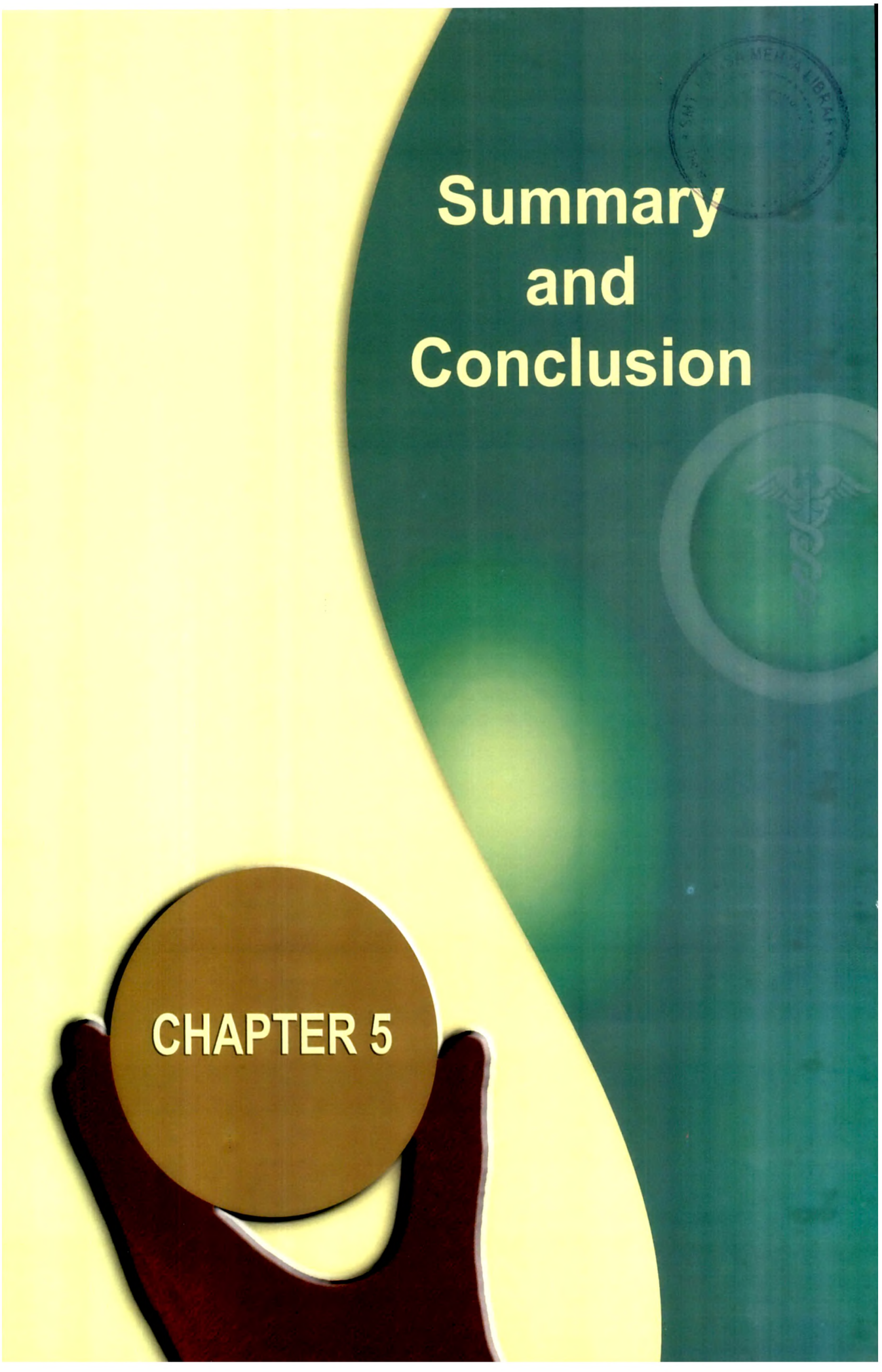
Summary
and
Conclusion
CHAPTER 5

In the last few decades there has been an exponential growth in the field of herbal medicine. It is getting popularized in developing and developed countries owing to its natural origin and lesser side effects. In olden times, *vaidyas* used to treat patients on individual basis, and prepare drug according to the requirement of the patient. But the scene has changed now; herbal medicines are being manufactured on a large scale in mechanical units, where manufacturers come across many problems such as availability of good quality raw material, authentication of raw material, availability of standards, proper standardization methodology of single drugs and formulations, quality control parameters, etc. Herbal drugs, singularly and in combinations, contain myriad compounds in complex matrices in which no single active constituent is responsible for the overall efficacy. This creates a challenge in establishing quality control standards for raw materials and the standardization of finished herbal drugs.

A big quantum of research work in the area of authentication of correct plant source has been undertaken to provide means of differentiation among many controversial available plants sources. These include the methods developed using morphological, histological, physico-chemical; especially heavy-metal estimation and radiobiological contamination in plants. However, over the years the nature and scope of these evaluations have changed. Initially it was considered sufficient to authenticate the plant material by comparison with a standard botanical description or monograph whereas in recent requirements detection of adulterants and utilization of the phytoconstituents as chemo or bio markers has been taken up as practice of prime importance. Estimation of the content of marker in Ayurvedic botanicals is of the utmost important in evaluating the phytochemical entity of the herb.

Ambiguity of our own system of medicine – the Ayurveda, is reflected in the interpretation of names and description of drugs given in the books like *Charaka Samhita* and *Sushruta Samhita*, etc. Before the establishment of British rule, like the other books, ayurvedic treatises were also hand written. This might be one of the reasons due to which ayurveda could not stand parallel to the western medicine Due to lack of scientific names in the original texts, under one name, different plants are

known in different parts of the country as per the description, which makes the drug controversial, e.g. *Jivanti*, *Brahmi* and *Shankhpushpi*.

Shankhpushpi is an ayurvedic drug used for its action on the central nervous system, especially for boosting memory and to improve intellect. Quantum of information gained from ayurvedic and other Sanskrit literature revealed the existence of four different plant species under the name of *Shankhpushpi*, which is used in various ayurvedic prescriptions described in ancient texts, singly or in combination with other herbs. The sources comprise of entire herbs with following botanicals viz., *Convulvulus pluricaulis* Choisy. (Convulvulaceae), *Evolvulus alsinoides* Linn. (Convulvulaceae), *Clitorea ternatea* Linn. (Papilionaceae) and *Canscora decussata* Schult. (Gentianaceae).

These are pre-European names that are applied to a medicinal plant. These synonyms have caused controversy in the identification of plants and hence the correct source sometime is misleading with a fictious plant. It has become terror and an important task to generate parameters of identification as well as differentiation among different plant sources having similar name. Since herbal product are prepared using the extracts of plant known for particular activity, the controversial source sometimes lead to inefficacious preparation. Hence generation of parameters based on characterization and identification of chemical component and their biological activity, using modern method may provide a solution for solving out the controversy. The current study was performed to develop an effective method for the identification and differentiation above mentioned botanicals claims of *Shankhpushpi*. The developed methods are summarized in briefs are:

- Comparative morphological and microscopical differencitiation
- Identification of various differencitiation parameters according to WHO guidelines
- Comparative TLC fingerprinting of different species
- Isolation of differentiation chemical component of various species
- Development of analytical methods for the qualitative and quantitative assessments:

- ✓ HPLC
- ✓ HPTLC
- ✓ Spectrofluriometry., etc

➢ Comparatives toxicity profiling of isolated compounds, fractions and extracts.

- ✓ Brine Shrimp toxicity assay
- ✓ MTT cytotoxicity assay on cell line

➢ Comparative in vitro antioxidant studies of isolated compounds, fractions and extracts.

- ✓ DPPH
- ✓ Ferric chloride
- ✓ Phsphomolybedate

➢ Comparative nootropic activity of isolated compounds, fractions and extracts.

In vitro model

- ✓ AChE inhibition assay
- ✓ AChE TLC bioautography
- ✓ β-amyloid induced neuroprotection on brain cell line.
- ✓ Serotonin receptor assay

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* anti-microbial studies.

PHARMACOGNOSTICAL AND PHYTOCHEMICAL INVESTIGATIONS

In present study, the parameter of differentiation based on comparative pharmacognosy and phytochemistry of various botanicals of ayurvedic medicine *Shankhpushpi*, reputed as brain tonic for its proper authentication has explored. The detailed systematic pharmacognostical and phytochemical evaluation of plant and plant material provides means of standardization of a herb that can be used as drug or as raw material. The macroscopic features, anatomical sections, surface preparations of the fresh leaves, stems, roots and powdered samples for the microscopy were carried out of the fresh plant of EA, CP, CD and CT according to methods reported by WHO. The major morphological identification parameters observed among plants were similar with previous reports. On comparison with the observations made on EA, CP, CD and CT usually available in commerce as *Shankhpushpi*, it becomes evident that there is a great similarity in habit, habitat and in the macro and microscopical features of their stem, leaves and root. They are small herbs with several branches bearing sessile and shortly petiole leaves. There are certain salient diagnostic characters (marked by underline) by which these plants can be differentiated from one another. Although EA and CP is much similar in characteristic, as they belongs to same family. The marked difference between EA and CP was absence of phloem fiber in former. There is well defined diagnostic profile of CD among all. The CT resemblance in some characters to EA and CP, but differ in lamina and stomata type. The maximum content of iron in EA and CD makes there use as drug of choice for iron deficient diseases. Formulation containing CD, although proves its potential in problems related with post menopausal disorders.

All the extracts of *Shankhpushpi* botanicals were evaluated for physical parameters such as consistency, color, odor and taste. Qualitative chemical tests were carried out for the methanolic extracts of various *Shankhpushpi* botanicals. The results

Table 5.1 Identification of presence of Known Markers by Co-TLC in *Shankhpushpi* botanicals

CP	CTB	EA	CD	SS	BT	CTW
Stigmasterol	Stigmasterol	Stigmasterol	Stigmasterol	Stigmasterol	Stigmasterol	Stigmasterol
Scopoletin	Lupeol	Ursolic acid	Lupeol	Ursolic acid	Lupeol	Lupeol
	Scopoletin	Scopoletin	Betullinic acid	Scopoletin	Betullinic acid	Scopoletin
			Ursolic acid		Ursolic acid	
			Scopoletin		Scopoletin	
			Mangiferin		Mangiferin	

Various HPLC fingerprint of all available *Shankhpushpi* samples along with two marketed formulation were shown that EA has similar fingerprinting profile with *Shankhpushpi* syrup and the fingerprint of CD also matches in major peaks with Brain tab.

The column chromatography analysis for petroleum ether and methanolic extracts of *Shankhpushpi* botanicals leads of various fractions, some of which were on further processing shows the presence of a single spot. These fractions were mixed and crystallization was done with methanol. This was further purified with the aid of preparative chromatography or washing in fresh column and ultracentrifugation to get pure compound. Various isolated crystals obtained were recrystalized with acetone and collected.

These crystals showed R_f value similar to that of various identified chemical used for co-TLC drug. Further the FT-IR, analysis of the isolated compound confirmed its identity with the standard markers. The FTIR analysis of a mixture of pure compound and isolated compound also showed similar peaks. This indicated that the isolated compound is same to that of standard. Furthermore the melting points, HPTLC and UV absorption maxima of the standard and the isolate were found to be in the same range, indicating their similar chemical identity. EA-1, EA-2, EA-3 and scopoletin were isolated from *Evolvulus alsinoids* and characterized as stigmasterol, betulinic acid, β-carotene and scopoletin. CP-1 and scopoletin were isolated from *Convolvulus pluricaulis* and characterized as stigmasterol and scopoletin. CT-1, CT-2,

CT-3 and rutin were isolated from *Clitorea ternatea* and characterized as stigmasterol, lupeol, ursolic acid and rutin. CD-1, CD-2 and mangiferin were isolated from *Canscora decussata* and characterized as stigmasterol, ursolic acid and mangiferin.

These studies, thus, proved to be successful for the differentiation of *Shankhpushpi* botanicals on the basis of their morphological, microscopical and phytochemical basis. These studies paved the way for the development of standardization parameters for the different varieties of *Shankhpushpi.*

BIOLOGICAL SCREENING

Brine shrimp is a primitive organism and most porn to toxicity. The Percentage cell survival is tested for various purified compounds and methanolic extracts of various *Shankhpushpi* botanicals. The majority of the extracts and compound tested showed good brine shrimp larvicidal activity according to Meyer et al. (1982), who classified crude extracts and pure substances into toxic (LC50 value < 1000 μg/ml) and non-toxic (LC50 value > 1000 μg/ml). Among the tested compound ursolic acid and stigmasterol has significant percentage toxicity then other compounds. Among the botanicals of *Shankhpushpi* the CP has greater toxicity then other. The order of activity among compounds towards toxicity are ursolic acid > Stigmasterol > Scopoletin > β-carotene > Mangiferin > Lupeol > Betaine > β-sitosterol > Chlorogenic acid. Among methanolic extracts of *Shankhpushpi* botanicals the order of activity toward toxicity were found to be CT > CP > EA > CD.

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. 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.

nicotine > stigmasterol. Among methanolic extracts of *Shankhpushpi* botanicals the order of activity toward DPPH were found to be CT > CP > EA > CD. Among isolates scopoletin and mangiferin were found to be most active.

The result shown by phytonutrients from commonly used plant based human food, FCRP 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 DPPH were found to be CT > CP > EA > CD. Among isolates chlorogenic acid, scopoletin and mangiferin were found to be most active.

The result shown by phytonutrients from commonly used plant based human food towards antioxidant by Phosphomolybdenum 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 antioxidant mechanism of various biomarker are depicted in figure 5.1

DPPH radical scavenging compounds appeared as yellow spots against a purple background. Densitogram of all reference standards (β-carotene, rutin, gallic acid and curcumin) after DPPH derivatisation exhibited concentration dependent reduction in peak area. 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.

chlorogenic acid could provide valuable inference. The potency of identified antioxidants followed the order scopoletin>rutin>β-carotene>chlorogenic acid in this method. Mangiferin was found to react very slowly with DPPH providing very minute reduction in peak area hence it was not quantified. However mangiferin identification with DPPH derivatization can be utilized for qualitative purpose. The differentiable antioxidant markers were quantified before and after DPPH derivatization to find if identification and quantification can be estimated simultaneously, but the quantification after DPPH derivatization showed lot of variation. However quantification after DPPH can be used to assess the relative antioxidant potency within the individual constituents of extracts/fractions. The marketed formulation (Brain tab) showed the presence of scopoletin and mangiferin providing the evidence for the presence of *Canscora*. The analyses of marketed formulation (Brain tab) proved again the utility of this method in identifying the claimant used as *Shankhpushpi*.

Table 5.2 Antioxidants for differentiating the four claimants of *Shankhpushpi*

Botanical claimants of Shankhapushpi and its marketed Formulation	β-carotene	Rutin	Mangiferin	Chlorogenic acid	Scopoletin
Convolvulus pluricaulis	-	-	-	-	+
Evolvulus alsinoides	+	-	-	+	+
Clitorea ternatea	+	+	-	-	+
Canscora decussata	+	-	+	-	+
Brain Tab	-	-	-	-	+

*(+) – present; (-) – absent

Acetylcholine (ACh) is one of the major neurotransmitters in the central nervous system (CNS). The cerebral cortex contains a dense plexus of cholinergic axon terminals that arise from the cells of the basal forebrain including the nucleus basalis of Meynert. Degeneration of this cholinergic projection is recognized as one of the most prominent pathologic changes in Alzheimer's disease (AD).

The results for AChE assay suggest that EA have great affinity to inhibit AChE then other. The order of inhibitory activity was EA>CD> CP> CT. In literature all plants exhibit strong potential for memory enhancement. In order to find solution for this ambiguity the piracetam were evaluated for AChE activity. It was found in results that there is no or null activity of this molecule in AChE inhibition. So from this, it is hypothesized that rest of the plant extracts may act in the memory other then AChE inhibition. The AChE inhibition activity observed for the potent extracts partly offers evidence for use of these herbs in traditional medicine to enhance cognition or to correct cognitive decline. *Evolvulus alsinoids*, which showed good activity is a very popular plant used for improvement of memory and cognition enhancement in Indian traditional medicine. The AChE inhibitory principles in the methanolic extracts are reported to be scopoletin. It is also is shown to be neuroprotective especially to the hippocampal region in stress-induced neurodegeneration. In light of the present results one could expect *Evolvulus alsinoids* to be helpful in neurodegenerative conditions like AD.

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 Rf. TLC bioautography of active plants revealed active spots at R_f 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*). The white spots pertained to EA and CD appeared more rapidly.

Alzheimer's disease is characterized by the presence of two types of abnormal deposits, senile plaques (SPs) and neurofibrillary tangles, and by extensive neuronal loss. β-amyloid is a major element of SPs and one of the candidates for the cause of the neurodegeneration found in AD. It has been shown that the accumulation of Ab precedes other pathologic changes and causes neurodegeneration. The results for β-amyloid induced neuroprotection suggest that EA and CD have great affinity to protect the neurons. The order of activity was EA>CD> CP> CT.

5-HT2B receptor was found mainly in brain and act for most of brain related disorder. Systemic administration of *Shankhpushpi* botanicals shows that CT and EA have agonist action at 5-HT2B receptor with some selectivity over other sites tested.

extract of EA and CD produced better retention and recovery 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 of EA treated groups was significant as compared to the groups receiving same doses. Thus, antiamnesic effects of EA and other *Shankhpushpi* botanicals on scopolamine-induced amnesia were successfully demonstrated through the study.

The improvement in memory-dependent learning by Moris water maize in all treated mice and 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.

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. The findings of the present study clearly indicate that the EA and CD at doses of 400 mg/kg and methanolic extract of marketed formulation at a dose of 400 mg/kg significantly improve the acquisition and retention of memory of the learned task as evident from the increase in the number of ARs, thus demonstrating nootropic activity.

Lipoxygenases (LOXs) comprise a family of non heme iron-containing dioxygenases, representing the key enzymes in the biosynthesis of leukotrienes that have been postulated to play an important role in the pathophysiology of several inflammatory and allergic diseases. The products of LOXs catalysed oxygenation [hydroperoxyeicosatetraenoic acids (HPETE), hydroxyeicosatetraenoic acids (HETE), leukotrienes and lipoxins] apparently are involved in the development of rheumatoid

arthritis, psoriasis, asthmatic responses and glomerular nephritis. The results of Lipoxygenase Inhibition again suggest entirely new story other then above mentioned i.e., CP exhibit significant inhibition then rest. The order of activity were found to be CP>CT>CD> EA.

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.

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. 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.*

ANALYTICAL METHOD DEVELOPMENT

Spectrofluorimetric method

Instruments that measure the intensity of fluorescence are called fluorimeters. Those that measure the fluorescence intensity at variable wavelengths of excitation and emission and are able to produce fluorescence spectra are called spectrofluorimeters. The spectrofluorimetric study was carried out with a Shimadzu RF- 5301 PC spectrofluorimeter, to determine levels of fluorescence in the coumarins in a stationary state. A preliminary analysis was carried out to determine the wavelength at which maximum intensity is shown by pure scopoletin and mangiferin. For these purpose, 100µg/ml samples of pure scopoletin and mangiferin were separately prepared in methanol. On the basis of this analysis a range of excitation and emission wavelength was determined for scopoletin and mangiferin. The λ_{max} shown by scopoletin for excitation was 430nm, and for the emission is 460 nm; On the other hand λ_{max} shown by mangiferin for excitation is 248 and for the emission is 520 nm. Standard curve of scopoletin and mangiferin was prepared in methanol. First

of all, stock solution containing 100µg/ml of scopoletin and mangiferin was prepared in methanol. Then this stock solution was used for preparing required dilutions containing 5µg, 10µg, 15µg, 20µg, 25µg and 30µg of scopoletin and mangiferin. The quantity of stock solution used was 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 ml respectively. The samples were analyzed in the spectrofluorimeter against solvent blank (methanol). The wavelength and intensity for each sample was recorded and standard curve was prepared between concentration and intensity of fluorescence.

Once the standard curve for both scopoletin and mangiferin were prepared using series of standard dilutions from 5 µg/ml to 30 µg/ml, the dilutions covering the detection limit of the instrument, it became feasible to estimate scopoletin and mangiferin in the herbal extracts by measuring their fluorescence intensity within the range of excitation and emission wavelengths for scopoletin, i.e. 430 to 460 nm and for mangiferin, i.e. 248 to 520 nm

After the success of spectrofluorimetric analysis in determining the concentration of scopoletin and mangiferin in methanolic extracts of *Shankhpushpi* botanicals, it was thought worthwhile to develop a method for the determination of scopoletin and mangiferin concentration in crude drug samples.

Thus a simple analytical method was developed which proved to be very crucial in estimating concentration of scopoletin and mangiferin simultaneously in various drug samples. The developed method was validated for specificity, reproducibility and accuracy. The method was found to be specific for scopoletin and mangiferin since both are fluorescent. Linearity range was found to be in the range of 5-30 µg/ml. The correlation coefficients (r) were 0.9919 and 0.9937 indicating good linearity between fluorescence intensity and concentration. Repeated scanning of the samples three times checked precision of the method. Carrying out a recovery study checked reproducibility and accuracy of the method. A known concentration of scopoletin and mangiferin were added to varying concentrations of the aqueous extract i.e. 0.1, 0.2, 0.5 and 1.0 µg/ml. The sample of known concentration was added in equal volume to the various dilutions of the extract and analyzed spectrofluorimetrically to see whether the practical concentration obtained is in

correspondence with the theoretical or hypothetical concentration from the standard curve. The percentage recovery for scopoletin and mangiferin was found to be in the range of 98- 102%. Hence this developed spectrofluorimetric method is quick and reliable for simultaneous quantitative monitoring of scopoletin and mangiferin in raw material, processed powder and in herbal preparations containing said botanicals of *Shankhpushpi.*

Simultaneous HPTLC method for estimation of scopoletin, mangiferin and rutin

The selected mobile phase simultaneously resolved scopoletin, mangiferin and rutin effectively. The R_f of scopoletin, mangiferin and rutin were found to be 0.75, 0.12 and 0.06 respectively (Figure. 4.3). The fingerprint of scopoletin, mangiferin and rutin in various differentiated shankhpushpi botanicals and marketed sample were shown in figure 4.5 and 4.6. The calibration plots (Figure 4.8) were linear in the range 200–1200 ng, and the correlation coefficient of 0.9579, 0.9979, and 0.9962 were indicative of good linear dependence of peak area on concentration. The calibration curve was represented by the linear equation $y = 177.4 + 1.78\ x$, $y = 486.2 + 9.846\ x$ and $y = 522.2 + 4.652\ x$ respectively (where y is the response as peak area and x is the concentration).

To ascertain the effectiveness of the method, suitability tests were performed on a freshly prepared mixture of standard stock solution of scopoletin, mangiferin and rutin spiked with preanalysed with identified extract of shankhpushpi botanicals and marketed formulation. The repeatability of sample application and measurement of peak area were expressed in terms of R.S.D. % and the percentage relative standard deviations for intra- and inter-day. Intra-day precision (%RSD) on the basis of content of scopoletin, mangiferin and rutin were between 0.035-0.150, 0.012-0.037 and 0.020-0.041. Inter-day precision (%RSD) on the basis of content were between 0.040-0.165, 0.009-0.045 and 0.015-0.077 respectively.

The standard deviation of peak areas was calculated for each parameter and R.S.D. % was found to be less than 2%. The low values of R.S.D. %, indicated robustness of the method.

The LOQ and LOD were calculated from the equations LOD = 3xN/B and LOQ = 10xN/B, where N is the SD of the peak area of the standard (n = 3), taken as a measure of the noise, and B is the slope of the corresponding calibration curve.

The peak purity of individual scopoletin, mangiferin and rutin were assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot, i.e., *r* (start, middle) = 0.9991 and *r* (middle, end) = 0.9993 and the overlain spectra. The peak purity of scopoletin, mangiferin and rutin were assessed by comparing the spectra of standard at peak start, peak apex and peak end positions of the spots, i.e., *r* (start, middle)= 0.9973 and *r* (middle, end) = 0.9979. Good correlation (*r* = 0.9994) was also obtained between standard and sample overlain spectra.

The proposed method may used for extraction and subsequent estimation of scopoletin, mangiferin and rutin from pharmaceutical dosage form after spiking it with 100 ng/spot and 200 ng/spot of additional standards. The recoveries obtained were in the range 99.67–100.95%, showing the reliability and reproducibility of the method.

Simultaneous HPTLC method for estimation of ursolic acid, betullinic acid, stigmasterol and lupeol

The selected mobile phase simultaneously resolved ursolic acid, betullinic acid, stigmasterol and lupeol effectively. The R_f of ursolic acid, betullinic acid, stigmasterol and lupeol were found to be 0.24, 0.31, 0.38 and 0.54 respectively. The calibration plots were linear in the range 100–600 ng, and the correlation coefficient of 0.98251, 0.96899, 0.99156 and 0.95121 were indicative of good linear dependence of peak area on concentration. The calibration curve was represented by the linear equation y = 3581 + 1.585 x, y = 4862 + 0.6321 x, y = 71.25 + 637.4 x and y = 2626 + 7.638 x respectively (where y is the response as peak area and x is the concentration).

To ascertain the effectiveness of the method, suitability tests were performed on a freshly prepared mixture of standard stock solution of ursolic acid, betullinic acid, stigmasterol and lupeol spiked with preanalysed with identified extract of shankhpushpi botanicals and marketed formulation. The repeatability of sample application and measurement of peak area were expressed in terms of R.S.D. % and