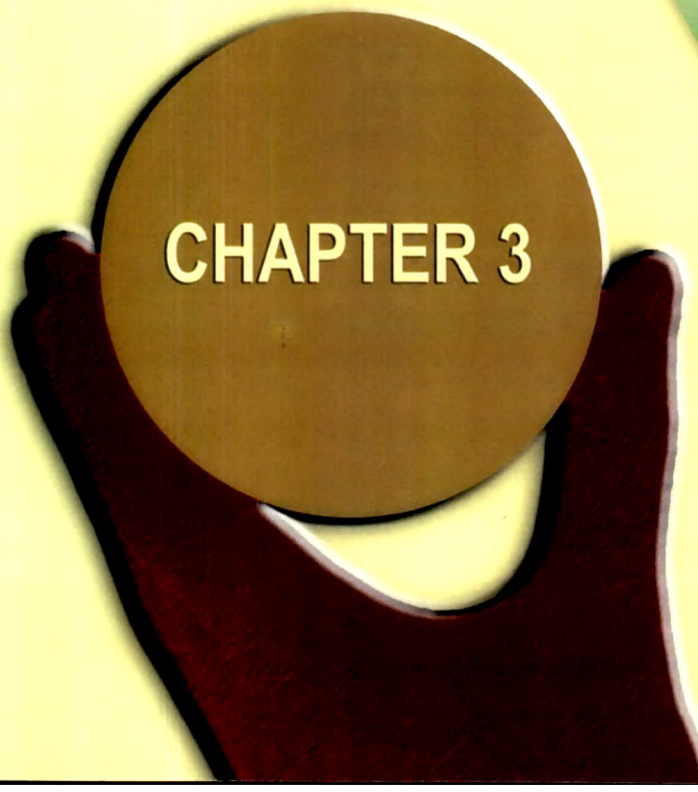
Comparative Biological Differentiation



In vivo model

- ✓ Pole climbing apparatus for condition avoidance test
- ✓ Step through model for passive avoidance
- ✓ Water maize test

➢ Comparative *in vitro* lipoxygenase (LOX) inhibition assay

➢ Comparative *in vitro* antimalarial (PfLDH) assay.

➢ Comparative *in vitro* antimicrobial studies.

3.1.1 PREPARATION OF TEST SOLUTION FOR IN VITRO STUDIES

The amount in stock and method of preparation was shown in table 3.1 and figure 3.1.

Table 3.1 List of samples used in the study (In vitro biological screening)

S. No.	Test drug	Stock solution (conc.- mg/mL)	Abbreviation
Standard Markers			
1	Ascorbic acid	1 mg/mL	-do-
2	Apigenin	1 mg/mL	-do-
3	Betulinic acid	1 mg/mL	-do-
4	Betaine	1 mg/mL	-do-
5	β-carotene	1 mg/mL	-do-
6	Chlorogenic acid	1 mg/mL	-do-
7	Curcumin	1 mg/mL	-do-
8	Ellagic acid	1 mg/mL	-do-
9	Gallic acid	1 mg/mL	-do-
10	Lupeol	1 mg/mL	-do-
11	Marmesin	1 mg/mL	-do-
12	Mangiferin	1 mg/mL or 3 mg/ mL	-do-
13	Morin	1 mg/mL	-do-
14	Naringin	1 mg/mL	-do-
15	Nicotine	1 mg/mL	-do-

S. No.	Test drug	Stock solution (conc.- mg/mL)	Abbreviation
16	Querecetin	1 mg/mL	-do-
17	Rutin	1 mg/mL or 3 mg/ mL	-do-
18	Stigmasterol	1 mg/mL	-do-
19	Scopoletin	1 mg/mL	-do-
20	Tocopherol	1 mg/mL	-do-
21	Ursolic acid	1 mg/mL	-do-
Marketed Ayurvedic Formulation			
22	Brain Tab	10 mg/mL	BT
23	Shankhpushpi Syrup	10 mg/mL	SS
Hydro- distillate extract			
24	Hydro Distillate of *E. Alsinoids*	100 mg/mL	HDEA
25	Hydro Distillate of *C. pluricaulis*	100 mg/mL	HDCP
26	Hydro Distillate of *C. ternatea*	100 mg/mL	HDCT
27	Hydro Distillate of *C. decussata*	100 mg/mL	HDCD
Petroleum Ether Extract			
28	Petroleum Ether Extract of *E. Alsinoids*	10 mg/mL	PEEEA
29	Petroleum Ether Extract of *C. pluricaulis*	10 mg/mL	PEECP
30	Petroleum Ether Extract of *C. ternatea*	10 mg/mL	PEECT
31	Petroleum Ether Extract of *C. decussata*	10 mg/mL	PEECD
32	Unsaponified Petroleum Ether Extract of *E. Alsinoids*	5 mg/ mL	UPEEEA
33	Unsaponified Petroleum Ether Extract of *C. pluricaulis*	5 mg/ mL	UPEECP
34	Unsaponified Petroleum Ether Extract of *C. ternatea*	5 mg/ mL	UPEECT
35	Unsaponified Petroleum Ether Extract of *C. decussata*	5 mg/ mL	UPEECD
Chloroform fraction			
36	Chloroform fraction of *E. Alsinoids*	10 mg/mL	CFEA
37	Chloroform fraction of *C. pluricaulis*	10 mg/mL	CFCP
38	Chloroform fraction of *C. ternatea*	10 mg/mL	CFCT
39	Chloroform fraction of *C. decussata*	10 mg/mL	CFCD

S. No.	Test drug	Stock solution (conc.- mg/mL)	Abbreviation
Defatted Methanolic Extract			
40	Methanolic Extract of *E. Alsinoids*	10 mg/mL	MEEA
41	Methanolic Extract of *C. pluricaulis*	10 mg/mL	MECP
42	Methanolic Extract of *C. ternatea*	10 mg/mL	MECT
43	Methanolic Extract of *C. decussata*	10 mg/mL	MECD
Successive Aqueous Extract			
44	Aqueous Extract of *E. Alsinoids*	10 mg/mL	AEEA
45	Aqueous Extract of CP *C. pluricaulis*	10 mg/mL	AECP
46	Aqueous Extract of *C. ternatea*	10 mg/mL	AECT
47	Aqueous Extract of *C. decussata*	10 mg/mL	AECD

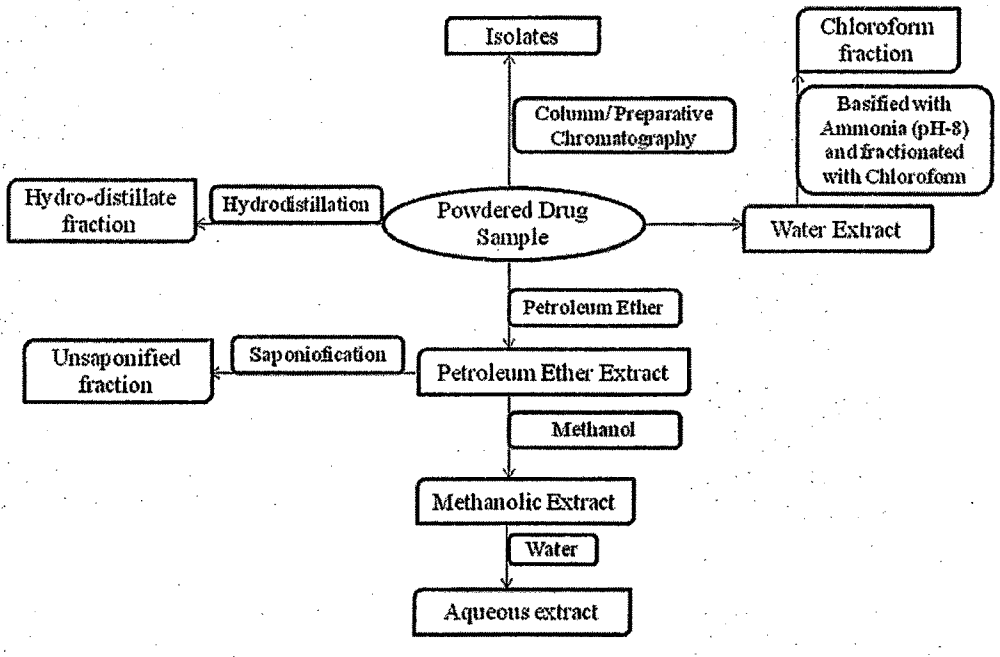


Figure 3.1 Scheme for preparation of isolates, fraction and extracts samples for biological screening.

Table 3.2 Composition of laboratory marine water (g/l)

Source	Composition	Content
Crystalline salts	NaCl	23,960
	$MgSO_4.7H_2O$	10,346
Stock solution (ZR-1) (20 ml/l)	$MgCl_2.6H_2O$	325,0
	NaBr	51,45
	KCl	29,80
Stock solution (ZR-2) (10 ml/l)	$CaCl_2$	29,98
	$NaHCO_3$	20,10
	$SrCl_2.6H_2O$	2,70
	H_3BO_3	0,60
	NaF	0,42

3.1.2.2 Test procedure

Freshly hatched individuals, so called nauplii, were used for testing. Hatched individuals are to be poured from a beaker into test tubes along with various concentrations of tested samples of isolates, fractions and extracts of *Shankhpushpi* botanicals. Brine shrimps are not fed during the test. Tested samples were not aerated. Mortality of nauplii is noticed as endpoint. Time for testing the mortality was 24 hours.

3.1.2.3 Test procedure by laboratory work

Hatching of eggs (run by assistant a day before)

- ✓ A high beaker is filled approximately 2/3 with marine water.
- ✓ A tea spoon of frozen dry brine shrimp eggs were added to beaker.
- ✓ The beaker with eggs was kept for 24 h with established aeration and light.

Testing

- ✓ 10 individuals were taken into each testing tube.
- ✓ Two control group were taken (Without test drug)
- ✓ Samples solutions were added in respective concentration.
- ✓ Test was carried out in light (lamp in cultivator)

Counting and evaluation after 24 and 48 hours

- ✓ Dead brine shrimps were visually counted (laying at bottom)

3.1.2.4 Evaluation of the test results

A graph on organism mortality (in %) related to logarithm of concentration of tested compound was plotted. Using the values on died individuals in given concentrations was used for determining the percent of mortality according to following formula:-

$$Mm_{ct} = \frac{N_{Mm}}{N_0} \times 100$$

Where:

Mm_{ct} is mortality of individuals in time t [%]

N_{Mm} is average number of died individuals

N0 is initial number of living individuals put into every concentration at the test start

Individual EC values were determined for each replicate of various isolates, fraction and extracts of Shankhpushpi botanicals.

3.1.3 MTT Toxicity Assay

The MTT assays are colorimetric assays for measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple color. (Mosmann, 1983).

3.1.3.1 Cell Lines

Two types of cell lines used in this study

(1) NIH-3T3

(2) Neuro 2a

3.1.3.1.1 NIH-3T3 cell culture

The Cell line isolated from mouse embryo fibroblast (NIH-3T3) was used in DMEM medium and incubated for 24 hours.

3.1.3.1.2 Neuro 2a cell culture

The cell line isolated from mouse neuroblastoma was used in Minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non- essential amino acids, and 90 % of 1.0 mM sodium pyruvate, 10 % of fetal calf serum in 37 C with 5 % CO_2.

3.1.3.2 Cell survival assay

The detailed protocol for assessment of assay is depicted in figure 3.3

3.1.3.3 Assessment of cell viability

MTT is an indicator of the mitochondrial activity of living cells and widely used as an index of cell survival. Experimentally, the culture medium was replaced with a DMEM high glucose buffer containing freshly dissolved MTT (0.25 mg/mL). Following 3-h incubation at 37 C living cells containing MTT formazan crystals were solubilized in a solution of anhydrous isopopanol-HCl 0.1 N. The optical density (OD) was determined at 570 nm using a micro-plate reader.

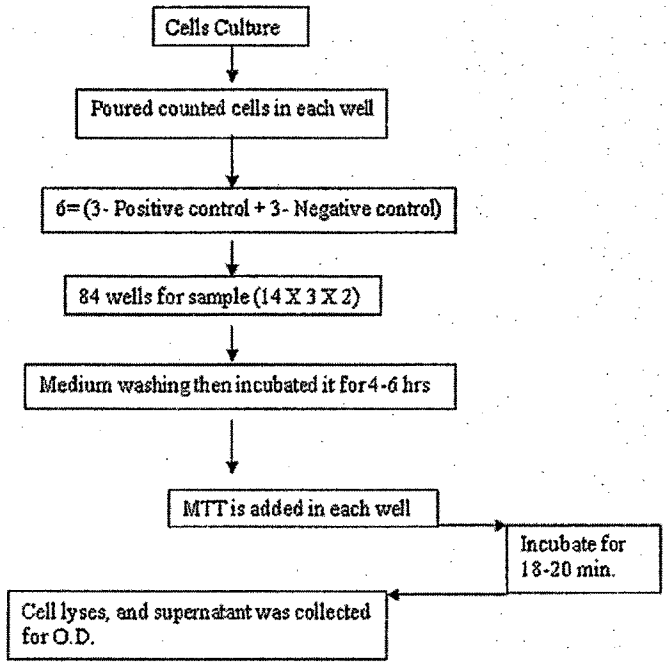


Figure 3.3 Protocol for cell survival assay

3.1.4 Evaluation of the Antioxidant Capacity

All experiments were done under subdued light. Before analysis, defined volumes of stock solution (1 mg/ml) were prepared in TBME/DMSO (v/v), respectively. All samples were analysed in triplicate at five different concentrations (10, 20, 30, 40, 50 µg/mL).

3.1.4.1 Reagents and chemicals

DPPH, curcumin, β-carotene, gallic acid and rutin were obtained from Himedia Laboratories Pvt. Ltd., Mumbai. Ascorbic acid, mangiferin, chlorogenic acid, β-sitosterol, querecetin, stigmasterol, urosolic acid, lupeol, betullinic acid, betaine and scopoletin were procured from Loba Chemie (Mumbai), Sigma Aldrich (USA), Sisco Research Laboratories (Mumbai) and Lailla Imprex Laboratory (Vijayawada) respectively. Other solvents and chemicals were of analytical grade and procured from Sd Fine (Mumbai). For *in vitro* biological assessment all the enzymes kits and chemicals were procured from Sigma Aldrich.

3.1.4.2 Sample preparation

Stock solutions of each extract/fraction (10 mg/ml) and pure compounds (1 mg/ml) were prepared in the specific solvent of each compound and stored at –30 ± 2 °C until analysis.

3.1.4.3 Comparative antioxidant potential of some phytonutrients from commonly used plant based human food, isolates, fractions and extracts of shankhpushpi botanicals

There is an increasing interest in the use and measurement of antioxidants in food industries. In addition, many states implement very rigorous regulations on the use of food preservatives, so that they only allow the use of natural antioxidants. The concern about the role of antioxidant in human diet from plants sources prompted us to search compounds from common foods as well as traditionally used medicinal plants. Routinely applied methods of our laboratory were used to evaluate the comparative total antioxidant capacity of various phytochemical (Flavonoid, phenolic, xanthone and alkaloid) vigorously shown their presence in commonly available food plants. The purpose of this study was to assess the antioxidant activity and validate the best biomarker standards for the antioxidation. Activities were performed by various in vitro methods. The various phytonutrients used in present studies were depicted in table 3.3 and figure 3.4

3.1.5.2 TLC bioautography for acetyl cholinesterase inhibition

Methanolic extracts of various botanicals of *Shankhpushpi* were applied on TLC plates and were developed in the solvent system of ethyl acetate–formic acid–acetic acid–water (100:11:11:26). After drying, the plate was sprayed with 5 mM ATCI and 5 mM DTNB in 50 mM Tris–HCl, pH 8 until saturation of the plate. The plate then sprayed with 3 U/ml AChE dissolved in 50 mM Tris–HCl (pH 8 at 37 °C). After a few minutes, white spots were appeared in the yellow background of the plate which indicates the presence of the compounds with AChEI activity. Another plate was done similarly for removing false positive reactions. White spots in yellow background indicated false positive reactions (Vinutha et al., 2007).

3.1.5.3 β-amyloid induced neuroprotection on brain cell line.

Neuro 2a cells isolated from neuroblastoma of mouse in the exponential phase of growth are exposed to a well reported neurotoxic compound β-amyloid. The duration of exposure is determined as the time required for maximal damage to occur. The compounds to be tested for protection against this damage were added, and then the cells were allowed to proliferate for two to three population-doubling times (PDTs) in order to distinguish between cells that remain viable and are capable of proliferation and those that remain viable but cannot proliferate. The number of surviving cells is then determined indirectly by MTT dye reduction assay. The amount of MTT-formazan produced can be determined spectrophotometrically once the MTT formazan has been dissolved in a suitable solvent (Bastianetto et al., 2000; Irie & Keung, 2003).

3.1.5.3.1 Materials

Neuro 2a, neuroblastoma cell line from mouse purchased from NCCS, Pune, India. Growth medium (MEM with 2mM L-Glutamine, 1mM Sodium pyruvate, NEAA and 1.5 gm per litre Sodium bicarbonate) , Fetal bovine serum (FBS) , Sterile antibiotic solution 100X (10,000 units penicillin and 10 mg streptomycin per ml in 0.9 % normal saline), Trypsin (0.25% + EDTA, 1 mM, in PBSA), MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 50 mg/mL, filter sterilized (Hi Media, Mumbai), Sorensen's glycine buffer (0.1 M glycine, 0.1 M NaCl adjusted to pH 10.5 with 1 M NaOH), Microtitration plates (96 well flat bottom; Tarsons,

Mumbai), Pipettor tips in an autoclavable tip box, Petri dishes (non-TC-treated), 5 cm and 9 cm as reservoir, Falcon tubes, 30 mL and 50 mL, Plastic box (clear polystyrene, to hold plates), Multichannel pipettor (Hi Media, Mumbai). Dimethyl sulfoxide (DMSO), ELISA plate reader (Bio Rad, USA)

3.1.5.3.2 Protocol

Plating out cells

- ✓ Subconfluent monolayer culture of neuro 2a was trypsinized, and collected in the growth medium containing serum.
- ✓ These cells were centrifuged (5 min at 200 g) and resuspended in the fresh growth medium, for counting.
- ✓ Seeded 7×10^3 cells per well in 96 well flat bottom microtitre plate (Tarsons, Mumbai)

Drug addition

- ✓ Dilutions of the neuroprotective test drug in growth medium were prepared in such a way that the highest concentration kills most of the cells and the lowest kills none of the cells. Three plates were used for triplicate determinations.
- ✓ The cells were feeded in the eight wells in columns 2 and 11 with 200 µL of fresh growth medium to serve as controls for the experiment.
- ✓ The neurotoxic compound β-amyloid to the cells in columns 3 to 88 were added.
- ✓ Sterile filter test samples of various *Shankhpushpi* botanicals were transferred in different concentration in 200 µL dilutions.

Growth period

- ✓ At the end of the sample exposure period, the medium from all of the wells containing cells were removed, and again feeded with 200 µL of fresh medium to the cells.
- ✓ Then it was daily feeded for 2–3 PDTs.

Estimation of surviving cell numbers

- ✓ At the end of the growth period, the plate with 200 µL of fresh medium were feeded and 50 µL of MTT to all of the wells in columns 1 to 11 was added.
- ✓ The plates were wrapped in aluminum foil, and incubated for 4 h in a humidified atmosphere at 37°C.
- ✓ Medium and MTT from the wells were removed and the remaining MTT-formazan crystals were dissolved by adding 200 µL of DMSO to all of the wells in columns 1 to 11.
- ✓ Glycine buffer (25 µL per well) to all of the wells containing DMSO were added.
- ✓ Absorbance at 570 nm was recorded immediately, because the product is unstable. The wells in column 1, which contain medium and MTT but no cells, was used to blank the plate reader.

3.1.5.4 Native 5-HT2B Receptor Functional Studies

The nomenclature of the 5-HT receptor subtypes has been altered to recognize the existence of an expanded 5-HT2 receptor family sharing very similar structure and pharmacology and the use of a common secondary messenger system. This family currently consists of 3 subtypes designated 5-HT2A (formerly 5-HT2), 5-HT2B (formerly the rat stomach fundus receptor) and 5-HT2C (formerly 5-HTIc) (Hoyer et al., 1994). Investigations of the function of the 5-HT2c receptor have led to the hypothesis that it is involved in the modulation of anxiety, (Kennett et al., 1996) Rat fundus is a sensitive tissue for studying the agonistic action of acetylcholine (ACh), 5 hydroxytryptamine (5-HT), histamine and bradykinin. This preparation is a slow contracting and slow relaxing tissue unlike ileum. Longitudinal fundal strips are employed by Vanel with high sensitivity for 5-HT. Recently a modified method of horizontally cut fundal strips was described and reported to be more sensitive to Acetylcholine. Wistar rat (150-200 g) fasting for 48 hrs were sacrificed by cervical dislocation. The abdomen was opened, fundus taken out and washed. The fundus was slit along the greater curvature and spread out. It was then cut horizontally in some and longitudinally in others leaving the adjoining ends uncut to yield a long strip. Different rats were used to obtain horizontal and longitudinal stomach strips. The cut strips about 4 cm long were suspended in a 20 ml bath containing tyrode solution at

37 °C, well aerated. Assay was started 30 min later. At least 6 longitudinal and 6 horizontal strip preparations were used for each test substance. The contractile response was recorded on a 2-channel recorder with an isotonic transducer (UGO Basile, Italy). A 3 min cycle was followed, including a 60 sec exposure time to test substance. The methanolic extracts of various *Shankhpushpi* botanicals were evaluated for its action against rats fundus strip.

3.1.5.5 In vivo animal model

Animals

Swiss albino mice (25-30 g) of either sex were used for the study. The animals were housed in groups of six in polypropylene cages, under standard laboratory conditions of temperature (25±2°C), lighting (0800-2000 h) and relative humidity (50±5%). The animals had free access to standard pellet chow (Brooke Bond-Lipton, India) and water. The animals were acclimatized for a period of minimum 7 days. Experiments were conducted between 0900 and 1400 hrs. The experimental protocol was approved by the Institutional Animals Ethics Committee (IAEC) of Pharmacy Department, The M S University of Baroda, Gujarat, India and care of laboratory animals was taken as per CPCSEA guidelines (Reg. No. 79/01/ab/CPCSEA).

Drugs and Chemicals

Piracetam (Neurocetam, 800 mg/tablet, Micro Labs, India) and Scopolamine butyl bromide (SBB) (Obtained as gift sample from Cadila Healthcare Pvt. Ltd., Goa) were used in the study. Piracetam was prepared for administration in the same manner as that for the plant extracts as explained below. SBB was dissolved in normal saline for i.p. injection.

Preparation of Extracts

Whole plants of Shankhpushpi botanicals were shade dried at room temperature. The shade dried plant materials were coarsely powdered and subjected to extraction with petroleum ether in a soxhlet apparatus. The extractions were continued till the defatting of the materials had taken place. The defatted marc of the various botanicals of *Shankhpushpi* was subjected to methanolic extraction for a period of 6-7 days. These methanolic extracts were utilized for the neuropharmacological investigation.

The present study was undertaken to investigate the effects of marketed formulation of shankhpushpi and methanolic extracts of various Shankhpushpi botanicals, acting on learning and memory in rodents.

Active Aviodance Paradigm or Condition Aviodance Response

Cook and Weidley's pole climbing apparatus

The nootropic activity was assessed using the active avoidance paradigm (Cook & Weidley, 1957). The apparatus consisted of a soundproof experimental chamber with a grid floor which could be electrified and with a provision for a buzzer tone. The enclosure had a covering lid at the top, through which the animal could be introduced into the chamber. A wooden pole, screwed onto the inner surface of the lid of the chamber acted as the shock-free zone. In the assessment of nootropic activity, the stimulus provided was a foot shock of 6 mA given for a period of 10 s from the electrified grid floor. Mice were initially trained to escape the foot shock by climbing onto the pole, i.e. the shock free zone. This initial trial was carried out by having three trial sessions interspersed with an interval of 10 s. During each of the initial trials the mice were allowed to explore the apparatus for 10 s. This was followed by a foot shock for 10 s. Only those mice, which were sensitive to the foot shock and could climb the pole, were included in the study. The animals were divided into eight groups, each group containing six animals. The control group receive vehicle only. Piracetam (100 mg/kg p.o.) was used as the standard reference drug for comparison. The methanolic extracts of EA, CP, CT, CD, SS and BT in doses of 400 mg/kg per oral were administered for a period of 7 days following which the training trial (TT) was conducted. This consisted of 10 trial sessions interspersed with an interval of 30 s. During each trial, the mice were allowed to explore the apparatus for 10 s, followed by a buzzer tone of 50 Hz (conditioned stimulus) for 10 s. This was followed by a foot shock for 10 s. The animal learned to associate the buzzer tone with the impeding foot shock and was capable of avoiding the foot shock on hearing the buzzer tone. Jumping onto the wooden pole, before the shock period, constituted an avoidance response (AR). The AR for 10 trials was noted on day 7 of training. Twenty four hours later, a relearning trial (RT) was conducted i.e. on day 8 and the number of ARs in the 10 trial sessions was noted.

Inhibitory (Passive) Avoidance Tests

The following parameters were used to assess effects on learning and memory.

Step Down Test

The test apparatus was a rectangular box (45 x 30 x 40 cm) with an electrified grid floor. It was made of transparent Plexiglass to permit observations. An 8 cm high wooden platform (17 x 12 cm) was fixed to the grid floor at the center of the apparatus. A mouse was placed on the platform and allowed to step down. Twenty four hours later, on Day 1 of the experiment, the mouse was again placed on the platform and foot shock (0.75 mA, 2 s) was delivered through the grid floor as soon as it stepped down. The mouse was given foot shock only when all the four paws were touching the grid floor. The mouse was given three more trials until the latency of the step down had stabilized. The test was repeated on Day 15. Memory retention score for each animal was calculated by determining "inflexion ratio" by the formula:

$$\text{Inflexion ratio} = L_{15} - L_1 / L_1$$

Where L_1 is the step down latency on day 1 in seconds and L_{15} is the step down latency on day 15 in seconds (Jaiswal et al., 1996; Bhattacharya & Muruganadam, 2003). For this experiment, the animals were divided into 8 groups containing six animals in each group. Test extracts (EA, CP, CT and CD), marketed formulation (SS and BT), Piracetam used as positive control and vehicle were administered once daily for 15 days, 45 min prior to stress.

Scopolamine Induced Amnesia in Rats

Cook and Weidley's pole climbing apparatus

This activity was performed using the Cook and Weidley's pole climbing apparatus. The apparatus consisted of a soundproof experimental chamber with a grid floor, which could be electrified and with a provision for a buzzer tone. The enclosure had a covering lid at the top, through which the animal could be introduced into the chamber. A wooden pole, screwed onto the inner surface of the lid of the chamber acted as the shock-free zone. The stimulus provided was a foot shock of 6 mA given for a period of 10 s from the electrified grid floor. Mice were initially trained to escape the foot shock by climbing onto the pole, i.e. the shock free zone. This initial trial was carried out by having three trial sessions interspersed with an interval of 10

and CD) and marketed formulation (SS and BT) in a dose of 400 mg/kg per oral. All treatments were administered for two consecutive weeks from the beginning of the experiment as an oral supplementation. All the treated groups except the Group II received 0.4 mg scopolamine/kg ip as one dose on the day proceeding the last day of the experiment (on 14th day) for inducing memory dysfunction and after treatment with the last dose for each group by 60 min. The test of Morris water maize began after 45 min of scopolamine injection. The rats were exposed to training sessions using Morris water maize for two consecutive days before decapitation. The maximum drug concentration occurs approximately 30 min after scopolamine administration

3.1.6 Lipooxygenase (LOX) Enzyme Inhibition Assay

Lipoxygenase enzyme inhibition assay was studied using linoleic acid as substrate and lipoxidase as enzyme. The basic metabolism involved in the generation of the enzyme from the diet is depicted in figure 3.5

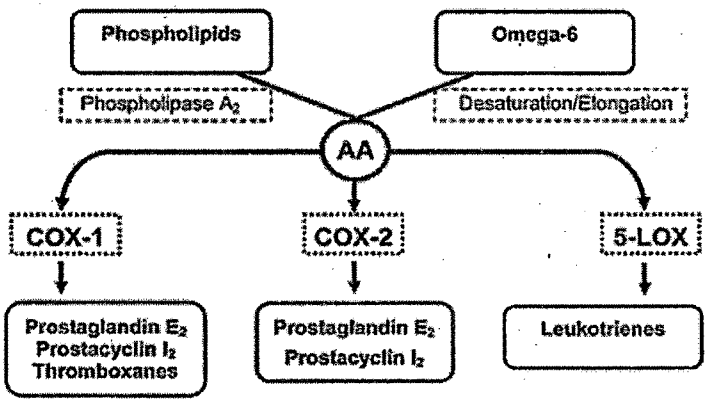


Figure 3.5 Enzyme metabolisms of membrane lipids and omega-6 fatty acids from the diet (Bruce et al., 2009)

Test solutions of isolates, fractions and extracts of various Shankhpushpi botanicals and marketed formulation of Shankhpushpi were dissolved in 0.25 ml of 2M borate buffer pH 9.0 and added 0.25 ml of lipoxidase enzyme solution (20,000U/ml) and incubated for 5 min at 25°C. After which, 1.0 ml of linoleic acid solution (0.6 mM) was added, mixed well and absorbance was measured at 234nm. Indomethcin was used as reference standard. The percent inhibition was calculated from the following equation:

% inhibition = [(Absorbance of control – Absorbance of test sample)/Absorbance control] x100

A dose response curve was plotted to determine the IC_{50} values. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum inhibitory capacity. All tests and analyses were run in triplicate and averaged (Kuaraswamy & Satish, 2008; Zenga et al., 2011).

3.1.7 Anti-malarial activity

P. falciparum specific L-lactate dehydrogenase (PfLDH) provides constant source of NAD+ required for glycolysis to continue in erythrocytic phase of parasite and has strikingly different structure and kinetic properties compared to other LDHs including Human LDH. This proves the enzyme as a suitable drug target (Lang-Unnasch and Murphy, 1998).To carry out enzyme inhibition studies, LDH was cloned from *P. falciparum* 3D7 strain using expression vector pET28a and expressed in *E. coli* BL21 (DE3). Protein purification was carried out by Ni-affinity chromatography. The methanolic extracts of Shankhpushpi botanicals (EA, CP, CT and CD) and marketed formulation (SS and BT) were dissolved in DMSO to produce a stock solution of 20 mg/ml, whereas for gossypol (Sigma Chemicals) was prepared in 1000 and 1 µg/ml, respectively. These stock solutions were subsequently diluted with 10% human serum before being transferred in triplicate of 10µl each at 22 concentrations of two-fold dilutions into two 96-well microtitre plates. Parasitised red blood cell suspensions (1–2% parasitaemia) of 190 µl were next added to each well. For the positive control wells, parasitised red blood cells were devoid of plant extracts and compounds whereas only non-parasitised red blood cells were prepared for the negative control wells. The plates were incubated in a candle jar for 72 h at 37 °C, and were subsequently cooled at −20 ◦C to lyse the red blood cells. The plates were next allowed to reach room temperature, and 20 µl of the supernatant blood suspension was dispensed into a new microtitre plate containing 100 µl Malstat reagent (Flow Inc., Portland, USA) and 25 µl nitro blue tetrazolium and phenazine ethosulfate (Sigma Chemicals) mixture. Absorbance was measured with an ELISA plate reader (Dynatech, USA) at 630 nm. The percentage inhibition at each concentration was determined and the mean of at least three IC_{50} values of parasite viability was calculated.

The inhibition of each extract or drug concentration was calculated as compared to the gossypol treated group.

Percentage Inhibition = [Control – Test/Control] × 100

3.1.8 Anti-Microbial Activity

The antimicrobial activity of the extracts (EA, CP, CT and CD) and formulation (SS and BT) were evaluated by determination of the diameter of zone of inhibition against both gram negative and gram positive bacteria using Agar diffusion method (Hugo and Russel, 1983). The test organisms were: *Bacillus subtilis, Staphylococus aureus, Salmonella paratyphi* B and *Escherichia coli.* Ampicillin (100 ppm) was used as controls (Arya et al., 2010; Elizabeth, 2005).

3.1.7.1 Inoculumn preparation

A loopful of isolated colonies was inoculated into 4 ml peptone water and incubated at 37 °C for 4 h. The turbidity of actively growing bacterial suspension was adjusted to match the turbidity standard of 0.5 McFarland units prepared by mixing 0.5 ml of 1.75% (w/v) barium chloride dehydrate with 99.5 ml 1% (v/v) sulphuric acid. This turbidity was equivalent to approximately 1-2×108 colony-forming units per milliliter (cfu/ml). This 2-h grown suspension was used for further testing.

3.1.7.2 Determination of activity index

The activity index of the crude plant extracts of Shankhpushpi botanicals (EA, CP, CT and CD) and marketed formulation (SS and BT) was calculated as

Activity index (A.I.) = Mean of zone of inhibition of the extract / Zone of inhibition obtained for standard antibiotic drug

3.2 STATISTICAL ANALYSIS

The data were expressed as mean ± SEM and statistically analyzed using ANOVA followed by Dunnet's tests. $P < 0.05$ was considered to be statistically significant and compared with control.

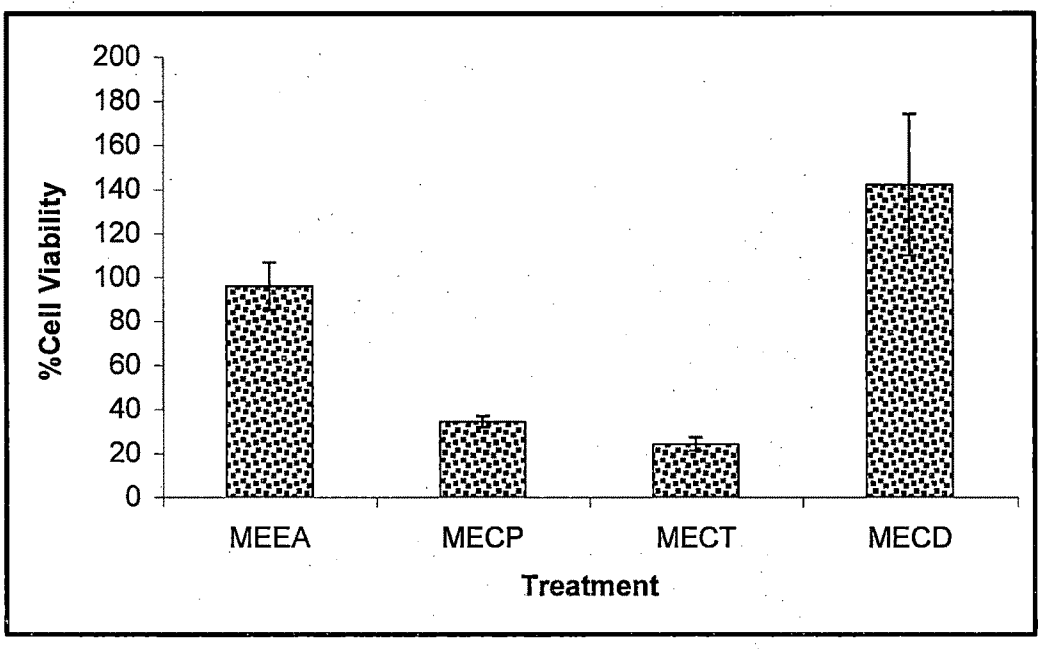


Figure 3.6 Comparative Brine Shrimp Toxicity assays of various extracts of *Shankhpushpi* Botanicals

3.1.3 MTT Toxicity Assay

A methylthiazol tetrazolium (3-[4,5-dimethylthiazol- 2-yl]-2,5-diphenyltetra-zolmm bromide; MTT) assay was performed to determine the amount of cell death (Tada et al., 1986).

After about 24 h of culture when cells reached 60–70% confluence, unattached cells were removed by gentle agitation and the medium was changed to serum-free medium containing various concentrations of isolates, fractions, methanolic extracts of various *Shankhpushpi* botanicals and marketed formulations or vehicle (DMSO) for control. The cells were treated for 24 h. Each concentration was tested in three different experiments in five replicates. The final concentration of DMSO in the test medium and controls was less than 1%. Although no apparent cytotoxic effect on cell viability was observed at lower concentrations (10 μM), although at higher concentrations, some of the samples are found to be toxic.

3.1.3.1 NIH-3T3 cell culture

The result shown by isolates, fraction and extracts of various Shankhpushpi botanicals are given in table 3.6 and figure 3.7. Among the tested compound ursolic acid and stigmasterol has significant percentage toxicity then other compounds. Among the botanicals of Shankhpushpi the CT has greater toxicity then other. The order of toxicity of compounds are ursolic acid > Stigmasterol > β-carotene > Scopoletin >Mangiferin > Rutin > Lupeol > Betaine > Chlorogenic acid > β-sitosterol. Among methanolic extracts of various *Shankhpushpi* botanicals the order of toxicity were found to be CT > CP > EA > CD.

Table 3.6 MTT toxicity assay on NIH-3T3 cell line by isolates, fraction and extracts of various *Shankhpushpi* botanicals

S. No.	Treatments	% Cell Viability 10 µg/mL
1	Control	100
Pure Compounds		
2	Lupeol	70.57± 2.84
3	Scopoletin	63.26± 5.44
4	Stigmasterol	55.52± 7.80
5	Betaine	71.08± 2.21
6	Mangiferin	68.10± 0.94
7	Rutin	70.26± 0.49
8	Chlorogenic acid	74.51± 3.15
9	β-sitosterol	88.89± 0.33
10	β-carotene	62.41± 6.04
11	Ursolic acid	51.22± 1.23
Methanolic Extract		
12	MEEA	85.77± 3.92**
13	MECP	55.54± 1.45***
14	MECT	52.90± 4.44***
15	MECD	100.53 ± 0.43*

All values are Mean ± S.E.M. (n=3),

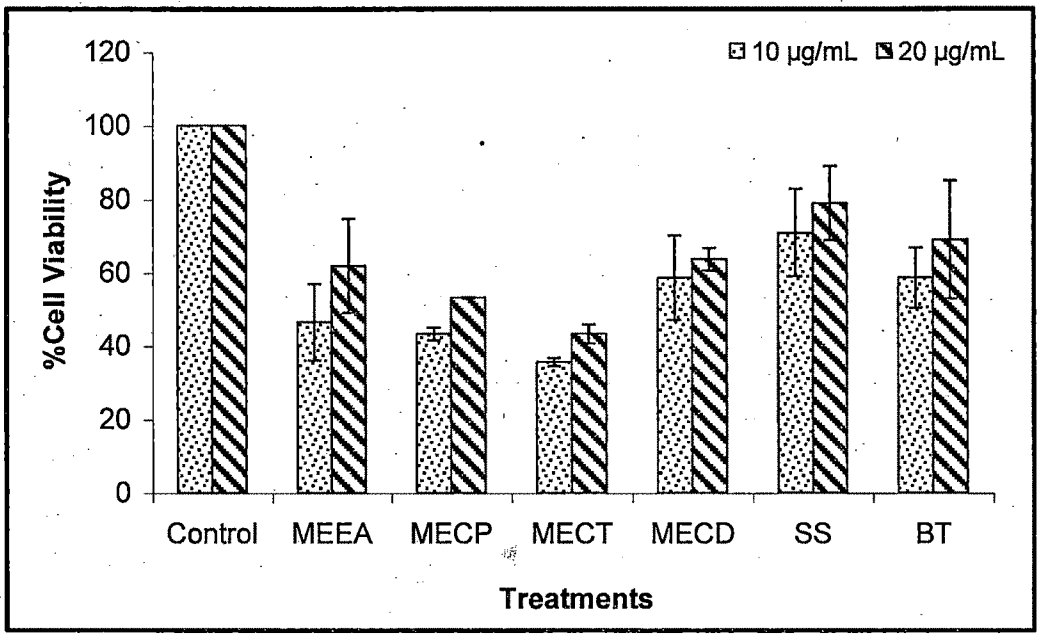
***p<0.0001, ** p< 0.001 and *p<0.05 compared to vehicle, One way ANOVA followed by Dunnett's Multiple Comparison post tests regardless of all p-values.

Table 3.7 MTT toxicity assay on neuro 2 a cell line by isolates, fraction and extracts of various *Shankhpushpi* botanicals

S. No.	Treatments	% Cell Viability	
		10 µg/mL	20 µg/mL
1	Control	100	100
Pure Compounds			
2	Lupeol	49.89±2.90	54.34±3.19
3	Scopoletin	48.23±13.23	55.43±14.80
4	Stigmasterol	58.52±14.51	60.93±15.97
5	Betaine	36.81±2.65	47.31±2.16
6	Mangiferin	48.12 ±8.53	59.17±16.54
7	Rutin	42.91± 16.91	49.33±17.89
8	Chlorogenic acid	39.67± 1.22	56.07±8.08
9	β-carotene	31.94± 5.35	38.23±3.50
10	β-sitosterol	49.97±20.72	55.27±17.75
11	Ursolic acid	54.93±23.76	41.51±4.89
12	Querecetin	43.35±3.53	48.02±8.62
13	Betulinic acid	29.04±2.12	33.16±5.17
Petroleum Ether Extract			
14	PEEEA	59.35±18.03	66.46±11.16
15	PEECP	28.81±1.75	31.13±2.14
16	PEECT	28.64±1.71	56.50±5.19
17	PEECD	55.38±1.49	62.55±2.94
Methanolic Extract			
18	MEEA	46.51±10.48**	61.91±12.81**
19	MECP	43.33±1.71***	53.21±0.15***
20	MECT	35.64±1.11****	43.34±2.53***
21	MECD	58.60±11.52**	63.67±3.02**
Marketed Formulation			
22	SS	70.82±11.87*	78.89±10.10*
23	BT	58.63±8.15**	68.98±16.00*
Hydro- distillate extract			
24	HDEA	66.65±14.94	83.07±2.64
25	HDCP	39.88±1.26	74.36±6.61
26	HDCT	36.95±18.75	56.83±6.19
27	HDCD	71.45±0.22	87.95±3.85

All values are Mean ± S.E.M. (n=3),

***p<0.0001, ** p< 0.001 and *p<0.05 compared to vehicle, One way ANOVA followed by Dunnett's Multiple Comparison post tests regardless of all p-values.



All values are Mean ± S.E.M. (n=3),
***p<0.0001, ** p< 0.001 and *p<0.05 compared to vehicle, One way ANOVA followed by Dunnett's Multiple Comparison post tests regardless of all p-values.

Figure 3.8 Comparative MTT toxicity evaluations on neuro 2a by methanolic extracts of various *Shankhpushpi* botanicals and their marketed formulation

3.1.4 Evaluation of the Antioxidant Capacity

3.1.4.3 Comparative antioxidant potential of some phytonutrients from commonly used plant based human food, isolates, fractions and extracts of shankhpushpi botanicals

(A). 2, 2-Diphenyl-1-picryl-hydrazil (DPPH) free radical-scavenging activity

DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. The color changes from deep purple to pink to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 518 nm. Radical scavenging activity increased with increasing percentage of the free radical inhibition. The degree of discoloration indicates the free radical scavenging potentials of the sample/antioxidant by their hydrogen donating ability. The electrons become paired off and solution loses colour stochiometrically depending on the number of electrons taken up. The result shown by phytonutrients from commonly used plant based human food, isolates, fractions and extracts of shankhpushpi botanicals are given in table 3.8; 3.9 and figure 3.9. The order of activity among compounds towards antioxidant by DPPH are Tocopherol > Ascorbic acid > Curcumin > Gallic acid > Ellagic acid > β-carotene > mangiferin > ursolic acid >

Table 3.9 Antioxidant potential of isolates, fraction and extracts of various shankhpushpi botanicals and their marketed formulation by DPPH method

S. No.	Treatment	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL	50 µg/mL	IC_{50} Value
1	Ascorbic acid	39.65 ± 0.42	47.31 ± 3.45	54.38 ± 3.81	64.77 ± 7.80	81.3 ± 2.24	13.64 ± 2.02
2	Vitamin E	46.32 ± 4.36	55.26 ± 1.62	60.55 ± 1.28	62.98 ± 1.79	70.71 ± 3.32	13.62 ± 2.03
Pure Compounds							
3	Lupeol	36.56 ± 0.87	39.62 ± 0.35	49.27 ± 0.59	54.36 ± 0.72	62.19 ± 0.62	32.39 ± 0.41
4	Scopoletin	37.55 ± 1.09	67.47 ± 0.61	73.28 ± 0.49	77.70 ± 0.51	82.62 ± 0.69	12.31 ± 0.88
5	Stigmasterol	27.23 ± 0.60	41.31 ± 0.54	47.33 ± 0.46	55.05 ± 1.02	72.35 ± 0.42	31.31 ± 0.30
6	Ursolic acid	27.14 ± 1.24	30.84 ± 0.62	34.11 ± 0.57	40.97 ± 2.06	47.20 ± 1.20	58.41 ± 3.36
7	Betaine	24.05 ± 0.10	30.62 ± 0.61	34.01 ± 0.20	40.02 ± 0.67	46.69 ± 0.69	57.37 ± 1.44
8	Mangiferin	35.83 ± 1.18	49.37 ± 0.52	73.64 ± 0.59	80.59 ± 1.26	85.32 ± 0.65	18.50 ± 0.18
9	Rutin	28.54 ± 0.40	35.67 ± 0.33	42.79 ± 0.48	56.72 ± 0.12	69.95 ± 0.42	33.13 ± 0.30
10	Chlorogenic	25.35 ± 0.30	35.82 ± 0.19	40.67 ± 0.26	45.58 ± 0.35	50.12 ± 0.25	47.69 ± 0.52
11	β-carotene	43.23 ± 1.77	47.21 ± 0.62	51.61 ± 0.42	56.91 ± 0.22	61.86 ± 0.40	25.13 ± 1.38
12	Betulinic	31.00 ± 0.74	35.03 ± 0.03	40.76 ± 0.60	47.29 ± 0.54	66.96 ± 0.87	36.89 ± 0.62
13	β-sitosterol	21.89 ± 0.18	31.60 ± 0.47	34.69 ± 0.34	43.58 ± 0.33	47.28 ± 0.55	52.60 ± 0.22
Unsaponified Petroleum Ether Extract							
14	UPEEEA	30.46 ± 0.64	32.39 ± 0.73	37.45 ± 0.09	41.39 ± 0.48	45.55 ± 0.40	62.22 ± 1.79
15	UPEECP	37.00 ± 0.96	40.70 ± 0.76	45.01 ± 0.01	47.79 ± 0.24	54.35 ± 0.49	42.07 ± 0.38
16	UPEECT	29.34 ± 0.62	34.41 ± 0.36	48.50 ± 0.61	55.52 ± 0.70	62.09 ± 0.56	34.63 ± 0.30
17	UPEECD	31.29 ± 0.82	36.60 ± 0.78	40.80 ± 0.97	45.98 ± 0.88	55.59 ± 0.45	42.50 ± 0.84

Petroleum Ether Extract							
18	PEEEA	37.29 ± 0.58	41.48 ± 0.63	47.81 ± 0.24	58.62 ± 0.53	67.56 ± 0.60	29.28 ± 0.18
19	PEECP	40.76 ± 0.61	53.39 ± 0.54	65.64 ± 0.38	73.75 ± 1.01	82.05 ± 0.66	17.22 ± 0.57
20	PEECT	22.19 ± 0.56	25.81 ± 0.34	31.52 ± 0.54	33.50 ± 0.42	38.86 ± 0.77	78.31 ± 3.87
21	PEECD	37.37 ± 0.53	41.14 ± 0.46	47.56 ± 0.34	50.54 ± 0.34	55.99 ± 0.15	37.43 ± 0.38
Chloroform fraction							
22	CFEA	25.74 ± 0.42	31.91 ± 0.12	37.73 ± 0.37	50.37 ± 0.52	82.16 ± 0.13	33.35 ± 0.20
23	CFCP	33.64 ± 0.42	43.97 ± 0.18	50.43 ± 0.45	55.65 ± 0.43	62.24 ± 0.65	31.19 ± 0.31
24	CFCT	30.39 ± 0.53	35.44 ± 0.40	54.21 ± 0.58	61.00 ± 0.60	64.71 ± 0.32	30.89 ± 0.26
25	CFCD	35.59 ± 0.26	37.78 ± 0.62	40.81 ± 0.25	48.28 ± 0.28	62.13 ± 0.57	37.98 ± 0.19
Methanolic extract							
26	MEEA	27.32 ± 0.61	44.22 ± 0.46	58.97 ± 0.93	68.74 ± 0.27	80.30 ± 0.54	25.46 ± 0.08***
27	MECP	31.12 ± 0.10	57.13 ± 0.51	69.66 ± 0.84	73.15 ± 1.01	74.89 ± 0.90	19.15 ± 0.35**
28	MECT	38.30 ± 1.08	49.52 ± 0.42	70.26 ± 0.53	79.56 ± 0.39	85.36 ± 0.33	18.21 ± 0.51*
29	MECD	22.27 ± 0.49	40.80 ± 0.24	44.62 ± 0.41	48.93 ± 0.28	60.28 ± 1.08	36.09 ± 2.10***
Successive Aqueous Extract							
30	AEEA	22.55 ± 0.69	30.75 ± 0.39	33.38 ± 0.52	41.76 ± 0.94	47.75 ± 0.61	54.08 ± 0.83
31	AECP	26.31 ± 1.13	32.00 ± 0.95	39.17 ± 1.06	57.00 ± 0.74	84.09 ± 0.10	31.62 ± 0.06
32	AECT	20.83 ± 0.95	34.40 ± 0.60	39.91 ± 0.11	45.67 ± 0.35	52.96 ± 0.91	44.22 ± 1.06
33	AECD	31.62 ± 0.59	37.59 ± 0.34	40.41 ± 0.38	47.38 ± 0.42	52.41 ± 0.33	45.85 ± 0.77
Marketed Formulation							
34	SS	16.49 ± 0.68	28.35 ± 0.57	32.27 ± 0.46	68.45 ± 0.53	79.70 ± 0.57	32.96 ± 0.31***
35	BT	11.99 ± 0.31	24.29 ± 0.52	30.44 ± 0.66	59.24 ± 0.62	78.15 ± 0.26	35.48 ± 0.09***

All values are Mean ± S.E.M. (n=3),

***p<0.0001, ** p< 0.001 and *p<0.05 compared to vehicle, One way ANOVA followed by Dunnett's Multiple Comparison post tests regardless of all p-values.

Table 3.10 Comparative antioxidant potential of some phytonutrients from commonly used food by FRAP method

S. No.	Treatments	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL	50 µg/mL	IC 50
1	Rutin	4.587 ± 0.28	12.02 ± 0.21	38.43 ± 0.35	53.44 ± 0.60	77.28 ± 0.60	36.88 ± 0.23
2	Quercetin	15.11 ± 0.46	26.36 ± 0.33	47.68 ± 0.28	57.92 ± 0.32	75.3 ± 0.49	33.6 ± 0.12
3	Chlorogenic	8.637 ± 0.31	18.47 ± 0.45	25.35 ± 0.54	54.95 ± 0.48	78.86 ± 0.24	37.26 ± 0.09
4	Tocopherol	39.22 ± 0.17	69.9 ± 1.01	72.24 ± 1.50	82.17 ± 0.58	94.41 ± 1.58	12.39 ± 0.17
5	Ellagic acid	43.18 ± 0.68	52.15 ± 0.58	62.17 ± 0.67	78.55 ± 0.61	87.71 ± 0.78	16.62 ± 0.63
6	Gallic acid	41.76 ± 0.34	57.04 ± 0.15	69.24 ± 0.44	83.9 ± 0.37	96.83 ± 0.41	15.58 ± 0.20
7	Betulinic	24.84 ± 0.17	35.33 ± 0.64	52.01 ± 0.24	64.57 ± 0.69	80.42 ± 0.29	28.98 ± 0.25
8	Ursolic acid	23.08 ± 1.05	65.76 ± 2.39	76.83 ± 1.12	84.26 ± 1.62	95.29 ± 2.05	18.32 ± 0.64
9	Morin	5.907 ± 0.34	16.79 ± 0.31	26.71 ± 0.34	46.75 ± 0.25	65.54 ± 0.43	41.83 ± 0.21
10	β- carotene	39.22 ± 0.49	50.19 ± 1.00	78.46 ± 0.58	86.11 ± 0.62	98.1 ± 0.10	16.75 ± 0.49
11	Ascorbic	43.22 ± 0.99	57.47 ± 0.22	70.19 ± 0.52	85.42 ± 0.42	93.45 ± 0.44	14.61 ± 0.56
12	Curcumin	38.85 ± 0.37	58.44 ± 0.31	75.45 ± 0.58	85.57 ± 0.48	91.27 ± 0.61	14.91 ± 0.08
13	Stigmasterol	4.303 ± 0.55	13.12 ± 1.05	16.15 ± 0.43	35.20 ± 0.61	64.22 ± 1.09	46.50 ± 0.55
14	Nicotine	10.60 ± 0.35	14.79 ± 0.27	18.28 ± 0.52	38.17 ± 0.46	73.56 ± 0.73	42.72 ± 0.29
15	Marmesin	6.613 ± 0.31	13.27 ± 0.33	24.18 ± 0.65	48.05 ± 0.40	83.26 ± 0.48	38.36 ± 14.49
16	Naringin	19.89 ± 0.16	31.85 ± 0.48	41.27 ± 0.11	58.64 ± 0.49	70.21 ± 0.90	34.41 ± 0.15
17	Mangiferin	38.24 ± 0.45	56.84 ± 0.29	66.2 ± 0.53	79.2 ± 0.29	92.1 ± 0.57	17.29 ± 0.34
18	Apigenin	5.997 ± 0.67	13.12 ± 0.38	32.33 ± 0.54	58.2 ± 0.58	83.19 ± 0.61	35.73 ± 0.22

All values are Mean ± S.E.M. (n=3).

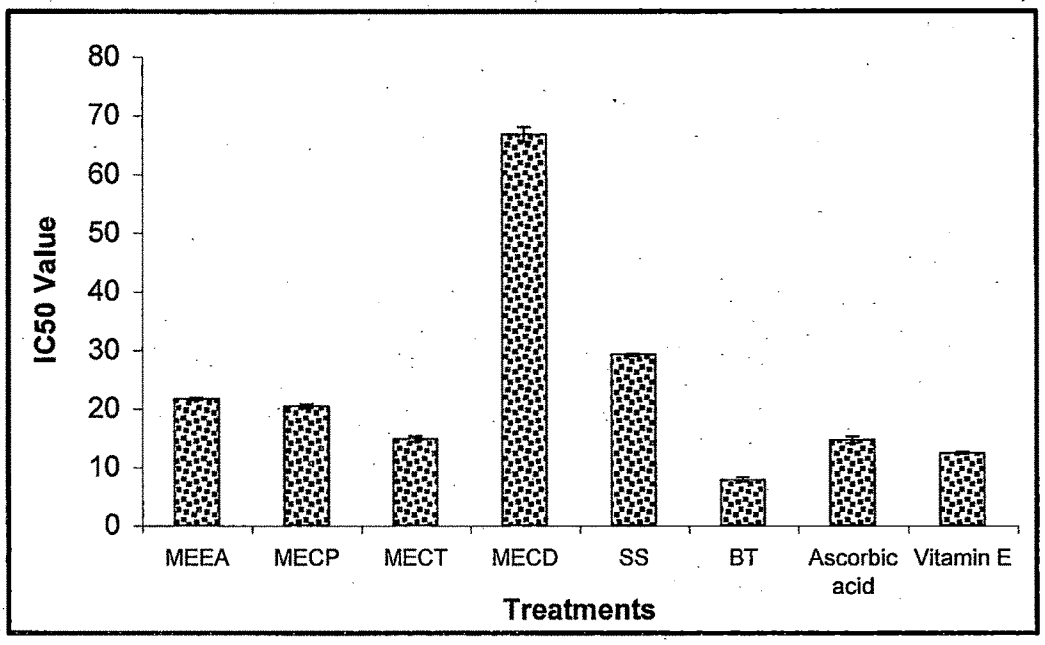
Table 3.11 Antioxidant potential of isolates, fraction and extracts of various *Shankhpushpi* botanicals and their marketed formulation by DPPH method

S. No	Treatment	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL	50 µg/mL	IC_{50}
1	Ascorbic acid	43.22 ± 0.99	57.47 ± 0.22	70.19 ± 0.52	85.42 ± 0.42	93.45 ± 0.44	14.61 ± 0.56
2	Vitamin E	39.22 ± 0.17	69.9 ± 1.01	72.24 ± 1.50	82.17 ± 0.58	94.41 ± 1.58	12.39 ± 0.17
Pure Compounds							
3	Lupeol	30.15 ± 0.28	32.38 ± 0.37	39.13 ± 0.32	41.28 ± 0.50	65.62 ± 0.55	40.37 ± 0.23
4	Scopoletin	38.48 ± 0.41	58.59 ± 0.34	69.60 ± 0.43	80.52 ± 0.59	85.53 ± 0.46	15.74 ± 0.09
5	Stigmasterol	25.43 ± 0.54	36.20 ± 0.25	44.91 ± 0.31	56.93 ± 0.34	72.78 ± 0.23	32.35 ± 0.27
6	Ursolic acid	16.17 ± 0.51	31.51 ± 0.74	44.73 ± 0.64	52.86 ± 0.28	67.87 ± 0.28	35.89 ± 0.16
7	Betaine	6.72 ± 0.48	10.20 ± 0.65	23.76 ± 1.39	43.64 ± 1.06	52.30 ± 0.55	48.22 ± 0.66
8	Mangiferin	41.32 ± 2.75	52.88 ± 0.27	62.50 ± 0.66	67.83 ± 1.81	75.79 ± 0.37	17.76 ± 1.83
9	Rutin	41.15 ± 3.22	60.32 ± 0.94	70.62 ± 0.54	76.94 ± 0.57	82.62 ± 1.06	13.18 ± 2.30
10	Chlorogenic	49.16 ± 0.16	59.10 ± 0.30	70.41 ± 0.65	75.94 ± 0.49	81.45 ± 0.49	8.84 ± 0.52
11	β-carotene	32.64 ± 0.39	35.89 ± 0.28	37.67 ± 0.12	44.90 ± 0.29	48.33 ± 0.65	55.16 ± 1.39
12	Betulinic acid	15.76 ± 0.76	23.89 ± 0.40	34.37 ± 0.38	46.17 ± 0.24	57.87 ± 0.31	43.50 ± 0.05
13	β-sitosterol	18.82 ± 0.99	22.32 ± 1.53	37.12 ± 0.54	44.45 ± 0.82	50.33 ± 0.38	48.06 ± 0.17
Unsaponified Petroleum Ether Extract							
14	UPEEEA	24.84 ± 0.66	28.17 ± 0.42	43.93 ± 0.23	48.49 ± 0.45	58.96 ± 0.24	40.29 ± 0.03
15	UPEECP	2.89 ± 0.49	7.34 ± 1.78	20.89 ± 0.13	27.47 ± 0.91	36.08 ± 0.13	65.96 ± 0.83
16	UPEECT	11.94 ± 1.64	18.51 ± 1.08	41.96 ± 0.45	58.05 ± 0.88	66.86 ± 1.25	37.08 ± 0.46
17	UPEECD	10.32 ± 0.74	16.20 ± 0.50	30.59 ± 0.59	45.58 ± 0.47	52.13 ± 0.73	46.83 ± 0.18

Petroleum Ether Extract							
18	PEEEA	29.19 ± 0.31	32.68 ± 0.37	41.39 ± 0.39	52.88 ± 0.27	60.43 ± 0.59	38.07 ± 0.20
19	PEECP	2.33 ± 0.76	7.37 ± 1.19	20.76 ± 1.06	27.39 ± 0.94	33.19 ± 1.02	69.27 ± 3.02
20	PEECT	6.03 ± 1.80	17.51 ± 1.42	37.04 ± 0.50	57.44 ± 0.30	67.48 ± 0.58	23.65 ± 2.33
21	PEECD	6.10 ± 1.89	17.61 ± 1.12	22.81 ± 0.60	29.36 ± 0.19	36.87 ± 1.32	67.482 ± 1.04
Chloroform fraction							
22	CFEA	39.67 ± 0.37	67.45 ± 0.83	75.45 ± 0.62	80.15 ± 0.25	84.55 ± 0.38	11.00 ± 0.16
23	CFCP	44.29 ± 2.31	61.19 ± 0.61	75.74 ± 1.05	80.86 ± 0.25	83.88 ± 0.35	10.38 ± 1.72
24	CFCT	24.14 ± 0.65	29.36 ± 0.49	42.99 ± 0.33	47.48 ± 0.48	53.66 ± 0.30	43.61 ± 0.41
25	CFCD	7.19 ± 1.22	15.53 ± 0.25	23.03 ± 0.13	45.21 ± 0.21	58.00 ± 0.40	45.38 ± 0.08
Methanolic extract							
26	MEEA	34.03 ± 0.27	48.99 ± 0.50	63.14 ± 0.47	72.47 ± 0.25	77.08 ± 0.55	21.65 ± 0.15***
27	MECP	35.54 ± 0.73	50.66 ± 0.33	63.42 ± 0.47	73.89 ± 0.33	87.99 ± 0.86	20.38 ± 0.37***
28	MECT	40.62 ± 0.40	57.15 ± 1.03	74.34 ± 1.20	81.77 ± 0.95	90.00 ± 0.13	14.78 ± 0.41**
29	MECD	30.64 ± 0.94	33.48 ± 0.45	37.43 ± 0.59	40.61 ± 0.42	44.40 ± 0.29	66.71 ± 1.28***
Successive Aqueous Extract							
30	AEEA	38.17 ± 0.28	47.90 ± 0.14	49.14 ± 0.32	56.31 ± 0.52	69.23 ± 0.59	26.94 ± 0.14
31	AECP	41.15 ± 1.36	76.09 ± 1.57	82.25 ± 1.58	84.45 ± 0.85	88.00 ± 0.30	5.96 ± 1.82
32	AECT	14.28 ± 1.26	17.32 ± 1.16	19.05 ± 0.79	23.80 ± 0.60	28.86 ± 0.93	117.08 ± 14.97
33	AECD	10.66 ± 1.04	37.15 ± 0.47	52.05 ± 0.34	64.77 ± 0.79	69.70 ± 0.29	32.14 ± 0.27
Marketed Formulation							
34	SS	15.49 ± 0.52	28.36 ± 0.80	57.87 ± 0.45	73.14 ± 0.57	82.26 ± 0.15	29.19 ± 0.18***
35	BT	48.70 ± 5.03	71.46 ± 1.51	77.40 ± 0.57	86.09 ± 0.06	88.92 ± 0.14	7.79 ± 0.42***

All values are Mean ± S.E.M. (n=3),

***p<0.0001, ** p< 0.001 and *p<0.05 compared to vehicle, One way ANOVA followed by Dunnett's Multiple Comparison post tests regardless of all p-values



All values are Mean ± S.E.M. (n=3),
***p<0.0001, ** p< 0.001 and *p<0.05 compared to vehicle, One way ANOVA followed by Dunnett's Multiple Comparison post tests regardless of all p-values

Figure 3.10 Comparative antioxidant potential of methanolic extracts of various *Shankhpushpi* botanicals and their marketed formulation by FRAP method

(C). Phosphomolybdenum Complex Method

The result shown by phytonutrients from commonly used plant based human food; isolates, fractions and extracts of shankhpushpi botanicals for Phosphomolybdenum complex method are given in table 3.12; 3.13 and figure 3.11. The order of activity among compounds towards antioxidant by DPPH are Tocopherol > Ascorbic acid > Curcumin > Gallic acid > Ellagic acid > β-carotene > mangiferin > ursolic acid > betulinic acid > querecetin > Naringin > apigenin > rutin > Chlorogenic acid > Marmesin > nicotine > stigmasterol. Among methanolic extracts of Shankhpushpi botanicals the order of activity toward Phosphomolybdenum complex method were found to be CT > CP > EA > CD. Among isolates chlorogenic acid, scopoletin and mangiferin were found to be most active. The difference in result by DPPH and FCRP may be attributed to the fact that all the extracts have significant antioxidant capacity. The degree may changes in their performance by due manual errors of the method. It may be also concluded that both CP and EA have antioxidant action.

Table 3.12 Comparative antioxidant potential of some phytonutrients from commonly used food by Phosphomolybdenum Complex Method.

S. No	Treatments	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL	50 µg/mL	IC 50
1	Rutin	16.25 ± 0.59	46.22 ± 0.53	56.29 ± 0.60	74.28 ± 0.55	79.14 ± 0.49	27.12 ± 0.36
2	Quercetin	36.23 ± 0.15	60.00 ± 0.12	64.45 ± 0.19	66.56 ± 0.27	70.39 ± 0.12	17.28 ± 0.10
3	Chlorogenic	30.33 ± 0.10	33.34 ± 0.19	47.25 ± 0.11	69.66 ± 0.03	72.17 ± 0.46	29.54 ± 0.11
4	Tocopherol	44.30 ± 0.11	79.16 ± 0.01	84.11 ± 0.07	91.61 ± 0.30	93.11 ± 0.05	4.41 ± 0.08
5	Ellagic acid	38.38 ± 0.56	75.42 ± 0.76	80.48 ± 0.45	87.46 ± 1.11	90.09 ± 0.03	8.88 ± 0.49
6	Gallic acid	42.34 ± 0.64	72.40 ± 0.55	88.51 ± 1.10	94.10 ± 0.62	97.17 ± 0.53	7.98 ± 0.53
7	Betulinic	44.42 ± 0.69	54.87 ± 0.91	55.37 ± 0.70	58.13 ± 0.36	61.33 ± 0.64	16.90 ± 1.31
8	Ursolic acid	43.38 ± 0.60	53.57 ± 0.27	70.82 ± 0.09	78.54 ± 0.33	81.30 ± 0.64	14.65 ± 0.19
9	Morin	16.32 ± 0.28	28.38 ± 0.34	39.04 ± 0.25	59.34 ± 0.34	61.33 ± 0.37	37.51 ± 0.10
10	β- carotene	44.23 ± 1.52	62.10 ± 0.58	76.22 ± 0.57	95.37 ± 0.63	95.75 ± 0.85	11.82 ± 0.48
11	Ascorbic	48.44 ± 0.34	71.08 ± 0.28	78.24 ± 0.31	86.09 ± 0.06	90.08 ± 0.07	4.77 ± 0.18
12	Curcumin	41.20 ± 0.10	78.21 ± 1.01	90.48 ± 0.57	91.40 ± 0.53	94.78 ± 0.17	5.72 ± 0.24
13	Stigmasterol	18.05 ± 0.03	23.32 ± 0.42	38.39 ± 0.34	49.10 ± 0.58	50.51 ± 0.60	45.59 ± 0.66
14	Nicotine	38.52 ± 0.23	41.17 ± 0.01	44.23 ± 0.12	52.45 ± 0.20	61.59 ± 0.26	44.20 ± 0.08
15	Marmesin	21.48 ± 0.73	39.49 ± 0.48	48.25 ± 0.74	59.50 ± 0.36	62.74 ± 0.97	33.61 ± 0.14
16	Naringin	47.20 ± 0.10	50.62 ± 0.27	53.12 ± 0.06	56.54 ± 0.01	59.59 ± 0.32	18.89 ± 0.23
17	Mangiferin	36.33 ± 0.71	65.65 ± 0.23	87.26 ± 0.13	94.08 ± 0.10	95.02 ± 0.02	12.39 ± 0.19
18	Apigenin	19.21 ± 0.21	51.41 ± 0.20	63.41 ± 0.01	68.42 ± 0.05	71.18 ± 0.09	26.09 ± 0.06

All values are Mean ± S.E.M. (n=3).

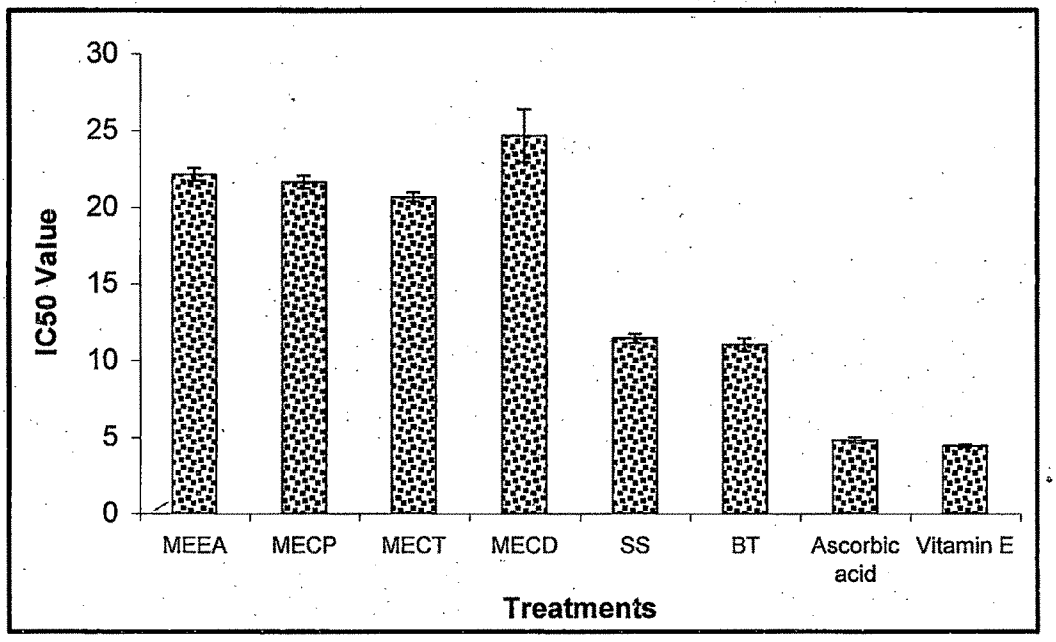
Table 3.13 Antioxidant potential of isolates, fraction and extracts of various shankhpushpi botanicals and their marketed formulation by Phosphomolybdenum Complex Method.

S. No	Treatment	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL	50 µg/mL	IC_{50}
1	Ascorbic acid	48.44 ± 0.34	71.08 ± 0.28	78.24 ± 0.31	86.09 ± 0.06	90.08 ± 0.07	4.77 ± 0.18
2	Vitamin E	44.30 ± 0.11	79.16 ± 0.01	84.11 ± 0.07	91.61 ± 0.30	93.11 ± 0.05	4.41 ± 0.08
Pure Compounds							
3	Lupeol	10.22 ± 0.58	24.06 ± 0.59	40.83 ± 0.17	46.89 ± 0.33	76.10 ± 0.62	36.71 ± 0.15
4	Scopoletin	16.43 ± 0.39	28.27 ± 0.20	47.27 ± 0.43	57.28 ± 0.53	79.42 ± 0.43	32.74 ± 0.17
5	Stigmasterol	22.85 ± 0.81	36.76 ± 0.39	57.67 ± 0.52	64.81 ± 1.01	78.48 ± 0.66	28.47 ± 0.21
6	Ursolic acid	41.41 ± 1.67	53.15 ± 0.97	59.46 ± 0.37	66.55 ± 0.61	73.09 ± 0.28	18.48 ± 1.49
7	Betaine	38.80 ± 1.29	46.98 ± 0.21	54.43 ± 0.73	62.31 ± 0.38	71.32 ± 0.45	24.01 ± 0.58
8	Mangiferin	42.18 ± 0.69	62.36 ± 1.41	81.17 ± 1.46	88.89 ± 0.79	93.50 ± 0.37	11.70 ± 0.67
9	Rutin	43.44 ± 0.59	66.22 ± 1.42	84.49 ± 0.94	92.83 ± 0.18	96.35 ± 0.88	9.85 ± 0.68
10	Chlorogenic	44.56 ± 0.44	63.58 ± 0.89	76.69 ± 0.27	84.13 ± 1.26	95.58 ± 0.61	14.63 ± 3.03
11	β-carotene	33.19 ± 0.75	52.39 ± 1.29	64.64 ± 1.10	85.48 ± 1.66	90.46 ± 0.42	19.67 ± 0.71
12	Betulinic acid	25.83 ± 1.39	45.40 ± 1.09	54.15 ± 1.19	61.80 ± 1.34	71.66 ± 0.70	28.33 ± 1.05
13	β-sitosterol	32.09 ± 0.19	44.70 ± 0.42	65.49 ± 1.62	80.13 ± 0.24	85.52 ± 1.58	21.85 ± 0.38
Unsaponified Petroleum Ether Extract							
14	UPEEEA	32.58 ± 0.62	65.09 ± 2.35	83.41 ± 2.04	87.47 ± 2.41	94.26 ± 0.49	14.48 ± 0.62
15	UPEECP	30.30 ± 0.38	52.34 ± 0.57	66.45 ± 0.92	73.54 ± 0.49	80.16 ± 0.54	21.26 ± 0.25
16	UPEECT	10.36 ± 0.68	44.36 ± 4.14	66.08 ± 1.38	79.53 ± 0.86	84.18 ± 0.52	26.20 ± 0.70
17	UPEECD	26.59 ± 1.65	57.35 ± 0.71	69.89 ± 0.90	80.16 ± 0.80	88.06 ± 0.89	20.08 ± 0.46

Petroleum Ether Extract							
18	PEEEA	36.45 ± 0.62	63.54 ± 0.72	82.54 ± 0.61	90.29 ± 0.43	93.42 ± 0.58	13.46 ± 0.08
19	PEECP	34.31 ± 0.39	51.80 ± 1.54	63.78 ± 0.36	74.84 ± 0.36	84.44 ± 0.55	20.38 ± 0.53
20	PEECT	30.43 ± 1.46	49.10 ± 0.59	73.79 ± 0.77	83.16 ± 1.49	88.08 ± 0.73	17.00 ± 2.48
21	PEECD	34.72 ± 0.61	52.20 ± 1.09	67.31 ± 0.57	80.55 ± 2.25	89.46 ± 0.34	19.21 ± 0.45
Chloroform fraction							
22	CFEA	43.66 ± 0.74	66.04 ± 1.22	86.26 ± 0.91	90.26 ± 0.54	94.91 ± 1.05	9.30 ± 0.51
23	CFCP	42.52 ± 0.55	63.96 ± 0.38	81.88 ± 0.28	90.10 ± 0.55	94.73 ± 0.80	11.12 ± 0.31
24	CFCT	18.14 ± 1.03	44.41 ± 1.48	58.20 ± 1.22	71.67 ± 0.65	74.82 ± 0.74	27.55 ± 0.49
25	CFCD	41.23 ± 0.63	59.83 ± 0.75	70.38 ± 0.62	80.31 ± 0.55	92.27 ± 0.16	14.64 ± 0.40
Methanolic extract							
26	MEEA	33.49 ± 0.59	50.36 ± 0.54	61.24 ± 1.17	70.16 ± 0.52	76.15 ± 0.67	22.11 ± 0.41***
27	MECP	33.87 ± 1.13	47.05 ± 0.52	64.76 ± 0.65	74.20 ± 1.06	80.51 ± 0.44	21.62 ± 0.40***
28	MECT	35.84 ± 0.89	48.94 ± 0.12	61.06 ± 0.24	79.36 ± 0.39	85.14 ± 0.59	20.63 ± 0.30***
29	MECD	35.17 ± 0.51	43.13 ± 0.08	62.16 ± 0.22	72.07 ± 0.55	83.13 ± 0.69	24.64 ± 1.73***
Successive Aqueous Extract							
30	AEEA	34.68 ± 0.78	65.52 ± 2.08	78.50 ± 2.31	85.97 ± 1.92	92.80 ± 1.29	14.26 ± 1.14
31	AECP	42.78 ± 1.11	64.67 ± 0.85	83.22 ± 0.76	90.57 ± 0.75	94.62 ± 0.94	10.56 ± 0.75
32	AECT	35.94 ± 0.87	54.67 ± 2.01	65.10 ± 1.22	73.21 ± 1.01	80.27 ± 0.46	18.96 ± 0.59
33	AECD	40.45 ± 0.40	55.75 ± 1.81	67.57 ± 1.32	76.35 ± 0.92	90.51 ± 1.20	16.61 ± 0.87
Marketed Formulation							
34	SS	42.24 ± 0.50	62.87 ± 0.27	81.97 ± 0.23	90.18 ± 0.18	93.90 ± 0.41	11.43 ± 0.30***
35	BT	42.84 ± 0.23	64.81 ± 1.14	81.39 ± 0.59	90.50 ± 0.25	95.93 ± 0.13	11.00 ± 0.42***

All values are Mean ± S.E.M. (n=3),

***p<0.0001, ** p< 0.001 and *p<0.05 compared to vehicle, One way ANOVA followed by Dunnett's Multiple Comparison post tests regardless of all p-values



All values are Mean ± S.E.M. (n=3),
***p<0.0001, ** p< 0.001 and *p<0.05 compared to vehicle, One way ANOVA followed by Dunnett's Multiple Comparison post tests regardless of all p-values

Figure 3.11 Comparative antioxidant potential of methanolic extracts of various shankhpushpi botanicals and their marketed formulation by Phosphomolybdenum Complex Method.

3.1.4.4 HPTLC Fingerprinting with marker and DPPH-HPTLC method for bioactive marker determinations

DPPH radical scavenging compounds appeared as yellow spots against a purple background. When RP-TLC plates were used with DPPH as detecting agent, the developing color proved to be very unstable (Yrjonen et al., 2003), but in normal TLC plates the coloration produced after spraying with DPPH has been proved to be relatively stable, enabling the identification of radical-scavenging activity after a period of 30 min (Klujer et al., 1997; Kumar, 2007). Densitogram of all reference standards (β-carotene, rutin, gallic acid and curcumin) after DPPH derivatisation exhibited concentration dependent reduction in peak area. Polynomial second-degree calibration equation calculated for the reference standards were found to give satisfactory correlation between concentration and percentage area reduction (Figure 3.12). Concentration dependent reduction in peak area of all reference standards (β-carotene, rutin, gallic acid and curcumin) after DPPH derivatisation proved that concentration at 50% reduction in peak area can be used to assess the antioxidant potency of compound. Gallic acid was found to be most active DPPH radical scavenger and β-carotene exhibited the least activity in this method.

can also be utilized for the bioassay guided isolation of unidentified natural antioxidants and can be used for selection of potential antioxidants from a group of structurally diverse compounds. The current application also demonstrates the versatility and adaptability of a standard HPTLC system to serve an additional purpose in the drug discovery arena. Although DPPH spectrophotometeric methods are ubiquitously available, the proposed method provides an edge in terms of identification and quantification of antioxidant constituent/s in a multi-component system, a simple and cost-effective alternative to the established methods.

3.1.5 COMPARATIVE NOOTROPIC ACTIVITY

3.1.5.1 Acetylcholinesterase enzyme (AchE) Inhibition assay

The result shown by isolates, fractions and extracts of shankhpushpi botanicals for AchE inhibition assay were given in table 3.15 and figure 3.13.

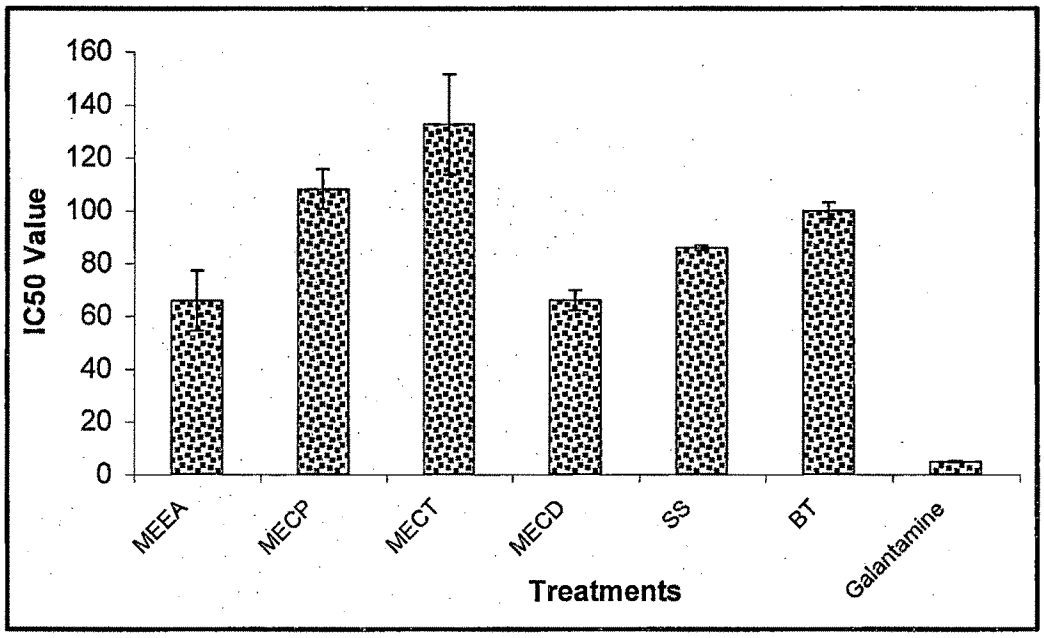
Table 3.15 AchE inhibition potential of isolates, fraction and extracts of various *Shankhpushpi* botanicals and their marketed formulation.

S.No.	Treatments	5 µg/mL	10 µg/mL	15 µg/mL	20 µg/mL	25 µg/mL	IC_{50}
1	Galantamine	44.33 ± 0.50	67.84 ± 0.80	73.56 ± 0.30	80.03 ± 0.59	89.06 ± 0.63	4.68 ± 0.16
Pure Compounds							
2	Lupeol	22.47 ± 1.05	29.30 ± 0.44	30.96 ± 0.62	33.88 ± 0.60	37.06 ± 0.55	43.63 ± 1.04
3	Scopoletin	9.86 ± 0.37	11.56 ± 0.16	13.87 ± 0.42	15.66 ± 0.65	18.82 ± 0.49	97.00 ± 3.82
4	Stigmasterol	21.80 ± 1.12	27.64 ± 0.34	30.69 ± 0.47	33.17 ± 0.39	35.06 ± 0.51	46.80 ± 0.96
5	Ursolic acid	26.88 ± 0.92	30.57 ± 0.29	33.06 ± 0.64	34.77 ± 0.57	38.73 ± 0.80	45.89 ± 1.23
6	Betaine	21.44 ± 1.24	23.13 ± 0.53	24.15 ± 0.20	26.63 ± 0.62	29.67 ± 0.90	79.20 ± 7.16
7	Mangiferin	34.88 ± 0.09	36.38 ± 0.35	38.02 ± 0.33	39.60 ± 0.52	40.76 ± 0.06	55.42 ± 1.52
8	Rutin	11.66 ± 0.14	16.03 ± 0.39	17.90 ± 0.34	20.99 ± 0.70	24.24 ± 0.76	68.27 ± 3.49
9	Chlorogenic	18.42 ± 0.66	18.62 ± 0.62	19.35 ± 0.22	21.22 ± 0.20	23.40 ± 0.33	134.97 ± 8.17
10	β-carotene	20.34 ± 0.61	22.31 ± 1.15	23.62 ± 1.17	24.61 ± 1.42	26.00 ± 1.28	116.44 ± 14.65
11	Betulinic	25.98 ± 0.49	27.81 ± 0.40	30.02 ± 0.29	31.88 ± 0.02	33.39 ± 0.19	68.69 ± 2.35
12	β-sitosterol	13.08 ± 0.27	14.52 ± 0.31	15.86 ± 0.35	17.35 ± 0.18	18.27 ± 0.47	130.99 ± 17.70

Unsaponified Petroleum Ether Extract							
13	UPEEEA	22.00 ± 0.96	23.43 ± 0.76	25.46 ± 0.78	28.36 ± 0.53	30.51 ± 0.70	69.91 ± 1.03
14	UPEECP	56.93 ± 0.84	58.40 ± 0.88	59.68 ± 0.55	62.23 ± 0.41	63.27 ± 0.57	14.92 ± 2.00
15	UPEECT	16.68 ± 0.39	17.69 ± 0.39	20.15 ± 0.55	21.38 ± 0.57	23.31 ± 0.66	104.55 ± 5.47
16	UPEECD	12.11 ± 0.05	13.81 ± 0.24	15.01 ± 0.24	17.05 ± 0.34	18.27 ± 0.47	127.80 ± 7.67
Petroleum Ether Extract							
17	PEEEA	9.31 ± 0.25	11.89 ± 0.74	13.36 ± 0.87	16.24 ± 1.50	18.66 ± 1.33	96.60 ± 12.21
18	PEECP	27.21 ± 1.14	29.63 ± 1.39	31.86 ± 1.76	33.89 ± 1.55	35.56 ± 1.92	61.65 ± 10.59
19	PEECT	16.52 ± 0.33	18.02 ± 0.34	21.65 ± 1.20	23.34 ± 1.55	26.42 ± 2.27	79.72 ± 16.68
20	PEECD	9.09 ± 0.37	10.93 ± 0.16	12.40 ± 0.50	13.98 ± 0.14	15.89 ± 0.27	128.04 ± 1.98
Chloroform fraction							
21	CFEA	11.72 ± 0.36	14.87 ± 0.40	16.64 ± 0.71	18.95 ± 0.82	20.61 ± 0.71	92.12 ± 5.22
22	CFCP	25.23 ± 0.94	26.59 ± 0.94	28.07 ± 0.72	29.84 ± 0.95	31.40 ± 1.06	85.10 ± 4.09
23	CFCT	33.76 ± 0.65	35.68 ± 0.43	37.22 ± 0.58	38.20 ± 0.19	40.31 ± 0.37	56.75 ± 1.24
24	CFCD	nd	nd	nd	nd	nd	nd
Defatted Methanolic Extract							
25	MEEA	35.32 ± 2.13	36.80 ± 2.16	38.12 ± 2.27	38.99 ± 2.15	40.32 ± 2.21	65.67 ± 11.32***
26	MECP	17.13 ± 0.11	18.47 ± 0.43	20.48 ± 0.43	21.77 ± 0.44	23.56 ± 0.60	107.99 ± 7.57***
27	MECT	17.08 ± 1.02	18.26 ± 1.02	19.60 ± 1.29	21.32 ± 1.53	22.30 ± 1.66	132.40 ± 18.96***
28	MECD	26.03 ± 0.71	27.94 ± 0.33	29.79 ± 0.19	32.15 ± 0.36	33.88 ± 1.13	65.88 ± 3.78***
Successive Aqueous Extract							
29	AEEA	13.21 ± 0.72	16.66 ± 0.21	18.03 ± 0.20	19.80 ± 0.13	20.29 ± 0.12	108.94 ± 8.99
30	AECP	11.75 ± 0.52	13.57 ± 0.94	15.32 ± 0.87	17.37 ± 0.87	18.83 ± 0.76	112.52 ± 7.69
31	AECT	10.77 ± 0.11	13.14 ± 0.05	14.46 ± 0.27	16.41 ± 0.26	17.96 ± 0.27	116.12 ± 4.04
32	AECD	21.78 ± 0.29	23.61 ± 0.54	24.26 ± 0.84	25.18 ± 0.34	27.36 ± 0.68	117.19 ± 8.41
Marketed Formulation							
33	SS	2.51± 0.30	7.31 ± 0.29	10.44 ± 0.29	12.51 ± 0.26	14.28 ± 0.14	85.69 ± 0.86***
34	BT	6.40 ± 0.30	8.11 ± 0.10	8.83 ± 0.33	12.81 ± 0.42	15.77 ± 0.39	99.73 ± 3.18***

All values are Mean ± S.E.M. (n=3),
***p<0.0001, ** p< 0.001 and *p<0.05 compared to vehicle, One way ANOVA followed by Dunnett's Multiple Comparison post tests regardless of all p-values

Out of the 6 methanolic extracts tested MECD and MEEA have significant potent inhibition against enzyme. Out of 2 marketed formulation both have more or less similar action and have potent effect after the EA and CD.



All values are Mean ± S.E.M. (n=3),
***p<0.0001, ** p< 0.001 and *p<0.05 compared to vehicle, One way ANOVA followed by Dunnett's Multiple Comparison post tests regardless of all p-values

Figure 3.13 Comparative AchE inhibition potential of methanolic extracts of various *Shankhpushpi* botanicals and their marketed formulation

3.1.5.2 TLC bioautography for acetyl cholinesterase inhibition

TLC bioautography of the plants with AChEI activity exhibited white spots on a yellow background. The methanolic extracts of various botanicals of shankhpushpi showed several white spots in different R_f. TLC bioautography of active plants revealed active spots at Rf values 0.85, 0.66, 0.54, 0.24 (*EA*), 0.43, 0.35, 0.24 (*CP*), 0.54, 0.43, 0.24, 0.35 (CT) and 0.8, 0.7, 0.59, 0.48 (*CD*) (Figure 3.14). The white spots pertained to EA and CD appeared more rapidly.

of the Ab precursor protein are found in familial AD, and these mutations are involved in amyloidogenesis (Citron et al., 1992). Also, familial AD mutations of presenilin 1 (PS-1) enhance the generation of Ab 1–42 (Tomita et al., 1997). We used the 25–35 fragment of the Ab peptide because of the reported neurotoxic effects of this fragment (Yankner et al., 1990). A 48-hour exposure to 20 mmol/L Ab caused a significant reduction in the neuronal cells. The result shown by isolates, fractions and extracts of shankhpushpi botanicals for Phosphomolybdenum complex method are given in table 3.16 and figure 3.15.

Table 3.16 Neuroprotection assay of isolates, fraction and extracts of various shankhpushpi botanicals and their marketed formulation against β- amyloid induced neurotoxicity.

S. No.	Treatments	% Cell Protected Viability by β- amyloid	
		10 µg/mL	20 µg/mL
1	Control	14.83±0.47	17.41±0.62
Pure compounds			
2	Lupeol	17.14±0.14	19.12±1.02
3	Scopoletin	20.32± 0.44	24.44±1.26
4	Stigmasterol	18.63±0.43	20.70±0.62
5	Betaine	17.08±0.39	20.45±0.72
6	Mangiferin	17.91±0.19	59.79±27.72
7	Rutin	18.81±0.46	20.10±3.01
8	Chlorogenic acid	18.22±0.42	18.27±0.49
9	β-carotene	24.95±2.97	47.20±19.13
10	β-sitosterol	22.25±0.37	29.75±3.09
11	Ursolic acid	18.94±0.85	19.30±0.92
12	Querecetin	27.79±1.42	60.79±8.88
13	Betulinic acid	18.79±0.37	18.68±0.39
Petroleum Ether Extract			
14	PEEEA	25.81±3.66	44.33±12.11
15	PEECP	39.61±9.62	41.69±9.01
16	PEECT	22.24±0.82	25.15±0.46
17	PEECD	73.41±1.59	92.50±3.38

Table 3.17 Effects of methanolic extracts of *Shankhpushpi* botanicals on CS induced learning retention (memory) deficit in the step-down test in rats

Treatment# (mg/kg)	Step down latency on Day 15 Inflexion ratio (L_{15}- L_1/ L_1)
Vehicle	4.06±1.21
Piracetam-100	33.13± 0.63
MEEA	31.17±0.51
MECP	19.26±0.32
MECT	17.72±0.20
MECD	28.06±0.09
SS	26.86±0.11
BT	25.75±0.17

All values are Mean ± S.E.M.(n=6),

***p<0.001 compared to vehicle, ANOVA followed by post tests regardless of all p-values

The test extracts and the vehicle were administered once daily for 15 days in the unstressed group or 45 min before stress

Scopolamine induced amnesia in rats

Administration of scopolamine produced amnesia as seen from the reduction in the number of avoidance responses. However, continued treatment of methanolic extract of EA and CD produced better retention and recovery in a dose dependent manner than the vehicle treated animals. Animals receiving only scopolamine butyl bromide on day 7 showed a substantial loss of memory and amnesia produced was also persistent. The retention of memory and retrieval as seen in the 400 mg/kg methanolic extract treated groups was significant as compared to the groups receiving same doses. There is also a significant increase in retention by increase in dose of the extract suggesting the effect of the drug on memory and its retrieval in a dose dependent manner. Thus, antiamnesic effects of isolated EA and CD on scopolamine-induced amnesia were successfully demonstrated through the study. Results are shown in table 3.18.

Table 3.18 Effect of methanolic extracts of Shankhpushpi botanicals on scopolamine-induced amnesia in rats

Day	No. of Avoidance Response								
	Vehicle	Scopolamine	Piracetam	MEEA	MECP	MECT	MECD	SS	BT
7	2.89±0.13	-	6.48±0.16**	6.02±0.23**	4.76±0.12**	4.38±0.14**	5.84±0.03**	5.32±0.02	5.02±0.14
8	2.97±0.21	-	6.89±0.14**	6.33±0.13**	4.91±0.21**	4.74±0.16**	6.16±0.21**	5.89±0.13	5.73±0.22
9	3.14±0.18.	1.84±0.21**	7.12±0.21**	6.74±0.03**	5.14±0.23**	5.03±0.12**	6.59±0.14**	6.11±0.31	6.01±0.14
10	3.42±0.16	1.96±0.22**	7.84±0.32**	7.12±0.0.13	5.56±0.12**	5.34±0.21***	6.91±0.24	6.64±0.21	6.56±0.12
11	3.91±0.21	2.14±0.12**	7.98±0.18**	7.56±0.12**	5.87±0.14**	5.64±0.16**	7.38±0.31	6.97±0.14	6.84±0.23
12	4.21±0.12	2.42±0.13	8.24±0.21**	7.88±0.23**	6.23±0.21**	5.92±0.13**	7.53±0.21	7.19±0.16	7.14±0.41
13	4.58±0.14	2.51±0.02	8.86±0.13**	8.24±0.08**	6.47±0.03**	6.06±0.24**	7.72±0.12	7.64±0.32	7.61±0.36
14	5.01±0.18	2.60±0.11	9.27±0.16**	8.62±0.14**	6.79±0.14**	6.16±0.21**	8.32±0.31	8.29±0.24	8.23±0.15
15	5.64±0.21	2.69±0.21	9.76±0.12**	9.01±0.16**	7.04±0.21**	6.34±0.23**	8.97±0.02	8.82±0.11	8.79±0.12

All values are Mean ± S.E.M.(n=6),

***p<0.001 compared to vehicle, ANOVA followed by post tests regardless of all p-values

The test drugs and the vehicle were administered once daily for 7 days in the unstressed group or 45 min before stress

Moris Water Maize

The data depicted in Table 3.19 showed the improvement in memory-dependent learning in all treated mice. The results in Table 3.19 revealed a significant increase in latency time in EA and CD in comparison with the corresponding control. All treated groups exhibited a significant decrease in the latency time as compared to the corresponding scopolamine treated mice. All the treated mice showed a significant decrease in the number of crossing over the platform position as compared to the corresponding control. The mice treated with SS and BT exhibited a significant increase in the number of this activity as compared to the scopolamine treated mice.

Table 3.19 Effect of methanolic extracts of Shankhpushpi botanicals on scopolamine induced amnesia by Moris water maize

S. No.	Treatment # (mg/kg)	Escape Latency	
		60 Seconds	Crossing the platform in 7 trials
1	Vehicle	32.89 ± 6.74	5.66 ± 0.33
2	Piracetam-100	19.22± 0.21	6.33 ± 0.33
3	MEEA	23.08± 0.02	5.33 ± 0.33
4	MECP	36.05± 0.12	2.33 ± 0.33
5	MECT	30.02± 0.09	2.66 ± 0.33
6	MECD	26.03± 0.07	4.66 ± 0.33
7	SS	27.26± 0.11	4.33 ± 0.33
8	BT	28.04± 0.09	3.66 ± 0.88
9	Scopolamine	48.52± 0.25	1.66 ± 0.33

All values are Mean ± S.E.M. (n=6),

*p<0.5, **p<0.01, ***p<0.001 compared to vehicle, ANOVA followed by post tests regardless of all p-values

The test extracts and the vehicle were administered once daily for 7 days. The trials were undertaken 45 min after the administration

Test for nootropic activity

The Cook and Weidley's pole apparatus used the number of ARs as an index for studying the nootropic activity. Piracetam (100 mg/kg p.o.), the marketed formulation (400 mg/kg p.o.), and methanolic extracts of various Shankhpushpi botanicals (400 mg/kg p.o.) of the drugs administered for 7 days showed a statistically significant increase in the number of ARs in the TTs as well as in the RTs.

Petroleum Ether Extract						
19	PEEEA	37.27 ± 0.67	52.20 ± 0.52	62.57 ± 0.80	79.55 ± 0.67	58.24 ± 2.72
20	PEECP	38.03 ± 0.53	54.21 ± 0.45	67.38 ± 0.65	83.90 ± 0.35	48.75 ± 1.35
21	PEECT	34.08 ± 0.08	53.44 ± 0.85	68.83 ± 0.30	78.36 ± 1.38	55.49 ± 1.72
22	PEECD	25.36 ± 0.55	53.20 ± 0.55	75.89 ± 0.86	80.31 ± 0.65	62.09 ± 2.10
Chloroform fraction						
23	CFEA	24.91 ± 0.68	56.29 ± 1.37	66.60 ± 1.06	73.26 ± 0.56	70.13 ± 4.36
24	CFCP	25.93 ± 0.84	52.56 ± 1.42	70.61 ± 0.97	82.49 ± 0.78	66.00 ± 3.61
25	CFCT	44.64 ± 0.50	64.29 ± 0.30	73.23 ± 0.21	89.32 ± 0.38	14.66 ± 1.79
26	CFCD	24.71± 0.51	56.43 ± 1.01	67.37 ± 1.14	75.00 ± 0.86	68.69 ± 3.58
Methanolic extract						
27	MEEA	10.86 ± 0.95	28.65 ± 1.15	64.28 ± 1.08	69.55 ± 0.53	114.35 ± 0.99***
28	MECP	40.96 ± 0.86	63.51 ± 0.32	83.76 ± 0.52	86.84 ± 0.40	19.04 ± 0.78***
29	MECT	41.92 ± 0.18	59.37 ± 0.26	71.33 ± 0.48	84.08 ± 0.13	28.39 ± 1.24***
30	MECD	10.65 ± 0.37	54.73 ± 1.09	70.52 ± 0.57	79.43 ± 0.68	81.76 ± 2.07***
Successive Aqueous Extract						
31	AEEA	31.18 ± 0.56	51.35 ± 0.67	59.23 ± 0.26	67.10 ± 0.54	80.91 ± 2.51
32	AECP	24.09 ± 0.38	44.32 ± 0.63	61.15 ± 0.58	67.29 ± 0.53	97.34 ± 1.13
33	AECT	28.24 ± 0.68	46.43 ± 0.61	60.53 ± 0.45	70.09 ± 0.47	87.48 ± 2.20
34	AECD	24.92 ± 0.71	57.86 ± 0.85	66.66 ± 0.34	73.36 ± 0.57	67.88 ± 2.41
Marketed Formulation						
35	SS	23.75 ± 1.22	55.71 ± 0.72	65.39 ± 1.34	82.73 ± 1.03	69.60 ± 2.74***
36	BT	39.90 ± 1.05	63.94 ± 0.45	71.85 ± 1.03	84.20 ± 0.44	23.65 ± 4.80***

All values are Mean ± S.E.M. (n=3),

***p<0.0001, ** p< 0.001 and *p<0.05 compared to vehicle, One way ANOVA followed by Dunnett's Multiple Comparison post tests regardless of all p-values

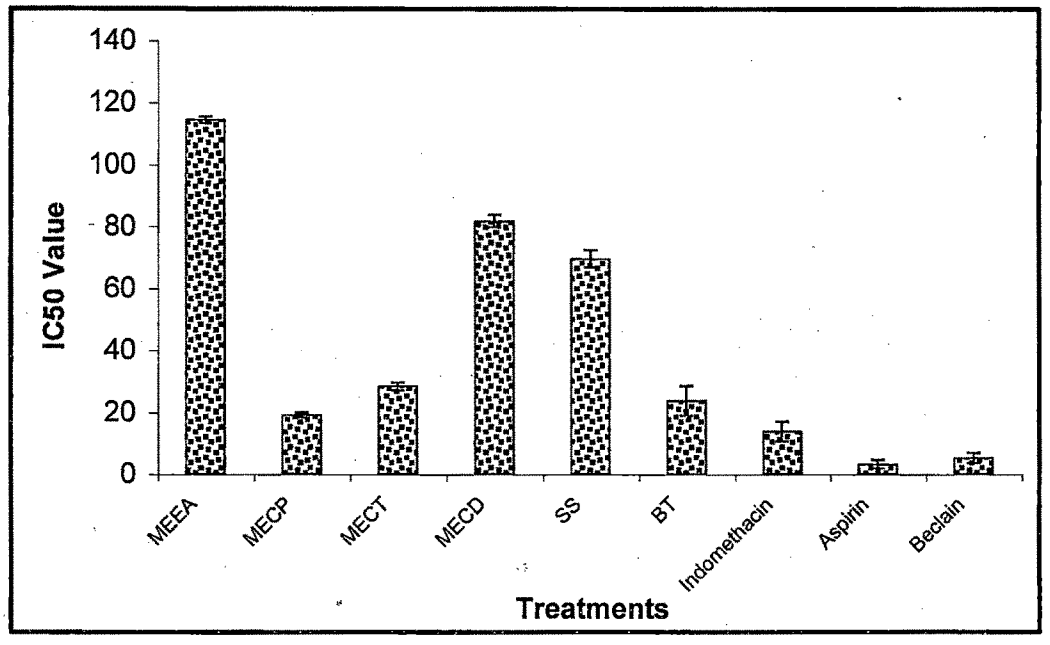


Fig. 3.16 LOX inhibition potential of various extracts of *Shankhpushpi* botanicals and their marketed formulation

3.1.7 Anti-malarial activity

In India plants have always been used for the treatment of malaria in traditional medicine. However, they need scientific validation in laboratory. The order of activity were found to be EA>CP>CD> CT. Brain tab, a marketed formulation was also find to be effective against this enzyme (Table 3.22 and Figure 3.17).

Table 3.22 Anti-malarial activity of various extracts of *Shankhpushpi* botanicals and their marketed formulation

Treatment# (mg/kg)	Step down latency on Day 15 Inflexion ratio (L_{15}- L_1/ L_1)
Vehicle	4.06±1.21
Gossypol	49.24±0.52
MEEA	25.04±0.51
MECP	18.32±0.24
MECT	17.32±0.12
MECD	19.26±0.32
SS	23.86±0.12
BT	24.05±0.17

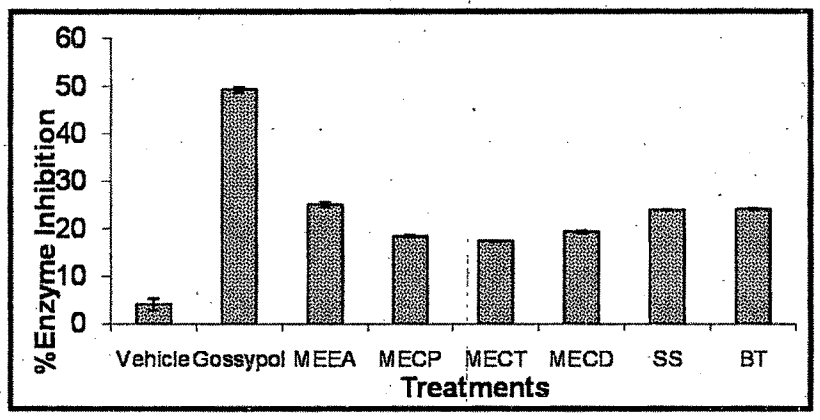


Fig. 3.17 Anti-malarial activity of various extracts of *Shankhpushpi* botanicals and their marketed formulation

3.1.8 ANTI-MICROBIAL ACTIVITY

Antimicrobial activities were performed among various species of *Shankhpushpi*. In this investigation, each fraction of methanolic extracts was screened against four strains of pathogenic bacteria by using Agar Well Diffusion Method (Table 3.23). Inhibition zone of diameter in millimeter was represented as the degree of activity. Out of these tested extracts only CD shows some inhibition zone against *Bacillus subtilis.*

Table 3.23 Anti-microbial activity of various extracts of shankhpushpi botanicals and their marketed formulation

S. No.	Treatment	Zone of clearance			
		Bacterial strain			
		Gram positive		Gram Negative	
		Bacillus subtilis	*Staphylococus aureus*	*Salmonella paratyphi B*	*Escherichia coli*
1	MEEA	NA	NA	NA	NA
2	MECP	NA	NA	NA	NA
3	MECT	NA	NA	NA	NA
4	MECD	1.40±0.08	NA	NA	NA
5	SS	NA	NA	NA	NA
6	BT	NA	NA	NA	NA
7	Ampicillin	7.69±0.51	---	----	---