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TITRE DE LA THÈSE - TITLE OF THE THESIS

Phytochemical Basis for the Anxiolytic Activity of the Ayurvedic Medicinal Plant *Centalla Asiatica* (L.) Urb. (Gotukola)

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PHYTOCHEMICAL BASIS FOR THE ANXIOLYTIC ACTIVITY OF THE AYURVEDIC MEDICINAL PLANT *CENTELLA ASIATICA* (L.) URB. (GOTUKOLA)

Priyantha Wijeweera

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies

in partial fulfillment of the requirements for the degree of
Master of Science

in the Ottawa-Carleton Institute of Biology

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Abstract

Gotukola (*Centella asiatica* L. Urban) (Apiaceae), its extracts and the pure compound asiaticoside were studied for anxiolytic activity in thirteen standardized rat trials. High performance liquid chromatography (HPLC) was used to conduct the phytochemical analysis. Among different models tested, the most promising positive response for anxiolytic activity was observed in the elevated plus maze test conducted with: a) whole plant materials, b) ethyl acetate and methanol fractions and c) asiaticoside. The results show for the first time that asiaticoside and triterpene enriched fractions of gotukola have anxiolytic effects in animal models. Therefore, they are recommended for clinical trials. The findings of this study also support the ayurvedic use of gotukola for psychiatric disorders.

Other supplementary investigations conducted show that methyl jasmonate and full sunlight enhance the expression of asiaticoside in gotukola plants. The stolon explants were more successful compared to the leaf explants in *in vitro* propagation of gotukola.

Résumé

Les extraits phytochimiques de gotukola (*Centella asiatica* L. Urban) (Apiaceae) et le composé pur, asiaticoside, ont été étudiés pour leur activité anxiolytique à l'aide de treize tests standardisés sur des rats. Les analyses phytochimiques ont ensuite été effectuées par chromatographie liquide haute performance (HPLC). Les rats ont été traités avec la plante entière, les extraits d'acétate d'éthyle et de méthanol ou l'asiaticoside pur et parmi les différents modèles expérimentaux, la réponse la plus prometteuse quant à l'activité anxiolytique a été observée lors du test « elevated plus maze ». Les résultats montrent pour la première fois que l'asiaticoside et les fractions riches en triterpènes de gotukola possèdent des effets anxiolytiques dans les modèles animaux. Par conséquent, ils sont recommandés pour des tests cliniques. Les découvertes de cette étude supportent aussi l'utilisation en science ayurvédique de cette plante pour les désordres psychiatriques.

D'autres investigations ont démontré que les stolons sont plus efficaces que les feuilles pour la propagation *in vitro* de gotukola, et que le jasmonate de méthyle ainsi que le plein soleil stimulent l'expression de l'asiaticoside dans la plante.

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Since the inception of this project concept, I had to overcome several inevitable challenges to bring it to a successful completion. Being a researcher started a fresh life in a new country without any initial personal or professional contacts it was never easy at the beginning. However, I was privileged to get the support of several institutes and individuals to make the project concept a reality on its own merits.

I am grateful to the International Development Research Center (IDRC) for approving and providing me with funds for 3 years for my initial project proposal with the United Nations Association in Canada (UNAC) and also later extending the project to the University of Ottawa (UOO), enabling me to conduct research work leading to a graduate degree during 2000-2002. The professional assistance of Dr. Gisèle Morin-Labatut of IDRC and Drs. Harry Qualman and Alan Clarke of UNAC was an inspiration to develop and implement the project. The early support extended by the Ottawa-Carleton District School Board, Canadian Agriculture Museum, Experimental Farm, Ottawa Botanical Garden Society and several community groups in Ottawa is commendable.

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CHAPTER 1

GENERAL INTRODUCTION, LITERATURE REVIEW AND OVERVIEW OF THE STUDY OF GOTUKOLA (*CENTELLA ASIATICA* L. URBAN)

1.1 Introduction

Plants that have proven to be useful in the treatment of psychological disorders are identified as psycho-active medicinal plants. Herbal remedies provide an alternative approach to conventional psychiatric treatments. These herbal remedies may produce changes in mood, thinking capability and behaviour. They may also interact with psychiatric medications (Bloomfield, 1998). In bioassay studies, psychoactive plants are assessed for their phytochemistry, safety issues and side effects, drug interactions and efficacy in treating target symptoms or diagnosis.

There has been a worldwide growing demand for natural products. At present, about 25% of the pharmaceutical drugs contain phytochemicals directly extracted from higher plants or their derivatives (Soejarto and Farnsworth, 1989). It is estimated that 25- 42% of the Canadian population uses the services of complementary medicine systems or self medicate with natural health products. In particular, many of them use herbs or herbal products. The booming Canadian market for herbal products is estimated at over US$ 350 million per year (Brevoort, 1998) and during the year ending September 2000, the market increased by 20% compared to the same period in the previous year (Briggs and Briggs, 2001). In the highly multicultural Canadian society the influence of different ethnic groups and their inherent herbal medicine practices have

a significant impact on the health care system. The First Nations holistic medicine system, European herbalism, Chinese medicines and the ayurvedic system are some of more prominent practices.

Gotukola (*Centella asiatica* L. Urban) (Apiaceae) is an ayurvedic medicinal plant used for centuries, in several therapeutic applications. Its uses for mental disorders and enhancing memory are well documented in the ancient ayurvedic literature (Sushruta ~600 BC). Its application in wound healing is well known and one of its triterpenic compounds – asiaticoside, possesses significant wound healing activity (Shukla et al, 1999a and Shukla et al, 1999b). The scientific basis of these well documented ayurvedic applications is also supported by several studies conducted by different research groups during the last 40-50 years. One study conducted by researchers at the Royal Ottawa (psychiatric) Hospital was a double blind placebo-controlled clinical trial which suggested that gotukola may impart anxiolytic activity in humans, as revealed by the acoustic startle response test (Bradwejn et al, 2000). For this investigation 12 g of gotukola dried plant materials (equivalent to more than twenty-four 500 mg commercial tablets) had to be administered orally in a single dose, which is cumbersome with human subjects. If such studies on gotukola are to be further continued, it is important to search for plant materials or extractions with a higher concentration of the suspected psychoactive compounds.

The focus of the current study was to further investigate the phytochemistry and anxiolytic properties of gotukola that may potentially lead to discover a novel pharmaceutical drug for anxiety disorders in the future.

1.2 Literature Review

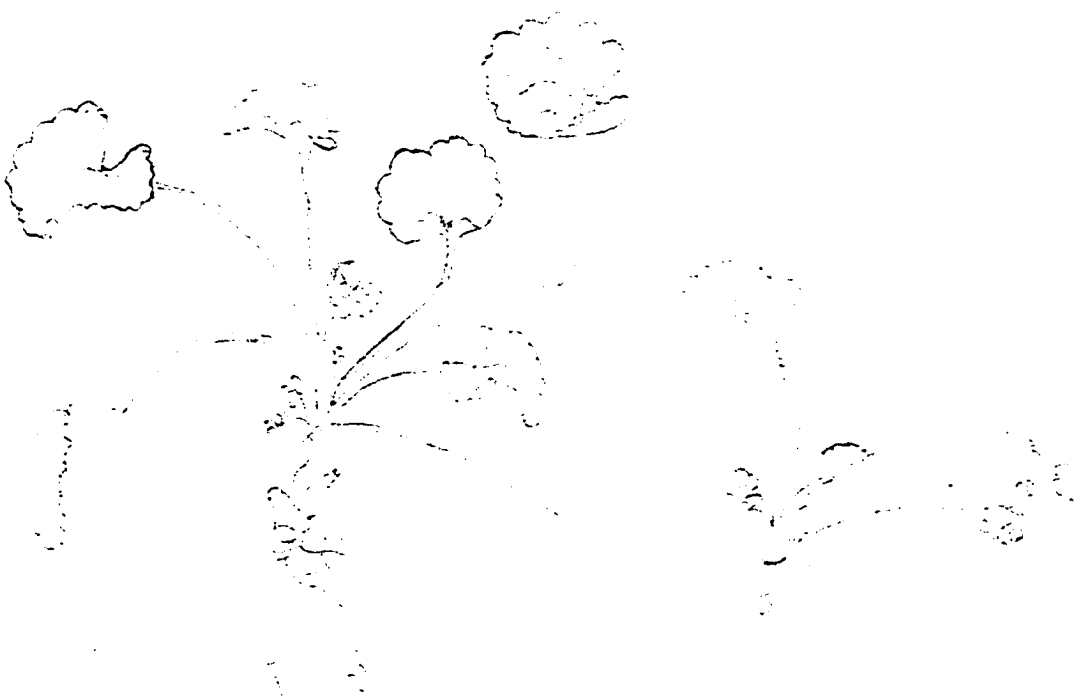
1.2.1 Botany, Ethnobotany, Distribution and Agronomy of Gotukola (*Centella asiatica*)

Gotukola is a perennial creeping herb that belongs to the family Apiaceae (carrot family) and subfamily Hydrocotyloideae. The leaves possess long slender petioles (5-15 cm) arising from a common node and nodes are connected by slender stolons (runners) (Figure 1.1). The leaves are greenish in color, thin with slightly lobed leaf margins, 2–5 cm in diameter, palmate in venation and hairless or with only a few hairs. The flowers are light violet in color. The umbels have short pedicels and originate from the leaf axils. The 2-5 fruits of each umbel are surrounded by a pericarp. Very poor germination problems in propagation of plant by seeds are associated with polyploidy (Lopes et al, 1996).

The plant is widely distributed in Southeast Asian countries (India, Sri Lanka, Bangladesh), China, Malaysia, Madagascar, South Africa and East Africa. It is also found in Southeast parts of the USA, Mexico, Venezuela, Columbia and Brazil (Brinkhaus et al., 2000). Gotukola is a tropical plant, which thrives well in moist rich soil and tolerates shady conditions. It is usually propagated by runners. Under good agronomic conditions the crop can be harvested 2-3 months after planting. Three varieties of gotukola exist; v. *abyssinica* (East Africa), v. *typica* (South Asia and Madagascar) and v. *floridana* (America) (MMP Inc., 2001). Other than the more frequently used common name 'gotukola', different botanical synonyms as well as common names are recorded in the literature (Table 1.1).

Other species of the genus Centella include; *C. crytocarpa*, *C. gymnocarpa*, *C. longifolia*

Figure 1.1: Illustration of gotukola (*Centella asiatica* L. Urban) x ½ (Drawn by U. L. Dharmasena).



(Shubert and Wyk, 1995a), *C. rupestris*, *C. restioides* and *C. thesioides* (Schubert and Wyk, 1995b). At present, only the *asiatica* species of the genus *Centella* is commercially important as a medicinal plant (Brinkhaus et al, 2000).

In the Sinhalese language "gotu" means cup-shaped, "kola" means leaves, suggesting the morphological features of the leaves. In ancient ayurvedic texts written in Sanskrit, it is referred to as "manduka parni". Since gotukola is also identified as "brahma-manduki", sometimes it is confused with "brahmi" - which is another psychoactive ayurvedic medicinal plant (*Bacopa monniera*).

'Gotukola-kenda' is a traditional Sri Lankan herbal drink where gotukola juice is cooked with unpolished red-rice, curry-leaves, garlic, salt and milk or coconut milk and usually served with 'kithul jaggery'. The herb is believed to help awaken the gateway to spiritual awareness. In Buddhist monasteries 'gotukola-kenda' is served as a vegetarian breakfast for monks, who stop consuming any solid food from noon until the next morning and engage in deep meditation. In Sri Lanka, fresh gotukola is a popular salad vegetable and a mixture of fresh gotukola and red onion is included in the diet of catarrh patients.

In ayurveda, the therapeutic properties of the plant are identified to treat *vata doshas* that create mental disorders. *Manduka-parni Rasayana* is such a gotukola-based ancient medication (Sushruta, ~600 BC). Other such ancient uses include as a remedy for infectious, dermatological and venous system diseases, wound healing and as a nerve tonic for longevity. It is listed in the Indian Pharmaceutical Codex, British Homeopathic Pharmacopoea (BHP 83) and Homoeopathisches Arzneibuch 1, 1978, Germany (HAB 1) (Gunther and Wagner, 1996).

Table 1.1: Synonyms and common names of *Centella asiatica* (adapted from Brinkhaus et al, 2000 and Plants for a Future Database, 2000 and modified).

Synonyms:	*Hydrocotyle asiatica* L., *Hydrocotyle lunata* Lam., *Centella coriacea* Nannfd., *Centella cordifolia* (Hooker) Nannfd., *Centella dusenii* Nannfd., *Centella floridana* (C. et R.) Nannfd., *Centella repanda* (Pers.) Small., *Centella triflora* (R. et P.) Nannfd., *Centella uniflora* (Col.) Nannfd.
Common Names:	
Chinese	Luei Gong Gen, Tungchian
English	Indian Pennywort
French	Hydrocotyle asiatique
German	Asiatischer Wassernabel
Indonesian;	
Jawa	Kaki kuda, Pegagan, Antanan gede, Gagan-gagan, Gang-gagan, Kerok batok, Panegowan, Rendeng, Caligan rambat, Kos tekosan
Sulawesi	Pagaga, Tungke-tungke
Bali	Papaiduh, Pepiduh, Piduh
Flores	Puhe beta, Kaki kuta, Tete karo, Tete kadho
Italian	Idrocotile
Japanese	Tsubo-kusa
Mauritius	Bavilacqua
Sanskrit	Manduka-parni
Sinhalese	Gotukola
Spanish	Blasteostimulina

1.2.2 Phytochemistry of *Centella asiatica*

1.2.2.1 Biosynthesis of Triterpenes

Triterpene biosynthesis in gotukola (*Centella asiatica*) occurs via the mevalonic acid pathway. This metabolic pathway proceeds from acetyl CoA via mevalonic acid. The basic five-carbon units are synthesized from acetyl CoA, where each five-carbon unit needs three molecules of acetyl CoA. Acetyl CoA condenses with acetoacetyl CoA to give 3-hydroxy-3-methylglutaryl CoA after hydrolysis. Then mevalonic acid is formed via mevaldic acid by the two-step reduction of 3-hydroxy-3-methylglutaryl CoA with NADPH. The mevalonic acid is pyrophosphorylated and then decarboxylated and dehydrated to produce isopentanyl pyrophosphate (IPP). IPP and its isomer dymethylallyl pyrophosphate (DMAP) are the activated five carbon starter units of terpenes that combine to form more complex larger molecules. The ten-carbon precursor of almost all the monoterpenes is produced as the result of the reaction between IPP and DMAP. The precursor of nearly all the sesquiterpenes is synthesized when geranyl pyrophosphate links to another molecule of IPP to yield farnesyl pyrophosphate (FPP). Finally, triterpenes are produced from two FPP units (Taiz and Zeiger, 1998 and Torssell, 1983).

In the mevalonic acid pathway, several regulatory enzymes have been identified. They are: (i). Thiolase (converts acetyl CoA to β-ketobutyrl CoA), (ii). HMG-CoA synthase (converts β-ketobutyrl CoA to β-hydroxy- β-methyl-glutaryl CoA-HMG-CoA), (iii). HMG-CoA reductase (converts HMG-CoA to mevalonic acid), (iv). MVA-kinase (converts mevalonic acid to mevalonic acid-5PP-MVA-PP), (v). Anhydrodecarboxylase (converts MVA-PP to IPP) and (vi). IPP isomerase (converts IPP to DMAP) (Taiz and Zeiger, 1998). The genes for these enzymes are now cloned from fir or peppermint. The specific enzymes that produce asiaticoside have not

been characterized.

1.2.2.2 Proximate Analysis and Phytochemical Constituents of *Centella asiatica*

Nutritional analysis of fresh gotukola leaves (100 g) reveal: 34 calories, 89.3 g water, 1.6 g protein, 0.6 g fat, 6.9 g carbohydrate, 2.0 g fibre, 1.6 g ash, 170 mg Ca, 30 mg P, 3.1 mg Fe, 414 mg K, 6580 μg β-carotene, 0.15 mg thiamine, 0.14 mg riboflavin, 1.2 mg niacin and 4 mg ascorbic acid (Duke, 1989).

Several saponins (Table 1.2), triterpenic acids (Table 1.3), polyacetylenes, sterols, flavanoids and nitrogen containing constituents have been identified from the gotukola plant. Several phytochemical methods have been developed to analyse and identify the important secondary plant metabolites of gotukola (Srivastava et al, 1997b). Kuroda et al (2001) isolated five new triterpene glycosides together with kaempferol and quercetin. In 1940, the most important saponin of the plant – asiaticoside was isolated and purified by Bontemp (Brinkhaus et al, 2000). Asiaticoside(2,3,23-Trihydroxyurs-12-en-28-oic acid *O*-6-deoxy-α-L-mannopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl ester) (Figure 1.2) is a saponin steroid, synthesized as rhamno-glucosyl derivative of the triterpenic acid – asiatic acid; (Budavari et al, 1989). The structures of asiaticoside-A and asiaticoside-B were elucidated by Sahu et al (1989).

Asiaticoside has been isolated also from *Schefflera octophylla* bark and identified by comparison of ^{1}H NMR and ^{13}C NMR data with those of asiaticoside isolated from gotukola. *S. octophylla* is used as an anti-inflammatory and analgesic tonic in Vietnam (Maeda et al, 1994). Table 1.2 lists saponins isolated from gotukola.

Several pentacylic triterpenic acids including asiatic, madasiatic and brahmic acid (Singh and Rastogi, 1968) have been isolated and characterized from *Centella asiatica.* They occur either in free state or as aglycones of the naturally occurring saponins (Table 1.3).

A fatty green colour essential oil found in gotukola possesses the strong odour of the original herb. The fatty oil consisted of glycerides of oleic, linoleic, linolenic, palmitic, stearic and lignoceric acid. The bitter principle, vellarine and peptic acid were reported from leaves and roots. The herb contains ascorbic acid in a concentration of 13.8 mg/100g. The plant also possesses the phytosterols sitosterol, stigmasterol, stigmasterone and stigmagsterol-β-D-glucopyranoside. An unsaturated acid dotriacont-8-en-1oic acid and a cyclohexane derivative 11-oxoheneicosanyl-cyclohexane have also been isolated and characterized from this plant (Srivastava et al, 1997b).

Several nitrogen-containing constituents are identified from gotukola. An alkaloid hydrocotylin has been isolated from the plant and it has also yielded glycine, aspartic acid, glutamic acid, alanine and phenylalanine. Glutamic acid, serine and alanine occurred in larger quantities than other amino acids in the leaves, petioles and stolons. In the roots, relatively large amounts of glutamic acid, threonine, alanine, glycine, histidine and aminobutric is found. Two-dimensional TLC and gas chromatography has demonstrated the presence of free lysine, glutamic acid and serine (Srivastava et al, 1997b).

Polyacetylenes are characteristic of the family Apiaceae. Several C_{15} polyacetylene compounds with alcoholic OH and acetyl functions have been isolated from the plant (Figure 1.3). The leaves contain hyperin a flavonoid compound, which is also found in St. John's wort. The leaves contain 3-glucosyl-quercetin, 3-glucosyl-kaemferol and 7-glucosyl-kaempferol (Srivastava et al, 1997b).

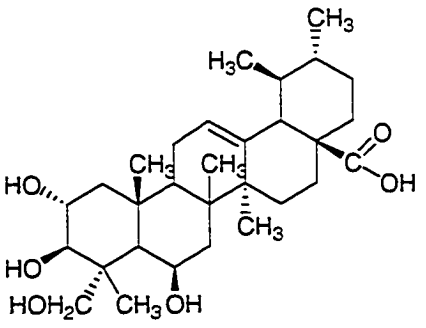
Table 1.2: Saponins isolated from *Centella asiatica* (prepared from Sahu et al, 1989 and Srivastava et al, 1997b)

Saponin	Constituent of saponin
Asiaticoside	Asiatic acid, glucose, rhamnose
Madecassoside	Madecassic acid, glucose, rhamnose
Centelloside	Centillic acid, glucose, fructose
Brahmoside	Brahmic acid, glucose, rhamnose, arabinose
Brahminoside	Brahmic acid, glucose, rhamnose, arabinose
Thankuniside	Thankunic acid, glucose, rhamnose
Isothankuniside	Isothankunic acid, glucose, rhamnose
Asiaticoside-A	6β-Hydroxyasiatic acid, glucose, rhamnose
Asiaticoside-B	Terminolic acid, glucose, rhamnose

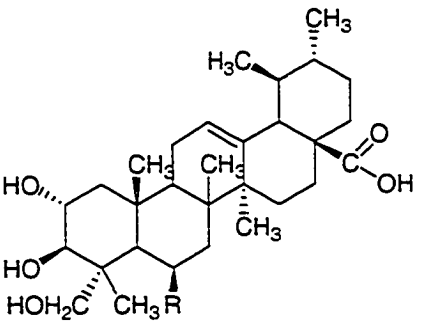
Table 1.3: Free triterpenic acids isolated from *Centella asiatica* (adapted from Srivastava et al, 1997b).

Triterpenic Acid
Asiatic acid
Madasiatic acid
Brahmic acid
Isobrahmic acid
Thankunic acid
Isothankunic acid
Betulic acid
Centoic acid
Centellic acid
6β-Hydroxyasiatic acid
Terminolic acid

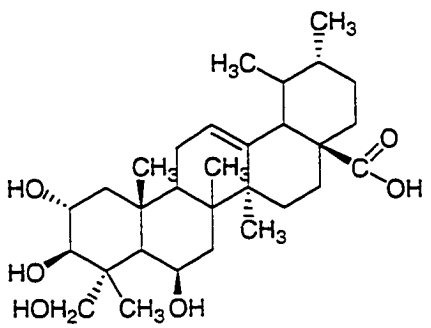
Figure 1.2: Several triterpenes isolated from *Centella asiatica* (adapted from Srivastava et al, 1997b).



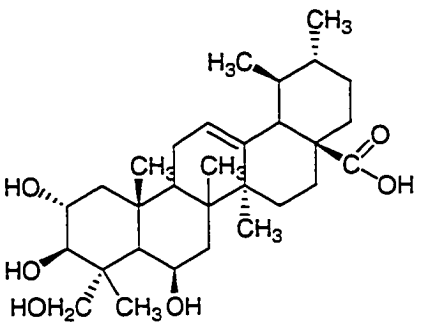
MADECASSIC ACID



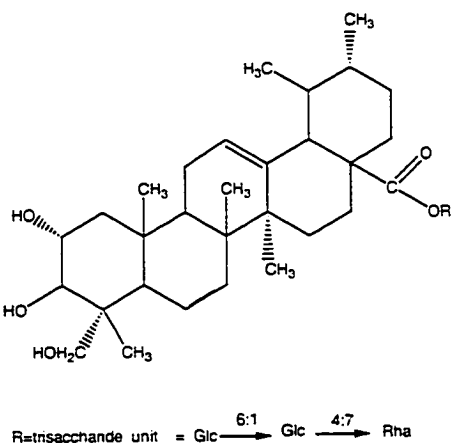
ASIATIC ACID R = H
6-HYDROXY ASIATIC ACID R= OH



BRAHMIC ACID

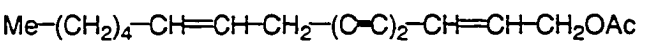


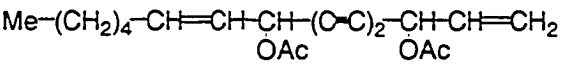
TERMINOLIC ACID

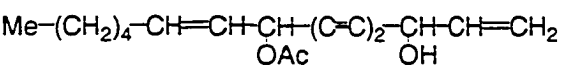


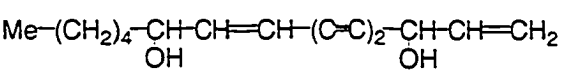
ASIATICOSIDE: 2,3,23-Trihydroxyurs-12-en-28-oic acid *O*-6-deoxy-α-L-mannopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl ester

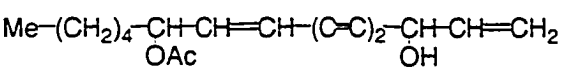
Figure 1.3: Polyacetylenes isolated from *Centella asiatica* (adapted from Srivastava et al, 1997b).

1 

2 

3 

4 

5 

1.2.2.3 Quality Control and Standardization of Commercial Gotukola Products

Since the commercial preparations of gotukola products in the market represent a wide spectrum of variation, adopting proper standards for active compounds is important in quality control. A quantitative analysis of gotukola raw plant materials of Indian origin and commercial product *Akrokapsulae* capsules (Akrofarm srl, Italy) - (dry weight basis) has revealed total triterpenic content of 3.19% and 2.29% and asiaticoside content of 0.37% and 0.23% respectively. The liquid extract of *Farmacia Dalle Due Torre* (Bolognia, Italy) contained 339.0 mg/100ml total triterpenes and 26.8 mg/100ml asiaticoside (Gunther and Wagner, 1996). Setting up proper standards requires comparison of clinically verified data with gotukola commercial materials.

1.2.2.4 HPLC Analysis of *Centella asiatica* for Asiaticoside

Reversed phase High Performance Liquid Chromatography (HPLC) is a powerful elution chromatographic method which employs a liquid mobile phase and a C_{18} stationary phase. This technique is highly versatile and widely used in separation, identification and determination of components in organic, inorganic and biological materials. The sample solution is injected to the mobile phase and it flows through the stationary phase with the mobile phase. The sample mixture is then distributed between two phases in the chromatographic column based on the chemical interactions of the components in the sample with the mobile phase and the stationary phase. The differences in the extent to which solutes are distributed between two phases determine the separation of components in the sample. Most of the HPLC separations are currently

performed with reversed-phase in which the stationary phase is non polar, often a C_{18} hydrocarbon and the mobile phase is a relevantly polar solvent such as water, acetonitrile or methanol. In reversed-phase chromatography, the most polar component is eluted first. In contrast, the normal phase chromatography employs a polar stationary phase and a less polar mobile phase. Thus less polar components elute more quickly than do more polar components (Meyer, 1998).

1.2.3 Pharmacology and Biological Activity of Gotukola

1.2.3.1 Introduction

Gotukola has been in use in Asia and Africa for a wide range of therapeutic applications as follows: bronchitis, diarrhea, dysentery, epilepsy, fever, gastritis, hepatitis, inflammations, leprosy, leukoderma, mental disorders, nerve tonic, pain, rheumatism, skin diseases, syphilis and wound healing (Brinkhaus et al, 2000). Several dosages of gotukola are in practice for such applications (Table 1.4).

In ayurvedic medicine gotukola has been used for the treatment of leprosy, varicose ulcers, lupus and certain obstinate eczemas. It is also considered a herb to improve memory and other psychological functions. During the last four decades there has been a continuous research interest on gotukola. According to an analysis of number of papers using the Agricola database, research activities on gotukola have been increasing with highest number of papers / mentions published during mid-1990s (Australian New Crops, 1997).

Apparently, based on the data of two studies conducted in 1969 and 1972 the Health Protection Branch (HPB) of Health Canada rated asiaticoside from gotukola under suspicion as a potential carcinogen. Despite this conclusion by HPB, gotukola and its constituents are used widely in Asia and Europe for several medicinal applications (Awang, 1998). However it is suggested that rather than asiaticoside, propylene glycol and benzene used in these studies caused the reaction. In a more elaborative study conducted with guinea pigs, it was found that frequent application of gotukola raw extracts or its triterpenes (asiaticoside, asiatic acid and madecassic acid) to damaged skin (e.g. keloids, leg ulcers, phlebitis, slow healing wounds, leprosy, surgical lesions, striae

distensae and cellulitis), show a very low risk of acquiring contact sensitivity (Hausen, 1993).

1.2.3.2 Psycho-neuropharmacological Applications

In a recent preliminary clinical study it was revealed that 12 g of orally administered gotukola significantly attenuated the peak acoustic startle amplitude 30 and 60 minutes after treatment. But the treatment had no effect on self-rated mood, heart rate or blood pressure. These observations support the anxiolytic properties of gotukola in human subjects (Bradwejn, 2000).

Only the aqueous extract of gotukola (200 mg/kg for 14 days) showed an improvement in learning and memory compared to the same treatment with methanolic and chloroform extracts in a recent rat trial with shuttle box and step through test paradigms. The aqueous extract at both 200 and 300 mg/kg dosages caused a significant decrease in the brain levels of malondialdehyde (MDA) with simultaneous significant increase in glutathione levels. At 300 mg/kg a significant increase in catalase levels, but no significant change in superoxide dismutase (SOD) levels were observed. The observations of this study indicate cognitive enhancing effect of the aqueous extract of gotukola and an antioxidant mechanism involved (Veerendra and Gupta, 2002).

The alcoholic extract that resulted in tranquillizing effects in rats was found non-toxic up to a dose of 350 mg/kg, i.p. (Ajthal and Sirsi, 1961). The bramoside-fraction was found to possess sedative action in rats and this action appeared to be mainly of cholinergic mechanism (Ramaswamy et al, 1970). In a previous study, the same action

was shown in rats with the glycosidal-fraction (Malhotra et al., 1961). Gotukola has been reported to significantly improve in both general ability and behavioral pattern in mentally retarded children (Appa et al., 1973).

In a study conducted with gotukola ethanol extract, the pentobarbitone induced sleeping time was increased and the immobility in behavioural test reduced. The study supports significant effect of its sedative-antidepressant activity (Sakina and Dandiya, 1990).

1.2.3.3 Dermatological Applications in Wound Healing and Ulcerous Skin Abnormalities

Although gotukola is effectively applied to skin for dermatological conditions such as leg ulcers, leprosy, slow-healing wounds, surgical lesions and cellulitis, its ethanol and diethyl ether extracts and triterpenic constituents – asiaticoside, asiatic acid and madecassic acid show a low risk of causing contact sensitivity (Hausen,1993). Morganti et al (1999) describe preparation and HPLC analysis of a new transdermal therapeutic delivery system for controlling cellulitis in the form of a cosmetic patch prepared with 5 mg of a gotukola extract containing asiaticoside, asiatic acid and madecassic acid at 40, 30 and 30% respectively.

Boiteau and Ratsimamanga (1956) studied the action of asiaticoside on the cictrization of experimentally induced wounds. They found that asiaticoside substantially improved the process of wound healing. They also confirmed that asiaticoside works selectively, stimulating rapid and healthy growth of the reticuloendothelium.

Treatments were given to mice, rats, guinea pigs, and rabbits, orally, by intramuscular injection or implantation. The treatment strengthened the skin, stimulated growth of hair and nails, promoted vascularization of the connective tissue, increased formation of mucus and enhanced local and general leukocytosis. The asiaticoside subcutaneous delivery of 0.04 - 0.05 g per kg body weight was toxic to mice and rabbits, while doses of 0.20 - 0.25 g per kg body weight lengthened the blood coagulation time (Boiteau et al, 1951a).

The drug "Titrated Extract from *Centella asiatica*" (TECA) is a mixture of 3 terpenes extracted from the plant: asiaticoside (40% w/w), asiatic acid (30% w/w) and madecassic acid (30% w/w). TECA increased the collagen synthesis in a dose-dependant trend. It was also found that asiatic acid was the only component accountable for collagen synthesis (Maquart et al, 1990).

Topical application of 0.2% asiaticoside twice daily for 7 days to excision-type cutaneous wounds in rats resulted in increased enzymatic and non-enzymatic antioxidants such as superoxide dismutase (35%), catalase (67%), glutathione peroxidase (49%), vitamin E (77%) and ascorbic acid (36%) in newly composed tissues. The lipid peroxide level was decreased by 69% as measured in terms of thiobarbituric acid reactive substance in the treated animals. Continued application of the treatment for 14 days showed no significant difference in these antioxidant levels, compared with the control group (Shukla et al, 1999a). Since asiaticoside promoted induction of antioxidant levels at an early stage of wound healing, this may be an important contributory factor of wound healing properties of gotukola as practised in ayurveda.

1.2.3.4 Antimicrobial Activity

Hexane and EtOAc extracts of gotukola indicated a significant antibacterial activity. Researchers observed the inhibitory activity these extracts were effective against *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Pseudomonas cichorii.* Similarly, they also found the stigmasterol and dotriacont-8-en-1-oic acid obtained from hexane extract inhibited *B. subtilils, E. coli* and *P. aeruginosa* (Srivastava et al, 1997a). The methanolic extract of gotukola have shown antifungal activity against the following fungi: *Alternaria alternata, A. brassicae, A.solani, A. tenuissima, Cercospora blumae, Curvularia lunata, C. penniseti, Drechslera monoceras, D. oryzae, D.turitica, Fusarium albizziae* and *F. udum* (Singh et al 2000).

1.2.3.5 Activity in Healing Duodenal Ulcer

Oral applications of gotukola asiaticoside were given to Wistar rats, 3 hours after rats were given mercaptoethylamine (65 mg/ 100 g body weight) to induce ulcers. The illness rate was reduced by 50%. (Ravokatra et al, 1974). Evidently, asiaticoside accelerated scar formation in the duodenal tissue through production of new connective tissue that had suffered a deregulation after the poisoning with mercaptoethylamine.

1.2.3.6 Activity in Recovery from Tuberculosis

The recovery of guinea pigs with experimentally caused tuberculosis was hastened by treatment with asiaticoside of gotukola extract. The bacteriostatic activity

and stimulation of reticuloendothelium by asiaticoside seems to promote the healing process of tuberculosis (Boiteau et al, (1951b).

1.2.3.7 Other Biological Activities of Gotukola

Investigators found protective activity of gotukola against radiation-induced conditioned taste aversion. This activity was as efficient as a standard antiemetic drug – ondansetron. Gotukola may be helpful in preventing radiation-induced behavioural changes during clinical radiotherapy (Shobi and Goel, 2001).

In a study by Del Vecchio et al (1984), it was found that the growth rate of cultured, human embryo fibroblast cells was not modified at concentrations of the triterpene component of gotukola of 25 mg/ml, but the growth rate was inhibited by higher concentrations. The triterpene fraction in the culture medium stimulated the incorporation of proline into proteins and lipids and acetate into glycosaminoglycans in the cells.

The insecticidal activity (Lin et al., 1972) and the cytotoxic activity (Chin et al., 1944) of the freshly pressed gotukola sap have been observed. Asiaticoside 0.01% solution has been observed to promote germination and growth of some plants, while a 0.1 - 1.0% solution inhibited germination and growth of some plants. (Boiteau and Ratsimamanga, 1958).

Table 1.4: Some dosages for medicinal uses of gotukola and its constituents.

(Adapted from Awang, 1998).

Disease / Condition	Dosage
Leprosy	0.5 g powdered dried leaf in capsules, 3 times daily
Leg ulcers	10 mg tablets 6 times daily of standardized extract for 3 – 8 weeks
Ulcerous skin	20 mg asiaticoside daily, intramuscularly or 40 mg extract every 2nd day
Liver disease	90 – 150 mg extract daily
Venous Insuffiency	2 – 6 tabs daily. Madecassol (10 mg stand. ext. per tab) for 1-3 months or 60 -120 mg TECA (extract) daily
Special skin Diseases	Salve with 0.5% asiaticoside or powder with 2% asiaticoside, once or twice daily
Wounds	Pure asiaticoside in powder form
Eye wash	1% solution of asiaticoside, 6 times daily

1.2.4 Introduction to Psycho-active Medicinal Plants and Their Ethno-pharmacological Uses

Most of the plant-based remedies for psychiatric disorders are associated with ancient medicines that are intended to bring about behavioural changes in the patient. Treatments may be based on cultural practices and beliefs and/or pharmacological activity based on bioactive phytochemicals. Contemporary bio-medical research can identify the ancient remedies that have pharmacological activity and can effectively treat diseases through this mechanism. There are several ethnobotanically distinctive psychoactive plants among different ancient medicine systems including: gotukola (*Centella asiatica*) of ayurveda, Reishi mushroom (*Ganoderma lucidum*) of Chinese medicine and Kava (*Piper methysticum*) of Polynesian cultures.

The ayurvedic medicinal plants affecting the central nervous system are categorized into several categories such as: analgesics, antidepressants, learning and memory enhancers, anticonvulsants, anti-stress remedies and anxiolytics (Vaidya, 1997) (Table 1.5). Sarpagandha (*Rauwolfia serpentina*) provides an example of contributions of ayurvedic science to the modern drug development process. The earliest record of this plant is in the ancient ayurvedic treatise known as "Charaka Samhita". Significant phytochemical and pharmacological studies carried out in India and Switzerland finally led to isolation of its principle sedative, reserpine in 1952. Besides acting as a tranquillizer, reserpine is also effective in lowering high blood pressure. Brahmi (*Bacopa monniera*) contains active components called baccosides, which promote brain cognitive functions (Sukh, 2001). Researchers have isolated three triterpenoid saponins -

Table 1.5: Central nervous system (CNS)-active ayurvedic plants (adapted from Vaidya, 1997).

Analgesics	Antidepressants	Enhancers of Learning & Memory	Anticonvulsants	Anti-stress	Anxiolytics
Corchorus depressus	*Mucuna pruriens*	*Bacopa monniera*	*Withania somnifera*	*Ocimum sanctum*	*Withania somnifera*
Embelia ribes	*Saraca indica*	*Centella asiatica*	*Convolvulus pluricaulis*	*Centella asiatica*	*Azaradicta indica*
Gossypium indicum	*Withania somnifera*	*Butea frondosa*	*Cynodon dactylon*	*Eleutheroco--ccus senticosus*	*Nardostachys jatamansi*
Azaradicta indica		*Celastrus paniculatus*	*Erythrina variegate*		*Acorus calamus*
Psidium guava		*Eclipta alba*	*Pongamia pinnata*		*Celastrus paniculatus*

bacopasaponins A, B and C of biological interest from brahmi (Garai et al, 1996).

Besides manduka-parni (gotukola), Sushruta (~600 BC) mentioned several other ayurvedic plants to prepare remedies for psychiatric disorders such as brahmi (*Bacopa monniera*), sarpagandha (*Rauwolfia serpentina*), rajani (*Curcuma longa*), chitraka (*Plumbago zelanica*), pippali (*Piper longum*), nilotpala (*Nymphea stellata*), padma (*Nelumbium speciosum*), vacha (*Acorus calamus*), vidanga (*Embelia ribes, E. robusta*) and trivrit (*Ipomea terpethum*).

The following formulations of elixirs and remedial agents ("Medhayushkamiyam Rasayanam") are also prescribed in ayurveda: "Manduk-parni-Rasayana" (gotukola, milk), "Svetavalguja-Rasayana" (avalguja seeds, treacle, clarified butter), "Vacha-Rasayana" (vacha, milk), "Sata-paka Vacha-Ghrita" (vacha, clarified butter), "Brahmi-Rasayana" (brahmi), "Brahmi-Grita" (brahmi, vidanga, trivit, clarified butter) (Sushruta, ~600BC).

Bopaiah et al (2000) showed the antidepressant activity of 50% ethanol extract of an ayurvedic formulation comprised of: *Acorus calamus* – vacha rhizome 8%, *Nardostachys* – jatamamsi root 25%, *Piper longum* – pippali fruit 10%, *Plumbago zelanica* – chitraka root 10%, *Withania somnifera* – ashwaganda root 35% and *Allium sativum* – bulb 12%. The investigators suggest that the antidepressant activity of this formulation as shown in the behavioral and biochemical tests with rats may be mediated by the dopaminergic and serotonergic mechanisms in the brain.

Bo (*Ficus religiosa* L.) Moraceae is the most sacred plant venerated by Buddhists, since the Buddha attained the enlightenment under the sacred bo-tree. The belief that this tree has anxiety relieving properties is supported by a recent rat trial study (Vogel test)

with an aqueous extract of trunk of the tree (Ratnasooriya, 1998).

There has been a significant development in plant based treatments for psychiatric and psychosomatic diseases in Africa since 1950's mainly due to the advent of *Rauwolfia* plant alkaloids (reserpine) for the management of such diseases (Iwu. 1993). Roots of the African serpentwood (*Rauwolfia vomitoria*), which is rich in ajmaline alkaloids, have been traditionally used in African medicine for calming mentally disturbed patients (Bown, 1995).

In North America and Europe following plants are used for their psychoactive calming effects: St.-John's-wort (*Hypericum perforatum*), valerian (*Valeriana officinalis*), chamomile (*Matricaria recutita*), California poppy (*Eschscholtzia californica*), hops (*Humulus lupulus*), passion flower (*Passiflora incarnata*), ginseng (*Panax ginseng*, *P. quinquefolius*), milk thistle (*Silybum marianum*) and *Ginkgo biloba* (Bloomfield, 1998). Skullcap (*Scutellaria spp.*), black cohosh (*Cimicifuga racemosa*) and evening primrose (*Oenothera biennis*) are some other plants with soothing effects (Bown, 1995).

Anise (*Pimpinella anisum*), deadly-nightshade (*Atropa belladonna*) and German chamomile (*Matricaria chamomilla*) are used in Egypt for their sedative effects (Demerdash, 2001). An alkaloid in *A. belladonna* – atropine dilates the pupil of the eye and the plant was important to make the patients unconscious for surgery, before the invention of modern anaesthetics (Bown, 1995).

The Maya, Inca and Aztec civilizations of the Central and South America also had an advanced pharmacopoeia of psychoactive medicinal plants. Cocoa (*Theobroma cacao*) contains caffeine and was the basis of traditional Aztec drink 'chocolatl'. Costa

Rican plants such as *Tabebuia rosea*, torchwood (*Bursera simaruba*), balsam (*Myroxylon balsamum*), strong-man's-weed (*Petiveria alliacea*), quassia (*Quassia amara*) possess neurological effects (Romero, 2001). Recently, a highly effective anxiolytic has been isolated by our group from a neotropical vine code named "Sun Susto" (unpublished).

The ancient Chinese medicinal system is an important knowledge base that uses materials from both plant and animal origin. Among more than commonly used 600 -700 medicinal materials, more than 70% are of animal origin. They include animals such as deer, musk deer, scorpion, centipede and tortoise (Ruili, 1990). The text 'Pen Tsao Ching' written by Chen Nong in 2800 BC is considered one of the oldest known Chinese pharmacopoeia with records of the use of medicinal plants. The dualistic Taoist philosophy is the basis for the ancient Chinese medicine system. According to this philosophy, all living beings including humans and medicinal plants are composed of two forces; *Yin* and *Yang*. *Yin* is feminine, cool, suppressed and dim, while *Yang* is masculine, warm, dominant and bright. Proper balance of *Ying* and *Yang* has to be maintained for a healthy life. Any imbalance of these two forces creates diseases and disorders. Ginkgo (*Ginkgo biloba*), Reishi mushroom (*Ganoderma lucidum*) and Licorice (*Glycyrrhiza glabra*) are important Chinese herbal medicines for anxiety and other psychological disorders, since they are capable of bringing a proper balance to the *Yin* and *Yang* forces governing such disorders (Bloomfield, 1998). *Bupleurum falcatum*, *Cyperus rotundus* and *Ephedra sinica* (ma-huang) are also used in Chinese medicine to treat mental illness (Walter and Rey, 1999).

In Kampo oriental medicines of Japan, preparations such as Hange-Koboku-to, Yoku-Kan-san, Saiboku-to and Kami-Kihi-to have been historically prescribed to treat

clinical depression and anxiety-related disorders such as anxiety neurosis, insomnia and anxiety hysteria. These preparations are mixtures of several medicinal plants (Maruyama et al, 1998) (Table 1.6).

The ancient Western medicine system categorizes the herbs used for psychiatric or neurological disorders as 'nervines'. There are three types of nerviness: a) nervine tonics said to strengthen and restore the nervous system, b) nervine relaxants to soothe the body and mind, c) nervine stimulants to directly stimulate nerve activity. In Europe, 10% of the herbal drug sales are herbal sedatives and anxiolytics in 1994. Compared to many western societies, Germany plays a leading role in therapeutic applications of herbs and other natural products. St. John's wort is more commonly prescribed for depression than the pharmaceutical drug fluoxetine in Germany. A recent community survey has revealed the willingness of the Australian consumers to use herbal remedies for mental illness: a treatment program that include herbal medicines was considered potentially helpful by 57% of respondents (Walter and Rey, 1999).

Some psychoactive plants are also capable of causing potentially negative psychological impacts such as hallucination but also play an important role in Shamanistic traditions. *Psychotria viridis* and *Diplopterys cabrerana* are two prominent psychoactive plants of amazon containing hallucinogenic tryptamines (Davis, 1997). Opium poppy (*Papaver somniferum*), marijuana (*Cannabis sativa*) and coca (*Erythroxylum coca*) are medicinally important psychoactive plants, which are also abused as street drugs. Cola (*Cola nitida*) and coffee (*Coffea arabica*) are popular beverages which contain the nervous system stimulant, caffeine. The neuro-physiological effect of tobacco (*Nicotiana tabacum*) is caused by a constituent alkaloid,

Table 1.6: The composition of Saiboku-to; the Japanese herbal preparation for anxiety-related disorders and depression (Prepared from Maruyama et al, 1998).

Plant Component (dried raw material)	Weight (g)
Bupleurum root (*Bupleurum falcatum*)	7
Hoelen (*Poria cocos*)	5
Pinellia tuber (*Pinellia ternata*)	5
Ginseng root (*Panax ginseng*)	3
Jujube fruit (*Zizyphus jujuba*)	3
Magnolia bark (*Magnolia obovata*)	3
Scutellaria root (*Scutellaria baicalensis*)	3
Glycyrrhiza root (*Glycyrrhiza uralensis*)	2
Perilla herb (*Perilla frutescens*)	2
Ginger rhizome (*Zingiber officinale*)	1

nicotine. Herbal medicines such as kava (*Piper methysticum*) and ma-huang (*Ephedra sinica*) should be used carefully, since they may create adverse effects due to toxicity and interactions with other drugs. St. John's wort appears to have monoamine oxidase (MAO) inhibitor and selective serotonin re-uptake (SSR) inhibitor properties and therefore should not be combined with other antidepressants (Walter and Rey, 1999).

Seeds of several culinary plants of the family Apiaceae (the carrot family, to which gotukola also belongs) possess hallucinogenic compounds in minute quantities. The oils of anise, dill, fennel and parsley may cause epileptiform madness and hallucination, apparently via *in vivo* transformation of such compounds into hallucinogenic amphetamines. Another member of the family, angelica which was traditionally used as a treatment for nervous diseases, contains furonocoumarins (psoralens) which may cause photo-dermatitis in susceptible individuals (Small, 1997).

1.2.5 Introduction to Anxiety Disorders

Mental disorders are among the highest causes for hospitalization in Canada. During 1995-96 the rate of hospitalization was 709 per 100,000; a rate equivalent to cancer and genitourinary diseases (Statistics Canada, 1999). One out of every five Canadians is likely to be diagnosed with a mental disorder at sometime in their life. Divorced or separated people tend to be affected more with mental disorders compared to their married counterparts. Women are reported to be twice as prone as males. Twenty-five percent of people affected with serious health concerns such as diabetes, heart disease and cancer are also prone to develop a psychological disorder. Anxiety disorders affect about twelve percent of the population (Canadian Health Network, 1999). Total direct and indirect costs incurred due to anxiety disorders per year in the United States in 1990 were estimated to be $42.3 billon US or $1,542 US per sufferer (Greenberg et al, 1999).

Fearful concern over actual dangers is normal for a healthy individual. It may be helpful to take precautions to avoid obvious and real dangers and will not interfere negatively in the individual's daily activities. Therefore, it is not considered a psychiatric disorder. An anxiety disorder (or pathological anxiety) can be defined as an exaggerated psychological response to a less obvious, ill-defined, irrational, distant or unrecognized source of danger. Anxiety disorders are manifested as an unpleasant psychological tension usually accompanied by physiological symptoms causing the patient to feel both mentally and physically helpless and also exhausted by being always on guard against an unidentifiable danger (Bloomfield, 1998).

Anxiety disorders may be a result of hereditary predisposition, major stress or trauma, painful childhood, medical illness and alcohol or drug abuse. It may also occur without any obvious or apparent reason. During the last 100 years, investigators have presented several theories of anxiety based on the behavioral, neurobiological and cognitive aspects of emotion. Fluoxetine (Prozac®), clomipramine (Anafranil®), paroxetine (Paxil®), sertraline (Zoloft®) and venlafaxine are some of the drugs prescribed for anxiety disorders. Anxiolytic drugs may cause side effects such as sedation, withdrawal problems and sexual dysfunction (Barlow, 2002). Therefore, the use of psycho-active herbal remedies may provide alternative treatment for these drugs.

The animal trial conducted for this thesis has shown the anxiolytic activity of an ayurvedic medicinal plant gotukola (*Centella asiatica*). *Withania somnifera* and *Acorus calamus* are some other examples of medicinal plants used as anxiolytic herbal remedies in ayurveda (Bhandari and Sharma, 1998). Several recent clinical studies support the use of gotukola (Bradwejn et al, 2000) and other herbs such as kava (*Piper methysticum*) (Connor and Davidson, 2002) and St. John's wort (*Hypericum perforatum*) (Taylor and Kobak, 2000) for their anxiolytic properties. The vast array of psycho-active medicinal plants (as described in Chapter 1.2.4) may provide potential for recovery of anxiolytic drugs in the future.

Based on the symptoms (feelings and thoughts patients complain of) and signs (behaviors and physical attributes physicians observe), the American Psychiatric Association has developed a diagnostic system for anxiety and other psychiatric disorders. These standards are described in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (American Psychiatric Association, 1994). As described in

the DSM-IV, some common types of anxiety disorders are:

1.2.5.1 Panic Disorder with or without Agoraphobia

The core features of panic disorder are recurrent unexpected panic attacks and anticipatory fear of future attacks. Panic attacks are discrete episodes of intense anxiety that reach a peak very quickly and are accompanied by a number of distressing physical and cognitive symptoms such as palpitations, sweating, difficulty breathing and fear of loosing control. A major complication of panic disorder is agoraphobia, which is fear of places or situations where the dreaded panic may occur. Typically, these are situations where in case of a panic attack, help is not available or escape is difficult. Common agoraphobic situations include being far away from home, being at home alone, crowded places, public transportation, restaurants, theaters and enclosed places. These situations are either avoided or endured with intense fear. Epidemiological studies indicate that the panic disorder with or without agoraphobia is common, with a life-time prevalence rate of 3.5% (White and Barlow, 2002). Women have a two-fold greater risk of developing panic disorder than men and the median age of onset of the illness is 24 years (Burke et al, 1990). Longitudinal studies indicate that panic disorder is a chronic condition that is frequently associated with comorbid conditions such as depression and other anxiety conditions (Bradwejn and Koszycki, 2002). Patients with panic disorder, especially those with agoraphobia, experience significant impairment in functioning (Leckman et al, 1983).

1.2.5.2 Agoraphobia without History of Panic Disorder

Most individuals who suffer from panic disorder also develop agoraphobia. However, when agoraphobia is not accompanied by panic attacks, it is diagnosed as agoraphobia without a history of panic disorder. Patients with agoraphobia with no history of panic disorder experience panic-like sensations, such as sudden spell of palpitations or dizziness, but never meet criteria for panic disorder. As a result of a fear of these sensations, the individual avoids situations where they may occur. The situations that agoraphobic patient with no history of panic disorder fear and avoid are similar to those of patients with a history of panic disorder. Epidemiological studies indicate that the lifetime prevalence of agoraphobia without a history of panic disorder is 5.3%, with the disorder being more common in females than males (Kesler et al, 1994).

1.2.5.3 Social Anxiety Disorder

Social anxiety disorder (also known as social phobia) is an anxiety condition characterized by intense fear and avoidance of situations where the person may be subject to scrutiny of others while performing a task. The individual fears that he or she will act in a way that is humiliating or embarrassing. Common social phobic situations include speaking in public, eating, drinking or writing in front of others, using public washrooms, using the telephone, meeting strangers and meeting people of authority. The DSM-IV describes two types of social anxiety disorder; the generalized subtype and the non-generalized subtype. In the generalized subtype, the patient fears most social situations whereas in the non-generalized subtype the patient fears one or two social situations.

Epidemiological studies indicate that social anxiety disorder is very common, with a lifetime prevalence of rate of 13.3% (Kessler et al, 1994). The age of onset of social anxiety disorder is adolescence and women are more likely to suffer from this disorder than men. Social anxiety disorder is associated with marked impairment in social, academic and work-related activities. Major complications of social anxiety disorder include depression and substance abuse (Lang and Murray, 2001).

1.2.5.4 Specific Phobia (Simple Phobia)

Simple phobia is characterized by the persistent, irrational fear of and compelling desire to avoid a specific object or situation other than agoraphobia and social phobia. Some common examples are fear of animals, heights, darkness, flying in planes, pointed objects, sight of blood, injections, loud noises, lightning, water, and illnesses. Usually, children affected with specific phobia do not recognize that their fears are excessive or unreasonable and rarely report distress about having the fear. Fears of objects in the natural environment (e.g. animals) are common during childhood. In the US population, about 20 - 30% of individuals have specific phobia for flying according to Boeing estimation (Dean and Whitaker, 1980). About 75 - 90% of affected individuals with phobias of animals and other objects in the natural environment are female. Only 55 - 70% of affected individuals with fear of heights are females. The lifetime prevalence of specific phobia is about 11% (Kessler, 1994).

1.2.5.5 Post-Traumatic Stress Disorder (PTSD)

Severe physical or psychological trauma causes this disorder. The symptoms of

PTSD include recurrent and intrusive recollections and dreams about the event, flashbacks of the trauma, avoidance, hyper-arousal and emotional numbing. Comorbidity of depression and substance abuse often occur with this disorder (Shalev, 2001). If the disaster is unexpected, the effects of the trauma event will be worsened. Natural disasters, plane crashes, automobile accidents, kidnapping, torture, rape and war are common traumatic incidences which may lead to the development of PTSD (Schreuder et al, 1998 and Shalev, 2001). People who have recently emigrated from locations of critically higher levels of social unrest and civil conflict may still have higher rates of PTSD. The prevalence of PTSD in the general population is about 1 - 9.2% and 11.3% women and 5.6% men were affected with PTSD (Breslau et al, 1991). In individuals who are at high risk of being exposed to traumatic events, such as war veterans, soldiers, police officers and rescue workers, the prevalence rate increases to 3 – 58%. (American Psychiatric Association, 1994).

1.2.5.6 Generalized Anxiety Disorder (GAD)

The central feature of generalized disorder is unrealistic or excessive anxiety and worry about several life circumstances that the person finds difficult to control. Typically, the patient worries about things that are unlikely to happen or if they did happen would be much more manageable than worrier believes. Associated symptoms of GAD include motor tension, autonomic hyper arousal, and vigilance and scanning. Epidemiological studies indicate that the lifetime prevalence of GAD is 4.2 – 4.6% (Ninan, 2001). The age at onset of GAD range from 2 to 61 years (Yonkers et al, 1996) and it prevails twice as much as in women compared to men (Wittchen et al, 1994).

GAD may be associated with impairment of role of functioning in social life as well as a variety of somatic complains such as irritable bowel movement and chest pain (Carter and Maddock, 1992).

1.2.5.7 Obsessive-Compulsive Disorder (OCD)

The core symptoms of OCD are obsessions and compulsions. Obsessions are persistent and distressful ideas, thoughts or images (e.g. becoming contaminated by shaking hands, wondering whether one has left a door unlocked) and compulsions are repetitive behaviors (e.g. hand washing) or mental acts (e.g. counting, repeating words silently). Repetitive activities of children and some culturally accepted rituals are distinguished from this disorder. It is equally common in men and women. As revealed by community studies 1.9 - 2.5% (life time prevalence) and 1.1 - 1.8% (one-year prevalence) are affected with the disorder (Weissman et al, 1994). About 80% of OCD patients may be also currently depressed (Steketee and Barlow, 2002).

1.3 Overview of the Study

1.3.1 Rationale

At the United Nations Association in Canada (UNAC) I initiated a 3-year project to promote knowledge and practice of medicinal plants with a multicultural perspective and the current study is a secondary extension of this project. While implementing the UNAC project, I realized that there is a remarkable interest in the public about ancient uses of medicinal plants and their effectiveness. When the audience raised questions such as "...is gotukola another kind of 'cola'?", or "isn't gotukola a toxic plant restricted in Canada?", or "is there a scientific basis for ayurveda?...", these gave an early inspiration to initiate this new project.

Despite the therapeutic claims in ayurveda and popular culinary uses in Asia, as well as widespread use in Europe and the US, Health Canada has designated gotukola as a potentially toxic plant that may not be sold in any form. The Food Directorate specified it as a plant not suitable for unrestricted consumption as foods. These decisions are debatable and apparently based on the results of old scientific reports using inappropriate methods (Awang, 1998). Recently, several research groups have shown interest in gotukola including Bradwejn et al (2000) of the Royal Ottawa (psychiatric) Hospital for its psycho-pharmacological profile. Ascertaining the active principles of the plant and their activity at the physiological-level is not yet fully understood. To assist further studies, selection of gotukola plant materials containing higher concentration of these active components is important. With the aforementioned background and focus for the research needs for the plant, this thesis research was conducted. During the study, I

investigated the phytochemical basis of gotukola for its anxiolytic properties with special attention given to one of its triterpenic compounds asiaticoside.

1.3.2 Hypotheses and Objectives

Hypothesis 1:

The anxiolytic activity of different commercial gotukola products may vary depending on their asiaticoside content.

Null Hypothesis 1:

There is no difference among the control and the treated groups with gotukola commercial products with different asiaticoside content.

Objective 1:

To assess the anxiolytic activity of Nature's Way and Solaray Madagascar gotukola products in rat test model at different dosages and post-drug intervals.

Hypothesis 2:

There might be variable anxiolytic activity in hexane, ethyl acetate and methanol extracts of gotukola containing groups of compounds with varying lipophilicity.

Null Hypothesis 2:

There is no difference of anxiolytic activity among the control group and the groups treated with hexane, ethyl acetate and methanol extracts of gotukola.

Objective 2:

To investigate the anxiolytic activity of hexane, ethyl acetate and methanol extracts of

gotukola.

Hypothesis 3:

Asiaticoside – a triterpenic compound isolated from gotukola may be an active anxiolytic principle.

Null Hypothesis 3:

There is no difference of anxiolytic activity among the control group and the asiaticoside treated groups.

Objective 3:

To investigate the anxiolytic activity of asiaticoside in rat test models at different dosages and post-drug intervals.

In addition to this hypothesis driven research, two additional experimental objectives of the thesis were:

4). Develop method for propagation of gotukola and increased expression of asiaticoside.

5). Develop validated HPLC methods for quantitative analysis of asiaticoside.

CHAPTER 2

FUNDAMENTAL CONCEPTS OF AYURVEDIC SCIENCE FOR MEDICINAL APPLICATIONS OF GOTUKOLA (*CENTELLA ASIATICA* L. URBAN) IN PSYCHIATRIC DISORDERS

2.1 Introduction

Although some scholars categorize ayurveda as a form of "traditional knowledge", it is more meaningful to describe it as an "ancient science". According to the Canadian Oxford English Dictionary, the word "traditional" may be used to describe an information system based on a custom, opinion or belief handed down to posterity orally or by practice, whereas the word "scientific" describes an information system based on systematically formulated knowledge (Barber, 2001). Ayurveda is not based on mythology, superstition or just common belief. Although it may not exactly fit into the definition of modern "scientific method", it reflects a logically developed and systematically well-documented science within its own context of a bygone era.

The perception that ayurveda is "traditional" but not "scientific", only hinders its true potential to serve humanity with a modern scientific perspective. Although ignorance about ayurveda may have created such a negative perception, it indirectly creates an opportunity for unethical exploitation of an already existing knowledgebase to re-invent it with a different technical jargon (modern concept of bio-piracy). Therefore, proper understanding of the ayurvedic knowledgebase and further studies of them with modern concepts of bio-medical

sciences is beneficial, because it creates a new complementary scientific concept. A practical outcome is the process of recovering ancient sciences as new pharmaceutical drugs.

In his English translation of one of the ancient ayurvedic texts Sushruta Samhita, Bhishagratna (1907) says "...The vast medical literature of ancient India practically remains as yet unexplored, and any undertaking, which has the object of making that *terra incognita*, known to the scientific world, is bound to be welcomed by the public..." Even a century later, this aspiration has progressed in a limited fashion. In this paper, the basic concepts of ayurveda are explored, with a focus on applications of the ayurvedic medicinal plant gotukola (*Centella asiatica*) in treatments for mental disorders.

The closest English translation of the Sanskrit term 'ayurveda' follows: *ayur* means life, *veda* means science – hence the science of life. The chronological development of ayurveda dates back to 1500 and 500 BC with the migration of the Aryan people into India and later into other neighboring countries such as Sri Lanka. Ayurvedic therapy includes not only plant and animal based natural medications. It is a comprehensive approach that consists of diet, exercises, meditation and spiritual cleansing along with medications tailored to the unique needs of individual patients (Bhishagratna, 1907).

The natural product based therapeutic knowledge of this science is well documented in comprehensive ancient ayurvedic texts, including Charaka Samhita (~900 BC; main focus – therapeutics), Sushruta Samhita (~600 BC; main focus – surgery), Ashtanga Hridaya (~700 AD; main focus – principles and practice of medicine) and Madhava Nidana (~800 to 900 AD; main focus – diagnosis). During the prime time of ayurveda, it was a cogent and scientifically organized discipline. Therefore, these ayurvedic texts were much respected in the technically advanced ancient contemporary world. Eventually, some of the texts were translated into Greek

(300 BC), Tibetan and Chinese (300 AD), Persian and Arabic (700 AD) (Sukh, 2001). The exact details of the period of publication or the author of some of these ancient texts are not clear. As an example the original Sushruta Samhita was written by Sushruta. The current version of the Samhita may be a re-written version by Nagarjuna during the 4th century BC (Bhishagratna, 1907).

2.2 The Ayurvedic Theory

All matters in the universe are composed of five *mahabhutas* (basic factors) for their existence. They are *akasha* (space), *tejas* (fire/energy), *prthivi* (earth / organic and inorganic compounds), *jala* (water) and *vayu* (air). According to Ayurveda, all biological processes are governed by *tridoshas* (three forces), namely; *vata, pitta* and *kapha*. These three forces are composed of different combinations of previously mentioned five basic factors as follows:

1. *Vata* - *Akasha* (space)
   - *Vayu* (air)
2. *Pitta* - *Tejas* (fire/energy)
   - *Jala* (water)
3. *Kapha* - *Jala* (water)
   - *Prthivi* (earth / organic and inorganic compounds)

The interactions of these *tridoshas* regulate all basic and complex physiological and psychological processes in living organisms. The basic foundation of good health is a well-balanced harmony of *tridoshas*. Any imbalance in them manifests diseases and disorders, characterized by their inherent symptoms and signs. A *dosha* possesses its own qualities to govern different bodily functions (Table 2.1).

Table 2.1: Qualities and functions of the three *Doshas* (prepared from Gerson, 1997 and Sushruta, ~600 BC).

Dosha	**Qualities**	**Functions**
Vata	Moving	Represent bodily functions concerned with
(space and air)	Quick	movement.
	Light	Controls the activities of the nervous system
	Dry	and the processes of elimination and
	Rough	respiration.
	Clear	
	Leads to other *doshas*	
Pitta	Hot	Represents bodily functions concerned with
(fire/energy and	Sharp	heat and metabolism.
water)	Light	Governs digestion and perception.
	Penetrating	
	Acidic	
	Slightly oily	
Kapha	Solid	Represents the structural aspects of the
(water and	Heavy	physiology and, is responsible for biological
earth / organic	Oily and Soft	strength, natural tissue resistance and proper
and inorganic	Cold	body structure.
compounds)	Sweet	
	Sticky	
	Immobile	

To understand ayurvedic *tridosha* concepts, they may be further explained as they correlate to allopathic medicinal concepts. However, since the ayurvedic principles address both body and mind energy concepts simultaneously, they are not directly translatable to allopathic interpretations. Although ancient concepts cannot be directly compared with modern biomedical concepts, such an attempt may be useful in the process of discovering new pharmaceutical drugs from ayurvedic medicinal plants.

As an example, the significance of *vata doshas* on nervous system may correspond to the modern concept of the effect mediated by the release of neurotransmitters such as serotonin and acetylcholine in the central nervous system. The *pitta doshas* may correspond to the sympathetic nervous system that largely utilizes the catecholamines epinephrine, nor-epinephrine and dopamine to activate functions regulating bodily energy release. The activity of histamine and the prostaglandins that in part regulates fluid balance in the tissues and also controls the permeability of capillaries may correspond to the effects of *kapha doshas* (Gerson, 1997). As an explanatory molecular approach to ayurvedic concepts, Tripathi (2000) specifies *vata* as the process that can be monitored in terms of membrane bound signal transduction, *pita* as the process of phosphorylation and de-phosphorylation of different proteins such as signaling moieties and enzymes and *kapha* as the process involved with protein synthesis and gene expression.

A well-balanced harmony of *vata, pitta* and *kapha* condition is expected in healthy living. Any imbalance of them will create disorders and diseases. Although anxiety is caused mainly due to unbalanced *vata doshas*, unbalanced *pitta* and *kapha doshas* also play a secondary role in inducing anxiety. Following tables are a simplified summary of the effects of balanced and unbalanced *tridoshas* (Tables 2.2, 2.3 and 2.4).

Table 2.2: Effects of balanced and unbalanced *Vata Dosha* (prepared from Gerson, 1997 and Sushruta, ~600 BC).

Effects of Balanced *Vata*	Effects of Unbalanced *Vata*
Proper regulation of all bodily functions	Bodily functions impaired
Mental activity controlled and precise	Mental inactivity and confusion; impaired memory
Excellent energy level	Non-specific fatigue, anxiety, worry, cold-intolerance, weakening of the Life Force
Desire to lead an active life; vitality and natural interest	Loss of energy and joy for life
Normal movements associated with eating digestion and excretion	Movements for eating, digestion and excretion inhibited
Control of the organs of perception and the organs of action	Perception and action are disturbed; senses are dulled, responses are slowed
Stimulation of digestive juices	Deficiency of digestive juices
Normal respiratory function	Respiratory disorders
Normal drying of excessive discharges	Persistent bodily discharges

Table 2.3: Effects of balanced and unbalanced *Pitta Dosha* (prepared from Gerson, 1997 and Sushruta, ~600 BC).

Effect of Balanced *Pitta*	Effect of Unbalanced *Pitta*
Strong and complete digestion	Poor digestion. Inefficient discrimination between nutrients and wastes
Normal heat and thirst mechanism	Irregular body temperature
Excellent vision	Impaired vision
Good complexion, generally healthy impression	Skin color variable, inflamed, unhealthy; Premature graying
Courageous, cheerful	Anxious, irritable
Stimulated intellect	Dullness of reasoning faculty
Steadfast concentration on the truth; disciplined	Spiritually impoverished
Efficient assimilation of foods	Heartburn, peptic ulcer, irritable bowels, diarrhea

Table 2.4: Effects of balanced and unbalanced *Kapha Dosha* (prepared from Gerson, 1997 and Sushruta, ~600 BC).

Effect of Balanced *Kapha*	Effect of Unbalanced *Kapha*
Excellent nutritional status, firm musculature, strong bones	Poor nutritional status, thin, flabby
Adequate moisture and lubrication in the body	Dry; decreased mucus and saliva
Well-knit joints	Loose joints
Stable, compact and strong physique	Soft and weakened physique
Sexual potency	Sexual impotency
Calm, forgiving, understanding	Intolerant, insecure, jealous
Strong digestion	Slow digestion
Physiological moisture to the respiratory tissues	Excess production of mucus

2.3 Principles of Ayurvedic Treatment Program

Although modern prescription drugs are usually composed of a single pure compound, ayurvedic formulations are either whole extract of the plant or combinations of poly-herbal mixtures. This ayurvedic strategy provides opportunity to exploit different plants for their additive, synergistic or adjuvant properties to enhance medicinal value of such preparations with reduced side effects to the patient.

The disease diagnosis process in ayurveda usually begins with the basic eight-point examination of the patient. They are: *nadi* (pulse), *akriti* (face), *sparsa* (skin), *drika* (eyes), *jihva* (tongue), *shabda* (voice), *mutra* (urine) and *malam* (faeces). If the following symptoms are diagnosed in this examination, *vaidya* (physician) will usually focus on a treatment program geared for balancing excessive *vata dosha*:

- *nadi*: thready, rapid and snake like
- *akriti*: poor darkened complexion
- *sparsa*: cool, rough, dry and bluish in color
- *drika*: lack luster and moisture, constricted pupils
- *jivha*: bluish with many furrows, dry lips
- *shabda*: rough, cracked, weak voice, a dry cough may prevail
- *mutra*: dark yellow
- *malam*: hard, dry and dark

Since both body and mind are considered together in ayurveda, a complete treatment package usually consists of several therapeutic methods. Such methods include *bhavana* (meditation), *leda aahara* (patient's diet), *sambhahana* (massage therapy), *dinacarya* (daily

routine), aromatherapy and herbal therapy. By adjusting diet, life style and environmental factors alone according to ayurvedic recommendations, 2/3 of diseases could be managed (Tripathi, 2000). Usually it may take a while to completely bring the *doshas* to their healthy, balanced status.

Meditation practice is an essential element in achieving the true potential of ayurvedic healing. It allows the human mind to settle in to a state of intense stillness while remaining awake. During the process of meditation the attention is turned inward and towards the very source of thought. This may have an impact on the activity of neurotransmitters of psychological disorders. Meditation appears to significantly influence the neuroendocrine system. In a recent study (Infante et al, 1998) it was found that the practitioners of meditation with similar levels of anxiety of the non-meditating control group, showed a different pattern in the daytime secretion of pituitary hormones. In the meditation group, cortisol levels had a normal pattern. This may be due to a change in feedback sensitivity caused by meditation.

Buddhist monasteries made wide use of medicinal plants in the historical period and usually monks were trained in ayurvedic medical practice as well. The Buddhist philosophy is mainly focused on training unhindered mental concentration. The Buddha himself attained the enlightenment (Buddha hood) after prolonged deep meditation under a bo-tree (*Ficus religiosa*). Ratnasooriya et al (1998) revealed the psychoactive properties of the bo-tree in an anxiolytic activity testing rat trial (Vogel test model) with the aqueous extract of the trunk of this tree. The *Ratana sutra* is a powerful disclosure originally recited by the Buddha to alleviate the three great anxieties in Visala, the capital city of Licchavi kingdom in ancient India. This disclosure is still in practice among Buddhists during *pirith*-chanting ceremonies, to calm down fear psychosis in mentally disturbed people.

2.3 **Gotukola as a Medication for Unbalanced *Vata Dosha***

In Ayurvedic therapy, gotukola has been identified as a treatment component for psychological ailments that correspond generally to modern concepts such as anxiety and depression. Since anxiety and depression is mainly due to unbalanced *vata dosha*, the effect of gotukola is to balance the bodily *vata dosha* paradigms. In Sushruta Samhita, the origins of these doshas are classified into five different categories - namely; *prana*, *udana*, *samana*, *vyana* and *apana*. They seem to correspond to the divisions of cerebro-spinal and sympathetic nerve system (Bhishagratna, 1907). An approximate English translation for the original (Sanskrit) ayurvedic classification of psycho-active substances would be: analgesics, sedatives, depressants, amnesiacs, anesthetics, mood-elevators, intelligence-enhancers, rejuvenating substances, neuro-harmonizers, neuro-humor disruptives, narcotics, anti-hypnotics, stimulants, memory-enhancers, consciousness-restoratives, anti-psychotics, anti-epileptics, intoxicants, anti-aging and mind-soothing substances (Vaidya, 1997).

Leng et al (1998) have shown the tendency of antioxidants for their anxiolytic and antidepressant activity in human subjects. Asiaticoside is a triterpenic compound isolated from gotukola and 0.2% topical application of asiaticoside twice daily for 7 days to wounds in rats had significantly increased the levels of antioxidants such as dismutase, catalase, glutathione peroxidase, vitamin E and ascorbic acid (Shukla et al, 1999a). The *vata-doshas* are caused due to imbalances in *akasha* (space) and *vayu* (air) factors, according to the ayurvedic concept and this concept may be related to the modern concept of antioxidant activity. Therefore, the anxiolytic and other biological activity (such as wound healing, anti-aging and rejuvenating) of gotukola may be related to the antioxidant properties of asiaticoside.

Vata dosha is believed to be the most influential in health management, as it also provides motion to *pitta* and *kapha doshas*. According to ayurvedic descriptions, people affected with unbalanced *vata dosha* are prone to anxiety, fear and nervousness. In verbal communication, they tend to talk fast and interrupt frequently. Sleep pattern is interrupted and may suffer from frequent headaches. They possess an erratic memory with poor concentration and behavior is restless. Their unbalanced *vata dosha* is also interpreted in their dreams as well, with images of flying (like birds), jumping, running and climbing tall trees and other tall objects.

Gotukola (*manduka-parni)* has been identified as an important ayurvedic remedial agent to improve memory and invigorate the mental faculties. The following is a gotukola-based treatment (*Manduka-parni Rasayana*) described in the Sushruta Samhita for such purposes (Sushruta, ~600BC):

First, the patient's *doshas* of the system should be properly cleansed with purgatives and emetics. The patient will be brought to his therapeutic chamber and will remain there during the entire course of treatment and also will be on a special ayurvedic diet comprised of *peya, yavagu* etc. An adequate dose of *manduka-parni* expressed juice, stirred in milk will be consecrated by reciting proper *mantras* a thousand times. This medication will be given to the patient with a meal of cooked barley grains with milk. Or the expressed juice of *manduka-parni* with an admixture of sesame seeds followed by a portion of milk. After digestion of the medicine, a meal of cooked rice with milk and clarified butter will be given. This medication should be administered for three consecutive months.

Alternatively, the patient should fast for three consecutive days and take only the expressed juice of *manduka-parni* during the fasting period. After this period, the patient should live on a paste of *manduka-parni,* stirred with milk for further ten consecutive days.

2.5 Discussion

The confusion in differentiating various kinds of knowledge systems may be derived from the use of the term "western science" to enunciate the meaning of "modern science", and "traditional knowledge" to express anything that does not fit into "modern science". This use of "western science" itself is not appropriate, since it gives an exaggerated predisposition that all recent scientific endeavors in ethno-pharmacology were accomplished in geographically or culturally western countries. To clarify confusions in the terminology of ethno-pharmacological studies, it would be more appropriate to classify different existing knowledge systems as: a) ancient science, b) modern science and c) traditional practices. Inevitably, where to draw the timeline to distinguish ancient and modern sciences is debatable.

A radioligand receptor-binding assay (RRA) conducted at the National Institute of Mental Health (NIMH), USA, has shown that gotukola crude methanolic extract indicates a very high affinity for γ-amino butyric acid – $GABA_A$ and $GABA_B$ neurotransmitter receptors (Misra, 1998). The ayurvedic interpretation of gotukola supports this neurotransmitter activity and/or antioxidant activity (Leng et al, 1998 and Shukla et al, 1999a) concepts applicable to anxiety and other psychiatric disorders. As revealed by the study conducted for this thesis (Chapter 4) as well as several other recent investigations (Bradwejn et al, 2000, Inhee et al, 1999, Sang-sup et al, 2000), the scientific basis of ayurvedic use of gotukola is concordantly demonstrated.

Gotukola is another example among various other ayurvedic plants such as sarpagandha (*Rauwolfia serpentina*) and brahmi (*Bacopa monniera*), with proven psychoactive properties. The humble ancient science of ayurveda should be properly credited for its contributions for the advancement of modern pharmaceutical sciences. Ayurveda is not a "tradition" to be exploited and neglected, but a "science" deserved to be protected and further nourished.

CHAPTER 3

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS OF GOTUKOLA (CENTELLA ASIATICA L. URBAN) FOR ASIATICOSIDE AND ASIATIC ACID

3.1 INTRODUCTION

Asiaticoside, asiatic acid and madecassic acid are the three principle constituents of the total triterpenic fraction (TTF) of Gotukola (*Centella asiatica*). The TTF is approximately comprised of 60% asiatic and madecassic acids together and 40% asiaticoside (Grimaldi et al, 1990).

Several HPLC procedures have been developed to analyze and quantitative determination of these plant compounds. Gunther and Wagner (1996) employed reversed-phase HPLC separation with water (solvent A) and acetonitrile (solvent B) each containing 0.05% H_3PO_4 as the mobile phase with a flow rate of 0.8 ml/min and sample injection volume of 5 µl to analyze the methanol extract of gotukola. The RP 18 (5µm), 125 x 4 mm column and 205 nm photodiode array detector was used in this study. In another investigation, hexane insoluble component of gotukola was extracted with methanol and a reverse-phase HPLC method was adopted with water (containing 1% trifluroacetic acid (TFA): methanol (30:70 v/v) as the mobile phase with a flow rate of 1 ml/min. The column CLC-ODS (M) 250 x 4.6 mm was used with 26 °C temperature and the detector wavelength was 220 nm. This method resulted in 97% recovery of

asiaticoside (Verma et al 1999). To analyze a cosmetic patch made of triterpenic fraction of gotukola, which is intended to control cellulitis, Morganti et al (1999) used methanol for extraction. A Varian MICROPAK C_{18} (5µm) 40 x 12.5 mm column at room temperature and 0.3% phosphoric acid (solvent A) and acetonitrile (solvent B) mobile phase was used in the study. During the first 4 min of the run, the mobile phase was constant (H_3PO_4 / CH_3CN, 70:30). From 4 to 20 min, the acetonitrile gradient was linearly increased from 30 to 50% and the flow rate was increased from 1 to 1.2 ml/min from the beginning to 20 min. In our current study of gotukola raw materials and extracts, an attempt was made to improve on these established methods using a micro-bore column using substantially reduced solvent.

Asiaticoside is transformed into asiatic acid *in vivo* and the relatively small amounts of these compounds in plasma require a suitable method to detect them. In a pharmacokinetic clinical investigation of oral administration of total triterpenic fraction of gotukola, an assay method was developed to analyze the asiatic acid concentration in blood plasma. A Perkin Elmer C_{18} pre-column and Perkin Elmer Cyanosil-X-10 column was used with a flow rate of 1 ml/min and Perkin Elmer LC-95 detector at 200 nm wave length. A mixture of (A) acetonitrile/methanol (700:200) and (B) water / H_3PO_4 (85%) at the ratio of 42:58 was used as the mobile phase (Grimaldi et al, 1990). A column-switching HPLC procedure was introduced for determination of asiaticoside in rat plasma and bile after i.v. administration of asiaticoside at a 10 mg/kg dose. Capcell Pak MF Ph-1, 150 x 4.6 mm (5µm) was employed as the clean-up column A, and Capcell Pak C_{18} UG120, 35 x 2.0 mm (5µm) was employed as the concentration of analyte column B in the study at 40 °C and asiaticoside was detected at 210 nm wavelength. Sodium

phosphate 10 mM (pH 6.86) (buffer A) and 50% acetonitrile in deionized water (v/v) (buffer B) was used as the mobile phase and the bile and plasma samples were kept at 10°C until injected (Baek et al, 1999).

In a recent preliminary clinical study, researchers at the Royal Ottawa Hospital have shown the anxiolytic activity of gotukola (Bradwejn et al, 2000). For this study a dosage of 12 g of dried gotukola materials per human subject had to be used. Since administering this bulky amount of plant materials is impractical with human subjects, it is important to investigate a more appropriate gotukola raw material or extract which contains higher concentration of biologically active materials for future such studies. The objective of this HPLC study was to determine the asiaticoside content of different commercial products including the one (Nature's Way) used in this clinical study as well as extracts and tissue cultured material. The other objectives of the study were to analyze the tissue cultured gotukola plants grown in the university greenhouse for the asiaticoside content also to conduct a recovery analysis for one of the extraction procedures.

3.2 MATERIALS AND METHODS

3.2.1 Commercial Products Analysis (Study 1)

Five different gotukola commercial products were purchased from the local market for the analysis. They are: Nature' Way (NW), Li Chang Yun (LCY), Organika (OG), Solaray Madagascar (SM) and Bulk powder from India (IND) (Table 3.1). Capsules were removed from the products to take the gotukola powder out. One-gram material each was first defatted with hexane (3 x 20 ml, shaker 1 h) and then extracted with methanol (3 x 20 ml, sonicater 15 min, shaker 6 h). The filtered methanol extracts were concentrated under vacuum in rotary evaporator and redissolved in 10 ml methanol. Then they were filtered through Chromspec 0.2 μm PTFE (Chromatographic Specialties, Brockville, Ontario) filter and subjected to HPLC analysis.

HPLC UV/MS (Agilent 1100 Series, Agilent Technologies, Waldbronn, Germany) apparatus was used to estimate the asiaticoside and asiatic acid levels in the samples. HPLC method: YMC 3 μm ODS (C_{18}) AM 2 x 100 mm column (Waters, Mississauga, Ontario); oven 50 °C; flow 0.2 ml/min; 0.05 % formate in water; gradient 10-80 % MeCN in 8 min; 80-100 % MeCN in 2 min; hold 2.5 min; 100-10% MeCN in 2.5 min; end time 15 min; post-time 5 min.

The ultra-violet (UV) signal was detected at 205 nm and standards of asiaticoside and asiatic acid were used to identify the elution of respective compounds from plant materials at given retention times. The mass spectrometric (MS) signal was monitored at ion 487 m/z, the M^{-1} pseudo-molecular ion for asiatic acid. Confirmation of asiaticoside identity was via presence of 957.6 m/z ion (the pseudo-molecular ion of asiaticoside in

negative ionization mode).

3.2.2 Hexane, Ethyl Acetate and Methanol Fractions Analysis (Study 2)

Aerial parts of the tissue cultured gotukola plants grown in the university greenhouse were harvested and cleaned. Then they were oven dried at 40°C for 24 hours and crushed. Gotukola powder (128 g) obtained from 750g of fresh plant material was used for extractions. The powder was first extracted with hexane: (3 x 2.5 L, sonicater 5 min, magnetic shaker 6h). The hexane insoluble material was then extracted with ethyl acetate (3 x 2.5 L, sonicater15 min, shaker 6h). From the hexane and ethyl acetate insoluble residue, a final extract was made with methanol: (3 x 2.5 L, sonicater 15 min, shaker 6h). The extracts were filtered and concentrated under vacuum. The HPLC method used for the study 1 was adopted also for this analysis.

3.2.3 Recovery Analysis for Asiaticoside (Study 3)

Four one-gram (dry weight) gotukola samples were spiked with 0, 500, 1000 and 1500 µg asiaticoside each. Then the extraction and HPLC procedure employed in the Study 1 was conducted to estimate the recovery percentage of asiaticoside.

3.2.4 Minimum Detectable Amount Analysis for Asiaticoside and Asiatic Acid (Study 4)

A study was conducted to determine the minimum detectable amount with five times serial dilution of asiaticoside (720 µg/ml) and asiatic acid (700 µg/ml) standard solutions.

Table 3.1: Gotukola products analyzed for their asiaticoside and asiatic acid content.

Commercial Product	Capsule Weight (mg)	Accession Number
Nature's Way	530	UO 3001
Li Chang Yun	400	UO 3002
Organika	500	UO 3003
Solary Madagascar	250	UO 3004
Bulk Powder from India	-	UO 3005

3.3 RESULTS AND DISCUSSION

Although both the HPLC/UV and HPLC/MS were used to detect the compounds, it was found that more reliable quantitative data could be obtained by the HPLC/UV than HPLC/MS despite the lower detection limit of the latter method. Therefore data presented here are the ones obtained from HPLC/UV. Asiaticoside and asiatic acid contents were calculated on dry weight basis of materials tested.

HPLC/MS analysis provided unequivocal confirmation of asiaticoside and asiatic acid in the samples via presence of the molecular ions at 957.6 and 487 m/z respectively. To validate the HPLC procedure, the standard error of mean (SEM) and coefficient of variance (CV) values were estimated. Asiaticoside and asiatic acid were eluted with retention times of 9.42 min (SEM = 0.004, CV = 0.21%) and 12.31 min (SEM = 0.004, CV = 0.24%) respectively (Table 3.2 and Figure 3.1). These values indicate a reliably consistent HPLC method for these two compounds. The CV values for lower asiaticoside containing NW product analysis (n = 5) were 5.48% and 4.76% for asiaticoside and asiatic acid respectively. The higher asiaticoside containing SM product analysis (n = 5) showed CV values of 1.02% for asiaticoside and 4.05% for asiatic acid. The recovery analysis for asiaticoside resulted in a recovery of 87 %, which is satisfactory for the method (Figure 3.2).

The method showed minimum detectable levels of approximately 1.1 μg/ml (asiaticoside) and 0.68 μg/ml (asiatic acid) with the HPLC/UV and 0.03 μg/ml (asiaticoside) and 0.02 μg/ml (asiatic acid) with the HPLC/MS. These estimations were extrapolated from the lowest concentrations of standards analyzed.

Figure 3.1: HPLC chromatograms (A: UV and B: MS) of typical sample (SM1).

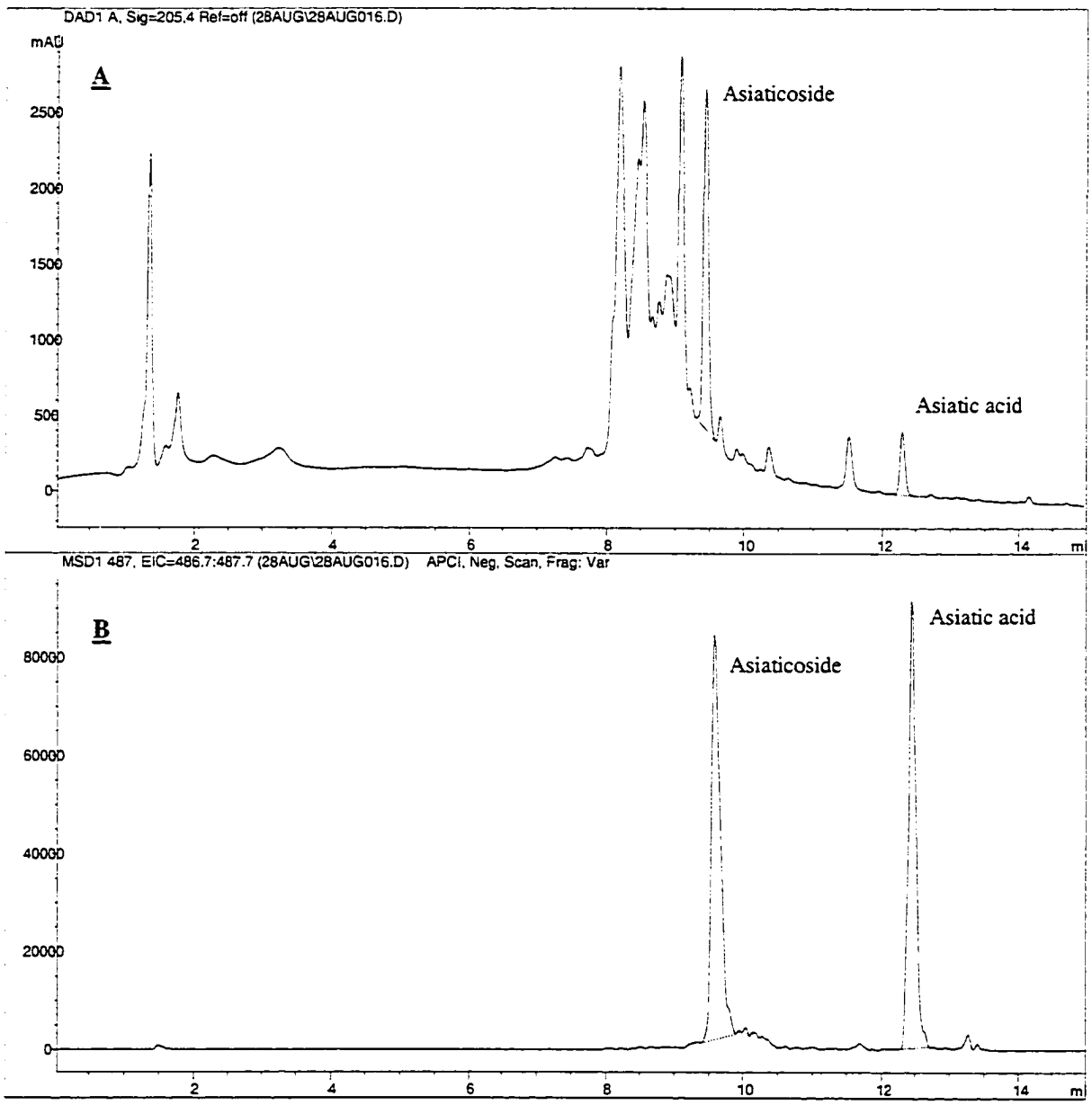


Figure 3.2: Amount of asiaticoside recovered from gotukola samples spiked with different amount of asiaticoside.

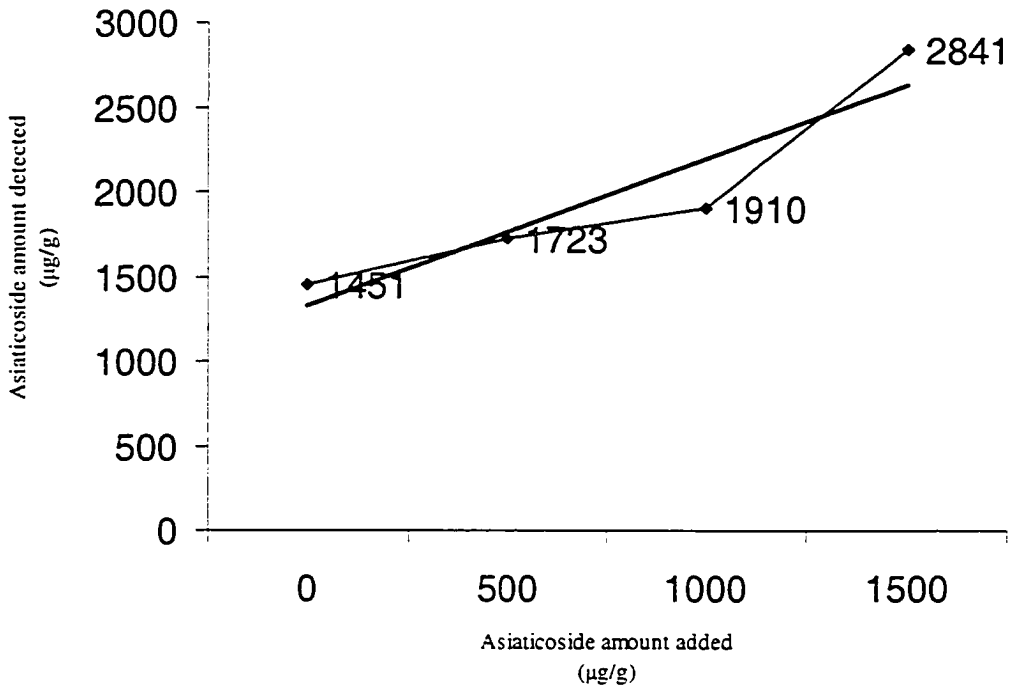


Table 3.2: Mean retention times for asiaticoside and asiatic acid in the HPLC procedure.

Compound	Retention Time (min)	Standard Error	Coefficient of Variance
Asiaticoside (n = 41)	9.42	0.004	0.21
Asiatic Acid (n = 44)	12.31	0.004	0.24

Out of the five commercial products, the asiaticoside content showed the highest amount in SM (2.72%), while the NW product contained a relatively low amount (0.37%). Both of these products were employed in the animal study for testing their anxiolytic activity. The other products LCY, OG and IND contained 0.13, 0.10 and 0.41% asiaticoside respectively. The asiatic acid content also varied among the products as indicated by NW (0.17%), LCY (0.10%), OG (0.02%), NW (0.17%) and IND (0.08%). Most of these differences among commercial products were significant ($P < 0.001$) in Tukey's HSD multiple comparison test (Table 3.3).

Genetic differences in the plant materials are known to be associated with their geographical location of origin and such differences may be reflected in the respective phytochemical contents. As an example, the SM materials originated in Madagascar contained significantly higher ($P < 0.001$) asiaticoside content of 2.4 % as compared to the asiaticoside content of materials from India (0.41 %). However, there was no significant difference in asiatic acid content of these two materials.

The study with sequential extraction of gotukola plant materials indicated that methanol, ethyl acetate and hexane extracts contained 83, 11 and 6 % of whole asiaticoside content and 32, 67 and 1 % of whole asiatic acid content respectively (Table 3.4). Most of the asiaticoside content was found in the methanol fraction in contrast to the fact that most of the asiatic acid content was found in the ethyl acetate fraction.

Table 3.3: Results of HPLC-UV analysis of different commercial gotukola products for the asiaticoside and asiatic acid content.

Commercial Product	Asiaticoside content (µg/g dry weight)	Asiatic acid (µg/g dry weight)
Nature's Way (n = 5)	$3700^{b} \pm 91$	$1735^{d} \pm 37$
Li Chang Yun (n = 3)	$1274^{a} \pm 69$	$991^{c} \pm 33$
Organika (n= 3)	$981^{a} \pm 7$	$237^{a} \pm 3$
Solaray Madagascar (n = 5)	$24{,}000^{c} \pm 110$	$1712^{d} \pm 31$
Bulk material from India (n = 3)	$4085^{b} \pm 90$	$799^{b} \pm 11$

Values are mean ± SEM. Means followed by a same letter (a – d) in a column are not significantly different in ANOVA / Tukey's HSD multiple comparison test ($P < 0.001$).

Table 3.4: Results of HPLC-UV analysis of hexane, ethyl acetate and methanol fractions of tissue cultured gotukola plants for their asiaticoside and asiatic acid content.

Fraction	Asiaticoside content (µg/g dry weight)	Asiaticoside (% of Total)	Asiatic acid (µg/g dry weight)	Asiatic Acid (% of Total)
Hexane (n = 3)	3135 ± 22	6%	148 ± 86	1%
Ethyl acetate (n = 3)	6019 ± 460	11%	12,509 ± 1014	67%
Methanol (n = 3)	43,467 ± 116	83%	6086 ± 15	32%

Values for weights are mean ± SEM.

CHAPTER 4

ANXIOLYTIC ACTIVITY OF WHOLE PLANT MATERIALS, EXTRACTS AND ISOLATED COMPOUND ASIATICOSIDE OF GOTUKOLA (*CENTELLA ASIATICA* L. URBAN)

4.1 INTRODUCTION

The ayurvedic use of gotukola whole plant materials for its psychoactive properties (Sushruta, 600 BC) is supported by several investigations including a recent double-blind, placebo-controlled study conducted by the Royal Ottawa Hospital (Bradwejn et al, 2000). In addition, the increasing in the pentobarbitone induced sleeping time and decreasing the immobility in the forced swim test (Sakina and Dandiya, 1990), gotukola also elicits anti-anxiety effects in the elevated plus maze, (Lucia et al, 1997) supporting the contention that gotukola ethanol extract may impart anxiolytic effects. In addition, the aqueous extract of gotukola was observed to have cognitive enhancing and antioxidant effects when administered to rats (Kumar and Gupta, 2002).

The most prominent group of biologically active compounds isolated from gotukola are the terpenes. Asiaticoside is the most abundant triterpene glycoside, which is effective in wound healing (Shukla et al, 1999a) and apparently acts by enhancing the induction of antioxidant levels at an early stage of wound healing (Shukla et al, 1999b). Asiaticoside is transformed into its aglycone asiatic acid *in vivo* by hydrolysis. Several

derivatives of asiaticoside (Inhee et al, 1999) and asiatic acid (Sang-sup et al, 2000) were found to have protective effect against beta amyloid (A-β)-induced neurotoxicity associated with dementia of Alzheimer's disease.

In the Bradwejn et al (2000) study, the effective dose in humans was estimated at 12 g crude leaf material per patient, based on scale up from animal trials. Administration of such large amounts of plant material is awkward, and impractical. One of the objectives of the present study was to identify the active fraction(s) and compounds from the plant as well as more active plant genotypes. The second objective of this study was to identify the efficacy of these compounds across a variety of animal models of anxiety including the elevated plus maze, open field test, social interaction test, locomotor activity test, Vogel test and novel environment test.

4.2 MATERIALS AND METHODS

4.2.1 Test Animals

Experiments were conducted in accordance with the guidelines stipulated by the local animal care committee and the Canadian Council on Animal Care. Male Sprague Dawley (SD) rats (300 – 325 g body weight) raised in a pathogen-free colony, were obtained from Charles River Canada Inc. (St-Constant, QC) a week before the tests. On arrival they were housed individually in standard rat cages measuring 45 x 24 x 20 cm (Lab Products, Pennsylvania, USA) and fed with standard rat chow (Purina; code 5012) and tap water *ad libitum*. In the rat housing facility ventilation (100 % fresh-air 20 exchanges per hour), lighting (12 h light-dark cycle; 7.00 am – 7.00 pm) and room temperature (21°C) were tightly regulated. Bedding material (Prochips; maple or birch hardwood chips) was changed once a week. During the habituation period of at least 5 days after arrival, rats were familiarized with the researcher(s).

Drug administration and testing of rats were conducted in a sound attenuated room. A mild detergent free of any strong odor (Quatsyl; 8ml/L water, Pharmacia & Upjohn Animal Health, Orangeville, Ontario) was used to clean the cages between tests.

4.2.2 Apparatus and Procedure

4.2.2.1 Elevated Plus Maze (EPM) Test

In the elevated plus-maze test, subjects were exposed to the inherent conflict and anxiety between the need to explore the novel area and the need to avoid more vulnerable (or aversive) areas of the EPM (heights and open spaces). The EPM is a wooden apparatus elevated off the floor at a height of 50 cm in a '+' array with two opposing

open-arms and two perpendicularly opposing closed-arms each measuring 50cm in length and 10cm in width. The closed-arms were enclosed by 40 cm high walls on three sides with open top, while the open-arms are completely open planks. The flooring was made of black rubberized runway and all interior walls were made of black plexiglas. An open central area measuring 10 x 10 cm separated each arm (Appendix 1). The apparatus was surrounded by a black curtain to minimize distractions. Light levels at the center, open-arm and closed-arm of the plus maze apparatus were 35, 40 and 4 lux respectively. A closed circuit camera was mounted on the ceiling positioned above the center of the maze and connected to a monitor for remote observation of animal's behavior.

Following drug administration, rats were returned to their home cages during the post-drug interval. Rats were then introduced into the open field arena for 5 minutes, just before placement into the plus maze. They were placed in the central open square facing the closed-arm and monitored for 5 minutes. The test was video taped for subsequent computer assisted data analyses. The behavioral parameters quantitated included; number of entries and time spent on the open-arm and the closed-arms, number of occurrences and time spent in protected head dips. Number of unprotected head dips was counted manually. All four paws must enter the arm to be counted as an entry into that arm. The protected head dips were operationally defined as the rat dipping its head over the sides of open-arm while part of the body is within the closed-arm. Head dips made from the open-arm (without contact with walls of closed-arms) were designated unprotected head dips. After five minutes of testing, rats were removed from the maze and returned to their home cages.

4.2.2.2 Open Field Test

In this test, the aversion of the central zone (or vulnerable area) of an arena is used as an index of the levels of anxiety. The open field apparatus constituted of a rectangular plexiglas arena measuring 60 x 60 cm with 35 cm high walls. The floor is marked with lines that divide it into 36 (6 x 6) squares (10 x 10 cm) (Appendix 1). The squares immediately adjacent to the walls of the test arena constitute the 'safer' peripheral zone, whereas the inner or more centrally positioned squares were identified as central (or vulnerable) zone. The behavior was monitored via a closed circuit video camera mounted on the ceiling. The test apparatus was surrounded by black curtains to minimize undue distraction. Light levels at the center and perimeter of the open field test arena were 22 and 12 lux respectively. Rats were introduced into the center of the arena to initiate the test. Over a period of five minutes, the number of squares crossed and time spent in the center and the perimeter was documented.

4.2.2.3 Social Interaction Test

In this test, the amount of time a pair of rats spending socially interacting with one another is thought to reflect the level of anxiety in these subjects. The duration of social interaction decreases with increased anxiety. Rats were placed individually in the test arena for 7 minute familiarization session on 2 consecutive days. During the test day, two randomly selected rats were administered the drug and placed in adjacent cages in the waiting area of the test room. One hour later, they were introduced together into the center of the test arena. Social interaction behavior was observed remotely for 7 minutes, via the video camera. The number of occurrences of social interactions (sniffing,

following, and grooming the partner) and time spent in them were scored.

4.2.2.4 Locomotor Activity Test

In order to monitor general locomotor activity, the distance traversed in their home cage was monitored via a computerized infrared tracking device. The locomotor activity was assessed by tracking the number of zones (6 per cage) crossed and the total distance traversed. Drugs were administered at 10 am and data collection initiated at 11 am lasted for 22 hours (until 9 am the next day). Light level around test cages was 90 lux with 12 h light-dark cycle; 7 am – 7 pm.

4.2.2.5 Vogel Test (Thirsty Rat Conflict)

In this test, thirsty rats are periodically given a mild shock during their attempts for drinking water. The lower the level of anxiety, the greater the number of shocks accepted during drinking. Thus despite the mild shock, less anxious rats will engage in higher number of licks. The Vogel cage (Habitest Operant Cage H 13-16, Coulbourn Instruments, Allentown, PA, USA) is made of plexiglas and measures 30 x 25 x 30cm. The box has a floor with metal rods spaced 2 cm. apart and is has a water spout tube connected with an external shocking device. The water bottle is placed on the outside of the test box (about 3 cm above the grid floor) and spout protrudes 2 cm into the cage (Appendix 1). A mild shock of 0.1 or 0.4 mA was delivered through the spout on every fifth lick. The number of licks emitted were tracked over the 10 minute test period via an optical beam (that is interrupted by the tongue when it contacts the spout).

Rats were previously trained for 2 consecutive days to locate and drink from the water spout, in the absence of shock delivery. Then they were deprived of water for 22 h

and randomly assigned to various test groups. The light level around the test cages was 92 lux.

4.2.2.6 Novel Cage Test

When rats are presented with a familiar palatable snack in their home cage, they immediately approach and consume it. However, when the same snack is presented in a novel environment, the latency to approach the snack is markedly increased, and the amount consumed is decreased. Rats treated with an anxiolytic agent would be expected to shorten the latency to approach the snack and increase the amount consumed. Rats were trained to eat a palatable snack (Graham Honey Maid brand) for 20 minutes each day for consecutive 8 days in their home cages. On the test day, rats were treated with the drug(s) and transferred into a novel cage and presented with the palatable snack. Then the latency to initiate snack consumption as well as the amount consumed was measured. The light level around the rat cages was 90 -100 lux.

4.2.3 Drug Preparation

Ayurveda recommends mixing gotukola with milk before its administration, to get the intended psychoactive effects (Sushruta, ~600BC). Dry powder and fresh leaf extracts of gotukola were sonicated for 15 minutes in 50% sweetened condensed milk solution (No Name brand, Sunfresh Ltd, Toronto, Ontario) or distilled water to make their suspensions. The treatments were given orally (*p.o.*) using a gavage tube (gage 18, length 5 cm). Asiaticoside was sonicated in peanut oil to make suspensions for intra-peritoneal (i.p.) administration (needle gage 25, length 1.6 cm). The volume of drug or

vehicle (control) was around 2 ml or less for *p. o.* and less than 1 ml for i.p. Rats were habituated to drug administration modalities for two consecutive days prior to the tests. Different dosages and post-drug time intervals (PDI) were tested as described under descriptions of particular tests. Immediately after the drug administration, rats were brought into the test room and placed in the holding area adjacent to the test apparatus.

4.2.4 Dosage, Post-drug Interval and Treatment Administration

4.2.4.1 Study(A) with Whole Plant Materials from Different Gotukola Products

Two commercial gotukola products containing relatively higher or lower concentration of asiaticoside were selected for this study. They were the Nature's Way product (NW) and Solaray Madagascar product (SM). As revealed by the HPLC analysis (Chapter 3, Table 3.2), NW and SM contain 0.37% and 2.40% of asiaticoside (dry weight basis) respectively. The elevated plus maze test (EPM) and the novel cage test (NCT) were conducted in this study with different test parameters (Table 4.1).

4.2.4.1.1 Elevated Plus Maze Test (A1):

The NW and SM products were tested at 200 mg/kg dosage with distilled water as the control / vehicle and 1 h PDI, during 9 am to 3 pm.

4.2.4.1.2 Elevated Plus Maze Test (A2):

The NW and SM products were tested at 500 mg/kg dosage with 50 % condensed milk as the control / vehicle and 2 h PDI, during 10 am to 5 pm.

4.2.4.1.3 Novel Cage Test (A3):

The NW and SM products were tested at 200 mg/kg dosage with distilled water as the control / vehicle and 1 h PDI, during 10 am to 11 am.

4.2.4.2 Study(B) with Gotukola Extracts of Different Polarity

The aerial parts of the tissue culture propagated gotukola plants grown in the university greenhouse were cleaned and oven dried at 40 °C for 24h and crushed into powder. Gotukola powder (128 g) obtained from 750g of fresh plant material was used for extractions. The powder was first extracted with hexane: (4L, sonicated for 15 minutes, magnetic shaker 6h) x 3. The hexane insoluble material was then extracted with ethyl acetate: (4L, sonicated for 15 minutes, magnetic shaker 6h) x3. From the hexane and ethyl acetate insoluble residue, the last extract was made with methanol: (4L, sonicated for 15 minutes, magnetic shaker 6h) x3. The extracts were filtered and concentrated under vacuum. The extracted fractions (hexane – 2.12 g, ethyl acetate – 1.11 g, methanol – 30.47 g) were then dissolved in 50 ml of 50 % condensed milk.

This test was conducted from 9 am to 4 pm with the elevated plus maze test model. The hexane extract (212 mg/kg), ethyl acetate extract (111 mg/kg) and methanol extract (3047 mg/kg) was tested with 50% condensed milk as the control / vehicle. Two hours of post-drug interval was employed in this test.

4.2.4.3 Study(C) with Pure Compound Asiaticoside

A pure compound of gotukola - asiaticoside was purchased from Indofine

Chemicals, Somerville, NJ, USA. The study was conducted with different test conditions as summarized in the Table 4.2.

4.2.4.3.1 Elevated Plus Maze Test (C1):

Asiaticoside was tested at 1 and 3 mg/kg dosages with peanut oil as the control / vehicle and 1 h PDI, during 8 am to 3 pm.

4.2.4.3.2 Elevated Plus Maze Test (C2):

Asiaticoside was tested at 3, 5 and 10 mg/kg dosages with peanut oil as the control / vehicle and 1 h PDI, during 8 am to 3 pm.

4.2.4.3.3 Open Field Test (C3):

Asiaticoside was tested at 3, 5 and 10 mg/kg dosages with peanut oil as the control / vehicle and 1 h PDI, during 8 am to 3 pm.

4.2.4.3.4 Social Interaction Test (C4):

Asiaticoside was tested at 1 and 3 mg/kg dosages with peanut oil as the control / vehicle and 1 h PDI, during 8 am to 3 pm.

4.2.4.3.5 Social Interaction Test (C5):

Asiaticoside was tested at 1 and 3 mg/kg dosages with peanut oil as the control / vehicle and 4 h PDI, during 11 am to 6 pm.

4.2.4.3.6 Locomotor Activity Test (C6):

Asiaticoside was tested at 1 and 3 mg/kg dosages with peanut oil as the control / vehicle. The treatments were administered at 10 am and hourly data were collected from 11 am to 9 am the next day.

4.2.4.3.7 Vogel Test (C7):

Asiaticoside was tested at 1 and 3 mg/kg dosages with peanut oil as the control /

vehicle and 1 h PDI, during 8 am to 3 pm. The shock level of the apparatus was at 0.4 mA.

4.2.4.3.8 Vogel Test (C8):

Asiaticoside was tested at 5 mg/kg dosage with peanut oil as the control / vehicle and 1 h PDI, during 8 am to 3 pm. The shock level of the apparatus was at 0.1 mA.

4.2.4.3.9 Vogel Test (C9):

The PDIs of 0.5, 1 and 2 h was tested with asiaticoside dosage of 5 mg/kg, during 8 am -2 pm. The shock level of the apparatus was at 0.1 mA.

4.2.5 Statistical Analysis

The main focus of the study was to investigate the anxiolytic activity of different phytochemical components of gotukola, compared to the controls. For this reason the t-test was chosen, as the most straightforward analysis of this comparison.

Results for the rats, which fell off the EPM were omitted from statistical analyses.

Table 4.1: Test parameters for studying anxiolytic activity of gotukola products with high and low asiaticoside content.

Test No.	A1	A2	A3
Test Model	EPM	EPM	NCT
Drugs and dosage	1. control / vehicle: distilled water 2. gotukola: NW 200 mg/kg 3. gotukola: SM 200 mg/kg	1. control / vehicle: 50% condensed milk 2. gotukola: NW 500 mg/kg 3. gotukola: SM 500 mg/kg	1. control / vehicle: distilled water 2. gotukola: NW 200 mg/kg 3. gotukola: SM 200 mg/kg
Post-drug Interval	1h	2h	1h
Time of test conducted	9 am to 3 pm	10 am to 5 pm	10 am to 11 am

EPM = elevated plus maze test, NCT = novel cage test, NW = Nature's Way product, SM = Solaray Madagascar product

Table 4.2: Test parameters for studying anxiolytic activity of asiaticoside.

Test No.	C1	C2	C3	C4	C5	C6	C7	C8	C9
Test Model	EPM	EPM	OFT	SIT	SIT	LAT	VT Shock: 0.4mA	VT Shock: 0.1mA	VT Shock: 0.1 mA
Drugs and dosage: (mg/kg)	1.control 2. asd 1 3.asd 3	1.control 2.asd 3 3.asd 5 4.asd 10	1.control 2.asd 3 3.asd 5 4.asd 10	1.control 2.asd 1 3.asd 3	1.control 2.asd 1 3.asd 3	1.control 2.asd 1 3.asd 3	1.control 2.asd 1 3.asd 3	1.control 2. asd 5	asd 5
Post-drug Intervals: h	1	1	1	1	4	1-22	1	1	1. 0.5 2. 1 3. 2
Time of test conducted	8am - 3pm	8am - 3pm	8am - 3pm	8am - 3pm	11am - 6pm	11am - 9am next day	8am - 3pm	8am - 3pm	8am - 2pm

EPM = elevated plus maze test, OFT = open field test, SIT = social interaction test, LAT = locomotor activity test, VT = Vogel test, control = vehicle (peanut oil), asd = asiaticoside

4.3 RESULTS AND DISCUSSION

4.3.1 Study with Whole Plant Materials from Different Gotukola Products (Tests A1 – A3)

In the EPM test with low dose (200 mg/kg) the NW group spent significantly more time on the closed-arm (244.5 seconds), compared to the control group (216.4 seconds) (Table 4.3). However, there were no other overt differences between groups in other behavioral parameters quantitated.

In order to ascertain whether the relatively small effect observed in the above experiment was due to a) inadequate dosage or b) inadequate PDI, test parameters were changed. With these new changes to the test parameters, a more pronounced anxiolytic effect was observed in the test A2 (Table 4.4). The positive observations for anxiolytic activity of gotukola were consistent in 6 out of 7 criteria tested (at $P < 0.001$ level) as reflected by:

a) significantly increased number of open-arm entries in the SM group (4.8), compared to the control (2.2); b) significantly increased duration of time (in seconds) spent on the open-arms of the EPM in the NW (62.4) and SM (66.0) treated groups compared to the control group (24.1); c) significantly decreased number of closed-arm entries in the NW (9.7) and SM (10.2) groups compared to the control group(22.6); d) significantly decreased duration spent on the closed-arm in the NW (143.7) and SM (136.7) groups compared to the control group(208.9); e) significantly increased number of protective head dips in the NW (18.6) and SM (20.9) groups compared to the control group(12.8); f) significantly increased time duration (in seconds) engaged in the protected head dip

Table 4.3: Comparison of low dose of different gotukola products based on the performance on the elevated plus maze: Nature's Way and Solaray Madagascar products (at 200 mg/kg dosage) with the control (distilled water), after 1 h post-drug interval.

Test A1

Treatment	# o.a.	Time o.a. (seconds)	# c.a.	Time c.a. (seconds)	# phd	Time phd (seconds)	# uphd
Control n = 13	4.4 ± .8	58.3 ± 8.1	11.7 ± .5	216.4 ± 10.0	6.8 ± .9	14.5 ± 2.7	9.5 ± 1.5
Nature's Way (NW) n = 13	2.8 ± .6	35.3 ± 8.0	11.4 ± .7	244.5 ± 7.2*	6.5 ± .6	11.7 ± 1.3	6.1 ± 1.2
Solaray Madagascar (SM) n = 12	3.4 ± .9	44.8 ± 12.6	11.2 ± .8	231.5 ± 14.4	5.9 ± 1.0	13.8 ± 2.6	7.3 ± 1.9

All values represent the group mean ± SEM (n = 12 - 13)

o.a. – number of open-arm entries, Time o.a. – time spent on open-arm, # c.a. – number of closed-arm entries, Time c.a. – time spent on closed-arm, # phd – number of protected head dips, Time phd – time spent on protected head dips, # uphd – number of unprotected head dips

* $P < 0.05$ vs. control, t-test

Table 4.4: Comparison of high dose of different gotukola products based on the performance on the elevated plus maze: Nature's Way and Solaray Madagascar products (at 500 mg/kg dosage) with the control (distilled water), after 2 h post-drug interval.

Test A2

Treatment	# o.a.	Time o.a. (seconds)	# c.a.	Time c.a. (seconds)	# phd	Time phd (seconds)	# uphd
Control n = 10	2.2 ± .9	24.1 ± 9.7	22.6 ± 11.2	208.9 ± 13.1	12.8 ± 1.4	45.6 ± 6.7	10.6 ± 4.0
Nature's Way n = 10	3.7 ± .6	62.4 ± 10.8*	9.7 ± 1.2	143.7 ± 18.5*	18.6 ± 1.6*	77.9 ± 9.4*	29.4 ± 5.6*
Solaray Madagascar n = 10	4.8 ± .7*	66.0± 9.3*	10.2 ± .5	136.7 ± 10.7*	20.9 ± 1.2*	86.0 ± 5.6*	33.7 ± 4.0*

All values represent the group mean ± SEM. (n = 10)

o.a. – number of open-arm entries, Time o.a. – time spent on open-arm, # c.a. – number of closed-arm entries, Time c.a. – time spent on closed-arm, # phd – number of protected head dips, Time phd – time spent on protected head dips, # uphd – number of unprotected head dips

* $P < 0.001$ vs. control; t-test

activity in the NW (77.9) and SM (86.0) groups compared to the control group (45.6); and g) significantly increased number of unprotected head dips in the NW (29.4) and SM (33.7) groups compared to the control group (10.6).

Although there was a trend for greater anxiolytic activity in the SM group, compared to the NW group, the differences were not statistically significant. The 500 mg/kg dose (delivered in 50% condensed milk; *p.o.*) with 2 h post-drug interval appeared to be more effective in the elevated plus maze test of anxiety. Compared to all the other tests conducted in this study, the test A2 was started a little late in the morning. It may be useful to investigate whether it is more suitable to conduct the elevated plus maze tests for anxiolytic drug testing later in the morning - from 10.00 am to 5.00 pm, instead of from 8.00 am to 3.00 pm.

There was no significant difference between the control and the NW and SM drug groups and also between the NW and SM drug groups in the novel cage test A3 (Table 4.5). Since the same low dose as in the test A1 was employed for this test, it is apparent that the 200 mg/kg dosage and 1 h post drug interval is not sufficient to demonstrate any activity.

Table 4.5: Comparison of the novel cage test performance of rats treated with different gotukola products: Nature's Way and Solaray Madagascar (200mg/kg) with the control (distilled water), after 1 h post-drug interval.

Test A3

Treatment	Latency (seconds)	Food intake (g)
Control (Distilled water) n=13	29.3 ± 9.1	2.2 ± .4
Gotukola Nature's Way n=12	22.8 ± 6.0	2.5 ± .8
Gotukola Solaray Madagascar n=12	35.3 ± 9.3	3.0 ± .7

All values represent the group mean ± SEM. (n = 12 - 13)

4.3.2 Study with Gotukola Extracts of Different Polarity (Test B)

Fresh gotukola materials comprised of 17.07 % of dry materials (and 82.93% water). As a percentage of dry weight of plant materials, the methanol extract represents the most abundant fraction (23.80%) of gotukola, followed by the hexane (1.66%) and ethyl acetate (0.87%) fractions (Table 4.6). As revealed in a separate investigation, the hexane, ethyl acetate and methanol extracts used in this study contained 0.31, 0.60 and 4.3 % asiaticoside and 0.01, 1.3 and 0.61 % asiatic acid respectively (Study 2, Chapter 3).

The test B indicates that compared to the control:

a) the number of closed-arm entries has been significantly reduced in the methanol extract group - 12.1 vs. 9.1; b) the number of protected head dips has been significantly increased with the ethyl acetate extract group - 11.3 vs. 17.7 and c) the time spent on protected head dips was significantly increased with ethyl acetate and methanol extracts - 36.3 vs. 67.5 and 63.6 respectively. Performance of the hexane extract group showed no significant difference with the control group (Table 4.7). The analysis of variance test showed no overall significant difference among three fractions under the tested conditions.

The result of this test is an indication of the anxiolytic properties of ethyl acetate and methanol fractions of gotukola and also suggests that the hexane fraction is pharmacologically not active as an anxiolytic agent.

Table 4.6: Yields of hexane, ethyl acetate and methanol fractions of gotukola used in rat trial.

Fresh materials	Dried materials	Hexane extract	Ethyl acetate extract	Methanol extract
750 g	128 g	2.12 g 1.66% *	1.11 g 0.87% *	30.47 g 23.80% *

* - as a percentage of dry weight of whole plant materials

Table 4.7: Comparison of the plus maze performance of rats treated with gotukola hexane, ethyl acetate and methanol extracts with the control (50 % condensed milk), after 2 h post-drug interval.

Test B

Treatment	# o.a.	Time o.a. (seconds)	# c.a.	Time c.a. (seconds)	# phd	Time phd (seconds)	# uphd
Control (condensed milk) (n=10)	3.9 ± .8	51.2 ± 12.8	12.1 ± 1.0	190.8 ± 16.3	11.3 ± 1.5	36.3 ± 5.3	21.5 ± 5.3
hexane (n=10)	4.3 ± 1.0	68.1 ± 17.2	11.0 ± .6	166.5 ± 14.5	13.5 ± 1.0	46.3 ± 5.7	32.4 ± 8.3
EtOAc (n=9)	2.9 ± 1.1	38.4 ± 15.0	10.0 ± .6	168.9 ± 16.3	17.7 ± 1.2*	67.5 ± 5.8*	16.9 ± 7.0
MeOH (n=11)	3.3 ± .8	41.4 ± 11.2	9.1 ± .9*	174.5 ± 16.2	15.7 ± 1.7	63.6 ± 7.5*	20.2 ± 5.2

All values represent the group mean ± SEM (n = 9 - 11)

o.a. – number of open-arm entries, Time o.a. – time spent on open-arm, # c.a. – number of closed-arm entries, Time c.a. – time spent on closed-arm, # phd – number of protected head dips, Time phd – time spent on protected head dips, # uphd – number of un-protected head dips

* $P < 0.05$ vs. control, t-test

4.3.3 Study with the Pure Compound Asiaticoside (Tests C1 – C9)

4.3.3.1 Elevated Plus Maze Test (C1, C2)

As indicated by the test C1 there was a significant difference between control and both asiaticoside 1 and 3 mg/kg drug groups for: a). number of closed-arm entries; 15.7 vs.12.0 and 11.1 respectively and b). time spent on the closed-arm; 211.5 vs. 160.4 and 168.4 respectively. For the number of unprotected head dips, the difference between the control (7.4) and the Asia.3 group (20.4) was significant ($P < 0.05$)(Table 4.8).

The test C2 was a dose-response study. Results for the test (Table 4.9) showed significant ($P < 0.05$) anxiolytic activity of all three dosage levels tested with asiaticoside compared to the control group, as reflected by: a) increased number of open-arm entries: control (2.4), asiaticoside treated groups; 3 mg/kg (4.8), 5 mg/kg (4.8) and 10 mg/kg (4.2); b) increased time spent on open-arm: control (32.1), asiaticoside treated groups; 3 mg/kg (68.9), 5 mg/kg (64.3) and 10 mg/kg (60.5); c) reduced time spent on the closed-arm: control (203.4), asiaticoside treated groups; 3 mg/kg (144.7), 5 mg/kg (143.6) and 10 mg/kg (156.6) and d) increased number of protected head dips: control (11.0), asiaticoside treated groups; 3 mg/kg (18.4), 5 mg/kg (18.7) and 10 mg/kg (16.4).

Other parameters in which a significant difference was observed, were: e) time spent for protected head dips: compared to the control (47.7), asiaticoside 5 mg/kg group (68.3) spent more time and f) number of unprotected head dips: compared to the control (13.9), asiaticoside 10 mg/kg group (25.5) made higher number of unprotected head dips. Although the test did not indicate a clear dose-dependant relationship, a trend for a higher activity was observed around 3-5 mg/kg dosage.

Table 4.8: Comparison of the plus maze performance of rats treated with asiaticoside 1 mg/kg and asiaticoside 3 mg/kg or the vehicle (peanut oil), 1 h post-treatment.

Test C1

Treatment	# o.a.	Time o.a. (seconds)	# c.a.	Time c.a. (seconds)	# phd	Time phd (seconds)	# uphd
control peanut oil (n=11)	2.6 ± .5	33.4 ± 8.5	15.7 ± 1.4	211.5 ± 7.9	11.2 ± 1.0	34.6 ± 3.6	7.4 ± 2.0
asiaticoside 1 mg/kg (n=9)	4.0 ± .8	58.2 ± 11.8	12.0 ± 1.5*	160.4 ± 10.6*	13.4 ± 2.3	43.6 ± 6.8	15.0 ± 3.3
asiaticoside 3 mg/kg (n=8)	4.2 ± 1.3	63.9 ± 16.1	11.1 ± .8*	168.4 ± 19.0*	11.4 ± .9	36.0 ± 3.2	20.4 ± 5.3*

All values represent the group mean ± SEM (n = 8 - 11)

o.a. – number of open-arm entries, Time o.a. – time spent on open-arm, # c.a. – number of closed-arm entries, Time c.a. – time spent on closed-arm , # phd – number of protected head dips, Time phd – time spent on protected head dips, # uphd – number of un-protected head dips

* $P < 0.05$ vs. control, t-test

Table 4.9: Comparison of the plus maze performance of rats treated with asiaticoside 3, 5 and 10 mg/kg with the control (peanut oil), after 1 h post-drug interval.

Test C2 (dose-response study)

Treatment	# o.a.	Time o.a. (seconds)	# c.a.	Time c.a. (seconds)	# phd	Time phd (seconds)	# uphd
Control (n=11)	2.5 ± .4	32.1 ± 7.0	10.0 ± 1.2	203.4 ± 12.2	11.0 ± 1.5	47.7 ± 6.7	13.9± 3.1
asiaticoside 3 mg/kg (n=12)	4.8 ± .5*	68.9 ± 8.1*	10.3 ± .5	144.7 ± 7.9*	18.4 ± 1.6*	66.3 ± 7.0	25.0 ± 2.9
asiaticoside 5 mg/kg (n=11)	4.8 ± .9*	64.3 ± 13.8*	10.1 ± .7	143.6 ± 11.9*	18.7 ± 1.4 *	68.3 ± 7.4*	23.0 ± 5.8
asiaticoside 10 mg/kg (n=13)	4.2 ± .5*	60.5 ± 9.2 *	10.6 ± .7	156.6 ± 8.7*	16.4 ± 1.1*	61.9 ± 4.8	25.5 ± 3.8*

All values represent the group mean ± SEM (n = 11 - 13)

o.a. – number of open-arm entries, Time o.a. – time spent on open-arm, # c.a. – number of closed-arm entries, Time c.a. – time spent on closed-arm, # phd – number of protected head dips, Time phd – time spent on protected head dips, # uphd – number of un-protected head dips

* $P < 0.05$ vs. control; t-test

4.3.3.2 Open Field Test (C3: dose-response study)

The test C3 conducted with the open field test model indicated significantly higher duration of time spent at the center (in seconds) in the groups treated with asiaticoside 3 mg/kg (35.9) and 5 mg/kg (41.3), compared to the control (24.8). This observation also supports for the anxiolytic activity of asiaticoside (Table 4.10). Similar to the test C2, a dose dependant response was not observed in the test C3.

4.3.3.3 Social Interaction Test (C4, C5)

In the test C4, a significant reduction in the number of non-interaction activity was observed with the 1mg/kg dosage (29.8) compared to the control (35.0). Although this observation supports the anxiolytic activity of asiaticoside, the significant increase in the non-interaction time with the 3 mg/kg dosage (251.7 seconds), compared to the control (207.5 seconds) does not support such an activity (Table 4.11).

In the test C5, no significant difference among drug groups compared to the control group was observed after 4h of post-drug interval (Table 4.12). It seems that 4h time interval is too late to still exhibit any possible biological activity under the tested dosage.

4.3.3.4 Locomotor Activity Test (C6)

During the 22h of test period, no significant difference between the control and the asiaticoside (1 mg/kg and 3 mg/kg) treated groups was observed for the number of squares crossed and total distance traversed (Table 4.13). Asiaticoside at 1 and 3 mg/kg dosage has not affected the ultradian locomotor activity; an indication that asiaticoside may not have any sedative effects (Figure 4.1).

Table 4.10: Comparison of performance in the Open Field Test of rats treated with asiaticoside 3, 5 and 10 mg/kg and the control (peanut oil) after 1 h post-drug interval.

Test C3 (dose-response study)

Treatment	# c	c.dur	# c.blocks	# p	p.dur	# p.blocks
Control (n = 10)	7.9 ± 1.2	24.8± 3.0	20.6 ± 3.7	8.8 ± 1.2	271.0 ± 5.1	131.5 ± 5.5
Asiaticoside 3 mg/kg (n = 10)	9.2 ± .6	35.9 ± 3.4*	28.2 ± 3.0	10.1 ± .6	261.9 ± 3.8	130.1 ± 6.2
Asiaticoside 5 mg/kg (n = 10)	9.0 ± 1.1	41.3 ± 5.2*	27.0 ± 3.9	9.9 ± 1.1	257.4 ± 5.1	121.1 ± 7.1
Asiaticoside 10 mg/kg (n = 9)	8.1 ± 1.6	39.6± 7.8	27.3 ± 4.8	9.1 ± 1.6	259.5 ± 7.7	136.0 ± 12.4

All values represent the group mean ± SEM (n = 9 - 10)

c = center number of occurrences, c.dur = center duration (seconds), # c.blocks = center number of blocks crossed, # p = perimeter number of occurrences, p.dur = perimeter duration (seconds), # p.blocks = perimeter number of blocks crossed

* $P < 0.05$ vs. control; t-test

Table 4.11: Comparison of the social interaction test performance of rats treated with asiaticoside 1 and 3 mg/kg with the control (peanut oil) after 1 h post-drug interval.

Test C4

Treatment	# SI	SI time	# non-int.	non-int.time
Control (peanut oil) (n = 8 in 4 groups)	34.8 ± 2.3	206.9 ± 6.3	35.0 ± 2.0	207.5 ± 4.2
asiaticoside 1 mg/kg (n = 8 in 4 groups)	29.8 ± .5	209.5 ± 19.1	29.8 ± .5*	206.0 ± 19.3
asiaticoside 3 mg/kg (n = 8 in 4 groups)	31.8 ± 2.0	166.6 ± 16.5	32.5 ± 1.9	251.7 ± 16.6*

All values represent the group mean ± SEM (n = 4)

SI = number of social interactions, SI time = time spent on social interactions, # non-int. = number of non-interactions, non-int.time = time spent on non-interactions

* $P < 0.05$ vs. control; t-test

Table 4.12: Comparison of the social interaction test performance of rats treated with asiaticoside 1 and 3 mg/kg with the control (peanut oil) after 4 h post-drug interval.

Test C5

Treatment	# SI	SI time	# non-int.	non-int.time
Control (peanut oil) (n = 8 in 4 groups)	26.8 ± 3.6	212.4 ± 25.0	27.0 ± 3.8	205.5 ± 24.3
asiaticoside 1 mg/kg (n = 8 in 4 groups)	25.8 ± 2.2	189.2 ± 32.6	26.2 ± 2.2	228.5 ± 32.4
asiaticoside 3 mg/kg (n = 8 in 4 groups)	22.2 ± 1.9	219.6 ± 13.2	22.2 ± 1.9	195.2± 11.6

All values represent the group mean ± SEM (n = 4)

SI = number of social interactions, SI time = time spent on social interactions, # non-int. = number of non-interactions, non-int.time = time spent on non-interactions

Figure 4.1: Hourly distance traversed from 11 am to 10 am the next day by rats treated with 1 and 3 mg/kg asiaticoside and the control / vehicle (peanut oil) in the locomotor activity test.

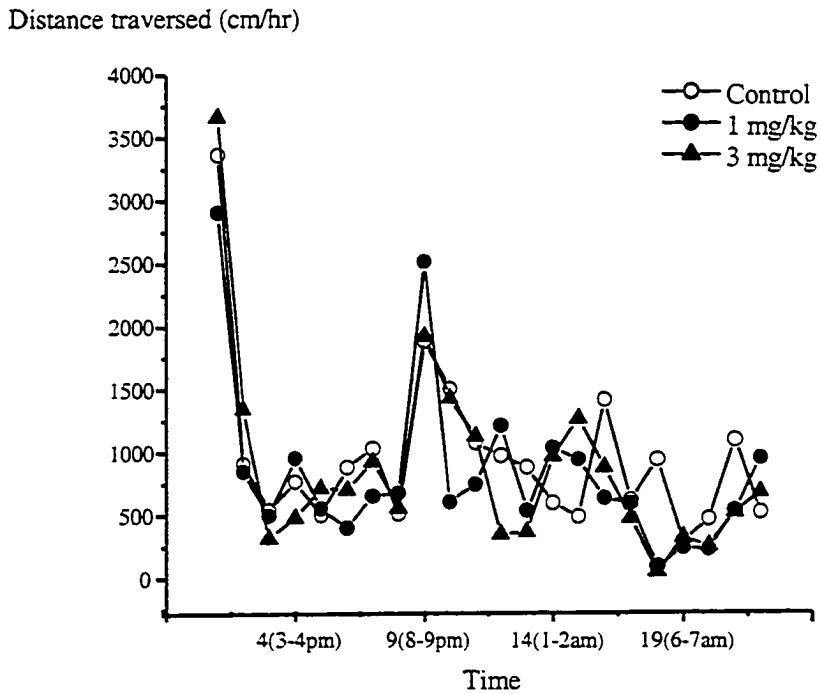


Table 4.13: Summary of locomotor activity data collected over 22 h (from 11 am to 9 am) of rats treated with asiaticoside 1 and 3 mg/kg or the vehicle (peanut oil).

Test C6

Treatment	Total number of squads crossed	Total distance traversed (cm)
Control peanut oil n = 13	63.7 ± 9.2	979.9 ± 140.2
asiaticoside 1 mg/kg n = 10	55.0 ± 9.2	848.4 ± 141.8
asiaticoside 3mg/kg n = 8	58.3 ± 10.2	892.3 ± 163.7

All values represent the group mean ± SEM (n = 8 - 13)

4.3.3.5 Vogel Test (C7, C8, C9)

No significant group differences in the number of punished licks emitted was apparent in the asiaticoside 1 and 3 mg/kg drug treated groups compared to the control group, after 1h post-drug interval (Table 4.14).

No significant difference in the response of the asiaticoside 5 mg/kg treated group and the control group with 1h post-drug interval was observed in test C8 (Table 4.15).

Although there was a trend to increase the number of licks compared to the 0.5 h post-drug interval (587.4), in the 1h (795.2) and 2h (602.1) post-drug interval groups, the difference was not statistically significant (Table 4.16). Compared to the test C7 where a shock of 0.4 mA was used, relatively much lower shock of 0.1mA was applied to the C8 and C9 tests. Other than the increase in the asiaticoside dosage level, the low shock applied for tests C8 and C9 may had an impact on the relatively higher number of licks compared to the test C7.

Table 4.14: Comparison of the Vogel test performance of rats treated with asiaticoside 1 and 3 mg/kg with the control (peanut oil), after 1 h post-drug interval.

Test C7

Treatment	Number of punished licks
Control (peanut oil) n = 10	180.3 ± 64.1
Asiaticoside 1 mg/kg n = 10	93.6 ± 10.5
Asiaticoside 3 mg/kg n = 10	107.0 ± 29.4

All values represent the group mean ± SEM (n = 10)

Table 4.15: Comparison of the Vogel test performance of rats treated with asiaticoside 5 mg/kg or the vehicle (peanut oil), after 1 h post-drug interval.

Test C8

Treatment	Number of punished licks
Control (peanut oil) n = 12	641.7 ± 145.2
Asiaticoside 5 mg/kg n = 12	795.2 ± 214.4

All values represent the group mean ± SEM (n = 12)

Table 4.16: Comparison of the Vogel test performance of rats treated with asiaticoside 5 mg/kg after 0.5, 1 and 2 h post-drug intervals.

Test C9 (time-response study)

Treatment	Number of punished licks
0.5 h (n = 13)	587.4 ± 150.4
1 h (n = 12)	795.2 ± 214.4
2 h (n = 12)	602.1 ± 209.6

All values represent the group mean ± SEM (n = 12 - 13)

4.4 CONCLUSIONS

The studies using several animal models of anxiety revealed that gotukola does impart anxiolytic activity. Furthermore, this anxiolytic activity appears to be attributable in part to asiaticoside within the plant extracts. The findings are concordant with those of previous other studies on the anxiolytic profile of gotukola by Sakina and Dandiya (1990) and Diwan et al (1991).

The observations reported herein using a variety of animal paradigms provide unequivocal evidence for the anxiolytic activity of the drugs tested. Future studies should be undertaken to compare these with standard anxiolytic drugs currently prescribed. Since there are similarities in brain chemistry, physiology and some emotional factors of test animals to those of humans (Gringauz, 1997), the gotukola based drugs administered in this study may possess the potential to be effective to humans with similar activity. In contrast, it is difficult to conclude whether any unforeseen factors involved in this study caused the state of anxiety, which may not identically resemble the mental and emotional states of humans.

The results of this study provide evidence to narrow down the anxiolytic drug search of gotukola to ethyl acetate and methanol fractions. These are the main fractions containing triterpene derivatives. The main triterpene asiaticoside has anxiolytic activity and may be considered as an active principle. It is probable that the other terpenes contribute to activity and these should be tested as well.

The fact that anti-anxiety effects were not necessarily detected in all the test models used may suggest that gotukola might be more effective in certain subtypes of

anxiety. However, to substantiate this claim one needs to conduct more extensive dose- and time-dependant studies across various test paradigms.

CHAPTER 5

DEVELOPMENT OF METHODS FOR PROPAGATION AND INCREASED EXPRESSION OF ASIATICOSIDE AND ASIATIC ACID OF GOTUKOLA (*CENTELLA ASIATICA* L. URBAN)

5.1 Introduction

Propagation of gotukola with seeds is not successful and *in vitro* methods are important as an alternative to vegetative propagation of the plant. Other than varietal screening of gotukola, physiological and agronomic factors of gotukola may be exploited to increase the production of its therapeutically important phytochemicals. The following preliminary studies were conducted to investigate tissue culture propagation of gotukola and to explore the possibilities of increasing asiaticoside and asiatic acid synthesis in gotukola plants with methyl jasmonate treatment and controlling light levels.

5.1.1 Seed Germination Problems and Tissue Culture Propagation

During the study for this thesis, attempts to propagate gotukola plants from commercial seeds (Ritchers plant nursery, Goodwood, Ontario) resulted in 0% success in a germination test. The problem of propagating gotukola by seeds is an inherent limitation with the plant and are associated with polyploidy. Therefore, the vegetative propagation by runners is the usual agronomic practice and tissue culture technique provides an alternative method to propagate gotukola.

An investigation with two Australian gotukola populations – "Mt. Archer" and "Mossman" and a Japanese population – "Omoto" showed the chromosome number of 2n = 18, with two satellite-chromosomes which suggested the plants were diploid. For two Japanese populations – "Shibusawa" and "Shibazaki" 2n = 36 with four satellite-chromosomes, suggested tetraploid plants (Goro et al, 1998). Similar observations were made in a meiotic behavior study conducted with five populations of gotukola from the Southern region of Brazil. Irregular chromosome segregation, abnormal spindles, chromosome transfer among microsporocytes and sticky chromosomes were revealed in them. The large number of chromosomes observed (2n = 54) in these five populations suggest hexaploidy (Lopes et al, 1996). The "Praia de Leste" population showed chromosome stickiness in 19.30 % microsporocytes studied. These irregularities may be the causes of low pollen fertility (0.30 %) and low production of normal fruits (1.0 %) (Lopes and Suely, 1996).

An alternative to seed propagation is tissue culture. In tissue culture, the ratio of auxin and cytokinin concentration in the growth medium influences formation of either roots or shoots. Higher concentration of auxin (2,4-D, IAA) relative to cytokinin (kinetin) induces root growth, whereas higher concentration of cytokinin relative to auxin induces shoot growth from callus. The tissue will grow as an unidentified callus at intermediate auxin:cytokinin ratios (Taiz and Zeiger, 1998).

The stem and leaf explants of gotukola on semisolid modified Murashige and Skoog's (MS) medium supplemented with kinetin 2 mg/L and alpha-naphthalene acetic acid (NAA) 4 mg/L induced callus formation. Shoot-buds were generated after 4 weeks of subculture on 6-benzyladenine 4 mg/L, kinetin 2 mg/L, NAA 0.25 mg/L, adenine

sulfate 20 mg/L. These shoot differentiated explants were rooted in 11 days in ½ strength MS–basal medium supplemented with indole-3-acetic acid (IAA) 0.5 mg/L and sucrose 2% (w/v) (Patra et al, 1998).

In another study, callus formation of gotukola leaf explants occurred on MS medium supplemented with 2,4 dichlorophenoxy acetic acid (2,4 D) 0.5 mg/L and kinetin 0.5 mg/L. Then buds were proliferated in ½ strength MS medium supplemented with benzylaminopurine (BAP) 2 mg/L, kinetin 0.5 mg/L and indolebutyric acid (IBA) 0.25 mg/L. Profuse rooting was resulted when transfer to ½ strength MS medium devoid of hormones. Prolonged culture on the shoot inducing medium also resulted in root initiation (Josekutty, 1999).

5.1.2 Effect of Methyl Jasmonate on Asiaticoside and Asiatic Acid Synthesis

Methyl jasmonate (MJ) is a highly active compound as a signaling molecule in plant communication. Direct application as well as volatile MJ can stimulate the plant defense mechanism and induce production of secondary compounds. As shown in some recent studies, the production of alkaloids, phenolics and diterpene glycosides of *Nicotiana attenuata* leaves (Keinanen et al, 2001), bilobalide of *Gingko biloba* callus tissue (Agrawal et al, 2001) and paclitaxel of *Taxus chinensis* cell suspension cultures (Zhang and Xu, 2001) have been increased with MJ treatment.

5.1.3 Effect of High and Low Sunlight Levels for Asiaticoside and Asiatic Acid Synthesis

In a genetic resources screening study in India with 16 accessions of gotukola, the herb and asiaticoside yields ranged from 470 to 2730 kg/ha and 1.0 to 9.8 kg/ha respectively. Thirteen out of 16 accessions tested had shading requirements for higher yields. Generally, 50% shading resulted in higher herbage and asiaticoside content. The accession CaShT at 50% shading and the accessions CaBp and CaCl at full sunlight resulted in higher yields compared to other accessions (Mathur et al, 2000).

5.2 MATERIALS AND METHODS

5.2.1 Tissue Culture Study

To overcome plant propagation problem by seeds and also to obtain plant materials for HPLC analysis and rat trials, *in vitro* propagation technology was successfully adopted. Tissues of stolons and leaves (4 mm) of gotukola plants (from Ritchers Nursery, Goodwood, Ontario) were cultured for callus induction in a MS medium supplemented with naphthalene-acetic acid and kinetin (Table 1). The callus-induced explants were sub-cultured to a medium of MS supplemented with kinetin, NAA, benzylaminopurine and adenine sulphate for shoot induction (Table 2) and then to a medium of MS supplemented with indol-butyric acid and sucrose for root induction (Table 3). The rooted plantlets were transferred to pots filled with sterilized potting mixture (1 soil: 1 sand: 1 organic manure) for 8 weeks of hardening and then transferred to the greenhouse.

The media were sterilized by autoclaving at 121 °C, 15 psi for 25 minutes. Plant tissues were sterilized by dipping them in 70% ethanol for 30 seconds and then in 10% bleach for 10 minutes. The tissue cultures were kept in a growth chamber where 27 °C temperature and 24 h light was maintained.

Table 5.1: Medium used for callus induction in *Centella asiatica* (Adapted from Patra et al, 1998)

Distilled Water	1 L
MS with sucrose	42.4 g
NAA	4 mg
Kinetin	2 mg

Table 5.2: Medium used to induce shoots from callus (Adapted from Patra et al, 1998).

Distilled Water	1 L
MS with sucrose	42.4 g
Benzylaminopurine	4 mg
Kinetin	2 ml
NAA	0.25 ml
Adeninsulphate	20 mg

Table 5.3: Medium used to induce roots from shoot-induced explants (Adapted from Patra et al, 1998 and modified).

Distilled water	1 L
MS Basal	21.2 g
IBA	0.5 mg
Sucrose	20 g

5.2.2 Methyl Jasmonate Study

The tissue cultured gotukola plants grown in the greenhouse were used in this study. Ten plants (in groups of 3, 3 and 4 each) were sprayed with 100 ppm solution of MJ (dH_2O 200 ml, dimethyl sulphoxide 19 µl, MJ 19 µl) and the control groups of ten plants (in groups of 3, 3 and 4 each) were sprayed without MJ (dH_2O 200 ml, dimethyl sulphoxide 19 µl). The plants were treated at 4:00 pm and then kept in closed growth chambers separately (temperature 27 °C, light 7400 lux) for 24 h. Then the aerial parts were harvested, oven-dried (40°C, 24 h) and three methanol extracts each from the treated and the control groups were prepared for HPLC analysis. The extraction and HPLC procedure is as same as with the procedure for analysis of gotukola commercial products (study 1-Chapter 3).

5.2.3 Light Levels Study

Three tissue cultured plants each were grown for 2 months (August – September) period in; a) greenhouse (full sunlight: > 2 x 10^5 lux) and b) indoor shady area away from windows to prevent direct sunlight (< 1000 lux). Then the plant materials were analyzed for the asiaticoside and asiatic acid content by using the extraction and HPLC procedure adopted for the analysis of gotukola commercial products (study 1 – Chapter 3).

5.3 RESULTS AND DISCUSSION

5.3.1 Tissue Culture Study

The globular and greenish callus formation occurred after 6 weeks. The success rate of callus formation was higher with explants from stolons (92%), compared to the lower success with explants from leaves (8%) (Table 5.4). The higher rate of success with stolons may have caused due to the optimum growth regulator levels in the tissue. Shoots were formed in calli-induced explants after 5 weeks. Roots were formed in these shoot differentiated explants 2 weeks after subculturing.. A collection of twenty plants was maintained in the greenhouse from these tissue cultures to obtain gotukola materials for the rat trial and the HPLC analysis.

5.3.2 Methyl Jasmonate Study

MJ treatment has significantly ($P < 0.001$) increased the asiaticoside level (95.56 µg/g dry weight) compared to the control (31.45 µg/g dry weight). The treatment also significantly increased the asiatic acid content (1928.67 µg/g dry weight) compared to the control (1671.39µg/g dry weight) (Table 5.5). This study indicates that MJ has effect on mediating asiaticoside and asiatic acid synthesis of gotukola.

5.3.3 Light Levels Study

In the plants grown in full sunlight, the asiaticoside and asiatic acid content was significantly higher (5631.6 and 1519.9 μg/g dry weight respectively), compared to the plants grown in shade (185.6 and 586.2 μg/g dry weight respectively) ($P < 0.001$) (Table 5.6). Both asiaticoside and asiatic acid synthesis in gotukola may be regulated by controlling the light levels.

5.4 Conclusion

The results presented here provide a method for producing gotukola by tissue culture and increasing its asiaticoside content by MJ and high light levels.

Table 5.4: Callus formation in tissue cultures from stolon and leaf explants of *Centella asiatica*.

Culture No.	Stolon: callus induced / total explants	Culture No	Leaf: callus induced / total explants
1S	10/10	1L	1/10
2S	10/10	2L	0/10
3S	8/10	3L	0/10
4S	8/10	4L	1/10
5S	10/10	5L	2/10
Success Rate	92%	Success Rate	8%

Success Rate = (Total number of calli induced / Total number of explants cultured) x 100

Table 5.5: Results of HPLC-UV analysis of the methanol extract of methyl jasmonate treated and control gotukola plants for the asiaticoside and asiatic acid content.

Treatment	Asiaticoside content (μg/g dry weight)	Asiatic acid (μg/g dry weight)
Control (dH_2O) (n = 3)	29.9 ± 1.7	2071.5 ± 41.6
Methyl jasmonate (n = 3)	86.0 ± 5.6*	2386.7 ± 35.4*

Values are mean ± SEM.

* $P < 0.001$ vs. control; t-test

Table 5.6: Results of HPLC-UV analysis for the asiaticoside and asiatic acid content of the methanol extract of gotukola plants grown under shade and full sunlight conditions.

Treatment (Light level)	Asiaticoside content (μg/g dry weight)	Asiatic acid (μg/g dry weight)
Shade (< 1000 lux) (n = 3)	185.6 ± 2.2	586.2 ± 2.8
Full sunlight (> 2 x 10^5 lux) (n = 3)	5631.6 ± 10.5*	1519.9 ± 5.3*

Values are mean ± SEM.

* $P < 0.001$ vs. Shade group; t-test

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

This thesis provides a clarification of ayurvedic medicine in a modern context, and its contribution of the gotukola plant to mental disorders. In addition, the thesis provides evidence for the action of the hydrophilic fraction of the plant in animal models of anxiety and specifically asiaticoside as an active principle.

Irrespective of the geographical or cultural background, almost every nation has an indigenous ancient medicinal system that reflects its own historical identity. These ancient health-care systems were based on natural products, especially medicinal plants and are distinguishable from modern medicinal practices, which mainly depend on (synthetic) pharmaceutical dugs. The vast knowledgebase of ayurveda is well documented in several comprehensive texts and one of them; Sushruta Samhita written during the ~600 BC was investigated for this study (Chapter 2). According to the ayurvedic principles, all disease causal factors (*doshas*) are categorized into three main groups: *vata, pita* and *kapha*. Any imbalance of them leads to health problems and unbalanced *vata-doshas* lead to psychological disorders.

The scientific ayurvedic system is still alive and popular in countries such as India and Sri Lanka, where it continues to enjoy a prominent role in healthcare with university-level training for physicians, fully pledged ayurvedic hospitals and ayurvedic research institutes equipped with modern research facilities and technically qualified staff. Therefore, the perception among some scholars that ayurveda is "traditional" but not

"scientific", demands an intellectual dialogue for clarification. As suggested in the Chapter 2, the term "ancient science" would be more appropriate to address ayurveda and other similar knowledgebases.

The high performance liquid chromatography (HPLC) analysis of gotukola commercial products revealed that there is a noticeable variation among them in the asiaticoside content. Since asiaticoside is one of the major active components of the plant, it may be an important marker compound for standardization of gotukola commercial products. The HPLC method adopted in this study is simple and effective in separation and detection of asiaticoside and asiatic acid of gotukola. It is a fully validated quantitative method.

The extensive animal trial, which altogether involved more than 430 rats in 13 different tests, revealed several important findings for the anxiolytic activity of gotukola. The 500 mg/kg dosage of gotukola whole plant material at 2 h post-drug interval clearly demonstrated the anxiolytic activity compared to the control. The gotukola ethyl acetate and methanol extracts showed anxiolytic activity compared to the hexane extract and the control. This observation suggests that the compounds involved in the anxiolytic activity of the plant are in these two fractions. There may be several such compounds. The main triterpenic compound asiaticoside revealed anxiolytic activity around 3 mg/kg dosage at 1 h post-drug interval and seems to be not dose-dependent at higher dosages. The quantitative HPLC analysis was useful in estimation of the asiaticoside and asiatic acid content of different drugs administered in the animal trial (Tables 6.1 and 6.2).

Based on the findings of this animal model study, a clinical study may be pursued with asiaticoside dosage of 150 mg per 50 kg subject. Since the methanol fraction of the

fresh plant contained 43.5 mg/g asiaticoside, 3.4 g methanol fraction would contain the intended dosage of asiaticoside (Table 6.3). Because of the other triterpenes in the fraction, the effective dose may be considerably less. Clearly, one of the recommendations of the thesis is to perform a dose response test on the methanolic fraction to determine if the human dose can be scaled down to 1-2 g / individual. If not high potency extracts can be prepared from high yield genotypes such as the Madagascar variety or by induction of triterpene levels with methyl jasmonate.

There seems to be a potential to formulate a pharmaceutical anxiolytic drug from gotukola. Although this study showed the effectiveness of gotukola in behavioral models, more research on the biological activity of its different fractions and pure compounds such as asiaticoside and asiatic acid is essential in future research. In particular, it would be of value to examine the related triterpenes for activity and study dose response of extracts.

Table 6.1: Asiaticoside and asiatic acid contents of commercial products and different fractions of gotukola administered in rat trials, as revealed by HPLC analysis.

Commercial Product / Fraction	Asiaticoside Content mg/g	Asiatic Acid Content mg/g
Nature's Way	3.7	1.7
Solaray Madagascar	24.0	1.7
Hexane Extract	3.1	0.1
Ethyl Acetate Extract	6.0	12.5
Methanol Extract	43.5	6.1

Table 6.2: Actual doses of asiaticoside and asiatic acid contained in different treatments of gotukola administered in animal trials, as revealed by HPLC analysis.

Drug / Dosage	Asiaticoside dose contained mg/kg	Asiatic acid dose contained mg/kg
Nature's Way (NW) 200 mg/kg	0.74	0.34
Solaray Madagascar (SM) 200 mg/kg	4.8	0.34
NW 500 mg/kg	1.85	0.85
SM 500 mg/kg	12	0.85
Hexane fraction	0.66	0.02
Ethyl Acetate fraction	0.67	1.39
Methanol fraction	132.5	18.6

Table 6.3: Drugs and their dosage used in rat trial, corresponding dosage for 50 kg body weight (of human subject) and drug performance in elevated plus maze test model as indicated by percentage increase in time spent on open-arm compared to the control group.

Drug / Dosage	Dose for 50 kg	Increase in time spent on open-arm compared to the control (%)
NW: 500 mg/kg	25 g	159% ***
SM: 500 mg/kg	25g	174% ***
Asiaticoside: 1 mg/kg	50 mg	74% NS (trend)
Asiaticoside: 3 mg/kg	150 mg	91% NS (trend)
Asiaticoside: 3 mg/kg	150 mg	115% *
Asiaticoside: 5 mg/kg	250 mg	100% *
Asiaticoside: 10 mg/kg	500 mg	88% *

NW = gotukola Nature's Way product, SM = gotukola Solaray Madagascar product

*** = the difference is significant at $P < 0.001$

* = the difference is significant at $P < 0.05$

NS (trend) = the difference is not significant at $P < 0.05$, but trend for increased activity

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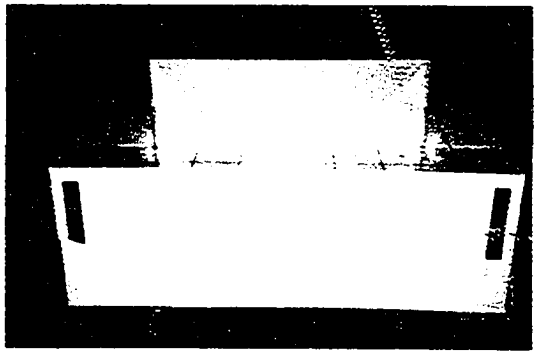
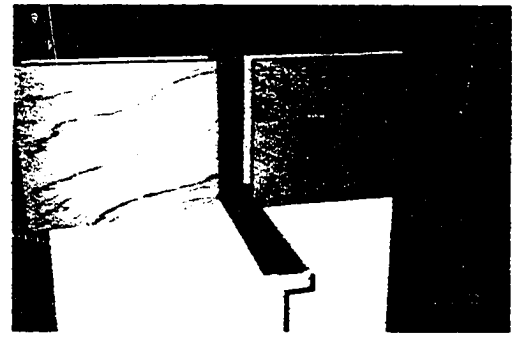
APPENDIX 1

Photographs of rat trial test models

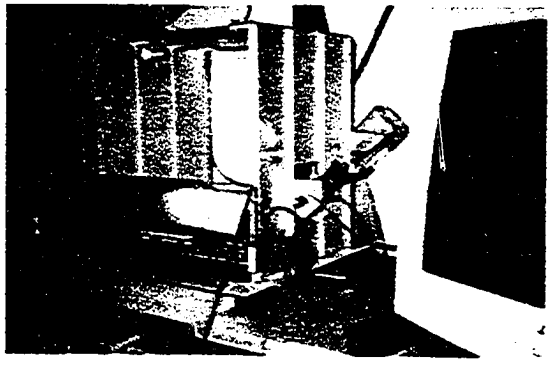
(by Priyantha Wijeweera)



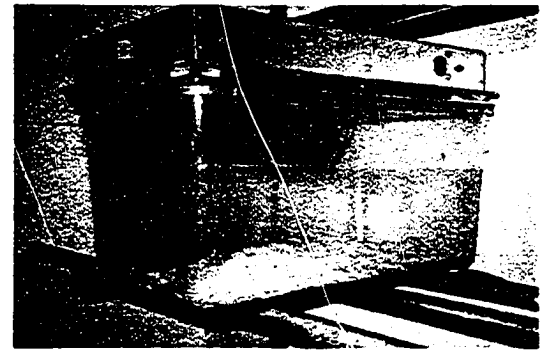
Arena for open field test and social interaction test



Elevated plus maze test apparatus



Vogel test apparatus



Tracking device for locomotor activity test assembled on a rat cage

APPENDIX 2

Antifungal Activity of Hexane, Ethyl Acetate and Methanol Fractions of Gotukola (*Centella asiatica* L. Urban): An Observation

Singh et al (2000) have shown the antifungal activity of gotukola methanolic extract against the following fungi; *Alternaria alternata*, *A. brassicae*, *A.solani*, *A. tenuissima*, *Cercospora blumae*, *Curvularia lunata*, *C. penniseti*, *Drechslera monoceras*, *D. oryzae*, *D.turitica*, *Fusarium albizziae* and *F. udum*.

The hexane (2.12 g), ethyl acetate (1.11 g) and methanol (30.47 g) fractions of gotukola extracted from 128 g dried gotukola plant materials were mixed in 50 ml of 50% condensed milk in 250 ml Erlenmeyer flasks (the drugs used in one of the rat trials; Chapter 4). A portion of this media was left open in the lab for 8h for natural contamination and then sealed and kept in the refrigerator (8 °C). After 4 months of observation period, no any visible signs of fungal growth were noticed in the methanol fraction medium, while a distinctively heavy growth of fungi was noticed in the media made from hexane and ethyl acetate fractions. Further formal study on this observation is planned for the future research work.