mosquito cage (90 x 90 x 90 cm) where the adults emerged. Adults were continuously provided with 10% sugar solution for 3 days. The adult females were then fed on rabbit blood for 2 days to ensure adequate blood feeding. On the following day, enamel trays with water were fitted at the bottom of each cage for oviposition.

3.9.8.2 Larval toxicity test

Laboratory reared larvae were used in this study. The colonies were maintained at 27±2 °C, 75-85% relative humidity under a 14:10 light/dark photo period cycle. The experimental set- up was as described by **Vahitha *et al*** (**2002**). Twenty five larvae of first, second, third and fourth instars were introduced into the 500 mL glass beaker containing 249 mL of dechlorinated water and 1 mL of desired concentrations of plant extracts were added separately. The control was setup by mixing 1 mL of acetone with 249 mL of dechlorinated water. The larvae exposed to dechlorinated water without acetone served as control. The control mortalities were corrected by using Abbott's formula (**Abbott's, 1925**).

$$\text{Corrected mortality} = \frac{\text{Observed mortality in treatment} - \text{Observed mortality in control}}{100 - \text{Control mortality}} \times 100$$

$$\text{Percentage mortality ty} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100$$

LC_{50}, LC_{90}, regression equation and 95 % confidence limit of lower confidence limit (LCL) and Upper Confidence Limit (UCL) were calculated using probit analysis (**Finney, 1971**).

3.9.8.3 Pupal toxicity test

A laboratory colony of *Culex quinquefasciatus* pupae were used for pupicidal activity. Twenty numbers of freshly emerged pupae were kept in 500 mL glass beaker containing 249 mL of dechlorinated water and 1 mL of plant extract concentrations (50-375 ppm). Five replicates were setup for each concentration and control was setup by mixing 1 mL of acetone with 249 mL of dechlorinated water. The control mortality was corrected by Abbott's formula

$$\text{Corrected mortality} = \frac{\text{Observed mortality in treatment - Observed mortality in control}}{\text{100 - Control mortality}} \times 100$$

$$\text{Percentage mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100$$

3.9.8.4 Mosquito repellant activity

The repellent dose - protection time response method was used (**WHO, 1996**). Approximately 1 h prior to the start of a test, 100, 3-4 day-old blood-starved female *C.quinquefasciatus* (100) were placed into a net cage (45 cm W x 45 cm H x 45 cm L). Then, both arms of a human test subject were washed with ethanol and allowed to air dry. Three doses of plant extract were tested (1, 2.5, and 5 mg/cm^2). A single dose was applied to the forearm skin of a test subject in each test (**Venkatachalam and Jebanesan, 2001**). The other forearm was used as a negative control. At the beginning of a test, the control and treated arms were introduced simultaneously into the cage. The number of mosquitoes that landed on the exposed skin on each arm in 3 minutes was recorded at 30 minutes interval between 6.00 pm and 6.00 am. Each dose of plant extract was tested 5 times for repellency. The effectiveness of the extract was assessed by determining the percent protection against mosquito landing that it provided on the treated arm compared with the untreated arm.

3.9.8.5 Statistical analysis

Data obtained for corrected mortality were subjected to probit analysis. The regression equation (y=a+bx); lethal concentration that killed 50% and 90% of larval population (LC_{50}, LC_{90}) and other statistics at 95% fiducidal limits of upper confidence limit

and lower confidence limit and chi-square values were calculated by statistical software sigmastat 4.1.

3.10 *In silico* studies

Molecular docking studies were performed using *Hex* 6.3 for eight ligands *viz.* stigma 5,22 diene-3 acetate, stigmast 22-ene-3,5,6-triol, stigma 5,22-ene-7 hydroxy-3-one, stigma 5,22-ene-3-hydroxy-6-one, Stigma 5,22-ene- 6-hydroxy-3-one, stigma 5,22-diene-3,7-diol, stigma 5,22-diene-3-one and stigmast-5ene-3 β-24 diol against three receptors including COX-1, COX-2 and SIRT1.

3.10.1 System Used

Intel® Atom Processor ® 4, 1.80 GHz, 512 MB RAM

3.10.2 Operating platform

Microsoft Windows 2007, Bioinformatics and Cheminformatics tools, biological databases and software were used in the present study. *Hex* 6.3 software was used for the docking of the ligands to the receptor. The pdb structures of the ligands and receptors were retrieved from PDB (Protein Data bank).

3.10.3 Marvin Sketch

Marvin Sketch, an advanced Java based chemical editor used for drawing, editing chemical structures and to make changes in the ligand structures.

3.10.4 Protein Data Bank

The structure of receptors was retrieved from Protein Data Bank. The PDB archive contains data about experimentally-determined structures of proteins, nucleic acids, and complex assemblies. A variety of tools and resources aids to perform simple and advanced searches based on annotations relating to sequence, structure and function.

3.10.5 *Hex* 6.3

The parameters used in *Hex* for the docking process are:

- Correlation type - Shape only
- FFT Mode - 3D fast lite
- Grid Dimension - 0.6
- Receptor range - 45
- Ligand Range - 45

- Twist range - 360
- Distance Range - 40
- Substeps - 2

3.10.6 Molecular docking

The anti-inflammatory and antiageing behavior of designed ligands on a structural basis was assessed by automated docking studies based on their binding affinities of the ligands at the active site of the COX-1, COX- 2 and SIRT1. The structures of the ligands were minimized using the Dreiding Force Field. The docking was performed using *Hex* 6.3 into the 3D model of the catalytic site of COX-1, COX-2 and SIRT1 enzyme by keeping program parameters to their default values. A comparative docking experiment of designed compounds with known non steroidal anti-inflammatory agent indomethacin and antiageing compound argireline was carried out. Molecules which show minimum dock score are said by definition to have more affinity for the foresaid receptors.