ALKALOIDS IN TISSUE CULTURE OF *HETEROSTEMMA TANJORENSE* W. & A. (ASCLEPIADACEAE)

THESIS

Submitted to the
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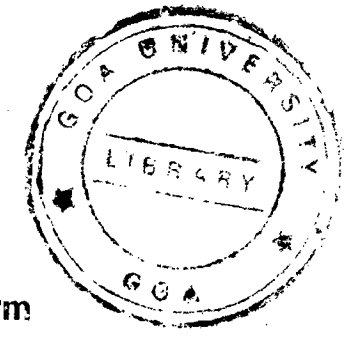

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CERTIFICATE

This is to certify that the thesis entitled " Alkaloids in Tissue Culture of *Heterostemma tanjorense*, W. & A. (Asclepiadaceae) " submitted by Shri. L. H. Bhonsle for the award of the degree of Doctor of Philosophy in Marine Biotechnology is based on the results of field surveys/laboratory experiments carried out by him under my supervision. The thesis or any part thereof has not previously been submitted for any other degree or diploma.

Place : Goa University
Taleigao Plateau

Date : 3/11/1995

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STATEMENT

As required under the University ordinance 0.19.8 (vi) I state that the present thesis entitled " Alkaloids in Tissue Culture of Heterostemma tanjorense, W. & A. (Asclepiadacae) " is my original contribution and that the same has not been submitted on any previous occasion. The present study is the first comprehensive study of its kind from this field.

The literature concerning the problem investigated has been cited. Due acknowledgements have been made whereever facilities have been availed of.

L. H. Bhonsle.

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(L.H.BHONSLE)

SYNOPSIS

ALKALOIDS IN TISSUE CULTURE OF HETEROSTEMMA TANJORENSE, W & A. (ASCLEPIADACEAE)

The science of plant tissue culture offers fascinating possibilities to produce natural compounds under microbiological conditions as this technique is in many aspects advantageous over traditional agricultural methods. It also offers a useful way to control production of the plant secondary metabolities of pharmaceutical importance on an industrial scale. Production of such plant metabolities can be augmented by subjecting callus cultures to physical, chemical and structural modifications. Precursors and amino acids in the medium help in increasing the yield. Among such metabolities are alkaloids which are most extensively investigated. The plant cultures that have been explored to study production of alkaloids include species of Atropa, Ephedra, Nicotiana, Rauwaolfia, Trigonella, etc.

The present work deals with the studies on plant tissue culture of Heterostemma tanjorense W. and A. belonging to family Asclepiadaceae. No reference has been cited regarding the use of this plant in medicine. However, Goan herbalists use decoction of the roots of this plant for cure of some systemic infections. This species grows very scarcely along the coastal belt of Goa especially in Bardez taluka. However, only Flora of Madras describes this plant as follows : " A slender twiner, with broadly ovate leaves, obtuse or cordate at base, and upto 4 in long, 2 in

broad, the linear follicles 4 in. long reflexed, hooked at apex." The plant was earlier investigated for its antibacterial and pharmacological actions, Which was mainly due to the presence of alkaloids. The active constituent and the major alkaloid heterstemmine was isolated from this species.

The present work shows unequivocal evidence of success in developing callus from the explants of H. tanjorense along with the demonstration of optimal yields of alkaloid in the callus. The study also deals with establishment of optimum conditions for the induction and production of alkaloid. Evidence of successful growth of suspended culture and the underlying results beneficial for commercial production of alkaloid are also discussed.

The thesis has been presented in six chapters and a brief outline is given below :

Chapter I deals with general introduction, review of previous work, nature and objectives of investigation.

Chapter II provides a general insight into the methodology of selection of different vegetative explants of H. tanjorense for initiation and growth of callus. The explants tested were leaf primordia, leaf, stem, flower buds, flowers, anthers and roots. The callus formation was observed only in flower buds. The basal media used were Murashige and Skoog (MS), White's, Eriksson, Gamborg and Nitsch. Auxins and cytokinins singly or in combination used were 2,4-Dichlorophenoxyacetic acid (2,4-D), Indole-3-Acetic acid(IAA), Indole-3 butyric acid (IBA) and Kinetin (Kn). The combination of 2,4-D, 1 mg/l and Kn, 0.5 mg/l was found to favour maximum callus growth. However, the callus tissue failed to give

any positive clue for the presence of alkaloids when callus extracts were subjected to extraction and subsequent thin layer chromatographic analysis using Dragendorff spray reagent.

Chapter III deals with raising the callus from the vegetative explants obtained from the seeds of H. tanjorense grown under asceptic conditions. The section deals with different physical and chemical parameters influencing the production of callus. The physical factors include : inoculum size, pH of media, light source, temperature and agar concentration. Chemical factors cover different basal media, growth hormones, inorganic nitrogen, vitamins, carbohydrates and natural extracts like coconut milk. It was observed that with an inoculum size of 100 to 200 mg of fresh weight per tube on MS basal medium 1/2 strength fortified with auxin, cytokinin combination of 2,4-D 1 mg/l, IBA, 0.5 mg/l and Kn, 0.5 mg/l, coconut milk 10% and incubation in fluroescent light of 1500 lux for 12 hours at $25^0 \pm 2^0C$ showed maximum production of callus. Callus initiation was observed in almost all the explants namely cotyledon, leaf, stem and root. Callus weight increase was maximum in six weeks. The standard error and growth index of the callus on wet weight basis were calculated.

In this chapter experiments were conducted to study the production of alkaloids by callus. This chapter also covers the standardisation method of solvent extraction from callus. The crude extracts were tested for the presence of alkaloids by thin layer chromatography using Dragendroff spray reagent. Quantitative estimation of total alkaloids was determined by devising a colorimetric method. The alkaloid heterostemmine was

isolated from total alkaloids by column chromatography on alumina and recrystallised from benzene.

Chapter IV deals with all the studies showing evidence of success in growth of callus in liquid media as cell suspension. Biosynthesis of alkaloid by the suspension culture and the optimised conditions for maximum yield of alkaloid is also presented in this chapter.

Chapter V deals with the chemistry of the isolated alkaloid. The isolated alkaloid reacted positive when tested with different alkaloidal reagents and it formed a reineckate derivative. It was cochromatographically identified as heterostemmine and its identity was further confirmed by melting point. The UV absorption (Emax) of the isolated alkaloid was comparable with the standard. The IR spectrum was superimposable with the standard. Also antimicrobial property of callus extract was studied.

Chaper VI synthesises the conclusion of the present study. This is followed by reference cited in the text.

LIST OF ABBREVIATIONS

ABA	abscisic acid
Approx.	approximate
BAP	benzyl amino purine
^0C	degree celcius
CM	coconut milk
Conc.	concentration
2,4-D	2,4-dichlorophenoxy acetic acid
Dist.	distilled
EDTA	ethylene diamine tetra acetic acid
eq.	equivalent
g	gram (s)
G.I.	growth index
G5	Gamborg's medium
GA3	gibberelic acid
hr	hour (s)
IAA	indole - 3 - acetic acid
IBA	indole butyric acid
i.d.	internal diameter
2ip	2-isopentenyl adenine
Kn	Kinetin
L	Litre (s)
mcg	microgram (s)
mg	milligram (s)
mg/g dry wt.	milligram per gram dry weight
mg/T	milligram per tube
min.	minute (s)
ml	mililitre
mm	millimetre
mol.wt.	molecular weight
MS	Murashige and Skoog's medium
NAA	naphthalene acetic acid
nm	nanometre
pH	hydrogen ion concentration
psi	pounds per square inch
PTLC	preparative thin layer chromatography
r.p.m.	revolutions per minute
R_f	relative to front
S.E.	standard error
Soln.	solution
TLC	thin layer chromatography
µl	microlitre

CHAPTER I
INTRODUCTION

The curative properties of plants from which many of the biologically active chemicals used in modern medicine are derived, have been recognized for centuries. Plants and their products appear to have been used in the treatment of infectious diseases at a time when some of the oldest of available human records were made and many centuries before micro-organisms were known (Abraham, 1949).

The oldest known herbal is Pen-Tsao written by Emperor Shen Nung about 3000 B. C. (Stuart, 1911). It contains 365 drugs one for each day of the year. The famous medical papyrus of Ebers (Bryan, 1930) written about 1700 B. C. shows that many of hundred drugs were used by the ancient Egyptians. From temple inscriptions (Ramstad, 1959), it is found that plants were commonly used to cure infections as far as 6000 years ago. Li-Shi-Chen (Ramstad, 1959) in China published in AD 1597 Pen-Tsao - Kang-Mu, a gigantic materia Medica in 52 volumes based on 800 previous authors. It contains about 2000 drugs. Dragendorff (1898) enumerates about 13000 different plants which were used medicinally by people in various parts of the world. Dioscorides (Ramstad, 1959) a Greek Surgeon dealt with the medicinal plants in De Universa Medicina. Indians left no stone unturned to examine and classify the herbs which they could come across into groups or gunas. Charaka classified 80 groups of ten herbs each.

Similarly, Sushruta has arranged 760 herbs in 37 distinct sets according to some of their common properties. Glossary of other writers have added to this list compiling the famous Materia Medica of India (Sanyal, 1964), Indian Materia Medica (Nadkarni, 1954), Indian Medicinal plants (Kirtikar and Basu, 1984), Indigenous drugs of India (Chopra, 1958), Glossary of Indian Medicinal Plants (Chopra et al., 1956) and a survey of Portuguese literature in Goa, (Dalgado, 1898; Barreto, 1967; Garcias Da Horta, 1891; Gracias, 1898a, 1898b), enumerates a number of plants and their parts used to cure infections.

Recent phytochemical analysis of plants used for the treatment of cancer has yielded a number of compounds with antitumor activity. Among these are usmic acid derived from lichens and podophylotoxin from Podophyllum hexandrum and related species. Isolation of an important alkaloidal anticancer drug from Madagascar periwinkle (Catharanthus roseus) was done, though this plant was used for treatment of diabetes by Chinese. Plants of a number of families have recently been shown to accumulate a class of alkaloids with anti HIV activity, notably castanospermine from the Australian Morten Bay chestnut tree (Stafford, 1991).

In addition to curative properties, plant products are used as raw materials for agro chemicals, perfumes, flavouring agents, dyes and gums.

Generally plant products of commercial interest are the secondary metabolites. Table 1.1. These secondary metabolites can be produced by plant tissue culture techniques which has two approaches.

1. As an aid for plant improvement.
2. For the direct production of chemicals in culture.

This science of plant tissue culture offers fascinating possibilities to produce natural compounds under microbiological conditions, as this technique is in many aspects advantageous over traditional agricultural methods. It also offers a useful way to control production of the plant secondary metabolites of pharmaceutical importance on an industrial scale. The term plant tissue culture therefore, broadly refers to the cultivation *in vitro* of all plant parts, whether it is a single cell, a tissue or an organ on a defined nutrient medium (Biondi, 1981).

The idea of growing plant cells and tissues *in vitro* was first recognised at the beginning of the 20^{th} century. Its potential application became a reality much later and in 1956, Nickell (1956) stated that in growing plant cells in culture it should be theoretically possible to produce any compound that is produced normally by the plant from which the culture was

Table 1.1

APPLICATION OF PLANT PRODUCTS IN INDUSTRY

Product	Application	Plant source
Medicinals		
Codeine	Analgesic	Papaver somniferum
Atropine	Anticholonergic	Atropa belladonna
Digoxin	Cardiatonic	Digitalis lanata
Quinine	Antimaterial	Cinchona ledgeriana
Vincristine	Antileukaemic	Catharanthus roseus
Food flavours and Additives		
Thaumaten	Sweetener	Taumatococcus damelli
Capsaiscin	Pungency	Capscicum annum
Lycopene	Red pigment	Lycopersicon esculentum
Crocin	Yellow pigment	Crocus saticus
Essential oils	Antispasmodic	Mentha piperata
Geraniol, Garlic oil	Flavour	Allium cepa
Menthol	Pharmaceuticals	
Insecticide		
Nicotine	Insecticide	Nicotiana tabacum
Pyrethrin	Insecticide	Chrysanthemum cinerariaefolium
Perfume		
Jasmine oil	Perfume	Jasminum sp.
Lavender oil	perfume	Lavendula vera

obtained. Such a system can produce a weather and disease resistant continuous homogenous supply of plant material in a uniform physiological state. Such material can be used to generate undiscovered novel compounds in addition to potentially known ones.

1.1. SECONDARY PRODUCT SYNTHESIS BY PLANT TISSUE CULTURE

The investigations of secondary metabolite biosynthesis by plant cell and tissue culture has gained momentum for the reason that plant tissue culture techniques can be applied to most of the species though only around 2000 different plant-species have been investigated and secondary production of medicinally important compounds obtained. (Stafford, 1991 and Medicinal Plant Biotechnology Course Manual 1994). **Table 1.2.**

The plant tissue may modify or abbreviate the metabolic pathways from that of the plant and produce new compounds which are hitherto not found in intact plants (Stafford, 1991). **Table 1.3.**

1.1.1. Factors affecting variation in production of secondary metabolites.

Environmental variation : Verzar-Petri (1980) observed that the alkaloid content of root and stem callus tissues derived from D. innoxia cultivated in light was higher. Also the static and suspension cultures of the same plant showed irregular growth and alkaloid production depending on seasons. Spring was the most

Table 1.2

Examples of secondary products reported from plant cell and tissue culture

Compounds	Product	Species
Alkaloids	Atropine	Atropa belladonna
	Berberine	Coptis japonica
	Berberine	Thalictrum minus
	Caffeine	Camelia sinesis
	Campothecin	Camptothercea accuminata
	Choline	Cannabis sp.
	Ephedrine	Ephedra gerardiana
	Harringtonine	Cephalotaxus harringtonia
	Harmin	Phaseolus sp.
	Hyosciamine	Datura innoxia
	Monocrotaline	Crotalaria retusa
	Morphinane, codeine Thebaine	Papaver Somnifera
	Nicotine	Nicotiana tabacum
	Papaverine	Papaver somnifera
	Quinine	Cinchona sp.
	Reserpine	Rawolfia serpentina
	Serpentine	Catharanthus roseus
	Trigonelline	
	Nor-sanguinarine	Poppy sp.
	Vindoline	Catharanthus roseus
	Visnagin	Amni Visnaga
Coumarins		
	Bergapten	Amni majus
	Scopoletin	Physochlaina praealta
Steroids	Cholesterol	Datura detoidea
	Diosgenin	Datura detoidea
	Solasodine	Solanum nigrum
	Stigmasterol	Brassica napus
	Sitosterol	Artemisia abinthrum
	Panaxadiol	Panax ginseng
	Tigogenin	Trigonella occulta
Phenyl propanoids	Anthocyanins	Daucus carola
	Shikonin	Lithospermum erythrorhizon
	Ubiquinone - 10	Nicotiana sp.
	Anthraquinones	Cassia obtusiflora
	Capsaicin	Capsicum annum

Table 1.3

Compounds hitherto detected only in tissue cultures and not in corresponding intact plants

Compound	Cell culture
Pericine	Picralima nitdia
Pericalline	Picralima nitdia
Hinokiol	Picralima nitdia
Ferruginol	Thuja Occidentalis
Pleiocarpamin	Thuja Occidentalis
Akummilin	Catharanthus roseus
Vomilenin	Rauwolfia serpentina
Paniculid A	Andrographis paniculata
Tarennosid	Gardenia jasminoides
Ruteculin	Ruta graveolens
Harmin	Phaseolus sp.
Putricine	Tobacco sp.

Table 1.4

Total alkaloid content of E. foliata and E. gerandiana Stem and callus tissues

Plant samples	Locality	Sex	% of alkaloid
E. foliata	Stem tissue		
	Pratapnagar	Male	0.010
	Umaidsagar	Male	0.012
		Female	0.010
	Gulasani village	Male	0.013
		Female	0.012
	Aj mer	Male	0.010
		Female	0.010
	Callus tissues	—	—
E. gerardiana	Stem tissues		
	Leh	Female	1.720
	Callus tissues		0.160

productive season in alkaloid biosynthesis. However a negative correlation was established for increase in biomass and alkaloid production (Berznegovaskaya, 1976). Lack of control over light, different temperatures, pH of media, addition of antibiotics and even autoclaving times may vary the production of secondary metabolites. Overall the subject of variability cannot be left without a mention of the problem of variable results obtained by different workers at different times, use of different isolates, cultures at different passages, inocula of different sizes or different physiological ages (Fuller, 1984). **Table 1.5.**

Location, sex and species variation : It was found that alkaloid ephedrine obtained from male plants of E. foliata collected from Jodhpur contained more ephedrine than female plants of E. foliata collected from other localities of Rajasthan. E. geradiana tissues yielded large amount of alkaloids as compared to E. foliata (Arya, 1978), Table 1.4.

In Vitro and in Vivo variation : The static cultures of T. polycerata were more potent to produce steroidal sapogenis than their parts in vivo (Kamal, 1992). Whereas Zheng (1976), showed that the production of hyosciamine in S. acutangula was less in callus (0.025%) as compared to stems of intact plant (0.123%). The alkaloidal pattern of root cultures of A. belladonna was almost identical with the pattern of roots from intact plants. Only the pattern differed quantitatively (Hartman, 1986).

Explant variation : Seed callus of D. stramonium and D. innoxia contained more alkaloid than the root, stem and leaf callus (Chan,

Table 1.5

Examples of manipulation of cell cultures by medium and environmental variation.

Environmental factor	Effect	On	Species
Light	inhibits	Nicotine Shikonin	N. tabacum erythrorhizon
pH shift	selects for	hydrosylation	D. lanata
coconut milk	stimulates	Phaseollin	Phaseolus vulgaris
cytokinin	inhibits	caretenoid, anthocyanin, Nicotine	Daucus carda Daucus carda N. tabacum
cytokinin	increases	Berberine	Thalictrum minus
2, 4-D	inhibits	Berberine	Thalictrum minus
2, 4 Dimethyl Phenoxy acetic acid	increase by 40%	Rosmaric acid	Coleus blumei
2, 4-D	increases	Diosgenin	Dioscorea tokoro

Table 1.6

Some of the precursors found to influence the biogenesis of alkaloids in plants

Alkaloid	Chemical Classification	Precursor
Anabasine. sedamine, Lycopodine	Piperidine	L.lysine
Nicotine, tropine	Pyrrolidine	ornithine
Retreonecine		lysine
Ephedrine	Phenylethylamine	Phenylalnine
Morphine, papaverine, Berberine	Benzyl isoquinoline and related structures	Tyrosine
Colchicine	"	Phenylalanine
Emetine	"	Tyrosine (not confirmed)
Norbelladine	Amaryllidaceae	Tyrosine & Phenylalnine
Serpentine, Ajmaline, Vindoline	Indole	Tryptophan
Ergotamine	Ergoline	Tryptophan
Quinine	Quinoline	Tryptophan

1965).

1.1.2. Site of synthesis and elucidation of biosynthetic pathways

The plant organ or tissue within which secondary products are accumulated are not necessarily their site of synthesis. A number of techniques have been developed to investigate these diverse pathways and the sites of biosynthesis including grafting and the treatment of sterile tissue cultures or excised organs with radioactive precursors. The mode of biosynthesis of alkaloids in plants is based upon the idea that they are derived from relatively simple precursors like phenylalanine, tryptophan, acetate units, terpene units, methionine, ornithine, etc. The modern approach to biosynthetic studies of alkaloid involves administration of labelled precursors to selected plants and after a suitable period of growth, isolation of alkaloids. They are then degraded in a systematic fashion to determine the position of the labelled atoms. Using this technique many alkaloids e.g. morphine, nicotine, hyosciamine, pellotine, papaverine, colchicine, gramine etc. have been shown to be synthesised from amino acids (Spencer, 1970; Leete, 1967). Table 1.6.

1.1.3.Effect of precursors on production of secondary metabolites

According to Zenk et al. (1975) feeding of direct precursor is not necessarily effective in increasing the final yield. Tabata et al.(1976a) observed no effect of p-hydroxy benzoic acid on the production of Shikonin by Lithospermum

culture. Schmauder et al.(1985) reported 90 fold increase in quinine and quinidine production by feeding tryptophan to Cinchona pubescens suspension culture. However, compounds added to media as precursors or to be transformed to alkaloids or other compounds have been studied by both growing cultures and their cellular fraction. Table 1.7.

1.1.4.Biotransformation by plant tissue culture and suspension culture

Biotransformation studies have been reported for a variety of compounds but it is only recently that the fractional potential has been recognised. For example, it was observed that cell cultures of Datura species (Hiraoka, 1974) growing on a medium containing (2, 4-D) could esterify exogenously supplied tropine to acetyltropine. The biotransformation took place with tropic acid but with endogenous acetic acid. If the culture was grown with 2 mg/L, NAA instead of 2, 4-D, than hyosciamine was formed. It has been observed that culture unable to produce secondary compounds de novo, can often perform specific enzymic reactions of the pathway. Thus, non producing cultures of D. lanata hydoxylate digitoxin or even better β- methyl digitoxin to the corresponding commercially used digoxin (Reinhard, 1980). Plant tissue culture has made it possible now to study biotransformation of plant callus tissue or plant cell suspension culture. Precursors of desired cell products when added to nutrient medium have been successfully biotransformed to final products and this has helped in maximising secondary metabolism and better understanding of metabolic pathways as can be seen in

Table 1.7

Role of precusor in production of secondary metabolite in cell & suspension culture

Plant	Sec. metabolite	Precursor	Reference
Datura deltoidea	Diosgenin	Cholesterol	Kaul (1969)
Datura tatula	total alkaloids	Tyrosine Phenylalanine	Sairam (1971)
Cinchona pubescens	Quinine & Quinidine	Tryptophan	Koblitz et al. (1983)
Catharanthus roseus	Ajmalacine	Tryptamine	Brodelius (1979)
Ruta graveolens	Dictamnine	4-hydroxy-2 quinoline	Steck (1973)
Tagetes erecta	Pyrethrine	Ascorbic acid	Khanna et al. (1976b)
Solanum Xantho carpum	Solasodine	Cholesterol	Khanna et al. (1976 c)
Datura metel	atropine, hyosciamine	Phenylalnine & tyrosine	Khanna, et al. (1972 b)
Capsicum annum	Capsaicin	L ascorbic acid & D-limonene	Veeresham (1991)
Trigonella foenum	Trigonelline	Nicotinic acid	Khanna et al.. (1972a)
Nicotiana tabacum	Nicotine	Nicotinic acid	Chan et al.(1965)
Trigonella foenum graecum	Diosgenin, gitogenin tigogenin	cholesterol	Khanna et al. (1975)

Table 1.8

Plant cell culture and Biiotransformation reactions

Plant species	Reaction	Precursor	Product	Reference
Anethum graveolens Allium Cepa	}Hydroxylation	Agroclavine Elymoclavine	C-8 } compound	Hsu (1973)
Cannabis Sativa	Oxidation	Geraniol	Nerol	How (1973)
Nicotiana tabacum	Reduction	Testesterone	Androsterone -3-dione	Hirotoni (1974)
Datura sp.	Esterification	Tropine	Actyltropine	Hiraoka (1974)
Digitalis lanata Thevaetia sp.	Glucoeylation	Citoxigenin	Gitoxine	Doller (1978)
Mentha sp.	Reduction	(-)Menthone	(+)neomenthol	Aviv (1981)
Datura innoxia	Glucosylation	Dihydroxy - benzene (Hydroquinone)	mono β-D glucoside (arbutin)	Tabata (1976b)

(Thengane, 1987). Table 1.8.

1.1.5 . Nutritional requirements for plant tissue culture :

With the development of plant tissue culture many types of cultures have come up, namely, plant culture, embryo culture, organ culture, tissue or callus culture or cell suspension culture. These cultures will exist only when supplied with suitable nutrients. A number of basic nutrient media of varying composition have been devised by different workers (Bhojwani, 1983 and George, 1984). Basically, all these media contain mineral salts, vitamins, amino acids and carbon source. These are further modified and supplemented with growth hormones so as to make the medium most suitable for the particular cell, tissue or organ. The selection of nutrients is the most complicated and confused job in the plant tissue culture technique as the medium stimulating the induction of growth in a certain tissue may not be suitable for maintaining its growth. Need arises to bring further alterations in the medium when the tissue has to be transferred from solid media to liquid media.

The nutritional requirements of plant tissue culture reflect in the biosynthesis activity. So efforts have been made to vary the culture conditions by including various plant and natural extracts like yeast, malt, tomato, casein, coconut, banana etc., and fruitful results have been obtained. Weete & coworkers (1972) tried even lunar material from Apollo 12 while doing tobacco tissue culture studies and after twelve weeks period they found fluctuation of both the relative and absolute

concentration of endogenous sterols and fatty acids. The experimental tissues were found to contain higher concentrations of sterols than in the controls.

1.2. REVIEW OF TECHNOLOGY FOR USE OF SUSPENSION CULTURE FOR SECONDARY METABOLITES

Literature pertaining to production of secondary metabolites by cell suspension cultures of diverse plant groups is reviewed by (Constabel et al., 1974). Cell suspensions have been used to study the biotransformations of secondary metabolites (Steck and Constabel, 1974) and cardenolides (Reinhard, 1974; Alfermann, 1977). Chlorogenic acid, a phenol was produced by cell suspension of Haplopappus (Strickland and Sunderland, 1972). High contents of ubiquinone 10, have been found in tobacco cell cultures (Ikuta, 1976) and L-dopa in Mucuna pruriens (Brain, 1974). A serpentine content equal to that of normal drug material is reported from cell cultures of Catharanthus roseus (Doller, 1976). Also of interest are results of Jhang et al. (1974) with cell cultures of Panax ginseng producing high amounts of saponins and of Tamaki et al. (1973) with Glycyrrhiza glabra cultures that contain 3-4% of glycerrhizin. Coleus blumei suspension cultures produced 13 to 15% of rosmaric acid (Razzaque et al., 1977).

The suspension culture of Dioscorea deltoidea produced up to 1.5% of dry matter of diosgenin (Kaul et al. 1969). Other reports in the light of this subject are on berbenine production in Thalictrum minus (Nakagawa, 1986), Solasodine in Solanum

laciniatum (Chandler, 1983), alkaloids in Ruta graveolens (Steck, 1973), cinchona alkaloids in Genus Cinchona L. (Koblitz, 1983), reserpine in Rauwolfia serpentina (Yamamoto, 1986).

1.2.1. Secondary product accumulation by suspension cultures :

The basic technique of initiating cell cultures are well known. Cultures from any desired plant species can be established with some patience. Plant cell cultures with doubling times of 20 hours are classified as rapidly growing and such growth rates are only achieved in suspension cultures. Therefore, the only culture system of biotechnological relevance for the production of secondary metabolites is suspension cultures that can be grown in shake flasks as well as in large bioreactors.

These systems have attracted enormous interest largely because of their potential for scale-up. Suspensions frequently do not achieve the secondary product yields required of them and more often not produce much less than the whole plant on dry weight basis. The fact remains that yet liquid suspensions are the easiest plant culture systems to initiate, maintain and scale up. Another important fact is that even given low product yields, the levels and activities of secondary biosynthetic enzymes in cultures may often be high, relative to the whole plant, making cell cultures an excellent source of material for enzyme purification and investigation on the molecular regulation of biosynthetic pathways. Table 1.9 shows few cases, where the capacity of plant cell cultures to synthesise and accumulate secondary products has been remarkable (Stafford, 1991).

Table 1.9

Examples of high yields of secondary product achieved in plant cell culture

Species	Compound	Max. yield in culture % dry weight
Coleus blumei	Rosmaric acid	23% (5.6 g/L)
Lithospermum erythrorhizon	shikonins	23% (6.4 g/L)
Morinda citrofolia	Anthraquinone	10% (2.5 g/L)
Catharanthus roseus	Serpentine	2%
Coptis japonica	Berberine	15% (1.7 g/L)
Panax ginseng	Gingesenoides	2% (150 mg/L)
Nicotiana tabacum	Nicotine	2.1%

Table 1.10

Emperical method to manipulate secondary product yield in suspension culture

Source	Effective manipulations
Carbohydrate	a)Type b) concentration
Nitrogen	c)Inorganic d)Organic
Phosphate level	High phosphorus is inhibitory to alkaloid
Phytohormone	Adjustment of Auxin-Cytokinin
Light regime	Critical factor as some cultures grow in dark/light regime.
Temperature	Lowest temperature enhances product yield but depress growth rate.
Osmotic stress	Improved by high sugar high salt
Precursors	Variable effects
pH	Variable effects
Elicitation	Treatment of callus with autoclaved filtrate of fungal culture often causes increase in production.

1.2.2. Increase of secondary product yield in cell suspension :

Depending on the product type, plant species and cell lines, plant suspension cultures vary enormously in their capacity to produce and accumulate secondary products. A vast majority of effort is still being devoted to an empirical approach to the enhancement of product yield. When attempting to optimise culture conditions for the production of a given target compound by selecting a range of variables to manipulate vital changes, the chemical nature of the target compound and the plant family is considered (Stafford 1991). Table 1.10.

1.2.3. Commercial production of secondary metabolites :

The first commercial production of a secondary metabolite by plant cell culture has been made possible by employing a two-stage culture system. Shikonin produced from suspension culture of the roots of L. erythrorhizon is used in Asia both as an antiseptic and as a dyestuff. Cell cultures of this plant could be induced to produce shikonin when grown on selected media using agitated two stage air-lift system. Yields up to 15% of shikonin could be obtained (Fujita, 1982). The commercial production of this product was announced by the Mitsui Petrochemical company in Japan in 1983 (Scragg, 1991). Tobacco and Salt Corporation of Japan has grown tobacco cells in fermentors for production of ubiquinone -10 used in congestive cardiac diseases (Berlin, 1984).

Another noteworthy industrial application involving plant cell cultures is the 12 β-hydroxylation of β- methyldigitoxin involving strains of D. lanata using the highly toxic product digitoxin, a bye product in the extraction of cardenolides from

digitalis to produce digoxin, a highly valuable cardenodide (Kurz, 1986).

Production of ajmalicine by Catharanthus roseus cell cultures would also be of commercial interest as the compound is used in the treatment of circulatory diseases (Berlin, 1984).

1.3. AIM AND SCOPE OF PRESENT STUDY :

The western Ghats facing the vast stretches of Indian coast of Arabian sea are known to be rich in vegetation producing secondary metabolites that are widely used as medicinal plants. The existence of such plants in the coastal state of Goa was assumed considering its rich flora and scattered reference in old literature, information supplied by local people, Goan herbalists and some Portuguese literature on flora of Goa (Dalgado, 1898; Barreto, 1967) which gave an idea of the plants commonly found in Goa and their use in curing local or systemic infections. A study was taken earler (Bhonsle, 1973) and several plants were screened for their antibacterial effect. One such plant Heterostemma tanjorense belonging to Asclepiadaceae family was found to have biological activity which was attributed to the alkaloids present in the plant. The foregoing review indicates positively that the plant kingdom has been investigated for its active constituents as secondary metabolites raised through tissue culture technique.

Present work envisaged the need to explore some of the plants of Asclepiadaceae family. This family represents nearly 320 genera and 1700 species. In general, these plants are used as

diaphoretic, diuretic, antisyphilitic, anthelmintic, emetic and antiasthmatic (Kirtikar and Basu, 1984). Among the products isolated from the plants of Asclepiadaceae family of Indian origin are polyoxygenated compounds. These compounds are mainly polyoxysteroidal (Cardiac and Pregnane) glycosides and have strong biological activity like cardiovascular, antitumor and anticancer. Various oligosacharides of normal and deoxy sugars have also been isolated. Besides these compounds a number of terpenes, alkaloids, flavenoids have also been isolated (Deepak, 1995).

Attempts have been made to give a biotechnological approach to this work and callus cultures were raised from vegetative explants. Plants namely Heterostemma tanjorense , Tylophora dalzellii, Cosmostigma reacemosa, Hemidesmus indicus, Marsdenia volubilis and Holostemma rheedianum were screened for callus induction. Table 1.11 lists their use and reported bioactive constituents (Cooke, 1967; Gamble, 1967; Santapau, 1967). Emphasis on investigation on H. tanjorense was stressed since this plant was earlier investigated for its antibacterial and pharmacological action. An active constituent heterostemmine an alkaloid with empirical formula $C_{10}H_{17}NO_2$ was isolated from the roots which had biological activity (Bhonsle, 1973). Callus induction studies of this plant were carried out earlier (Bhonsle, 1991). The plant is described as follows :

Botanical Name : *Heterostemma tanjorense* W. & A.

Family : Asclepiadaceae

Description : Flora of Madras (Gamble, 1967) describes this plant as follows :-"A slender twiner, with broadly ovate leaves, obtuse or cordate at base, and up to 4 in. long, 2 in. broad, the linear follicles 4 in. long, reflexed, hooked at apex." (Fig 1.1)

Distribution :

The plant *H. tanjorense* is very specific in its distribution. It is found along coast of Goa, Konkan and Madras.

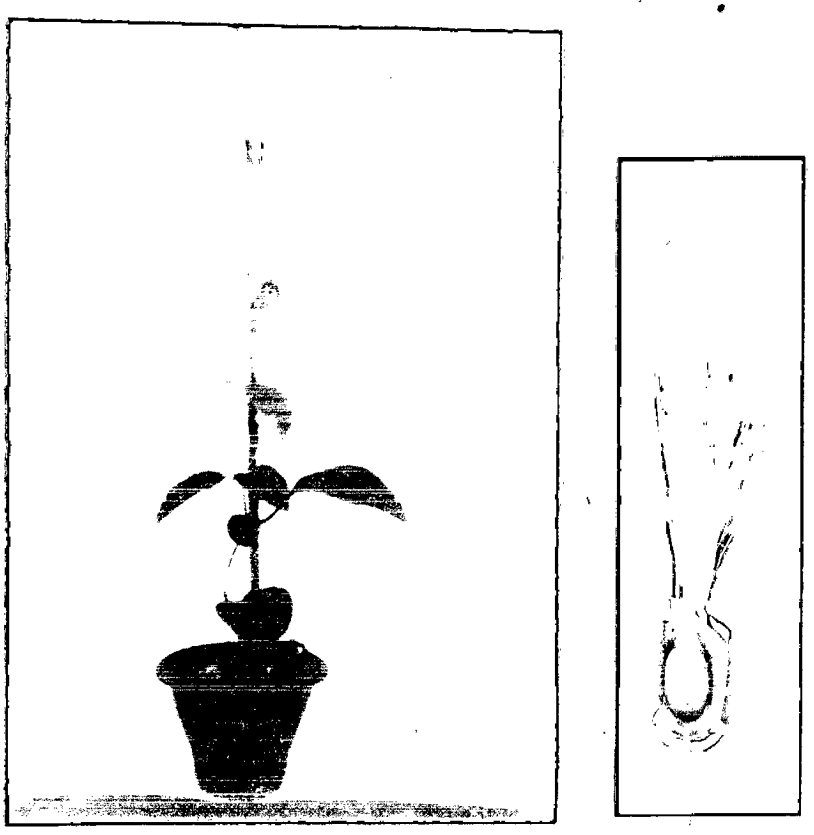
Considering the medicinal properties associated with the alkaloidal fraction of this plant, the research strategies emphasised the selection of plant material, formulation of suitable medium and environmental conditions for induction of callus optimisation for maximum yield of callus cultures and the detection of the bioactive alkaloid form *H. tanjorense*, formed the foundation of this work while characterization of alkaloid and its production in suspension cultures emphasised the later part.

Table 1.11

Plants screened for Callus induction, their biological component and medicinal use.

Plant	Biological component	Use based on information from local herbatists
Heterostemma tanjorense	alkaloids	antibacterial and anti-hypertensive.
Cosmostigma racemosa	alkaloid, glucoside	systemic infection
Tylophora dalzellii	alkaloid	antiasthmatic
Hemidesmus indicus	Coumarins	antisyphilitic & blood purifier
Marsdenia Volubilis	alkaloids glucoside	boils, abseces, emetic
Holostemma rheedianum	———	gonorrhoea, ophthalmia

Fig. 1.1



Heterostemma tanjorense and seed.

CHAPTER II
CALLUS INITIATION IN ASCLEPIADACEAE SPECIES

Members of the asclepiadaceae family are usually herbs or shrubs, frequently twining, often with milky juice. Their propagation is vegetative from roots or by seed dispersion. These are mostly tropical and some are temperate. They appear at the onset of the monsoon season.

To generate callus cultures of the plants, explants such as leaf primordia, leaf,stem, flower buds, anthers and seeds are generally selected and cell lines are generated on suitable growth medium. This chapter includes the preparation of explants from the members of the asclepiadaceae family such as Heterostemma tanjorense, Tylophora dalzellii, Cosmostigma racemosa, Hemidesmus indicus, Marsdenia volubilis and Holostemma rheedianum and subsequent development of callus cultures from the vegetative explants.

MATERIALS AND METHODS

2.1. Glassware

Glassware used was of Borosil brand. It was cleaned by soaking overnight in detergent solution of Labolene (Qualigens) and washed with tap water. It was rinsed with glass distilled water and dried at 150^{0}C for one hour.

2.2. Sterilization of Glassware

For all experiments 18 x 150 mm and 25 x 150 mm test

tubes, 100 and 250 ml. Erlenmeyer flasks and 100 x 15 mm petri dishes were used. All glassware and dissection instruments were steam sterilized at 15 p.s.i. for 20 minutes and dried at 150^0 C for 1/2 hr.

2.3. Chemicals

All chemicals, reagents, solvents, inorganic salts, carbohydrates, vitamins, amino acids etc. were obtained from BDH/Merck/Qualigens/Loba chemie/Hi Media and S.D. Fine Chem.

2.4. Media Preparation and sterilization

Media were prepared from ready made plant tissue culture media from Hi-Media using glass distilled water. The composition of Murashige and Skoog (MS), Gamborg (G5), White's, Errikson and Nitsch was as per media composition (Bhojwani, 1983). Whenever required media of different composition were prepared in laboratory making necessary alterations in the formula. MS medium 1/2 strength was prepared by diluting MS medium. Here the concentrations of sucrose was kept as 2% and agar 0.8%.

For static cultures media were distributed in 7 ml amounts in 18 x 150 mm and 15 ml in 25 x 150 mm test tubes. For suspension cultures 20 ml of medium was used in 100 ml Erlenmeyer flasks and 40 ml in 250 ml flasks. The media were sterilized at 15 p.s.i. for 20 mins.

2.5. Adjustment of pH

Prior to sterilization of media, pH was adjusted to 5.5 using either 0.1 N NaOH or 0.1 N HCL. Experiments involving different pH values, pH was adjusted with 1N NaOH or 1N HCL.

2.6. Agar Concentration

Except the studies involved in determining the percentage of agar required for optimisation of callus, all other media for static cultures contained 0.8% agar.

2.7. Preparation of Stock Solutions

The stock solutions of plant growth regulators, amino acids, vitamins etc. were prepared sterilized and stored as per Appendix 1. (Ahuja, 1994; Reynolds, 1982,1993).

2.8. Coconut Milk

It was pooled from several tender coconuts, boiled for fifteen minutes and filtered through Whatman No 1 filter paper. It was distributed in 50 ml amounts and stored at -20^0 C, whenever required it was thawed, mixed thoroughly and used (Allan, 1991).

2.9. Plant Material

A hillock near Betim village along the river Mandovi was chosen for the collection of plant specimens. The vegetative explants were collected during the rainy season June-August, cleaned with detergent solution and subsequently washed with distilled water. The explants were inoculated on basal media on the same day of collection.

2.10. Sterilization of Explant

The vegetative explants like leaf primordia,leaf,stem, flower buds, anthers, roots, seeds etc, were surface sterilized as per Appendix 2 (Bhojwani, 1983; Ahuja, 1994).

2.11. Seed Germination of H. tanjorense

The seeds of H. tanjorense were surface sterilized and placed in paper boats (5 seeds in each) in 25 x 150 mm test tubes

containing MS medium ½ strength.

2.12. Inoculation

All inoculations were done under laminar air-flow (Klen-Zeids).

2.13. Callus Initiation and Growth Determination

For callus initiation all the surface sterilized explants were inoculated on MS medium with 0.8% agar supplemented with 2,4-D (1mg/L). Visual observation was done to specifically note the period required for callus initiation and the morphological response of the callus.

A three day old germinated seedling of H. tanjorense was removed from paper boats and placed on MS medium for callus initiation.

The callus obtained from vegetative explants of all the plants was used for preliminary investigation after five subcultures.

2.14. Environmental Conditions

i. Temperature : The temperature adopted for all experiments was $25^0 \pm 2^0$ C. The experiments requiring low temperature were conducted in refrigerator. Experiments requiring high temperature of 30^0 and 35^0C were kept in incubators.

ii. Light intensity : All experiments were conducted in air-conditioned rooms with light intensity of 1500 Lux supplied from cool-daylight fluorescent tubes, measured by lux meter (Rajdhani make). The experiments requiring total darkness were covered with black paper and kept in air conditioned rooms.

iii. Humidity : Humidity though it was variable was maintained

between 60 to 80%

2.15. Extraction of Callus for Detection of Alkaloids

Callus obtained after 5 passages from different explants (5.0 g. of pooled wet mass approx. equivalent to 250 mg. dry wt.) of plants chosen for investigation was finely ground to a thin paste in a glass mortar and extracted in a soxhlet extractor with 75% alcohol containing 0.5% tartaric acid for 6 hrs. The solvent (200 ml) was concentrated in vacuum to a syrupy consistency. It was then diluted with water and rendered acidic (pH_2) by the addition of 0.5% tartaric acid. The aqueous acidic extract was filtered to remove resins and fatty acids. It was then extracted with solvent either (100ml) to remove neutral and acidic fractions which might have interfered in subsequent operations. The ether layer was discarded. The aqueous solution was made strongly alkaline (pH 10) with dilute NH_3 solution. The alkaline solution was extracted with chloroform (300ml). The chloroform extract was concentrated under vacuum and dried over fused calcium chloride. This extract was subjected to thin layer chromatographic analysis for the detection of alkaloids.

2.16. Thin Layer Chromatography System

TLC plates were prepared by conventional method using a stationary applicator, (Stahl, 1969a), (ACME MAKE), by using 10 x 20 cm plates of 0.2 mm thick silica gel GF 254 layer and activated at 110^0c for 30 min. Chromatograms were developed in trough type glass chambers for 30 mins in solvent system containing chloroform : Methanol (95:5) with 2% NH_3 and sprayed with Dragendorff reagent. The extracts were dissolved in

chloroform and applied on the chromatoplate with a micropipette (approx. 10mcg). The spots were air dried, the plates inserted in the chamber and the solvent allowed to run approximate for 30 mins. After removing the plates, the solvent front was marked, plates air dried and viewed under uv light. The plates were then sprayed with Dragendorff reagent. (Stahl, 1969b), Appendix 3.

RESULTS

2.17. Development of Callus on MS medium

To determine whether the Callus can be initiated from selected plants, the explants prepared as in 2.10 were inoculated on MS medium in a series of test tubes and incubated at $25^0 \pm 2^0$ C. The calli were initiated in the explants ranging from 4 days to 15 days. The results are depicted in Table 2.1 and represented by Fig. 2.1 to 2.6

It was observed that only flower buds of H. tanjorense formed callus, while callus initiation occurred in all the tested explants of the asceptically grown seedling of H. tanjorense. Callus initiation in case of I. dalzellii took place from the midrib portion of the leaf primordia. Profuse callusing was observed in all the explants tested of C. racemosa. Callus initiation in H. indicus was throughout the leaf primordia. In M. volubilis callus initiation started from the cut edge of leaf disc and later on spread to the whole leaf disc. In stem explant of H. rheedianum callus started from the cut ends of the stem.

2.18. Seed Germination and Callus Initiation in H. tanjorense

The seeds of H. tanjorense took nearly 7 to 10 days for

Table 2.1

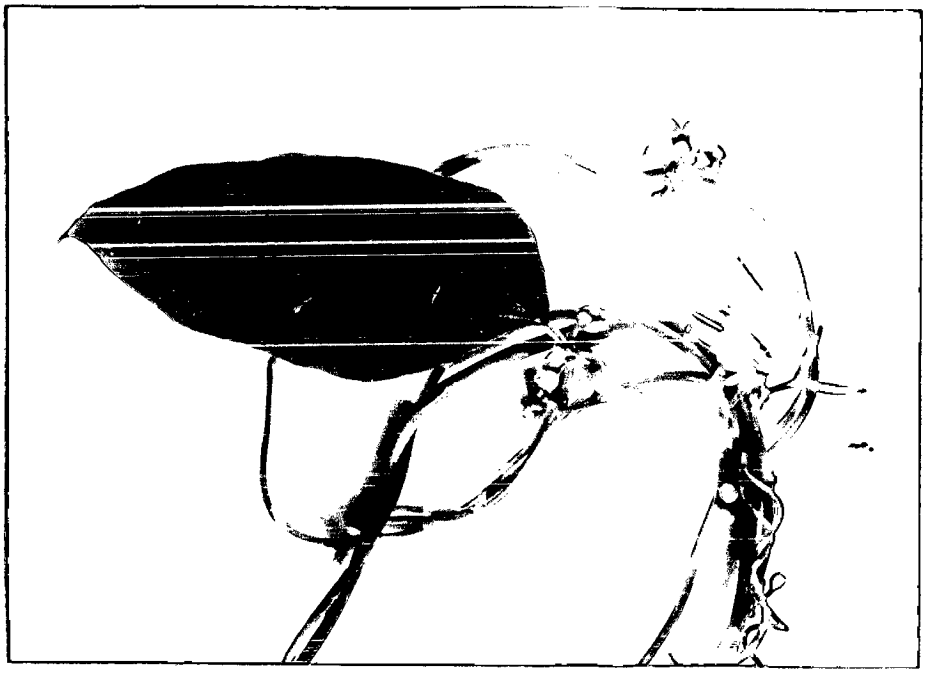
Different explants inoculated on MS medium containing 2,4-D (1mg/L) for callus induction.

Plant	Explants for callus induction							No. of days for callus initiation	Morphological response of callus
	LP	LD	ST	FB	AN	RT	GS		
H. taniorense	-	-	-	+	-	-	+	15 days for FB & 7 days for GS	White & hard for FB & Golden yellow for GS
H. taniorense seedling	+	+	+	NT	NT	+	+	7 days	hard, golden yellow
T. dalzellii	+	+	+	NT	NT	NT	NT	7 to 10 days	white & hard
C. racemosa	+	+	+	NT	NT	NT	NT	5 to 7 days	golden yellow soft
H. indicus	+	-	-	NT	NT	NT	NT	4 to 5 days	white & hard
M. Volubilis	-	+	-	NT	NT	NT	NT	10 to 15 days	golden yellow & soft
H. rheedianum	-	-	+	NT	NT	NT	NT	10 to 15 days	white & soft

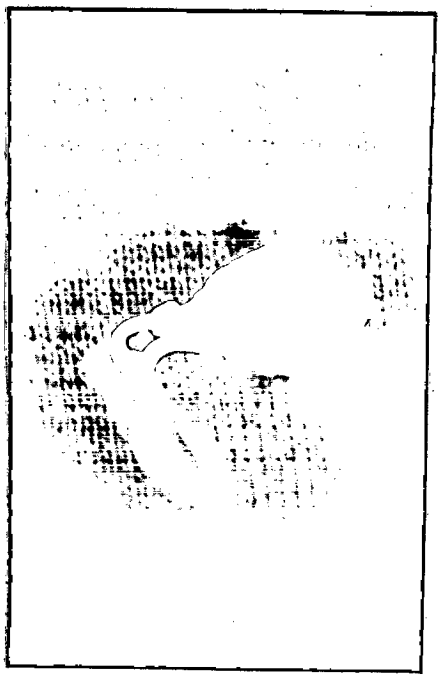
Abbreviations :

LP = Leaf primordia
LD = Leaf disc
ST = Stem
FB = Flower bud
AN = Anther
RT = Root
GS = Germinated seedling
NT = Not tested
\+ = Callus initiation
\- = No Callus formation

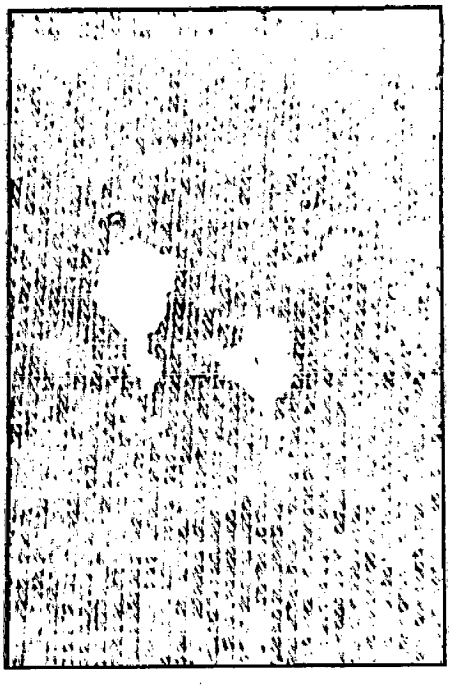
Fig. 2.1



Heterostemma tanjorense

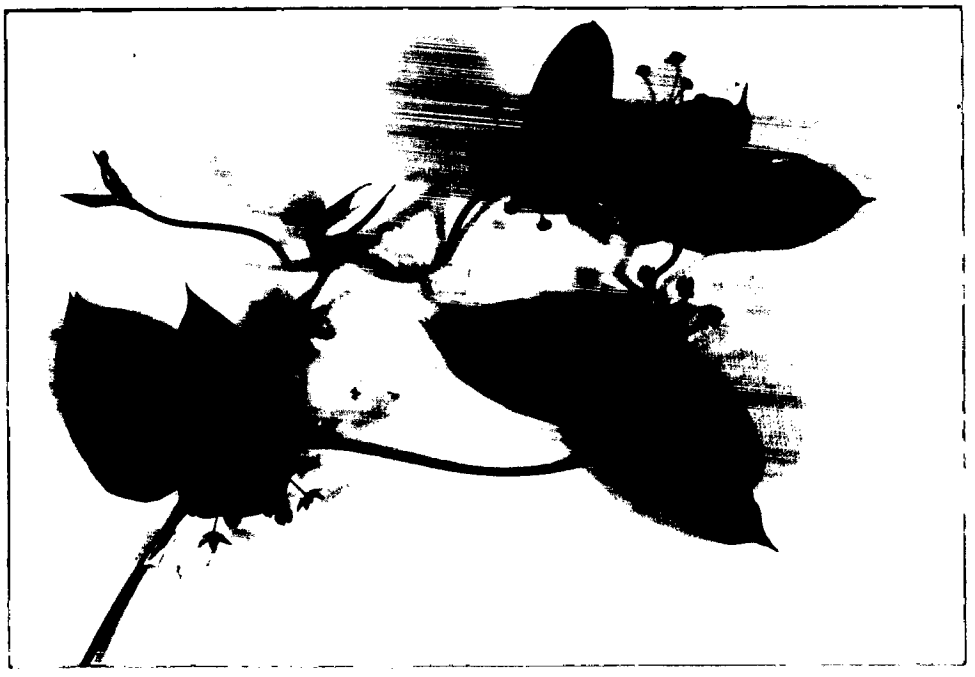


Germinated seedling Callus



Flower bud Callus

Fig. 2.2

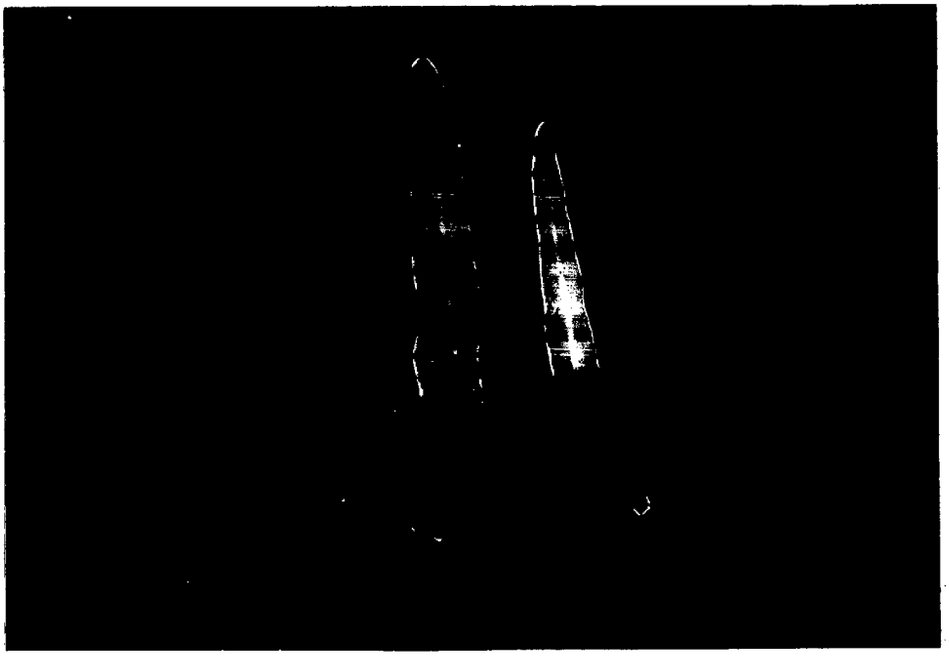


Tylophora dalzellii

Callus at the midrib

Fig. 2.3

Cosmostigma racemosa



Callus at leaf primordia

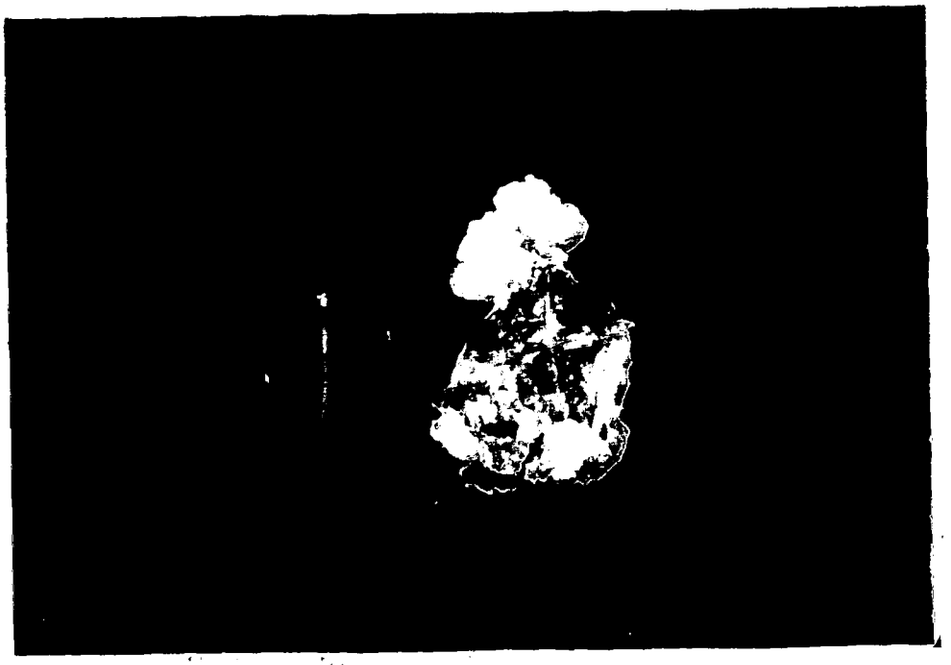
Fig. 2.4

Hemidesmus indicus

Callus at whole leaf

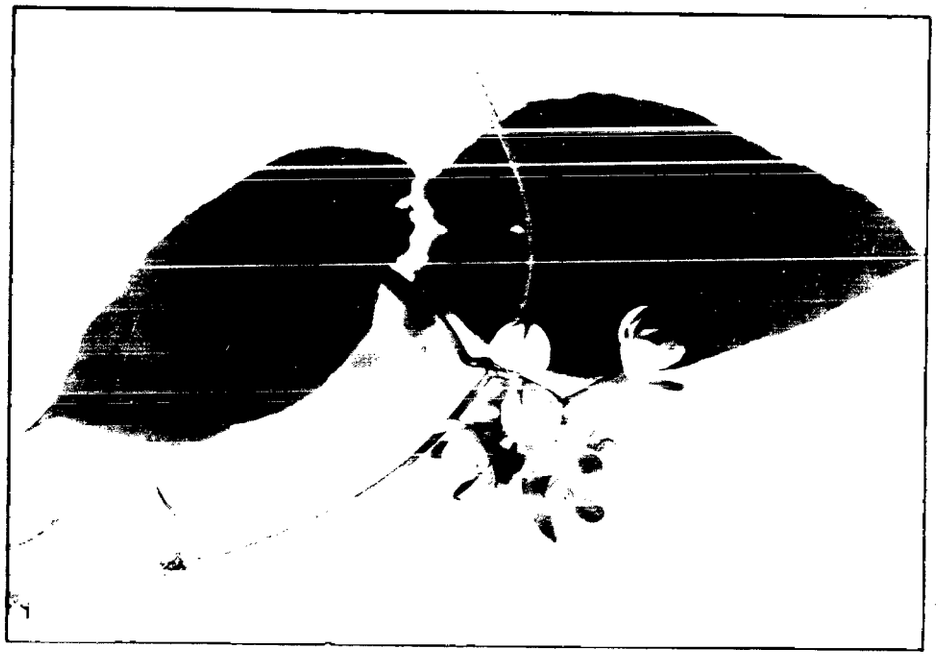
Fig. 2.5

Marsdenia volubilis

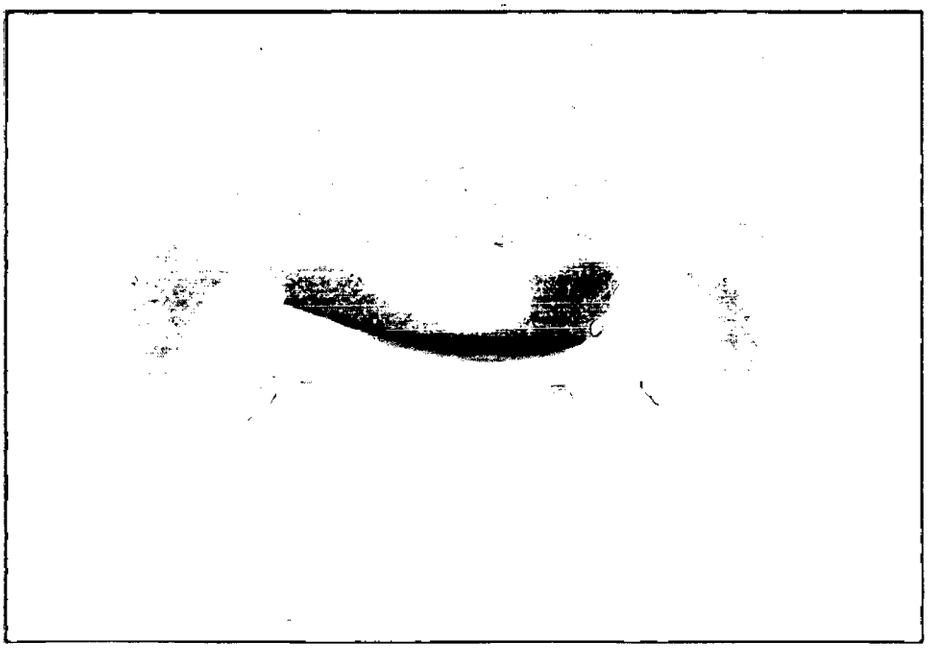


Callus at leaf disc.

Fig. 2.6



Holostemma rheedianum



Callus at stem ends.

germination Fig.2.7 and the callus formed on the germinated seedling started from the radicle portion of the root and later on extended to epicotyledonary and cotyledonary region. Fig. 2.8.

2.19. Selection of Media for Callus Induction in H.tanjorense

Amongst all the media tested for the induction of callus of various explants of H. tanjorense, MS medium supplemented with 2,4-D(1mg/L) was found to be most suitable. Table 2.2.

2.20. Detection of Alkaloids in Callus Extracts

The calli produced by various explants of the plants under investigation were subcultured every 4 wks and after 5th passage were extracted as per 2.15 and the extracts tested for the presence of alkaloids by TLC analysis. The results are represented by Table 2.3. Among all the callus extracts tested only callus of germinated seedling of H. tanjorense showed the presence of alkaloids. Fig 2.9.

DISCUSSION

It is observed from the results that callus initiation was observed in the explants of all the plants tested. The callus biomass formation varied from plant to plant and explant to explant. In C. racemosa callus formation took place from all the explants and the yield of the callus was maximum while H.tanjorense induced callus only in flower buds and germinated seedling. This may be because of the injury caused to the explants by the sterilizing agents. MS medium was found to be better for callus initiation than all other media tested. This may be because of its high salt contents. The calli produced from the in vivo

Table 2.2

Callus initiation on different basal media

Explant	Basal Media for callus formation				
	MS	whites	ER	B5	Nitsch
Leaf Primordia	-	-	-	-	-
Leaf Disc	-	-	-	-	-
Stem	-	-	-	-	-
flower buds	+++	-	-	+	+
Anthers	-	-	-	-	-
Roots	-	-	-	-	-
Germinated Seeding	+++			++	++

Media :

1. MS : Murashige & skoog
2. Whites :
3. ER : Eriksson
4. B5 : Gamborg
5. Nitsch.

(-) : No Growth
(+) : Callus initiation
(++) : Poor growth of callus
(+++): good growth of callus
(++++): Very good growth of callus

Table 2.3

Detection of alkaloids in callus extract of selected plants.

Chromotography system : TLC (Ascending)

TLC layer : Silica Gel GF 254

Solvent system : Chloroform : methanol 95:5 with 2% NH_3

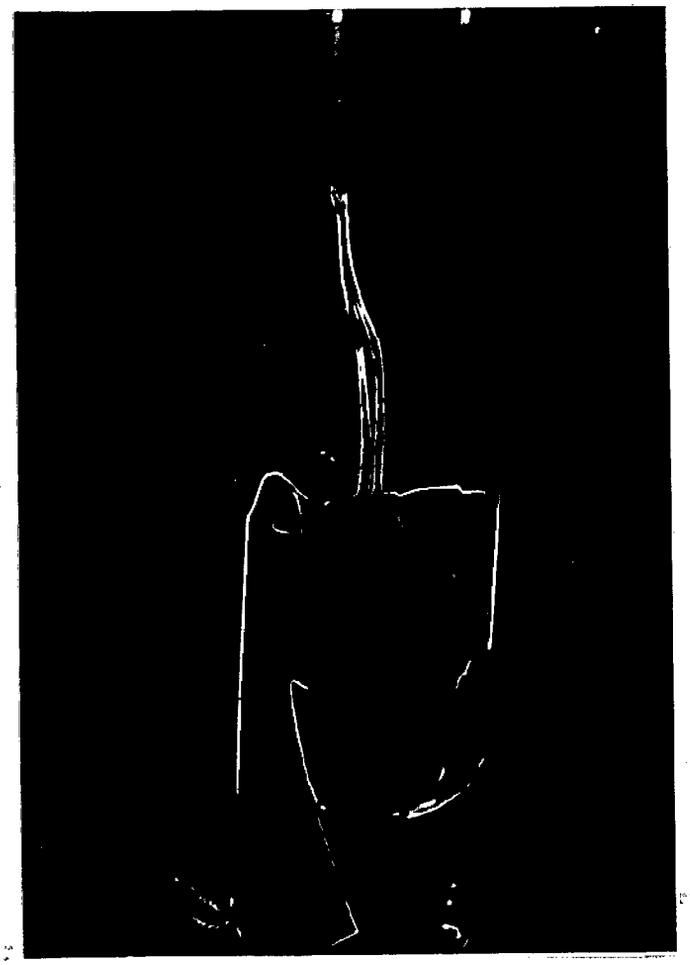
Detection Method : Dragendorff spray reagent

Plant	Colour reaction	Inference
H. taniorense flower bud callus	NIL	-ve
H. taniorense seedling Callus	Orange	+ve
T. dalzellii	NIL	-ve
C. racemosa	NIL	-ve
H. indicus	NIL	-ve
M.volubilis	NIL	-ve
H. rheedianum	NIL	-ve

-ve = Alkaloid absent

+ve = Alkaloid present

Fig. 2.7

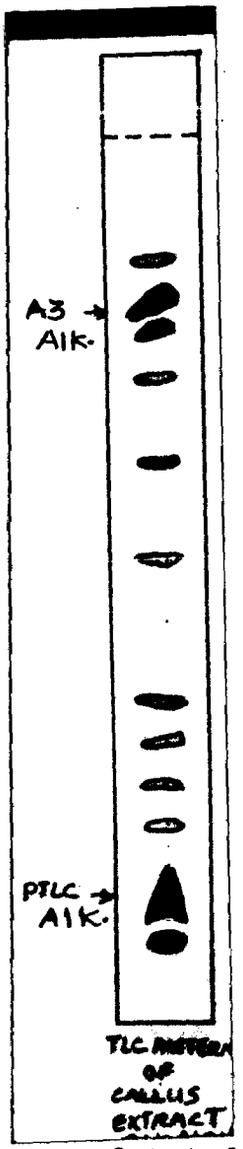


H. tanjorense germinated seedling.

Fig. 2.8

H. tanjorense Callus at epicotyledonary and cotyledonary region.

Fig. 2.9



TLC Pattern of total alkaloids of callus of H. tanjorense.

CHAPTER III
CALLUS OPTIMISATION AND ALKALOID PRODUCTION IN *Heterostemma tanjorense*

It is observed from the results of chapter II, that the flower buds of H. tanjorense produced callus that does not give any positive reaction for alkaloids. However, callus obtained from asceptically germinated seedling gave positive reaction for alkaloids. Hence attempts have been made in this chapter to set up optimum environmental conditions for maximisation of callus and alkaloid, extraction and isolation of pure alkaloids, study the production of total alkaloids from callus generated asceptically in vitro from different explants of germinated plantlet and in vivo production of alkaloids by H. tanjornense. The study also includes development of an analytical method for estimation of total alkaloids and effect of growth substances and photoperiod on production of total alkaloids in callus.

MATERIALS AND METHODS

3.1. Setting up of optimum environmental conditions for maximisation of callus

Unorganised callus tissue was established from asceptically grown seedling on MS medium 1/2 strength with 2% sucrose and 0.8% agar, supplemented with 2,4-D (1mg/L),IBA (0.5 mg/L) and Kn (0.5 mg/L). The tissues were subcultured after every 4 weeks and grown at $25^0 \pm 2^0$ c under fluorescent light of 1500 Lux intensity. Callus from 5^{th} subculture onwards was used in

developing the ideal conditions for growth so that the data obtained would be useful for further studies for the isolation of alkaloids.

Optimum conditions for growth of callus culture were established in ascertaining maximum growth with respect to varied conditions to select type of culture medium, inoculum size, pH, light (photoperiod), temperature, inorganic and organic nitrogen, carbohydrates as carbon source, growth factors and growth hormones.

3.2. Nutritional studies

For nutritional studies callus cultures were incubated at $25^0 \pm 2^0$ C with a photoperiod of 12 hr. light and 12 hr. darkness. The light intensity was 1500 Lux and humidity was maintained at 60 to 80%. Medium used for checking callus maximisation was MS 1/2 strength with 2%, sucrose, 0.8% agar and an auxin/cytokinin combination of 2,4-D (1mg/L), IBA (0.5mg/L) and Kn (0.5 mg/L). Wherever required these parameters were changed to suit the experimental conditions.

3.3.Measurement of growth

Fresh weights of callus were taken separately for three tubes after a period of six weeks. Dry weights were taken after drying the tissue to constant weight at 75^0 to 80^0 C for 24 hrs. The average wet and dry weights per tube were taken for determining the growth pattern of the callus. Standard Error (S.E.) was calculated according to the formula of Murashige and Skoog (1962), Phys. Plant. 15; 473.

$$\text{Standard Error} = \pm \sqrt{\frac{\Sigma \triangle^2}{n(n-1)}}$$

Where $\triangle$ is the deviation in weight from average weights, n is number of weights taken and Σ denotes summation.

Growth Index (G.I.) (Khanna, 1976d) was calculated from the formula :

$$\text{Growth Index} = \frac{\text{Final wet weight - Initial wet weight}}{\text{Initial wet weight}}$$

3.4.Extraction of total alkaloids from pooled callus

It is evident from chapter II that the preliminary investigation of callus extract of H. tanjorense tested positive for alkaloids. Hence studies were oriented in this section towards extraction and isolation of alkaloids. 100g (approx. eq. to 5g dry wt.) of wet pooled callus mass was extracted with 75% alcohol (1 litre) containing 0.5% tartaric acid in a soxhlet extractor as per section 2.15. The alcoholic extract was concentrated, diluted with water, made acidic (pH2), filtered and extracted with solvent ether (250 ml). The ether layer was discarded. It was then made alkaline (pH 10) with dilute NH3 solution and extracted with (1 litre) chloroform. The chloroform layer was concentrated, dried and the residue weighed (260 mg). TLC of the chloroform extract was carried out as per section 2.16 and the number of alkaloids present in the extract were noted by using specific Dragendorff reagent for alkaloids.

3.5. Isolation of the alkaloids by column chromatography

For the purpose of isolation of alkaloids from the crude extract of total alkaloids, column chromatography technique was employed. Neutral alumina was chosen as an adsorbent for it has been widely used for isolation and separation of alkaloids by several workers (Banerjee, 1972, Lederer, 1961).

For column chromatography a sample of 200 mg of total alkaloidal mixture dissolved in chloroform was column chromotographed over neutral alumina. It was then eluted successively with benzene, chloroform, methanol and a mixture of these solvents. Each 50ml of the fractions were collected and evaporated on waterbath to dryness. The solvent and their mixtures used for elution of total alkaloids, weight of each fraction collected were recorded. TLC analysis were performed of each fraction by observing the plates under ultra violet light and later on plates were sprayed with Dragendorff reagent to note the presence of alkaloids. The Rf values were calculated of the spots observed under ultra violet light and those obtained after spraying with the reagent.

3.6.Extraction of total alkaloids from calli developed from different explants asceptic conditions.

The callus cultures of the germinated seedling, leaf, stem and root were grown under optimum conditions as in Table 3.18 for six weeks and their growth indices calculated. 5.0 g of wet callus mass (approx. equivalent to 250 mg dry wt.) of each explant obtained from germinated seedling, leaf, stem and root was extracted as per section 2.15. The crude chloroform extracts

representing total alkaloids in each case were weighed and their estimation was done as per section 3.8.

3.7. Extraction of total alkaloids from roots of H.tanjorense

Dried powdered roots (10g) of wildly growing H. tanjorense were extracted with 75% alcohol (500 ml) containing 0.5% tartaric acid as per section 2.15. The alcoholic extract was concentrated, rendered acidic, filtered and extracted with solvent ether (200 ml). The ether layer was rejected. Acidic layer was made alkaline and extracted with chloroform (1 litre). The chloroform layer was concentrated, dried and weights recorded (220 mg). The extract was then subjected to TLC analysis as per section 2.16. The plates were viewed under ultra violet light and number of spots noted. The plates were then sprayed with Dragendorff reagent to observe the number of alkaloids. The respective R_f values of the spots observed under ultra violet light and after spraying with Dragendorff reagent were calculated. The crude chloroform extract was then estimated quantitatively for total alkaloids.

3.8. Development of analytical method for estimation of total alkaloids in H. tanjorense

The total alkaloids of H. tanjorense and the alkaloid heterostemmine forms a reineckate derivative soluble in acetone. The pink coloured complex is very stable and gives an absorption maxima at 520 nm. However this method requires large quantities of test samples and is time consuming.

These alkaloids also form an acid dye complex with most of the dyes, viz : bromothymol blue, bromocresol purple, bromocresol green, and thymol blue at a pH range from 3 to 4. The

yellow coloured complex is soluble in chloroform and has maximum absorption between 405 to 415 nm. The method is simple, quick and requires less quantity of sample.

Boromothymol blue was selected to study the various parameters affecting the reaction as the dye complex formed with bromothymol blue was completely soluble in chloroform, whereas the dye complex formed with other dyes was partially soluble. The various factors affecting the complex formation were standardised to obtain optimum conditions for the reaction.

3.8.1. Reagents and Solutions

Details for preparation of bromothymol blue solution, buffer solutions, and reagent buffer mixer are given in Appendix 3.

Solution A was prepared by dissolving 5 mg of heterostemmine alkaloid standard in 10 ml of chloroform to give a concentration of 0.5 mg/ml. Due to the paucity of the standard material, heterostemmine was used only for the preparation of the standard curve. In all other experiments, a stock solution of total alkaloids of H. tanjorense callus in chloroform was prepared.

Solution B was prepared by dissolving 50 mg of total alkaloids of callus in 50 ml of chloroform to give a concentration of 1 mg/ml.

Solution C was prepared by dissolving 10 mg of total alkaloids obtained from roots of H. tanjorense in 10 ml of chloroform to give a concentration of 1 mg/ml. This solution was

further diluted with chloroform to obtain a concentration of 0.1 mg/ml.

3.8.2. Standardisation of the method

A series of experiments conducted to standardise the parameters for quantitative estimation of total alkaloids are described below.

Spectral characteristics. The yellow coloured dye complex formed by reacting the drug solution with bromothymol blue at pH value of 3.5 was extracted with chloroform and absorbance was scanned on Shimadzu uv 150-02 double beam spectrophotometer from 350 to 500 nm. The absorbance values corresponding to the wavelength were recorded and a graph plotted.

Selection of optimum pH range. A series of experiments were conducted between pH 2.5 to 5.5 using 1 mg/ml of solution B. To this 10 ml mixture of buffer and dye solution prepared as stated earlier and 9 ml of chloroform was added. The mixture was shaken for one minute and allowed to separate. The chloroform layer was taken and dried adding a pinch of anhydrous Na_2SO_4. The yellow coloured complex of chloroform was measured at 410 nm and a graph plotted.

Effect of volume of dye solution. A series of experiments were carried using 1 mg/ml of drug solution B. In this experiment the volume of dye solution was varied, while buffer solution volume of 5ml was kept constant. To the mixture 9 ml of chloroform was added and shaken for one minute. Chloroform layer was separated and a pinch of anhydrous Na_2SO_4 was added. The coloured complex was measured at 410 nm.

Effect of volume of buffer. To a series of experiments fixed amount of drug solution B, 1mg/ml and varying amounts of buffer were added. To the mixture 9 ml chloroform was added and shaken for one minute. Chloroform layer was separated, dried with anhydrous Na_2SO_4 and measured at 410 nm.

Preparation of standard curve for heterostemmine. 10ml of mixture of buffer and dye solution in equal quantities (1:1) were taken in a series of separatory funnels. Varying aliquots of standard solutions of heterostemmine in chloroform ranging from 0.5, 1.0,- - - 2.5 ml in duplicate were added to the above mixture and the volume of chloroform was adjusted to 10 ml. The funnels were shaken for a minute, the chloroform layer was separated, dried with a pinch of anhydrous Na_2SO_4 and measured at 410 nm. The standard calibration curve showing absorbance against drug concentration was plotted.

Similar Standard Curves were obtained using varying aliquots of Solution B (total alkaloids from callus) and Solution C (total alkaloids from roots).

3.9. Quantitative method for estimation of total alkaloids in callus

The calli obtained under optimum environmental conditions were harvested after 2,4,6 and 8 weeks. Their initial and final wet weights were recorded to calculate growth index. 1 g of each of the samples collected after 2,4,6 and 8 weeks was dried to constant weight at 75-80^0 C for 24 hrs and their weights recorded to determine the total alkaloids present per g of dry tissue. Aliquot pooled wet samples of 5 g each were stored in 75% alcohol

at 5^0 C until used for the extraction of the total alkaloids. The sample of wet tissues were extracted as per section 2.15. The crude extracts representing total alkaloids were dissolved in chloroform and estimated quantitatively as per section 3.8, in two replicates of each of the sample in 2,4,6 and 8 weeks. For each series of experiments standard curve of total alkaloids of callus was plotted as per section 3.8 and the amounts of total alkaloids produced in 2,4,6 and 8 weeks were calculated as mg/g dry wt. in each case and expressed in terms of pure heterostemmine. The same procedure was adopted for further experiments where total alkaloidal contents were to be determined from callus.

RESULTS

3.10. NUTRITIONAL CONDITIONS FOR OPTIMAL YIELD OF CALLUS

3.10.1. Effect of Different basal medium

The media composition of MS, White's, Gamborg, Eriksson and Nitsch was as per media formulations (Bhojwani, 1983). The different media contained the mineral salts, vitamins and glycine. Maximum growth was obtained on MS medium followed by White's, Gamborg, Eriksson and Nitsch. It was also observed that when cultures were grown on MS medium with half the concentration of salts and vitamins with all other conditions remaining same, the callus showed maximum growth as shown in Table 3.1. Hence all further experiments were conducted using MS medium 1/2 strength.

3.10.2. Effect of inoculum size

Growth of callus was determined using various amounts of inocula per 15 ml of medium. It was found that the growth index

Table 3.1

Effect of Different Basal Media on callus growth
Basal medium : Different Basal media
Growth hormones : 2, 4-D(1mg/L); IBA(0.5mg/L), Kn(0.5mg/L)

Basal Medium	Fresh weight mg/T	Dry weight mg/T	Growth Index
Murashige & Skoog (MS)	742 ± 35	46	4.9
Murachige & Skoog MS ½ strength	737 ± 23	42	5.4
White's	568 ± 41	34	3.4
Gamborg	497 ± 19	27	3.0
Eriksson	465 ± 27	26	2.7
Nitsch	446 ± 55	26	2.4

Table 3.2

Effect of Inoculum size
Basal Meduim : MS ½ strength
Growth hormones : 2,4D (1mg/L), IBA(0.5mg/L),Kn(0.5mg/L)

Initial inoculum wet weight mg/T	Final growth wet weight mg/T	Growth Index
50	171 ± 9	2.4
100	563 ± 15	4.6
200	967 ± 64	3.8
300	1006 ± 56	2.5
400	1228 ± 13	2.0
500	1544 ± 34	2.0

was maximum at an inoculum of 100 to 200 mg per 15 ml followed by an inoculum of 50 mg. The growth increase was nearly 5 times with an inoculum of 100 mg. These conditions were followed in subsequent experiments. The inoculum used was from 5^{th} subculture and results recorded after 6 weeks. Table 3.2

3.10.3. Effect of pH

The growth of callus was determined at various pH values ranging from 4 to 9. The growth of callus on media was poor below pH 5 or above pH 7. Best growth was obtained at pH 5.5. The calli were golden yellow in colour while at higher and lower pH values there was stunted growth and calli turned brown. Table 3.3

3.10.4. Effect of agar concentration

The agar concentration in MS medium 1/2 strength was varied to study the optimisation of callus. Media prepared with different concentrations showed that growth index was almost the same for agar concentration ranging from 0.6 to 1.0%. The growth decreased with increase in agar concentration. However a concentration of 0.8% agar was used for all the experiments as it was found to be more suitable and used by most of the workers (Ramesh, 1990; Raj, 1991; Kackar, 1991) Table 3.4.

3.10.5. Effect of temperature

Effect of different temperatures ranging from 5^0 to 35^0 C was studied. Callus grown at 25^0C was golden yellow, hard and compact, whereas poor growth was observed at 35^0C. The callus here turned brown and showed signs of degeneration. At 5^0C there was no growth of callus whereas at 15^0 C there was no appreciable growth. Table 3.5

Table 3.3

Effect of pH
Basal medium MS ½ strength with varied pH
Growth hormones :2,4-D(1mg/L), IBA(0.5mg/L), (Kn(0.5mg/L)

pH	Fresh Weight mg/T	Dry Weight mg/T	Growth Index
4	238 ± 5	17	0.95
4.5	427 ± 24	24	2.5
5.0	607 ± 30	35	3.9
5.5	760 ± 10	42	5.3
6.0	745 ± 14	46	4.9
7.0	587 ± 20	38	3.5
8.0	495 ± 20	32	2.7
9.0	346 ± 36	26	1.9

Table 3.4

Effect of Agar concentration
Basal medium : MS ½ strength with varied agar concentrations
Growth hormones :2,4-D(1mg/L),IBA(0.5mg/L),Kn(0.5mg/L)

Agar %	Fresh Weight mg/T	Dry Weight mg/T	Growth Index
0.6	719 ± 20	47	5.0
0.8	840 ± 25	56	5.3
1.0	796 ± 22	45	5.2
1.5	675 ± 40	40	4.3
2.0	573 ± 35	34	3.5
2.5	559 ± 46	30	3.2
3.0	506 ± 36	29	3.1

Table 3.5

Effect of temperature
Basal medium : MS ½ strength
Growth hormones :2,4-D(1mg/L), IBA(0.5mg/L),Kn(0.5mg/L)

Temp-erature	Fresh Weight mg/T	Dry Weight mg/T	Growth Index
5	147 ± 7	8	0.14
15	343 ± 17	17	1.7
25	801 ± 12	52	5.4
30	643 ± 56	36	4.2
35	473 ± 50	28	2.7

Table 3.6

Effect of Photoperiod
Basal medium : MS ½ strength
Growth hormones :2,4-D(1mg/L),IBA(0.5mg/L),Kn(0.5mg/L)

Photo period	Fresh Weight mg/T	Dry Weight mg/T	Growth Index
12 hr.	773 ± 35	44	5.7
24 hr.	847 ± 23	52	5.3
Dark 24 hr.	743 ± 29	43	4.9

3.10.6. Effect of light (Photoperiod)

The callus cultures were exposed to different light intensity and time. The visual observation showed that the photoperiod and light intensity had a role to play on the nature of callus. Those calli subjected to 12 hr. photoperiod and 12 hr. darkness were golden yellow in colour, hard and compact while those exposed to 24 hr photoperiod were golden yellow upto 3rd week but slowly turned pale yellow upto 6^{th} week. Calli subjected to total darkness were white in colour. All calli were hard and compact. Table 3.6

3.10.7. Effect of inorganic nitrogen

MS medium 1/2 strength was prepared by omission of KNO_3 and NH_4NO_3. The potassium content was kept constant in all media by addition of KCL in an equimolar concentration of K. All the salts were added so that the nitrogen level was equivalent to that supplied by KNO_3 and NH_4NO_3 to MS medium 1/2 strength. The nitrogen content was kept at 0.42 g of nitrogen/litre which is equivalent to total nitrogen present in MS medium 1/2 strength. All the nitrogen containing compounds were added in concentration such that their content of nitrogen was 0.42 g/litre. It was observed that the growth of callus was best when KNO_3 and NH_4NO_3 were used in combination as in MS medium. None of the nitrogen sources could compare with this medium although moderate to poor growth of callus was observed on NH_4NO_3, KNO_3, $(NH_4)_2SO_4$, $Ca(NO_3)_2$ NH_4Cl and $NaNO_3$. Table 3.7.

3.10.8. Effect of organic nitrogen

Different complex organic compounds like yeast extract,

Table 3.7

Effect of Inorganic Nitrogen
Basal Medium : ½ MS without NH_4NO_3 & KNO_3
Growth hormones ; 2,4-D (1mg/L), IBA(0.5mg/L),Kn(0.5mg/L)
Suplement : Inorganic nitrogen as listed below

Nitrogen Source	Concentration g/L	Fresh weight mg/T	Dry weight mg/T	Growth Index
$NH_4 NO_3$	1.20	596 ± 28	29	3.9
KNO_3	3.03	549 ± 28	48	3.3
$Ca(NO_3)_2$	2.495	458 ± 21	23	2.8
$(NH_4)_2 SO_4$	1.98	540 ± 24	27	3.2
NH_4Cl	1.70	491 ± 39	24	2.9
$NaNO_3$	2.55	504 ± 17	29	3.2
KNO_3 + NH_4NO_3 MS ½ strength	1.775	778 ± 49	43	5.5

malt extract, peptone and casein hydrolysate were incorporated in MS medium 1/2 strength as a source of organic nitrogen in a concentration of 0.05%. No appreciable increase in callus weight was noted. However yeast extract showed more colour intensity on TLC plates when sprayed with Dragendorff reagent indicating that the alkaloid concentration was more in callus with incorporation of yeast extract in the medium. Table 3.8.

3.10.9. Effect of carbohydrates as carbon source

In this experiment the effect of different carbohydrate sources added at 2% concentration were studied. Optimum growth was observed with sucrose which was about 31/2 times higher than the other carbohydrates. Dextrose had comparatively less growth followed by fructose. All other carbohydrates gave poor response to the growth of callus. Table 3.9

3.10.10. Effect of sucrose

The optimum sucrose concentration was found to be 2% for culture growth. In absence of sucrose the growth was inhibited. The concentration of sucrose above 5% yielded calli which were brown or black in colour. 10% concentration almost inhibited the growth. Table 3.10

3.10.11. Effect of vitamins

The effect of vitamins was studied by adding different vitamins to MS medium 1/2 strength, individually and in combination. Optimum growth was obtained with a combination of vitamin B1, B_6 and niacin as in MS medium. Individually thiamine (vitamin B1) was found to be more effective Table 3.11.

Table 3.8

Effect of Organic nitrogen
Basal medium : ½ MS
Growth hormones :2,4-D(1mg/L),IBA(0.5mg/L) Kn(0.5mg/L)
Supplement : Different complex organic compound

Organic nitrogen source	Fresh Weight mg/T	Dry Weight mg/T	Growth Index
Yeast Extract	569 ± 14	28	3.4
Peptone	495 ± 18	26	3.2
Malt Extract	556 ± 10	30	3.3
Casein hydrolysate	684 ± 14	38	4.5

Table 3.9

Effect of Carbohydrates (Carbon Source)
Basal medium : MS ½ strength without sucrose
Growth hormones :2,4-D(1mg/L),IBA(0.5mg/L),Kn(0.5mg/L)
Supplement : Different carbohydrates at 2% concentration

Carbohydrate at 2% conc.	Fresh Weight mg/T	Dry Weight mg/T	Growth Index
0	235 ± 19	16	0.74
Sucrose	837 ± 24	58	5.6
Fructose	487 ± 31	24	2.6
Xylose	263 ± 34	15	0.93
Dextrose	603 ± 35	32	3.7
Lactose	267 ± 32	13	0.97
Starch	259 ± 34	12	0.90
Mannitol	242 ± 25	11	0.95

Table 3.10

Effect of Sucrose (Carbon Source)
Basal medium : MS ½ strength without sucrose
Growth hormones :2,4-D(1mg/L),IBA(0.5mg/L),Kn(0.5mg/L)
Supplement : Sucrose at different concentrations

Sucrose Concentration	Fresh Weight mg/T	Dry Weight mg/T	Growth Index
0	223 ± 23	11	0.7
1	424 ± 53	23	2.4
2	712 ± 15	46	5.4
3	692 ± 27	39	4.7
4	637 ± 32	33	4.0
5	456 ± 38	22	2.4
10	237 ± 34	13	0.83

Table 3.11

Effect of Vitamins
Basal medium : MS ½ strength without Vitamins
Growth hormones :2,4-D(1mg/L),IBA(0.5mg/L),Kn(0.5mg/L)
Supplement : Different Vitamins

Vitamins Added	Conc. mg/L	Fresh Weight mg/T	Dry Weight mg/T	Growth Index
B_1	0.5	714 ± 56	48	4.6
B_2	0.5	605 ± 10	37	4.0
B_6	0.5	668 ± 42	40	4.3
Niacinamide	0.5	600 ± 16	35	3.4
Folic acid	0.1	556 ± 31	27	3.2
Calcium pantothenate	0.5	554 ± 35	33	3.4
MS medium B_1 ½ strength B_6 Niacin	0.05 0.25 0.25	798 ± 44	54	5.3

3.10.12. Effect of coconut milk

Coconut milk was added at different concentrations ranging from 0 to 20%. It was observed that 10% had maximum callus formation. However growth decreased with increase in concentration. Table 3.12

3.10.13. Effect of different growth hormones

The different growth hormones like auxins, cytokinins and gibberellic acid were added in concentration of 0.5 mg/L. Of these, auxin 2,4-D showed maximum response followed by IBA. IAA and NAA showed poor response in growth of callus. Of the cytokinins tested kinetin showed good growth as compared to BAP. Gibberellic acid and abscisic acid did not show appreciable increase in growth Table 3.13.

3.10.14. Effect of 2,4-D

2,4-D initiated callus formation and enhanced growth in concentration upto 1 mg/L. The callus was white in the beginning but turned pale yellow on aging. Higher concentrations of 2,4-D inhibited the growth of callus and eventually callus turned brown. Table 3.14

3.10.15. Effect of IBA

Of the different concentrations tested of IBA, 0.5 mg/L was optimum for callus weight increase. Higher or lower concentrations decreased the growth rate. The callus was greenish in colour and showed no browning effect. Table 3.15.

3.10.16. Effect of Kinetin

A 0.5 mg/L concentration of kinetin was found to be ideal for callus formation. The callus was green in colour, hard and

Table 3.12

Effect of Coconut milk
Basal medium : MS ½ strength
Growth hormones :2,4-D(1mg/L),IBA(0.5mg/L),Kn(0.5mg/L)
Supplement : Coconut milk at different concentrations

Coconut milk Concentration	Fresh Weight mg/T	Dry Weight mg/T	Growth Index
0	550 ± 23	28	3.5
2.5	725 ± 29	39	4.6
5.0	761 ± 13	42	5.0
10.0	763 ± 25	40	5.6
15.0	635 ± 14	35	4.3
20.0	542 ± 31	27	3.2

Table 3.13

Effect of Different
Growth Hormones
Basal medium : MS ½ strength without growth hormones
Supplement : Different Growth hormones at 0.5 mg/L concentration

Growth Hormone	Fresh Weight mg/T	Dry Weight mg/T	Growth Index
Auxins			
2,4-D	633 ± 41	38	3.8
I B A	511 ± 28	25	2.9
I A A	294 ± 45	17	1.2
N A A	433 ± 33	22	2.5
Cytokinins	499 ± 34	26	2.7
Kinetin	411 ± 27	20	2.1
B A P			
Gibberelic Acid (GA_3)	454 ± 44	22	3.7
Abscisic Acid (ABA)	261 ± 65	15	1.1

Table 3.14

Effect of 2, 4-D
Basal medium : MS ½ strength without 2, 4-D, Kn & IBA
Supplement : Different concentrations of 2,4-D

Concentration mg/L	Fresh Weight mg/T	Dry Weight mg/T	Growth Index
0	253 ± 26	14	1.0
0.1	382 ± 38	20	2.2
0.5	560 ± 19	29	3.3
1.0	682 ± 42	42	4.4
2.5	528 ± 31	30	2.6
5.0	374 ± 24	21	2.2

Table 3.15

Basal medium : MS ½ strength without 2, 4-D, IBA & Kn
Supplement : Different concentrations of I B A

Concentration mg/L	Fresh Weight mg/T	Dry Weight mg/T	Growth Index
0	253 ± 26	14	1.0
0.1	462 ± 31	23	2.4
0.5	536 ± 32	30	3.3
1.0	514 ± 28	27	3.0
2.5	437 ± 24	22	2.2
5.0	329 ± 31	16	1.6

compact. No browning was observed on aging of the callus up to 6 weeks. Higher or lower concentrations gave poor response for growth. Table 3.16

3.10.17. Effect of combination of Auxin and cytokinin

The different combinations tried for observing the growth of callus, indicated that the ratio of 2,4-D:IBA:Kn as 1:05:05 was ideal for increasing the callus biomass. Also the callus remained golden yellow with slight greenish tinge. It was hard and compact. Hence all the further studies were directed for the use of this combination. Table 3.17

3.10.18. Growth on repeated subcultures

In some cases callus cultures grow initially and then stop growth, whereas in other cases growth increases on subculture. In H. tanjorense callus cultures, poor growth was observed in initial subcultures. However callus maintained a steady growth from 5^{th} subculture onwards.

3.10.19. Growth pattern of callus with optimum conditions.

Callus growth was observed with optimum conditions of growth at different time intervals of one week. Table 3.18. At specified periods, the wet and dry weights of 3 tubes were taken. The growth was almost nil in the first week (Lag phase), with the callus turning pale yellow. The growth slowly increased from second week and maximum growth was observed after 4 to 6 weeks and callus attained its natural golden yellow colour. (Phase of Rapid Growth). Then onwards upto 8 weeks, the growth remained steady and

Table 3.16

Effect of Kinetin (Kn)
Basal medium : MS ½ strength without 2, 4-D, IBA & Kn
Supplement : Different concentrations of Kn

Concentration mg/L	Fresh Weight mg/T	Dry Weight mg/T	Growth Index
0	253 ± 26	14	1.0
0.1	404 ± 45	22	2.0
0.5	522 ± 49	27	2.9
1.0	465 ± 30	23	2.5
2.5	395 ± 40	21	1.9
5.0	282 ± 24	15	1.0

Table 3.17

Effect of Combination of Growth hormones
Basal medium : MS ½ strength without Growth hormones
Supplement : Combination of different concentrations of 2,4-D, IBA & Kn

Auxin Kinetin Combination	Fresh Weight mg/T	Dry Weight mg/T	Growth Index
2, 4-D + Kn 1 + 0.5	748 ± 38	44	4.9
2, 4-D + IBA 1 + 0.5	711 ± 47	41	5.1
2, 4-D+IBA + Kn 0.1 + 0.1 + 0.1	667 ± 27	38	4.05
2, 4-D+IBA + Kn 0.5 + 0.1 + 0.1	688 ± 43	37	4.5
2, 4-D+IBA +Kn 1.0 + 0.1+0.1	718 ± 52	45	5.1
2,4-D+IBA+Kn 1 + 0.5 + 0.5	920 ± 19	63	5.6
2,4-D+IBA+Kn 1 + 1 + 1	666 ± 28	37	4.1

callus started turning brown showing signs of degeneration, (stationary phase). Occasionally few calli displayed the differentiation into shoots and roots that regenerated into full plantlets. Fig. 3.13.

The kinetics of growth were confirmed by using both, fresh and dry weights. Table 3.19 and Fig. 3.1a & 3.1b.

3.11. EFFICACY OF ANALYTICAL METHOD DEVELOPED FOR ESTIMATION OF ALKALOIDS FROM H.tanjorense

Fig. 3.2 shows the spectrometric absorption profiles of the yellow coloured dye complex obtained at section 3.8.2. It is observed that maximum absorption was obtained at 410 nm. Fig 3.3 shows that the coloured complex had maximum absorption at pH 3.5 at 410 nm. Also there was noted difference in absorbance values when different amounts of dye solutions were used to form the colour complex. It is noted from Fig. 3.4 that maximum absorption at 410 nm was obtained with 5 ml of dye solution. However, there was no appreciable change in colour intensity when volume of buffer solution was varied and maximum absorption was measured at 410 nm. Fig. 3.5. Whilst plotting the standard curve for pure drug heterostemmine Fig 3.6, it was observed that the colour complex obeyed Beer's law from 25 to 125 mcg/ml. Fig 3.7 denotes that total alkaloids from callus obeyed Beer's law at concentration ranging from 50 to 250 mcg while total alkaloids from roots of H. tanjorense obeyed Beer's law between 5 to 25 mcg. Fig 3.8. The total alkaloids of callus extract and root extract of H.tanjorense were calculated. All the concentrations of total alkaloids were expressed in terms of mg of heterostemmine after

Table 3.18

Optimum conditions for callus growth

Growth Parameters	Optimum conditions
Basal Media	MS ½ strength
Inoculum size	approx. 100mg
Temperature	25° ± 2°
Humidity	60 to 80 %
pH	5.5 ± 0.2
Light intensity	1500 lux
Photoperiod	12 hr light/ 12 hr dark
Sucrose	2%
Agar	0.8%
Growth hormones	2,4-D (1mg/L), IBA (0.5mg/L), Kn(0.5 mg/L)
Coconut milk	10%
Duration	8 weeks.

Table 3.19

Growth Pattern of callus under optimum conditions

Age Callus in weeks	Fresh Weight mg/T	Dry Weight mg/T	Growth Index
0	107	8	—
1	122	7	0.14
2	225	11	1.2
3	375	17	2.6
4	480	24	3.8
5	630	34	4.9
6	696	40	5.8
7	648	34	5.4
8	630	30	5.0

FIG. 3.1a

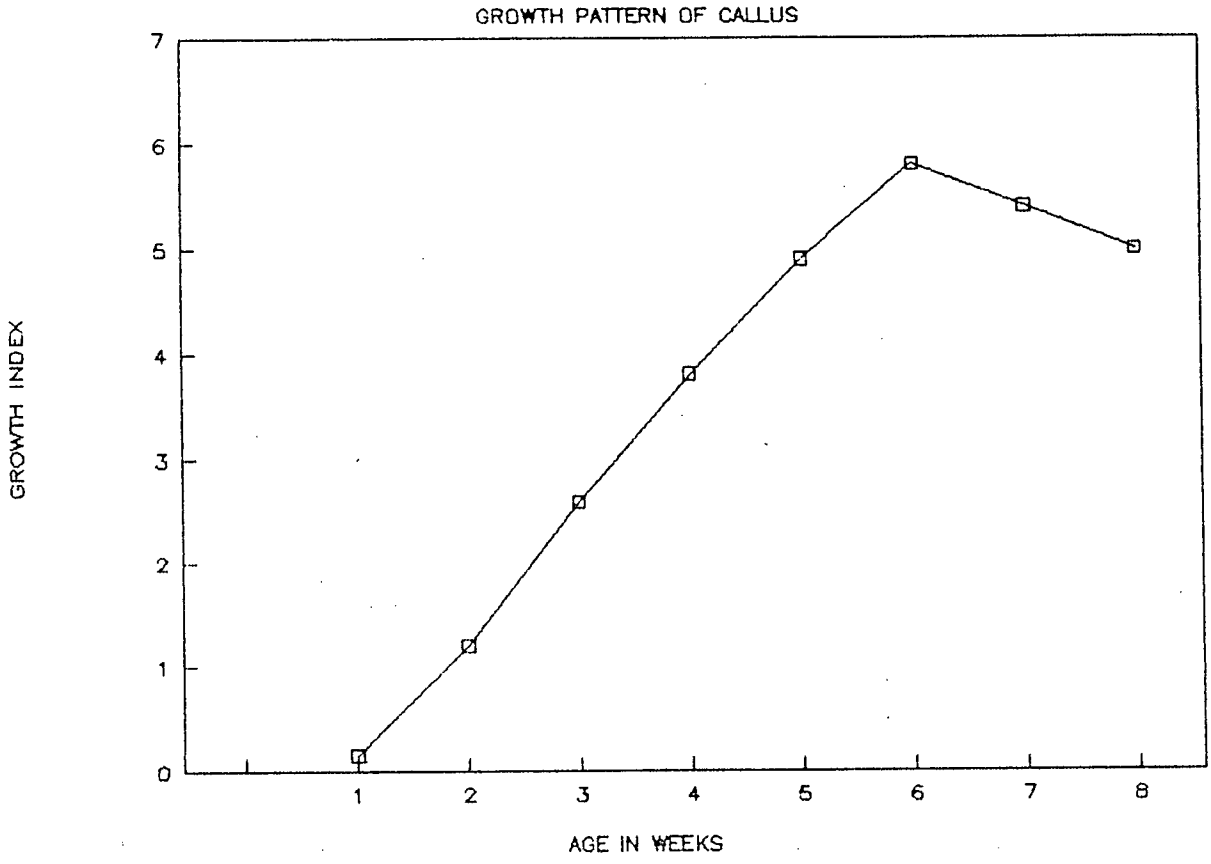

GROWTH PATTERN OF CALLUS
GROWTH INDEX
0
1
2
3
4
5
6
7
1
2
3
4
5
6
7
8
AGE IN WEEKS

FIG. 3.1b

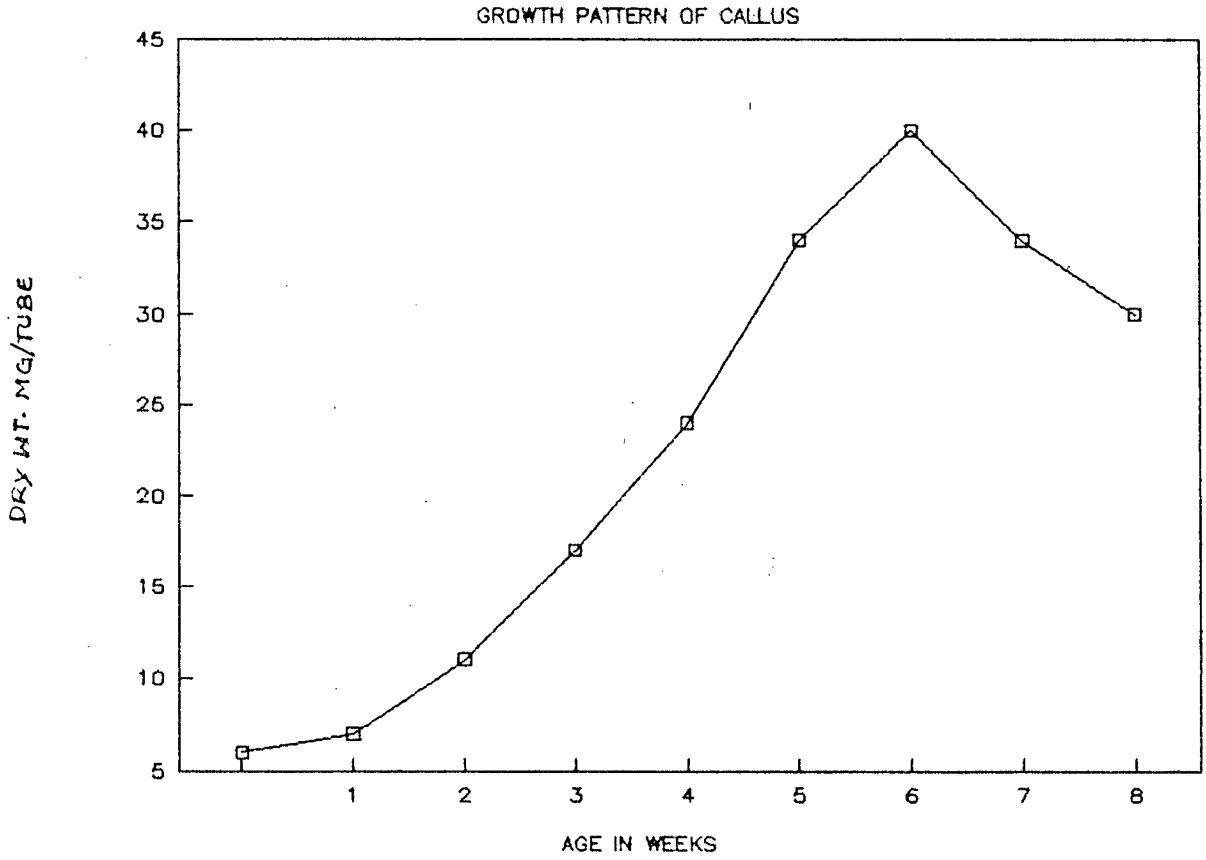

GROWTH PATTERN OF CALLUS
DRY WT. MG/TUBE
45
40
35
30
25
20
15
10
5
1
2
3
4
5
6
7
8
AGE IN WEEKS

FIG. 3.2

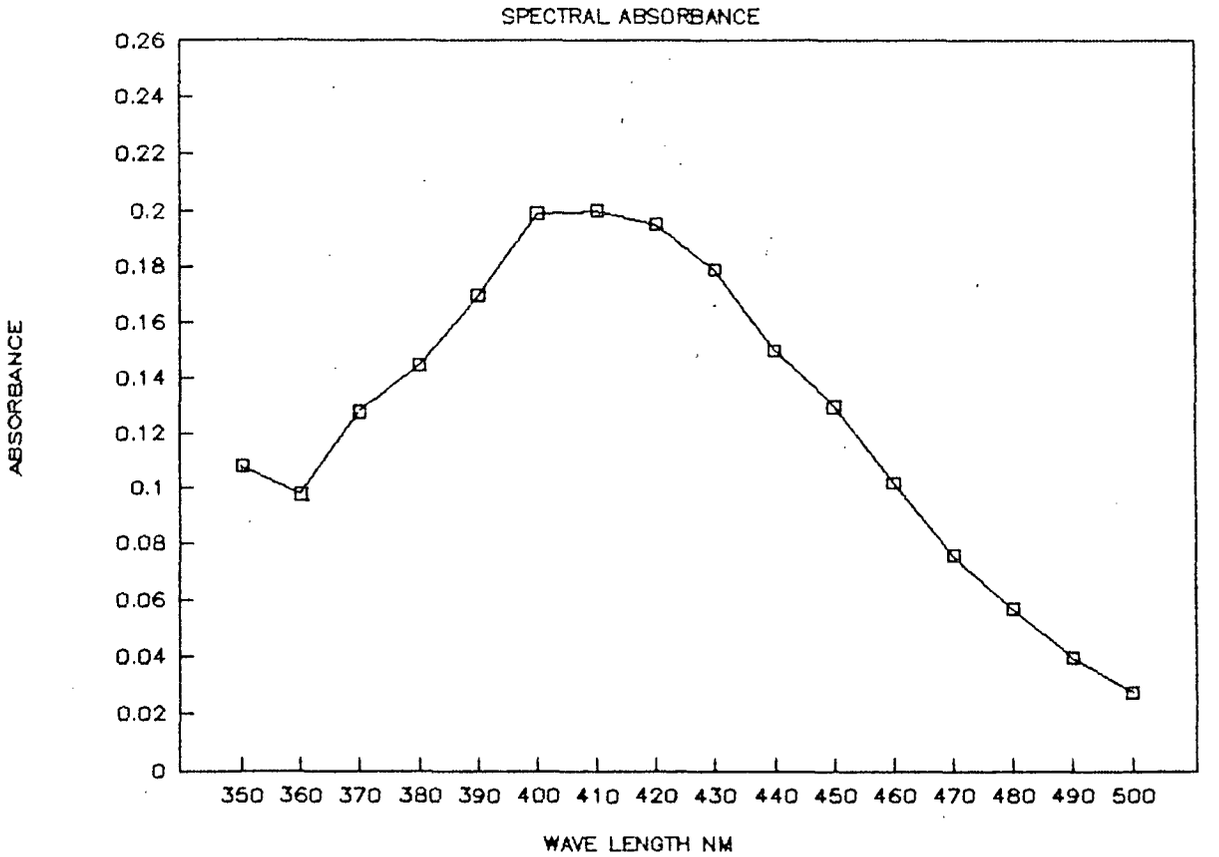

SPECTRAL ABSORBANCE
ABSORBANCE
0.26
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
350 360 370 380 390 400 410 420 430 440 450 460 470 480 490 500
WAVE LENGTH NM

FIG. 3.3

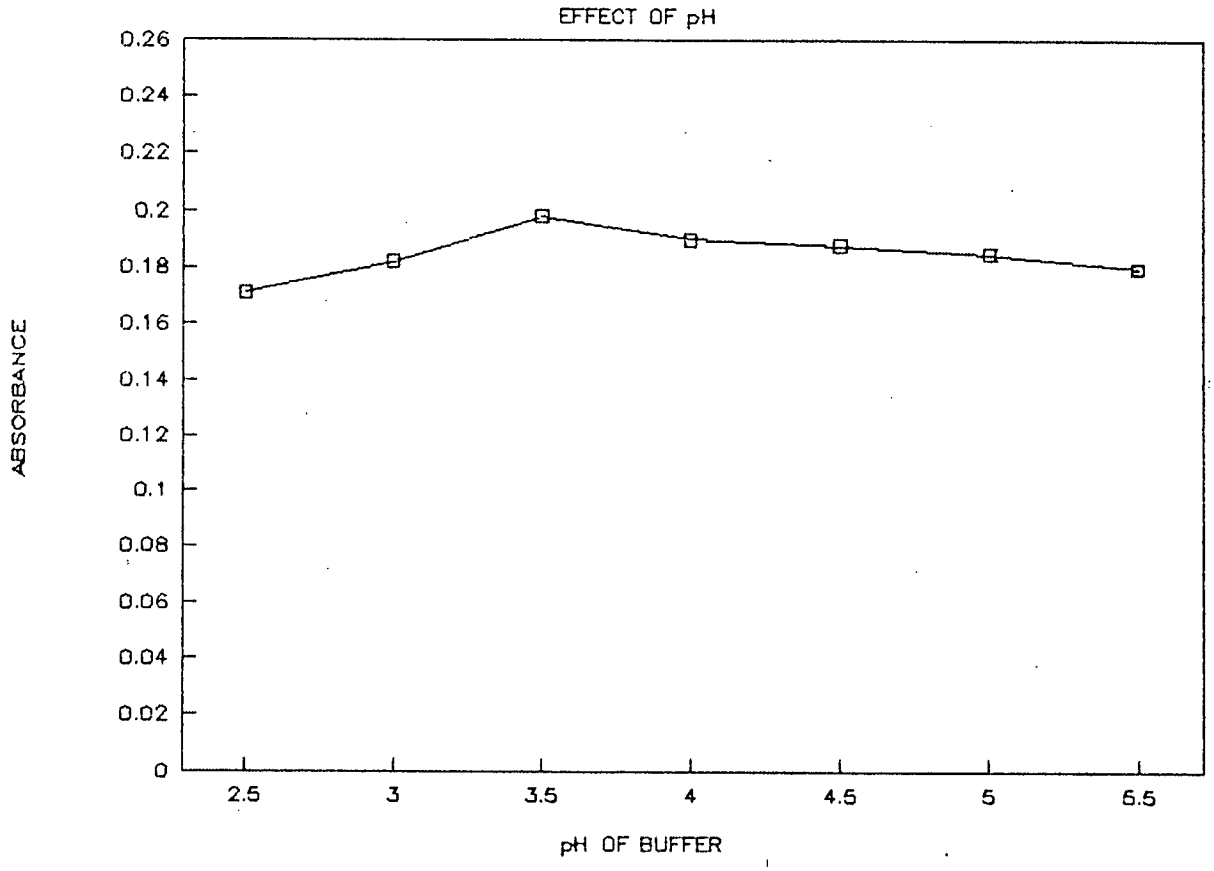

EFFECT OF pH
ABSORBANCE
0.26
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
2.5
3
3.5
4
4.5
5
5.5
pH OF BUFFER

FIG. 3.4

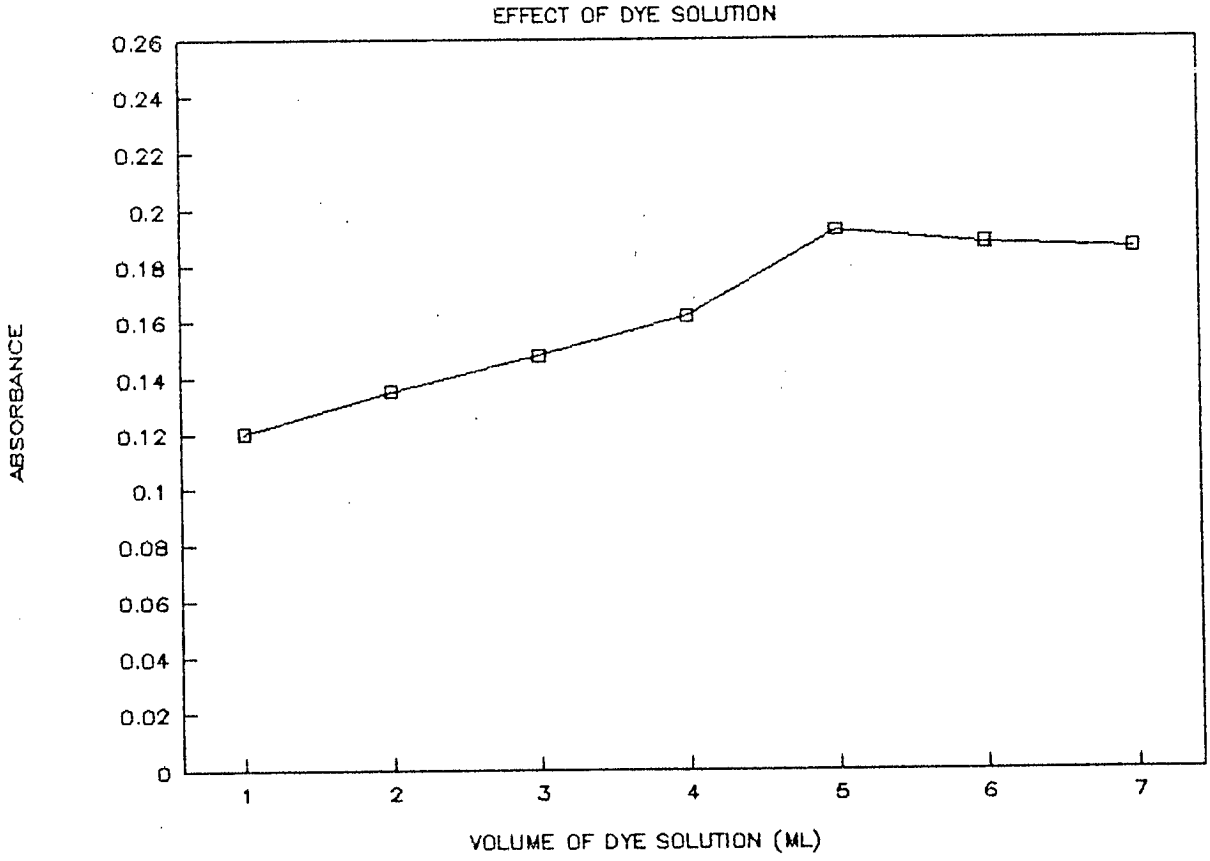

EFFECT OF DYE SOLUTION
ABSORBANCE
0.26
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
1
2
3
4
5
6
7
VOLUME OF DYE SOLUTION (ML)

FIG. 3.5

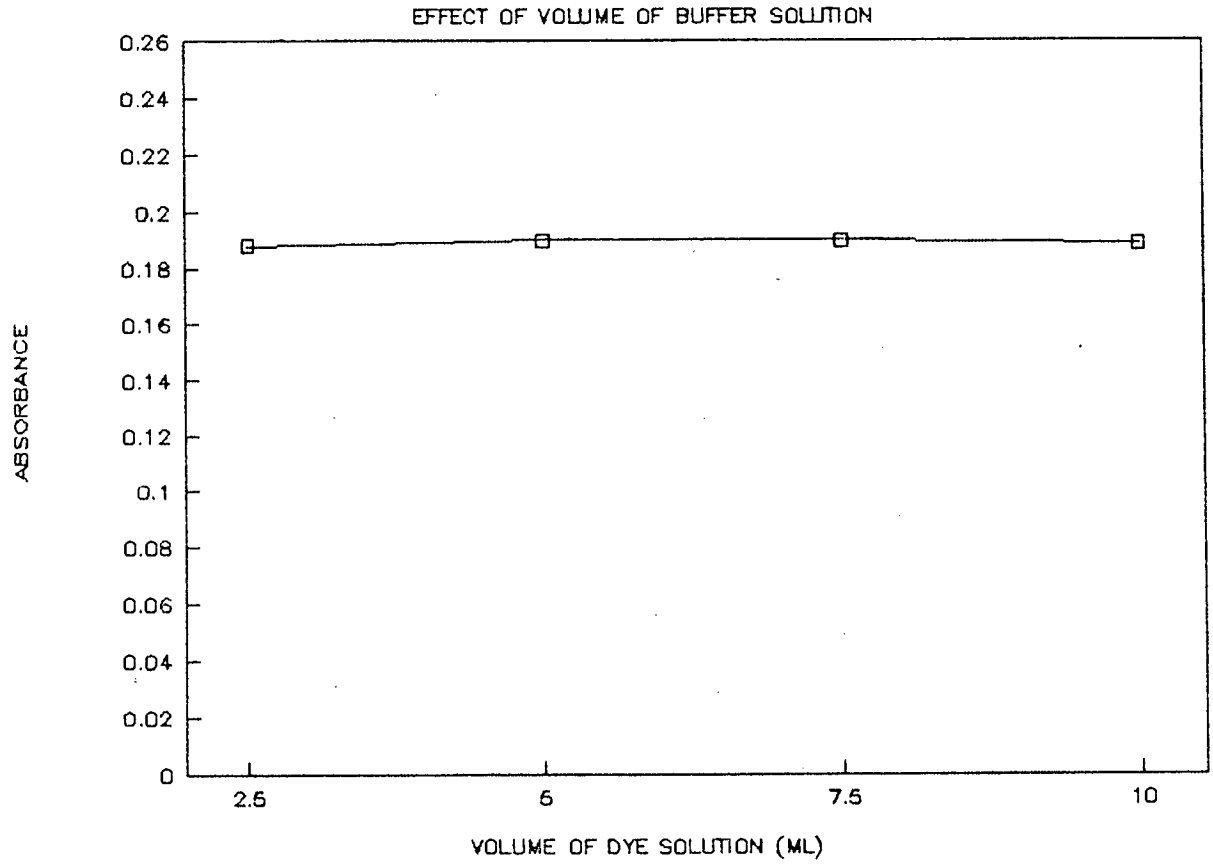

EFFECT OF VOLUME OF BUFFER SOLUTION
ABSORBANCE
0.26
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
2.5
5
7.5
10
VOLUME OF DYE SOLUTION (ML)

FIG. 3.6

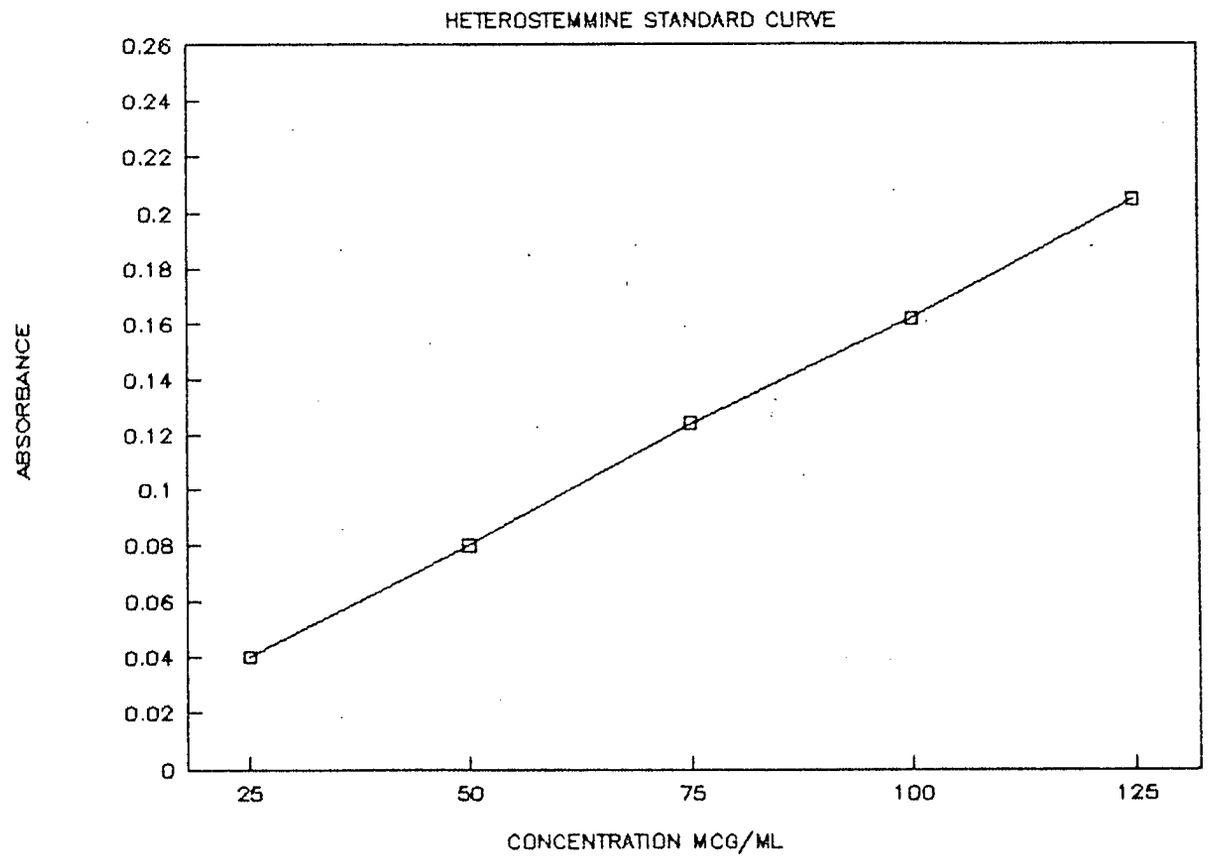

HETEROSTEMMINE STANDARD CURVE
ABSORBANCE
0.26
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
25
50
75
100
125
CONCENTRATION MCG/ML

FIG. 3.7

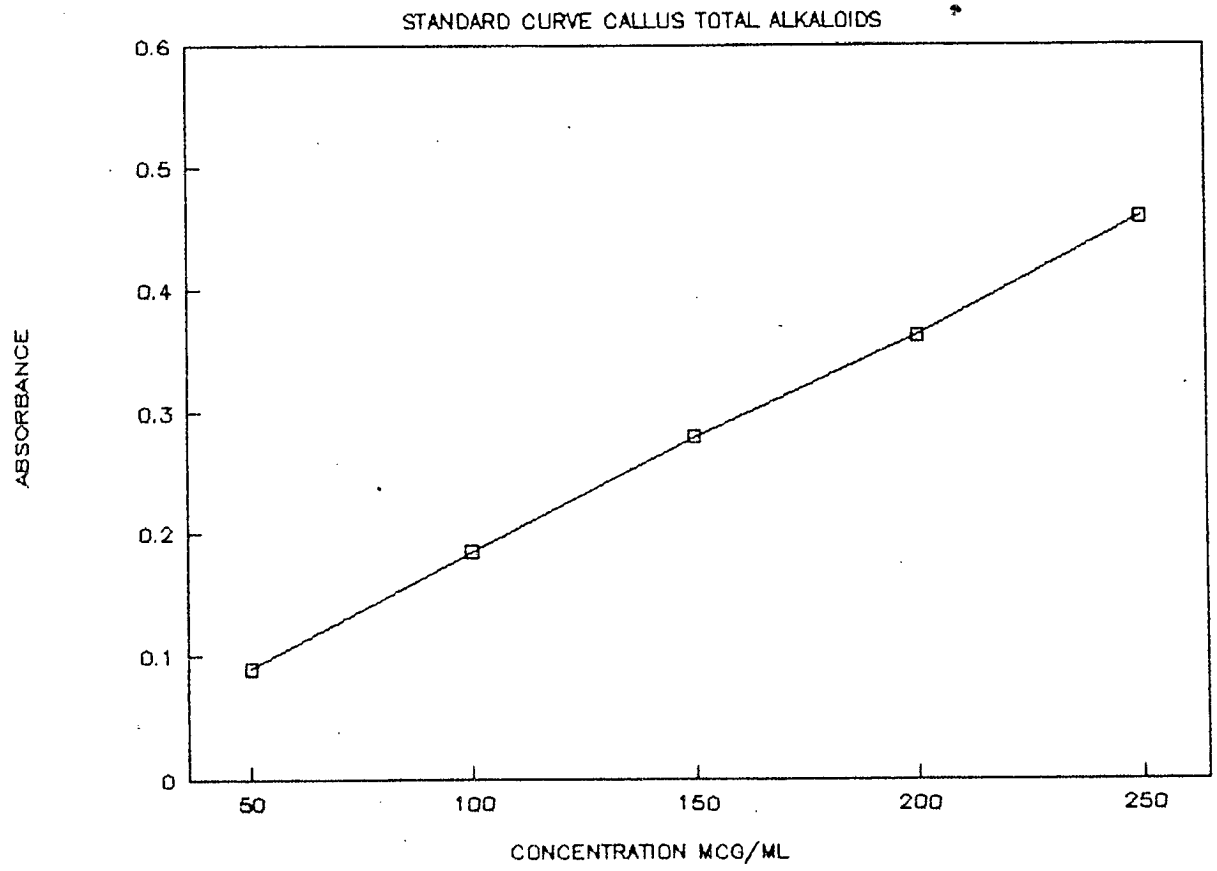
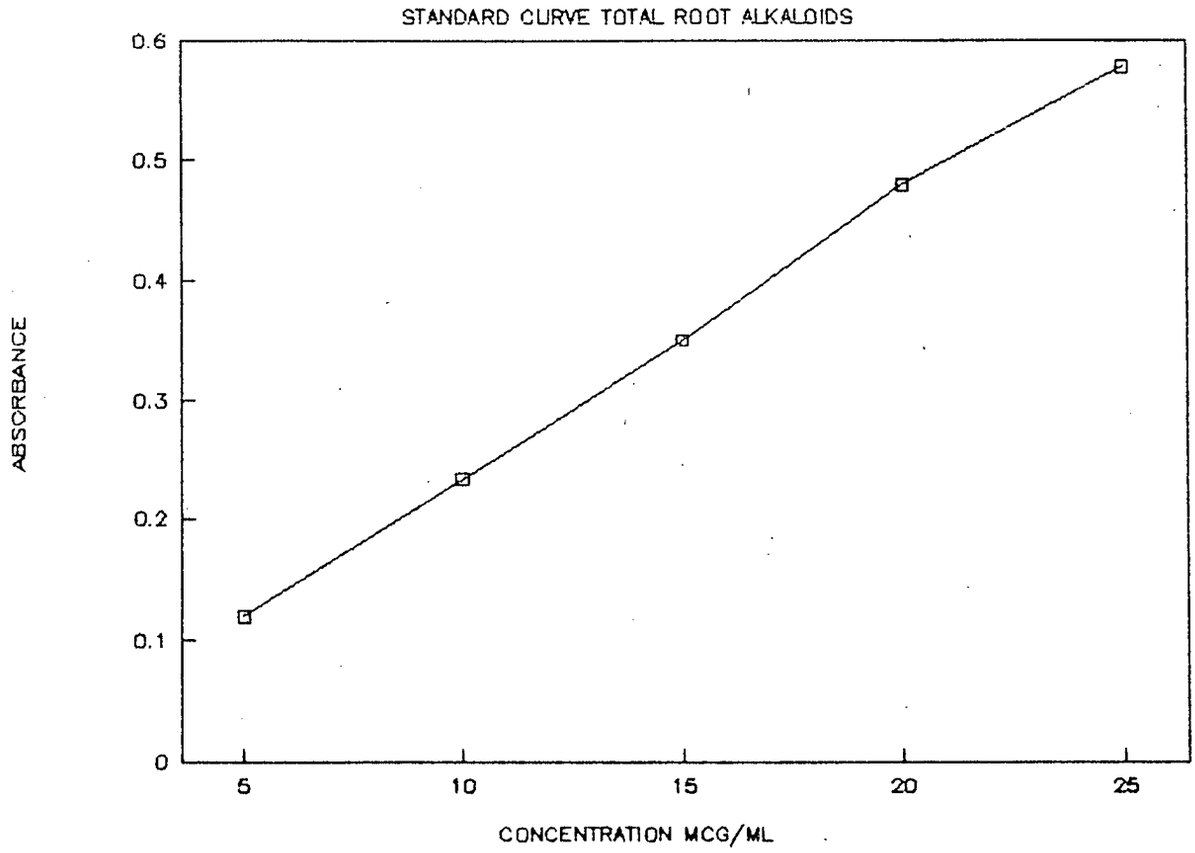

STANDARD CURVE CALLUS TOTAL ALKALOIDS
ABSORBANCE
0.6
0.5
0.4
0.3
0.2
0.1
0
50
100
150
200
250
CONCENTRATION MCG/ML

FIG. 3.8


STANDARD CURVE TOTAL ROOT ALKALOIDS
ABSORBANCE
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
25
CONCENTRATION MCG/ML

using the appropriate factor as shown in Table 3.29 and Table 3.30.

3.12. YIELDS OF TOTAL ALKALOIDS IN CALLUS UNDER OPTIMUM ENVIRONMENTAL CONDITIONS

It is observed from Table 3.20 and Fig. 3.9 that the total alkaloid contents in callus culture started increasing from the second week and reached optimum level at the end of sixth week. Thereafter the contents gradually decreased in seventh and eighth week. Also it can be noted that the alkaloid content increase with increase in growth index.

3.12.1. Effect of growth substances on production of alkaloids

Effect of growth hormones. Table 3.21, indicates the results of explants prepared during callus optimisation. It is clear that when growth hormones 2,4-D, IBA and Kn are added individually to MS medium 1/2 strength, the alkaloid contents are maximum with the incorporation of IBA followed by kn and 2,4-D. However the growth index was maximum with 2,4-D, followed by IBA and Kn.

Effect of Amino Acids. Table 3.22, shows that of all the amino acids tested, tryptophan showed maximum yield of alkaloids suggesting that it may be a precursor in the biogenesis of alkaloids. There was no change in alkaloidal contents when phenylalanine, tyrosine, glutamic acid, methionine and arginine were incorporated in the medium. However there was a marked decrease in contents and growth index when valine, lysine, adenine and leucine were incorporated in the medium indicating that they neither supported the growth of the callus tissue nor took part

FIG. 3.9

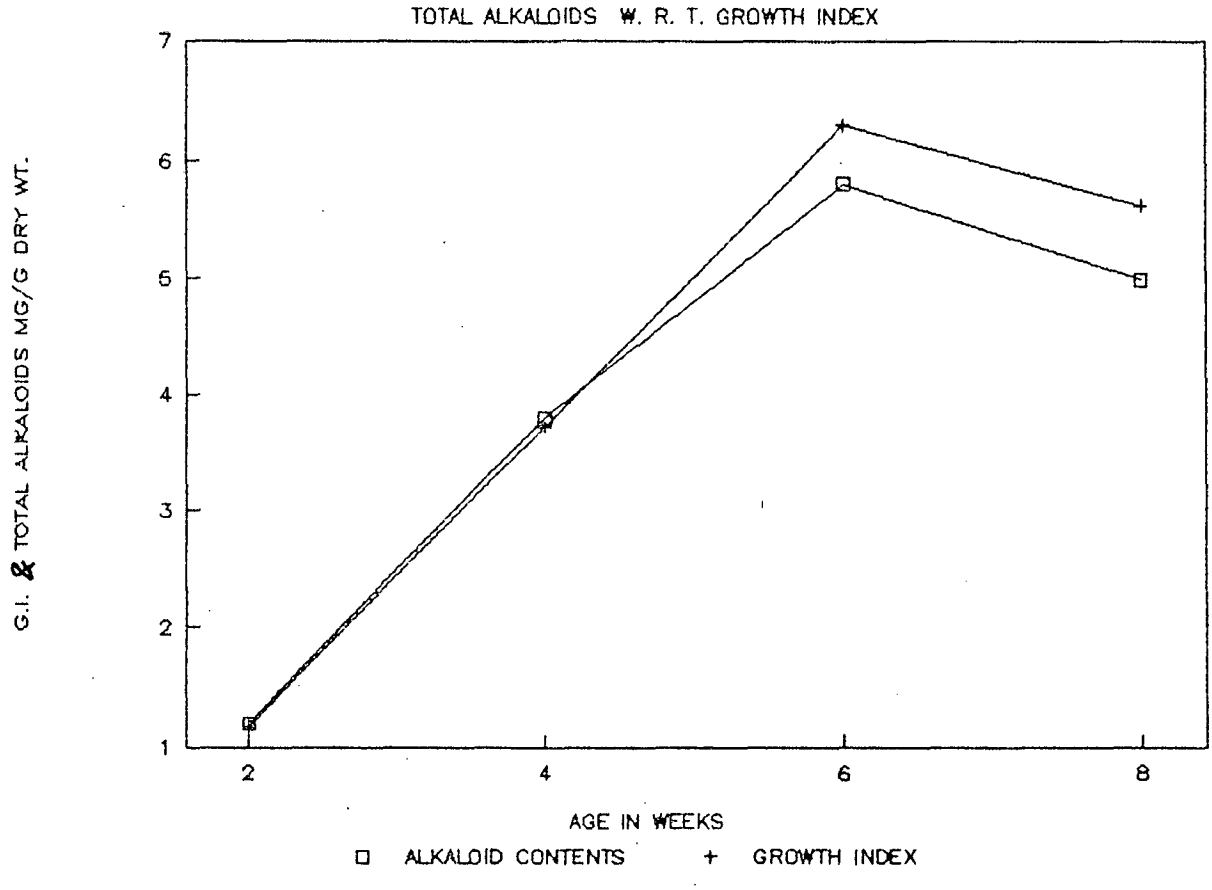

TOTAL ALKALOIDS W. R. T. GROWTH INDEX
G.I. & TOTAL ALKALOIDS MG/G DRY WT.
7
6
5
4
3
2
1
2
4
6
8
AGE IN WEEKS
□ ALKALOID CONTENTS
+ GROWTH INDEX

Table 3.20

Quantitative estimation of total alkaloids in callus under optimum environmental conditions

Age in weeks	Growth Index	Dry wt.mg/g	Total alkaloids mg/g dry wt. as heterostemmine
2	1.2	51	1.177
4	3.8	52	3.722
6	5.8	68	6.291
8	5.0	49	5.630

Table 3.21

Effect of Growth hormones on production of alkaloids

Growth hormone concentration	Growth Index	Dry wt. mg/g.	Total alkaloids mg/g. d. wt. as Heterostemmin
2, 4 - D 1 mg/L	4.4	55	4.66
I B A 0.5 mg/L	3.3	50	5.19
Kn 0.5 mg/L	2.9	52	4.706

Table 3.22

Effect of growth substances on production of alkaloids
Basal Medium : MS ½ strength with 10% CM
Growth Hormones : 2,4-D(1mg/L), IBA(0.5 mg/L),Kn(0.5 mg/L)
Supplements : Different concentration of growth substances
Harvesting period : 6 weeks

Growth substances	Growth Index	Dry wt.mg/g	Total alkaloids mg/g d.wt as Heterostemmin
Amino acids conc. 0.1%			
Tryptophan	6.8	55	7.605
Phenylalanine	7.3	58	5.178
Tyrosine	5.7	47	5.775
Glutamic acid	7.5	60	5.251
Methionine	4.2	54	5.109
Valine	2.0	56	3.738
Histidine	5.3	45	3.115
Threonine	6.2	56	2.383
Cysteine HCL	5.3	48	3.430
Aspargine	5.8	44	4.551
Arginine	5.1	51	5.460
Proline	6.8	47	4.970
L-lysine HCL	2.4	44	3.238
Adenine	1.8	48	2.603
L-leucine	1.4	42	2.330
Other growth substances			
Yeast Extract (0.05%)	3.4	58	4.526
Niacinamide (0.1%)	3.2	52	8.129
Ascorbic acid (0.1%)	5.6	48	4.357
Caffeine (0.1%)	0.43	50	0.2447
Cholesterol (0.1%)	0.37	52	0.2096

in production of alkaloids. The growth indices in the case of histidine, threonine and cysteine were normal but the alkaloidal content decreased considerably suggesting that they do not inhibit the growth of callus but decrease the production of alkaloids in tissue. Amino acids proline and aspargine did not have a marked effect either on growth indices or production of alkaloids. Table 3.23, indicates the effect of tryptophan in varying concentration of 0.025,0.05 and 0.1% on the production of alkaloids. The concentration of 0.05% gave maximum yield of the alkaloids.

Effect of Niacinamide. Table 3.22 and Table 3.24, show that when niacinamide was fed in the medium in different concentrations, there was a marked increase in the production of alkaloids. However the growth index was inversely proportional to the production of alkaloids. Of the different concentrations used (0.01,0.025 and 0.05%), 0.025% gave maximum yield. The callus showed signs of browning and degeneration when concentration of niacinamide was increased.

Effect of Yeast Extract. The effect of growth and production of alkaloids was studied by incorporating 0.05% of yeast extract in the medium. Table 3.22 indicates that yeast extract enhanced the production of alkaloids but decreased the growth index.

3.12.2. Effect of Ascorbic Acid

Ascorbic acid used in the concentration of 0.1% had no role to play in the production of alkaloids but definitely increased the growth index Table 3.22.

3.12.3. Effect of Caffeine

Inclusion of caffeine in the medium in the concentration of 0.1% decreased the growth as well as the alkaloidal contents indicating that it did not have any effect on production of alkaloids but has inhibitory effect. Table 3.22.

3.12.4 Effect of Cholesterol

Cholesterol in the concentration of 0.1% when added to the medium had a deleterious effect on both growth and alkaloid contents. Table 3.22.

3.13.5. Effect of Photoperiod

Table 3.6, indicates the results of explants prepared during optimisation of callus. The calli obtained from this set of experiment were harvested after 6 weeks, the G.I. determined and total alkaloids estimated. Table 3.25 shows that there was no remarkable change in the alkaloid content when tissues were exposed to different length of photoperiod.

3.13 EXTRACTION AND ISOLATION OF ALKALOIDS

It is observed from Fig. 3.10, that when the crude extract of total alkaloids was spotted on TLC a total number of 15 spots were detected when the plates were viewed under ultra violet light, out of which 12 spots responded positively for Dragendorff reagent, which is specific for alkaloids. The R_f values of the detected spots are listed in Table 3.26. The colour intensity of the spots obtained after spraying with Dragendorff reagent revealed that the spots representing alkaloids with R_f values 0.80, 0.76 and 0.05 were having maximum intensity while, colour intensity of other alkaloid spots was very low, indicating

Table 3.23

Effect of Tryptophan on production of alkaloids in H. tanjorense

Basal Medium : MS ½ strength with 10% CM.
Growth Hormones : 2,4-D (1 mg/L), IBA (0.5 mg/L), Kn (0.5 mg/L)
Supplements : Different concentrations of Tryptophan
Harvesting period : 6 weeks.

Tryptophan concentration %	Growth Index	Dry wt. mg/g	Total alkaloids mg/g dry wt. as heterostemmine
0.025	6.5	50	5.963
0.05	6.8	55	8.490
0.10	6.8	52	7.873

Table 3.24

Effect of Niacinamide on production of alkaloid in H. tanjorense

Basal Medium : MS ½ Strength with 10% CM
Growth hormones : 2, 4-D (1 mg/L), IBA (0.5 mg/L), Kn(0.5 mg/L)
Supplements : Different concentrations of niacinamide
Harvesting period : 6 weeks

Niacinamide concentration %	Growth Index	Dry wt. mg/g	Total alkaloids mg/g dry wt. as heterostemmine
0.01	3.5	58	8.439
0.025	3.2	56	10.330
0.05	1.2	52	4.107

Table 3.25

Effect of photoperiod on production of alkaloids

Photoperiod	Growth Index	Dry wt. mg/g	Total alkaloids mg/g dry wt. as heterostermmine
12 hr. light/ 12 hr. dark	5.7	49	5.812
24 hr. light	5.3	51	5.453
24 hr. dark	4.9	56	5.165

Table 3.26

Detection of spots on TLC b y UV lamp and Dragendorff reagent.
TLC Layer : Silica gel GF 254
Solvent system : Chlorofrom : Methanol (95:5) with 2% NH_3

Callus Extract		Root Extract	
R values of spots f detected under uv lamp	Alkaloid reaction with Dragendorff	R valus of f spots detected under uv lamp	Alkaloid reaction with Dragendorff
0.92	—	0.92	—
0.85	++	0.85	++
0.80	+++	0.80	+++
0.76	++	0.76	++
0.70	+	0.70	+
0.60	+	0.60	+
0.48	+	0.48	+
0.40	—	0.40	—
0.30	+	0.30	+
0.25	+	0.25	+
0.20	+	0.20	+
0.15	+	0.15	+
0.12	—	0.12	—
0.05	++	0.05	++
0.00	+	0.00	+

Note :- + Indicates faint intensity of alkaloid
++ Indicates fair intensity of alkaloid
+++ Indicates maximum intensity of alkaloid
— Indicates negative reaction for alkaloid.

that the concentration of the above alkaloids was more in total alkaloid concentrate.

The elution of the crude alkaloids from column chromatography with benzene, chloroform solvent mixture as described in section 3.5 yielded a single spot alkaloid (approx. 8 mg = 0.2% approx. on dry wt. basis). This alkaloid gave the same R_f value (0.80) when co-chromatographed with standard heterostemmine Fig. 3.11. It was further purified and crystallised from benzene. The other solvents yielded a mixture of alkaloids. The results are listed in Table 3.27.

3.13.1. Comparative study of total alkaloids present in calli from different explants

Table 3.28, indicates that the callus developed from asceptically germinated seedling gives maximum yield of alkaloids followed by calli developed from root, stem and leaf. Calli developed from these explants are shown in Fig. 3.14.

3.13.2. Extraction of total alkaloids from the roots of H.tanjorense (in vivo)

The total alkaloids from the crude chloroform extract of callus obtained from section 3.4, were subjected to TLC analysis along with the total alkaloid from root extracts obtained from section 3.7 for comparative studies. Additional three alkaloids were observed on TLC plate and colour intensity of the spots was more in the case of root extracts than in the callus extract. Fig. 3.12. The quantitative estimation of the extract was done as per section 3.8 and the contents calculated. Table 3.29 and Table 3.30.

Table 3.27

Column chromatography of total alkaloid present in Callus of *H. tanjorense*

Column size : 45 x 18 cms
Adsorbent : Neutral alumina
Quantity : 25 g
Wt. of sample: 200 mg

Fr. No.	Eluent	Physical appearance	Wt. in mg	R_f value under uv lamp	Alkaloid reaction
1	Benzene	Resinous yellow	20.4	0.92	—
2	"	"	18.6	0.92	—
3	"	"	20.2	0.92	—
4	"	"	10.9	0.92, 0.85	+
5	Benzene chloroform	yellow white	6.5	0.92, 0.85	+
6	1:1 "	"	4.2	0.85,0.80	++
7	"	white cryst.	2.5	0.80	++
8	"	"	3.5	0.80	+++
9	Chloroform	"	2.0	0.80	+++
10	"	"	2.0	0.80,0.76	++
11	"	yellow cryst	8.8	0.80,0.76,0.70	+++
12	"	"	6.4	0.76,0.70,0.6	++
13	Chloroform methanol	Pink cryst.	6.0	0.7,0.6,0.48	++
14	95:5 "	"	6.5	0.6,0.48,0.4	+
15	"	Red brown.	9.8	0.6,0.48,0.4,0.3	++
16	"	"	12.2	0.48,0.4,0.3,0.25	++
17	Methanol	yellow fluores.	16.2	0.4,0.3,0.25,0.2,0.15,0.05	++
18	"	"	6.4	0.3,0.25,0.2,0.15,0.05	++
19	"	yellow white	4.2	0.20,0.15,0.05	++
20	"	yellow white	3.5	0.05,0.00	++

TOTAL = 170.8 mg

Note :- + Indicates faint intensity of alkaloid
++ Indicates fair intensity of alkaloid
+++ Indicates maximum intensity of alkaloid
— Indicates negative reaction for alkaloid.

Table 3.28

Comparative study of total alkaloids present in calli developed from different explants of H. tanjorense

Explant	Growth Index	Total alkaloids mg/g dry wt. as heterostermmine
Germinated seedling	5.8	5.768
Leaf	4.6	5.085
Stem	5.6	5.409
Root	5.2	5.562

Table 3.29

Estimation of total alkaloids obtained from callus extract and roots of H. tanjorense in terms of heterostemmine standard.

Sample Concetration	Volume of soln. ml.	Absorb-ance	Concentration in terms of heterostemmine			
			concentration from absorbance	conc. mcg/ml	Average conc. mcg/ml	Conc. mcg/mg
Total Alkaloids of Callus Soln.B=1mg/ml	0.5 1.0	0.090 0.185	55 112.5	110 112.5	111.25	111.25
Total Alkaloids of roots Soln. C =0.1 mg/ml	0.5 1.0	0.060 0.117	37 70	74 70	72	720

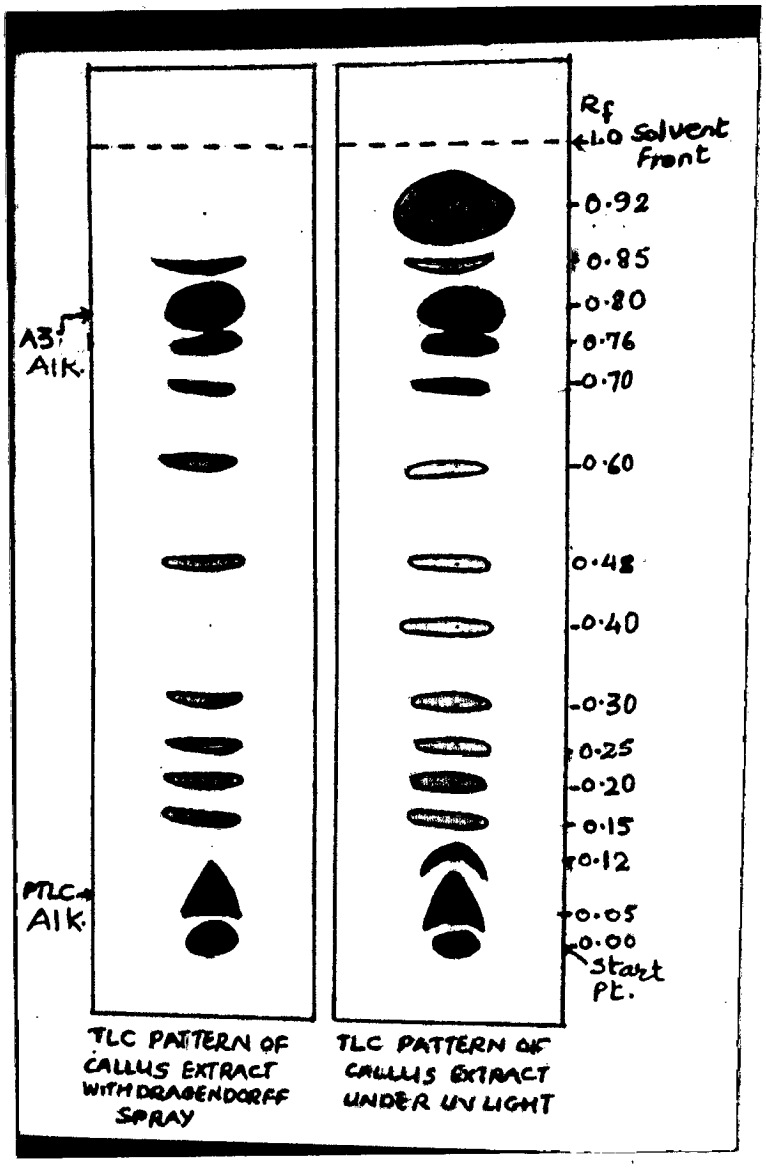
Table 3.30

Calculation for Estimation of total alkaloids in Callus and root extract in terms of heterostemmine

Sample dry weight	Total alkaloid yield mg	Yield/gm dry wt.	Average conc. mcg/mg	Conc./mcg/g dry wt.	Conc. mg/g dry wt(mg)
Callus (5g)	260	52	111.25	111.25X52 =5785	5.785
Roots (10g)	220	22	720.0	720X22 =15840	15.840

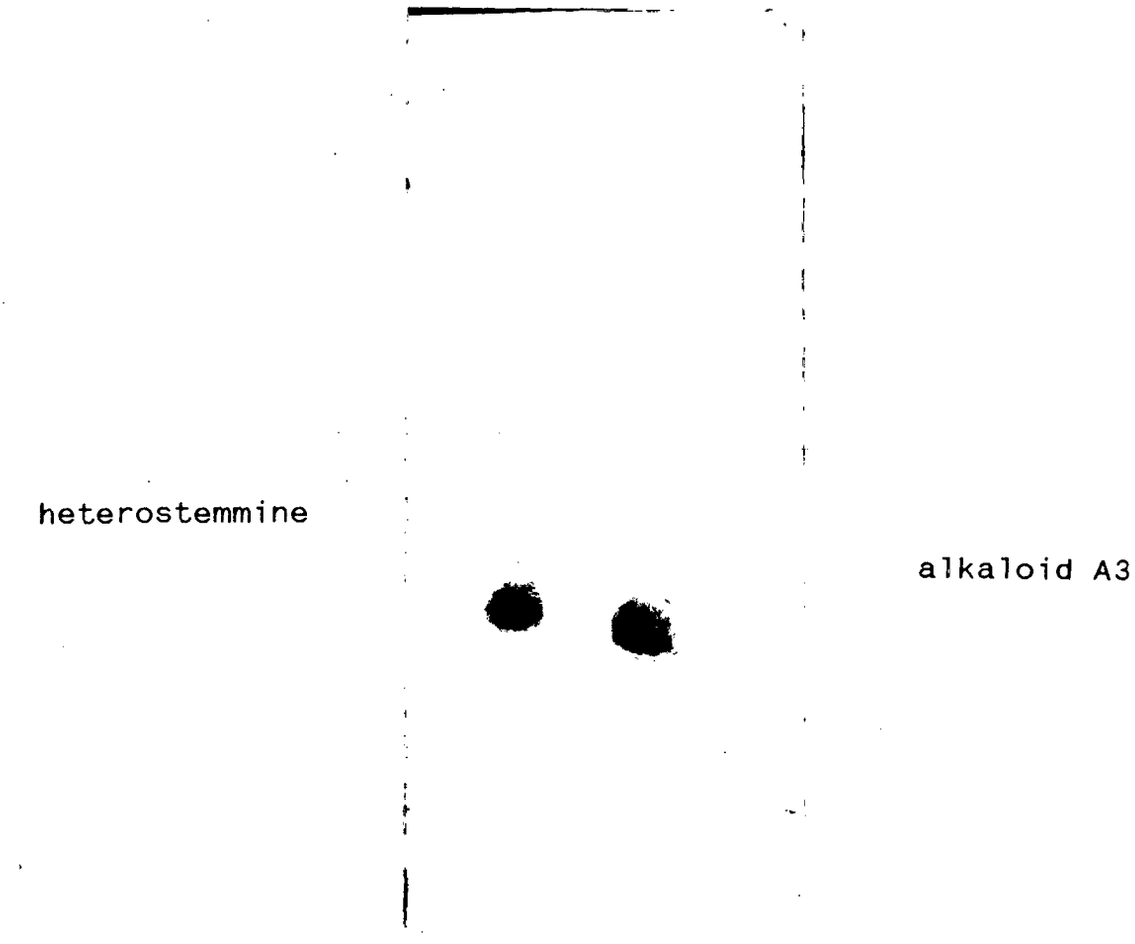
Derivation of factor for conversion of total alkaloids of callus in terms of heterostemmine.
1mg of total alkaloids of callus = 111.25 mcg of heterostemmine.
Hence 111.25 mcg is the conversion factor for all experiments where total alkaloids of callus are to be expressed as heterostemmine.

Fig. 3.10



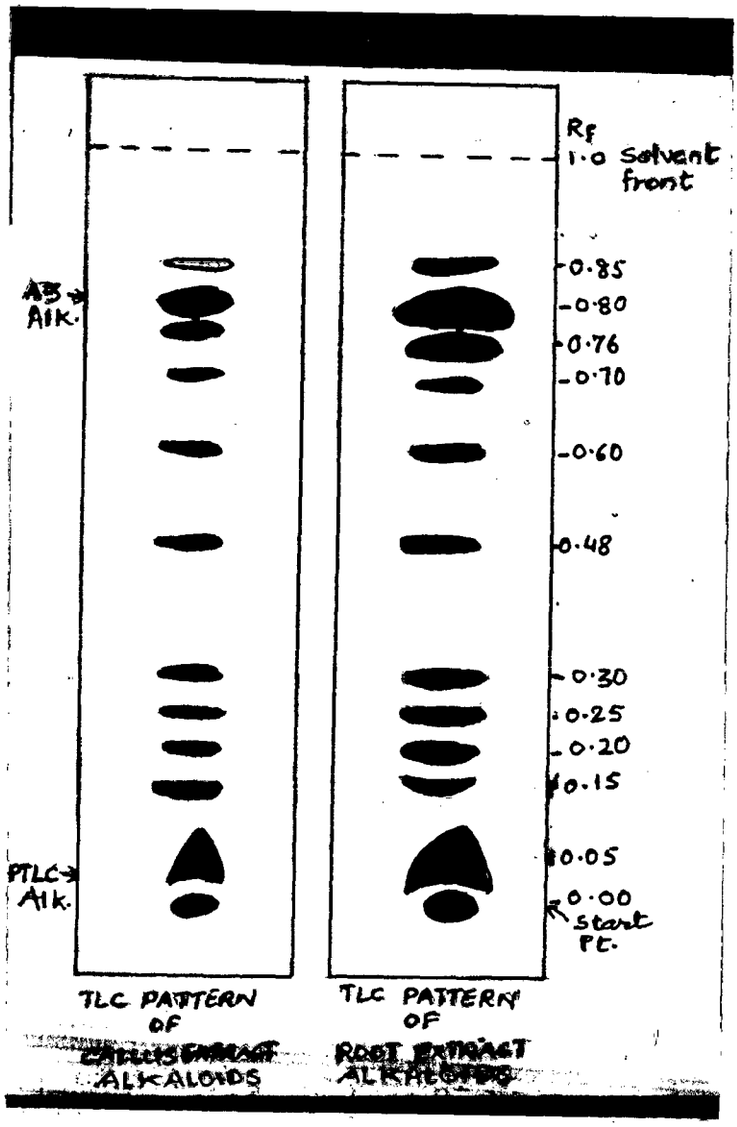
TLC Pattern of total alkaloids of callus of H. tanjorense.

Fig. 3.11



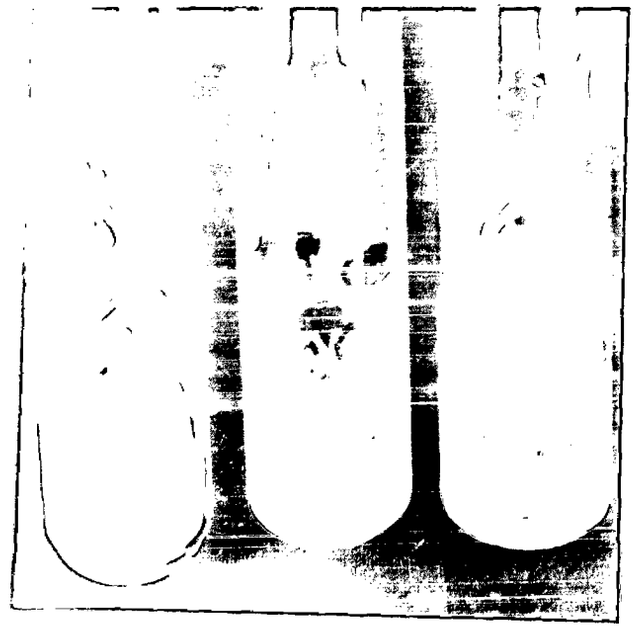
Co-chromatography of heterostemmine and alkaloid A3.

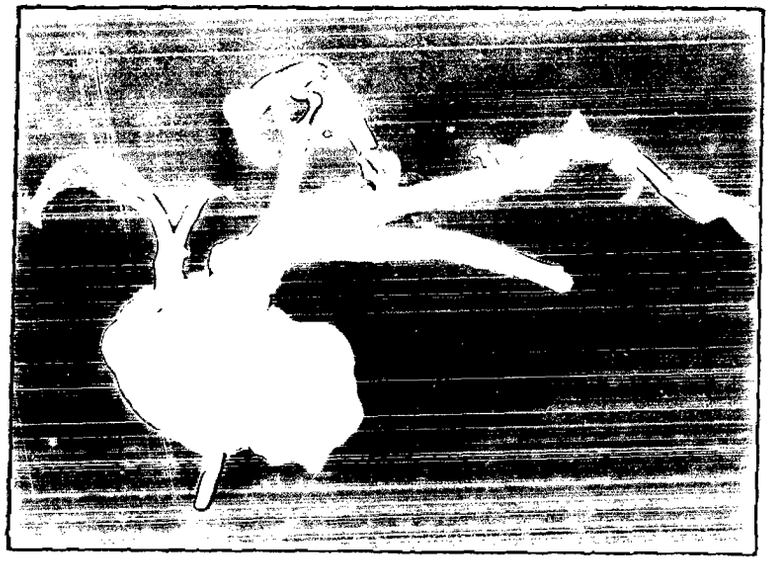
Fig. 3.12

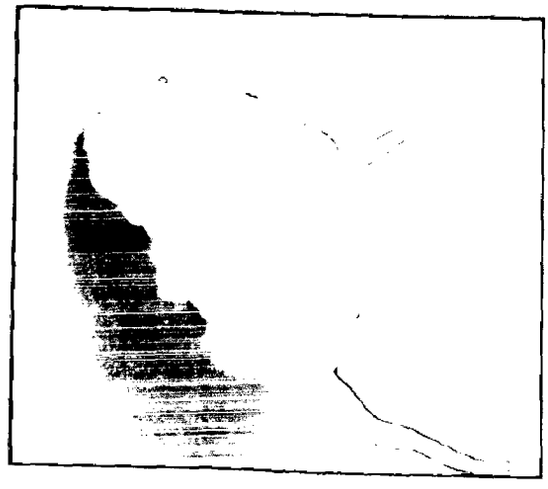


T. L. C. Pattern of total alkaloids of Callus and roots of H. tanjorense.

Fig.3.13

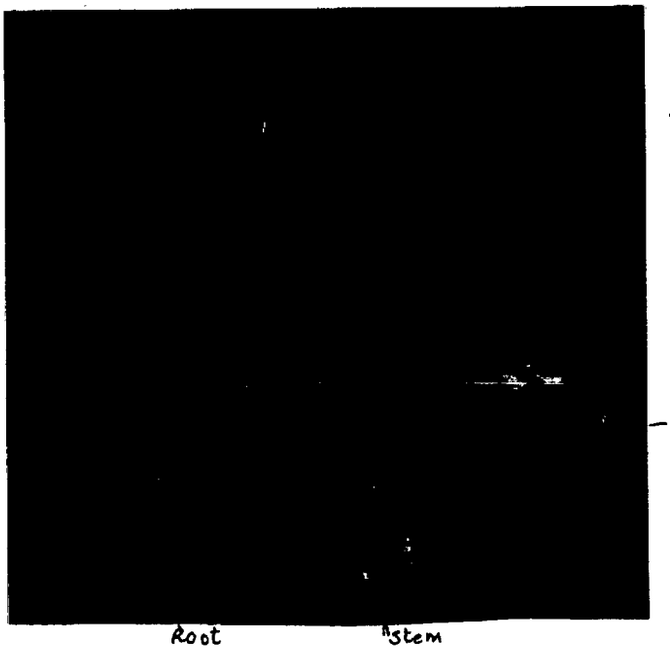






Organogenesis in callus of H. tanjorense.

Fig. 3.14



Callus from root, leaf and stem of H. tanjorense.

DISCUSSION

FACTORS AFFECTING OPTIMAL YIELD OF CALLUS CULTURE OF *H. tanjorense*

The foregoing results have allowed establishment of the optimal conditions for the growth of callus as well as the production of alkaloids. Earlier reports of production of secondary metabolites have shown that yield can be increased by standardizing the nutritional requirements. As far as nutritional conditions are concerned the composition of medium plays a major role.

Different standard nutrient solutions have been formulated by Gamborg(B5) (1968), White's (1963), Hildebrandt and Riker (1949), Burkholder and Nickell (1949), Nitsch and Nitsch (1956,1957), Heller (1953), Murashige and Skoog (1962), Eriksson (1965), Linsmaier and Skoog (1965), Nageta and Takebe (1971) for growing plant tissues. The most extensively used and most successful medium for a wide range of plants is Murashige and Skoog medium (MS), probably due to its high salt concentration and also to the presence of a chelating agent Fe EDTA, which ensures the availability of iron over a wide range of pH. *H. tanjorense* explants however showed confluent callus development on MS medium containing half the strength of all the salts depending on inoculum of *H. tanjorense* as can be seen in Table 3.1. Nitrates are the most widely used nitrogen source as is evident from several reports for sunflower Crowngall (Riker, 1948), Paul's Scarlet rose (Nash, 1972), Carrot (Reinert, 1967) tobacco and populus (Matsumoto et al., 1971). Nitrites cannot replace

nitrates. Sunflower crowngall tissue showed poor growth with nitrite, excellent growth with nitrate and good growth with ammonia (Riker, 1948). Reduced nitrogen in the form of ammonium can also be used in addition to nitrates. Murashige and Skoog (1962), used ammonium nitrate with potassium nitrate for tobacco callus growth. The growth of potato tissue (Yatazawa et al., 1968; Shantz et al., 1959) tissue was better on media containing ammonium and nitrate than on nitrate alone. Ammonium nitrogen was used for the growth of rice tissue (Yatazawa et al., 1968). Hendre et al (1975) studied the effect of different nitrogen sources on cereal tissue. A marked increase in growth was observed only with ammonium nitrate and not with other ammonium salt. Widely used plant tissue culture media contain both NO_3 and NH_4 as nitrogen sources (Dougall, 1981). Reduced nitrogen in the form of organic compounds is usually stimulatory to growth and morphogenesis if added in the form of amino acids, such as casein hydrolysate and yeast extract which were the two best nitrogen sources for callus growth in rice (Yatazawa, 1968). Dougall (1966) showed that exogenously applied amino acids such as alanine, glutamic acid, aspartic acid, glutamine and proline were available for protein synthesis in cultured cells of Paul's scarlet rose and other amino acids such as isoleucine, threonine, methionine, lysine, histidine, phenylalanine, serine, glycine, valine, leucine supressed their own biosynthesis. Khanna et al., (1972b) observed that the use of Phenylalanine and tyrosine increased the alkaloidal content in trigonella. It can be seen from the results at sections 3.10.7 and 3.10.8 that nitrogen

either in inorganic or in organic form plays an important role in production of biomass in the case of H. tanjorense . However, other nutritional conditions like presence of phosphorous, potassium, sulphur, calcium and micro elements influence the growth of callus. All these elements being present in MS medium help in growth maximisation as evident from the results. Lingappa (1957) studied the optimum concentration of phosphates and showed that the best growth of potato callus with 200 mg/L of KH_2PO_4. Phosphates is the primary buffering constituent in tissue culture media. Phosphorous levels greater than 0.06 mg/L were inhibitory to growth of tobacco pith tissue (Murashige 1962) whereas 0.07 mg/L phosphates were near optimum for Haplopappus gracillis suspension culture (Eriksson, 1965). Potassium is the accompanying cation for nitrate and phosphate salts and its concentration is not controlled separately. Potassium at a concentration of 0.03 mg/L was sufficient for growth of carrot suspension culture whereas a much higher level of 0.78 mg/L was necessary for full expression of the embryogenic potential (Brown, 1976). An increase of potassium in the medium can substitute various organic compounds normally required in media. For example Vinca rosea required glutamine with low K^+ levels in the medium. Higher K^+ levels were inhibitory (Wood, 1961). Certain organic forms of sulphur like L-cysteine, L-methionine and glutathione were found to be satisfactory source of sulphur for tobacco tissue (Hart, 1969). Growth of Rumex virus tumour tissue in culture was supported only by cysteine as a substitute for sulphates (Nickell, 1950). Calcium salts are among the least soluble one used in

tissue culture, thereby limiting their choice only to soluble salts like calcium chloride. Calcium reduces lignification in Helianthus culture (Stonier, 1971). Calcium was absolutely required for growth and pigment production in Morinda citrifolia (Zenk, 1975).

Microelements play an important role in nutrition even though they are required in very small amounts, they are necessary for growth. The essential elements required in micromolar concentration include Fe, Mn, Zn, B, Cu and Mo (Gamborg, 1976). Omission of Mn, Cu, Mo and B from the medium had very little effect on growth unlike omission of Fe and Zn in a test conducted on rice cultures (Ohira, 1975). Plant tissue culture media often contain cobalt, iodine, nickel, aluminium etc. (Ojima, 1978). Iron is the essential microelement in tissue culture media. Ferrous sulphate, ferric chloride or tartrate were used as source of iron in early media preparations. These caused precipitation and hence media were formulated by chelating with EDTA (Jacobson, 1951). This form was less toxic and more useful for in vitro cultures over a wide pH range. In the present study manipulation of salt concentrations was not attempted since the callus could be developed well at half the concentrations of constituents of MS medium, Table 3.1. The majority of higher plants are completely autotrophic being able to synthesise all the organic carbon compounds necessary for growth and survival. Sucrose is the most widely used source and was found to be the best sugar for the growth of poplar and tobacco callus (Matsumoto, 1971). It gave optimal growth of onion cultures whereas glucose was ineffective

(Fridborg, 1971). Sucrose is used for majority of plant tissue culture at a concentration of 2 to 5% for rice (Yatazawa, 1967), maize (Sheridan, 1975), sugarcane (Ahloowalia, 1983). Other carbohydrate sources have also been used for callus cultures of different plant species such as sorbitol for Rosaceae genus (Coffin, 1976). Carrot cells were able to utilise sucrose, fructose, galactose, mannose, maltose, raffinose and stachylose as a carbon source (Verma, 1977). Maximum callus of H.tanjorense was obtained at 2% concentration of sucrose, Table 3.9. The use of vitamins in plant tissue culture medium has been a matter of custom rather than a proven necessity (Seabrook, 1980). Thiamine hydrochloride is the only vitamin for which there seems to be a consistent requirement for growth of plant tissues in vitro. Other vitamins should be added to media formulations where enhancement of growth or morphogenesis indicated they are necessary (Huang, 1977). Pantothenic acid was used for the growth of Haworthia, Willow and Jumper tissues (Morel, 1946; Constabel, 1958; Gautheret, 1959). Folic acid was inhibitory to prine tissue (Reinert, 1956). Thiamine, Pridoxine hydrochloride, nicotinic acid were used in higher concentrations than that used in White's medium for optimum growth of cereals (Hendre, 1975). Revised tobacco medium of Murashige and Skoog (1962) containing vitamins cyanocobalamin, folic acid, riboflavin, ca-pantothenate, pyridoxine HCl, thiamine HCL and nicotinamide was used in dioscorea tissue culture (Kaul, 1968). Unlike carbon, sucrose and vitamins, certain compounds occurring naturally within plant tissues exert a regulatory effect on growth and development of

callus tissue. Synthetic chemicals with similar physiological activities to plant growth substances, or compounds having an ability to modify plant growth by some other means are termed as plant growth regulators (George, 1984). The most commonly detected natural auxins is IAA. Besides IAA, NAA, 2,4-D and IBA are widely used in plant tissue culture (Dougall, 1980). Some investigators have used mixture of many different auxins (Blackmon, 1981), but some prefer to use only one or at the most two. IBA alone or in combination with NAA has proved very effective with some tissues (Street, 1977; Hangarter, 1980). Species specificity for NAA, 2,4-D was better than IAA or other auxins for callus growth in Pinus (Brown, 1968), rice (Yamada, 1967; Wu and Li 1971), dioscorea (Rao, 1969). 2,4-D in general is very effective in callus induction, however in certain plants like Plumbago, auxins like IAA is more effective (Nitsch and Nitsch, 1967). Cytokinins regulate growth and morphogenesis in tissue culture. Kende (1971), Skoog (1970) and Fosket (1978) reported that a basic regulatory mechanism underlying organogenesis involves a balance between auxin and cytokinin. There are many tissues which require cytokinin for callus growth like stimulation of the growth of Jerusalem antichoke tuber tissue (Nitsch, 1957) but in general the absolute requirement is not as clear as the requirement for auxin, which applies practically to all tissues other than tumor tissues. Matsumoto et al. (1971) reported that of 39 species of Nicotiana cultures, only one, N. glutinosa required a cytokinin in addition to an auxin. In the case of cytokinin it is always a case of a cytokinin plus an auxin

for callus growth. The best known and widely used Gibberellin is GA3 (Brain et al., 1960). Gibberellin stimulated callus growth in tobacco (Murashige, 1965). Nickell and Tulecke (1959), studied the response of GA3 on 59 strains of callus tissues from 25 species and found that it was inhibitory for growth. (Hendre, 1975; Helgeson and Upper, 1970; Lance et al., 1970). Morgan and Hall (1962,1964), showed that in cotton, the production of ethylene is closely linked to the level of exogenous auxin applied and later Burg and Burg (1968) and Kand et al (1974), demonstrated that rate of ethylene production in tissues is related to their endogenous auxin level. Abscisic acid (Milborrow, 1974), provides a defence mechanism against physiological stresses by stopping growth and conserving energy (Rothwell, 1964). Though it is generally considered as a plant growth inhibitor, it has been shown to enhance the growth of certain plant tissues, aerial leaves and precocious flowering was induced in nodal explants of submerged shoots by ABA (Ram Mohan, 1982). ABA is usually widely inhibitory to cell growth. (Sankhla, 1968). Generally concentrations between 5 to 50 mg/L are necessary to cause 50% growth retardation of cell growth. (Li, 1970, Gamborg, 1971). In contrast several instances have been cited where ABA stimulates callus growth as in Cryptomeria (Isikawa, 1974), and citrus (Altman, 1971).

Exhaustive experiments were conducted to establish concentrations of phytohormones for optimal yield of callus and also production of alkaloids in the case of H. tanjorense Tables 3.13 to Table 3.17. Varying concentrations of growth hormones

tested in the present studies showed inhibition or increase in callus growth. GA3 did not show much effect on the callus of H. tanjorense. Also growth retardants like ABA showed deleterious effect on callus. Complex natural extracts, such as coconut milk (Van Overbeek, et al.; 1941), tomato juice (La-Rue, 1949; Nitsch, 1949), potato juice (Lingappa, 1957), malt extract (Steinhart, 1961), tobacco juice (Murashige,1962), Carrot juice (Syono, 1965), Water melon and prune juice (Guha and Maheshwari, 1967), yeast extract, (Yatazawa,1967), orange and banana juice (Murashige,1974), have been used in callus cultures. These are used as a source of amino acid and vitamins (Yatazawa, 1968). Amongst these natural extracts, coconut milk was widely used and was found to have stimulatory growth effect on Trapaeolum (Ball,1946), carrot (Steward, 1954), citrus species (Burnet, 1973) and Panicum miliaceum (Rangan, 1974). Vasil and coworkers (1981), used coconut milk in callus and suspension cultures. Natural extracts like coconut milk used in tissue cultures of H. tanjorense enhanced the callus growth due to stimulatory effect as is observed from the results. Table 3.12. The concentration of agar is another factor which influences growth in vitro Romberger et al., (1970), reported better growth of Picea meristem on Difco agar. White and Risser (1964), tested the effect of Difco Noble agar concentrations varying from 0.125 to 2 % on the growth of Picea glaucea callus and obtained best growth at 0.5 % agar concentration. As seen from results in Table 3.4, 0.8 % agar displayed maximum yield of callus of H. tanjorense.

Physical factors such as pH, temperature, light intensity, humidity, when properly manipulated yield maximum of callus biomass, (Nickell, 1950; Henderson, 1954; Gauthert, 1959 ; White, 1964 ; Gamborg, 1968; Strickland, 1972 ; Jones, 1974; Holt, 1975; Fridborg and Eriksson, 1975; Hendre, 1975; Narayanswami, 1977; Yeoman, 1977; Negrutiu, 1978; Gupta, et al., 1980; Barlass, 1982; George, 1984) as is evident from the result where different parameter were adjusted for initiation of callus in H. tanjorense and subsequent increase in callus biomass obtained. Hammerschlag (1978) observed that in geranium species the callus growth was depending on variety of plants from which explants were derived. In one case growth was best with 1000 or 5000 lux and in another, it was most rapid in 5000 lux intensity. However growth of all these was reduced with 10,000 lux intensity. Haramaki (1971) found that Gloxinia plant callus was present at 320 lux but absent at high flux density. H. tanjorense callus grew luxuriously at 1500 lux. Table 3.18 and Fig. 3.1 clearly indicate the established optimal conditions for maximum yield of callus culture. It was interesting to note that the callus growth increased from second to sixth week. These optimal conditions were in turn used for ascertaining maximum yield of secondary products which follows.

OPTIMAL YIELD OF ALKALOID IN CALLUS OF H. tanjorense

Literature review indicates that the growth hormones, growth promoters, precursors and photoperiod help in increasing the yield of alkaloids and other secondary metabolites in plant

tissue culture. The effect of 2,4-D, NAA,IAA,IBA, Kn was studied on berberine production in Thalictrum minus (Nakagawa, 1986). Effect of 2,4-D and Kn was studied in the production of reserpine in Rauwolfia serpentina (Yamamoto, 1986). The influence of cholesterol, yeast extract, 2,4-D, simazine and ascorbic acid on production of diosgenin in Dioscorea deltoidea has been reported (Kaul, 1969) The effect of IAA,IBA and 2,4-D has been reported in the production of solasodine in Solanum xanthocarpum (Heble, 1971). The effect of ornithine (Chan, 1965), glutamic acid (French, 1957), Proline (Gibson, 1963), Phenylalanine (Grass, 1963), and tropic acid (Stohs, 1969), has been reported in Datura species. Sairam and Khanna (1971), found a considerable increase in alkaloid contents in D. talula. The effect of phenylalanine and tyrosine on growth and alkaloid production in Datura metel was reported by Khanna and Nag (1972b). The effect of L-tryptophan was studied in Cinchona alkaloids (Koblitz 1983). Effect of nicotinic acid on production of Trigonelline from Trigonella foenum was observed by (Khanna 1972a). The effect of light on solosodine production in Solanum laciniatum was reported by Chandler and Dodds (1983). Use of yeast extract on alkaloid production in Scopalia parviflora was reported by Tabata et al. (1972).

In the present work, the study undertaken to see the effect of growth substances on production of alkaloids in H. tanjorense callus cultures positively indicates that these factors play an important role on production of alkaloids. Amino acids like tryptophan increased the alkaloid production while some had adverse effect. Also niacinamide, yeast extract helps in

production of alkaloids indicating that these substances may act as precursor in the biogenesis of alkaloids. Caffeine and cholesterol had adverse effect on both production of biomass and alkaloid formation which indicated that they have no role to play in these experiments. However ascorbic acid accelerated the growth of callus without actively taking part in production of alkaloids. All these results show that callus formation as well as metabolite synthesis largely depends upon the environmental conditions, growth factors, etc. With the manipulation of these substances an increase in the callus formation or metabolite generation can be achieved.

QUANTITATIVE ESTIMATION OF ALKALOIDS IN PLANTS.

Several methods for the quantitative estimation of alkaloids in plants have been reported Vitali-Morin, (Morin, 1936), reaction has been widely used for colorimetric estimation of alkaloids. This reaction has been applied to beladonna and its related products by Allport and Wilson (1939). It has been further investigated by James and Roberts (1945) and Ashley (1952), Carr (1953), F. M. Freeman (1955), Birner (1969), described a colorimetric method for determination of total glycoalkaloids in solanaceous species by colour complexing with methyl orange. He also reported that this method is also applicable to similar nitrogen containing alkaloids and colored complex can be formed with bromocresol purple and thymol blue at pH 3 to 4. Balakrishna et al. (1992) reported estimation of total alkaloids in solanaceous species by colour complexing with methyl orange.

Chandler and Dodds (1983) reported estimation of solasodine using bromothymol blue. Estimation of glycoalkaloids as solasodine from Solanum varbascifolium was done by Lamba et al. (1987) by colour complexing with bromocresol green. The total glycoalkaloids from solanaceous species have been determined by Fitzpatrick et al. (1974), Coxon et al. (1979), Mackenzie et al. (1979). These are either colorimetric, spectrophotometric or titrimetric methods.

The above mentioned methods have been made use of to determine alkaloids from tissue cultures, a review of literature is cited in Appendix 4. In the present work a colorimetric method was devised for the estimation of total alkaloids of H.tanjorense. The method is simple and fast and a number of samples can be tested at a time. The molar absorption obtained indicated that the reagent used is sensitive. The colour of ion pair complex formed is stable over a period of 30 min. The yellow colour ion associated complex can easily be measured at 410 nm against reagent blank which is almost negligible. The colour of the complex was found to be pH sensitive. Beer's law was obeyed by pure alkaloid heterostemmine in the concentration range of 25 to 125 mcg/ml, while total alkaloids of Callus had a range of 50 to 250 mcg/ml and total alkaloids of roots 5 to 25 mcg/ml.

In conclusion the results reported in this chapter reveal unequivocal evidence that the callus developed from H.tanjorense produced alkaloids. The optimum conditions for development of callus and obtaining maximum yield of the alkaloids is the main accomplishment besides the development of

a novel quantitative method for determining the extent of alkaloids produced by the callus cultures.

CHAPTER IV
ALKALOID PRODUCTION IN SUSPENSION CULTURES OF *Heterostemma tanjorense*

Extensive studies on the production of useful compounds such as alkaloids (Sato et al., 1982), Steroids, terpenoids, quinones etc. (Mizukami et al., 1978; Fujita et al., 1983) by cultured plant cells or organ cultures have been studied for possible industrial application. Cell suspension cultures of Coptis japonica are capable of producing large amounts of alkaloids (Fukui et al., 1982; Sato and Yamada, 1984). Studies of Sumaryomo et al. (1991), Yoshimatsu et al. (1991), Gertlowski et al., (1993) indicate that suspension culture is a means of better production of plant metabolites. In this chapter studies have been oriented towards the production of alkaloids by suspension culture and the parameters employed for the growth of callus and alkaloid optimisation in chapter III have been applied here with appropriate modifications to initiate suspension cultures for optimisation of biomass as well as alkaloid production.

MATERIALS AND METHODS

4.1. Generation of callus for cell suspension

Suspension cultures were initiated from seedling derived callus as in section 2.13. callus maintained on MS medium 1/2 strength under optimum conditions established at Table 3.18 was used for inoculation of suspension cultures after 25 passages.

4.2. Media for cell suspension

MS medium 1/2 strength containing 2% sucrose supplemented

with 2, 4-D (0.5 mg/L), IBA (0.25 mg/L) and kn (0.25 mg/L) was distributed in 20 ml amounts in 100 ml and 40 ml in 250 ml conical flasks and sterilized as per section 2.4. Conditions were varied whenever required as per MATERIALS AND METHODS in Chapter III.

4.3. Preparation of stock suspension culture

Initially 500 mg of callus was placed in 100 ml conical flasks containing 20 ml of media with glass beads (9 mm diam 6 in each flask) to break up the callus in pieces. The suspension cultures were allowed to grow under normal room light conditions on rotary shaker at 100 r.p.m. maintained at room temperature. After shaking for 4 weeks, 10 ml of the resulting suspension was diluted to 30 ml with fresh medium. After a further 4 week incubation 10 ml of this second subculture was inoculated in a 250 ml conical flask containing 40 ml of medium and incubated on rotary shaker to establish a stock suspension. This stock suspension was regularly subcultured every 4 weeks to maintain the cell line.

4.4. Inoculation of medium with stock suspension culture

A series of 250 ml flasks containing 40 ml of MS medium 1/2 strength were inoculated in triplicate with 10 ml of stock suspension and sealed with a square of aluminium foil. The cultures were allowed to grow on rotary shaker for 6 weeks at a speed of 100 r.p.m.

4.5. Detection of growth (Microscopic)

Increase in turbidity due to released plant cells was visually observed as the indication of growth. Also microscopic

examination of the cells was done. For this 1 ml of four week old suspended cells were centrifuged at 3000 r.p.m. for 10 mins. and the pellet obtained was used to prepare wet mount. The cells were stained with safranine and observed under microscope.

4.6. Measurement of Growth

The suspension cultures were harvested every week for six weeks to determine the growth response. Growth was measured as dry weight yield per flask. To obtain this data, fresh cells from individual flasks were collected in preweighed centrifuge tubes. The samples were centrifuged at 3000 r.p.m. for 10 mins and washed with water to free from media. Water was drained from the tubes with the aid of filter paper strips and the compact cells were weighed to obtain fresh wet weights. Dry weights were obtained by drying the cells at 80^0C overheight.

4.7. Estimation of total alkaloids in suspension culture

The dried samples of cells obtained in section 4.5, after 2,4 and 6 weeks were weighed and the dry weight equivalent to 200 mg was extracted in a soxhlet extractor using the same solvent as mentioned in section 2.15. The crude total alkaloids were spotted on TLC to observe the presence of alkaloids using the same procedure as in section 2.16. The total alkaloids were then estimated quantitatively as per section 3.8 and expressed as mg/g dry wt. of callus.

4.8. Extraction of stale media to observe the release of alkaloid by cells in liquid medium

The stale medium obtained in section 4.5 after centrifuging the cells was made alkaline with dilute NH_3 solution

to pH 9 and extracted with chloroform. The chloroform was evaporated and the residue obtained was tested for the presence of alkaloids by TLC as per in section 2.16.

RESULTS

4.9. Growth of H. tanjorense cells in suspension

The growth of callus cells of H. tanjorense in suspension form was measured after every week upto six weeks after inoculation in terms of fresh weight and dry weight. During the 1st week there was almost no growth (Lag phase). In the 2nd week turbidity started increasing indicating active cell division (exponential growth phase). Thereafter there was substantial increase in cells and growth reached maximum at the end of the 4th week. (Phase of rapid growth). At the end of the 5th and 6th week there was a decline in growth of cells. The results are indicated in Table 4.1. The growth response curve w.r.t. age in weeks was plotted and is represented by Fig. 4.1 . Fig. 4.2 shows a typical microscopic profile of suspended culture. Highly vacuolated morphology is depicted.

4.10. Estimation of total alkaloids in suspension culture

The dried chloroform extract of the cell mass obtained in section 4.6 was subjected to TLC analysis to observe the number of alkaloids present. Observation of TLC plates under UV light showed the presence of 15 spots. The plates were then sprayed with Dragendorff reagent which gave 12 orange spots on the plate indicating the presence of a total number of 12 alkaloids as in the extracts of callus cultures. The TLC pattern is represented

Table 4.1

Growth response and alkaloid contents in suspension culture of H.tanjorense

Age in weeks	Cell Growth		Growth Index	Total alkaloids mg/g dry wt.
	g fresh wt flask(50ml)	g dry wt/ flask(50ml)		
1	1.2	0.062	0.2	
2	2.1	0.115	1.1	4.227
3	4.6	0.220	3.6	
4	7.6	0.418	6.6	7.008
5	6.2	0.330	5.2	
6	5.1	0.226	4.1	5.451

Table 4.2

Effect of niacinamide on alkaloid production in suspension culture of H.tanjorense

Niacinamide conce. %	Cell Growth		Growth Index	Total alkaloids mg/g dry wt.
	g fresh wt/ flask(50ml)	g dry wt./ flask(50ml)		
0	8.1	0.438	7.1	7.342
0.01	4.20	0.216	3.20	11.450
0.025	3.40	0.184	2.40	6.897
0.05	5.68	0.269	4.68	9.345

Table 4.3

Effect of tryptophan on alkaloid production in suspension culture of H.tanjorense

Tryptophan conc. %	Cell Growth		Growth Index	Total alkaloids mg/g dry wt.
	g fresh wt/ flask(50ml)	g dry wt./ flask(50ml)		
0	8.1	0.438	7.1	7.342
0.025	8.4	0.412	7.4	9.567
0.05	8.2	0.392	7.2	8.688
0.1	7.8	0.378	6.8	7.676

FIG. 4.1

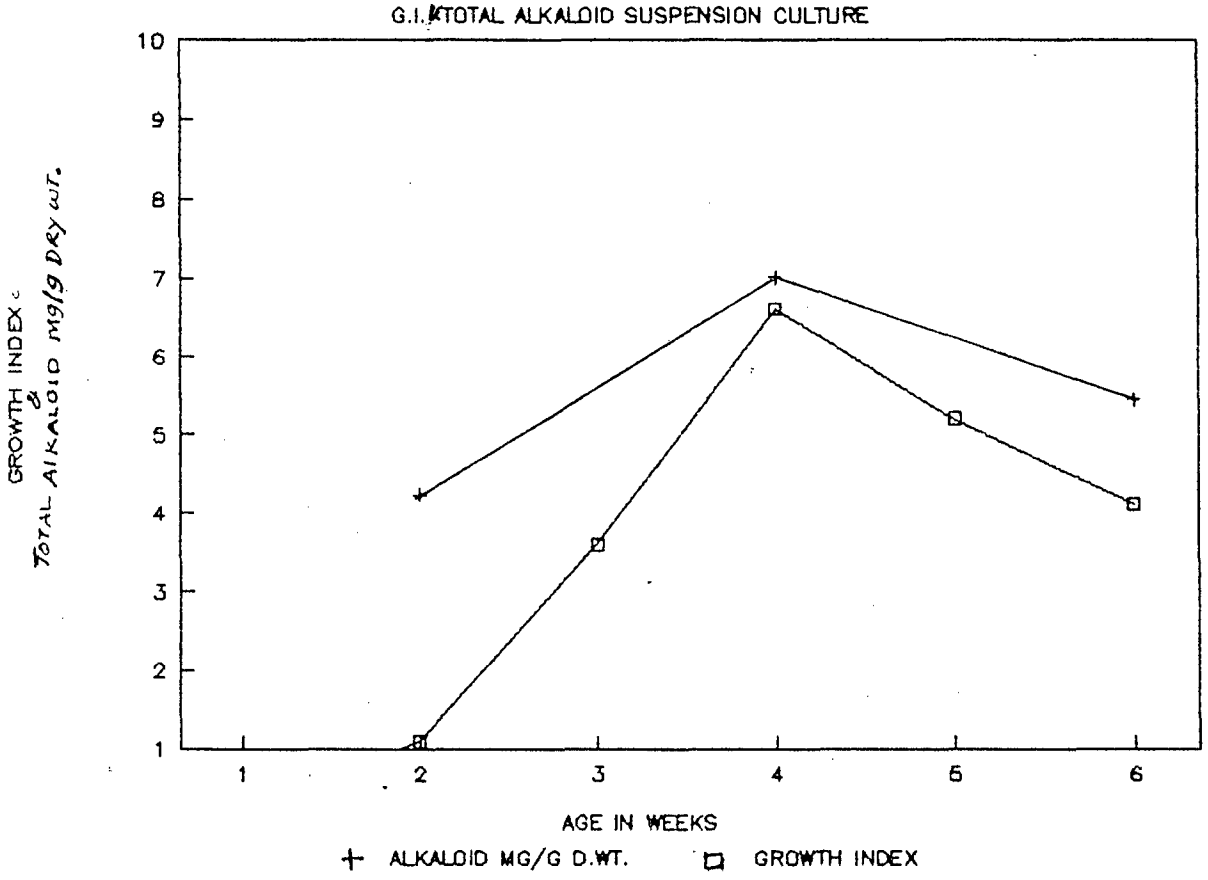

G.I. & TOTAL ALKALOID SUSPENSION CULTURE
GROWTH INDEX & TOTAL ALKALOID MG/G DRY WT.
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
AGE IN WEEKS
ALKALOID MG/G D.WT.
GROWTH INDEX

Fig. 4.2
Microscopic profile of cells of H. tanjorense suspension culture cells.

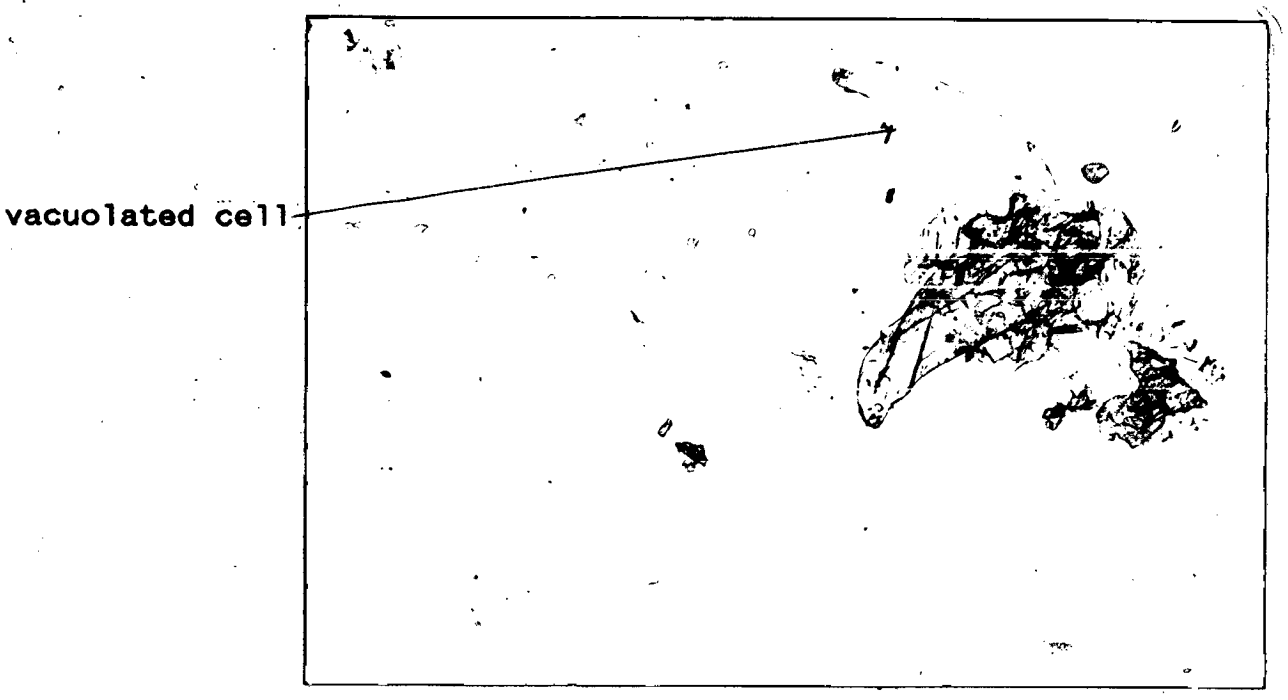
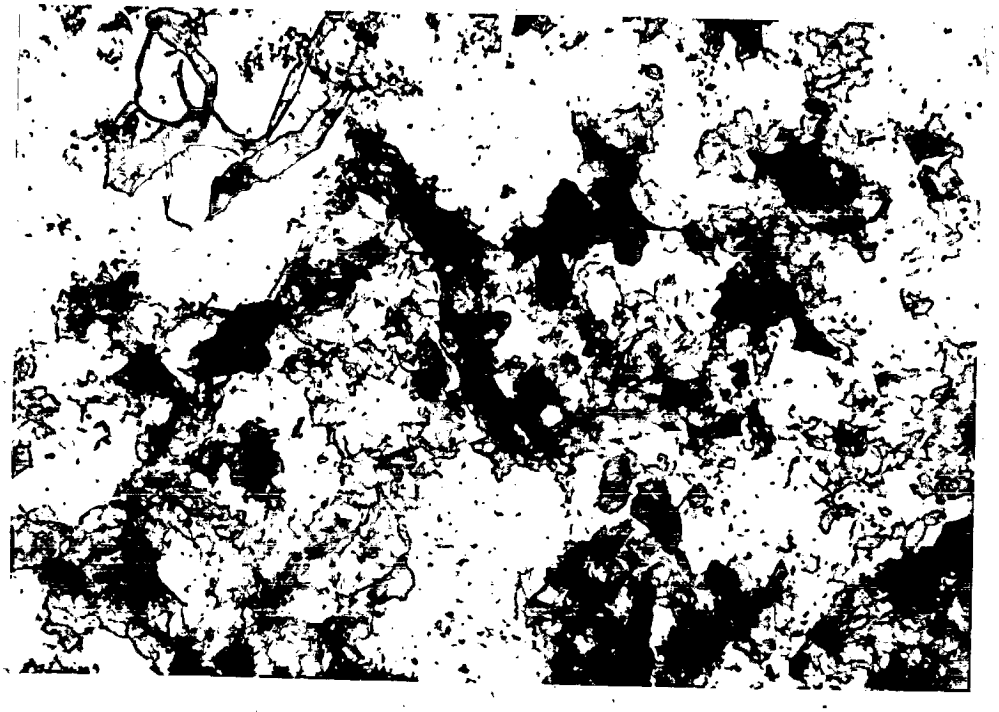


Fig. 4.2 Wet mount of suspension culture (vacuolated cell)



Cells stained with safranine.

by Fig. 4.5. The quantitative estimation of the total alkaloids indicated that the maximum total alkaloids were obtained after 4 weeks and the concentration decreased in 5^{th} and 6^{th} week Table 4.1, Fig. 4.1.

4.11. Observation of alkaloids in stale medium

The chloroform extract of the stale medium obtained in section 4.8 of MATERIALS AND METHODS when subjected to TLC analysis gave a very faint spot at the base level of the plate after spraying with Dragendorff reagent indicating that the amount of total alkaloids was negligible as compared to the extract of whole cells.

4.12. Optimisation of the yield of alkaloids in suspension cultures

Conditions for maximum yield of the alkaloids was studied based on the results obtained in Chapter III, section 3.12, where growth substances like niacinamide and tryptophan increased the alkaloidal contents to a considerable amount. Varying concentrations of niacinamide and tryptophan were fed in suspension cultures to optimise the yield. Concentrations of niacinamide used were 0.01,0.025 and 0.05 %, while that of tryptophan were 0.025, 0.05 and 0.1 %. It was observed that niacinamide did not favour the growth of cells in cell suspension culture but increased the total alkaloidal contents when the concentration of niacinamide was 0.025 %. However, tryptophan increased the cell biomass and also the total alkaloidal contents when a concentration of 0.05 % of tryptophan was used. The maximum yield of the alkaloids in the case of niacinamide and

tryptophan was obtained at the end of 4 weeks. The results are presented in Tables 4.2, Table 4.3 and in Fig. 4.3 and Fig. 4.4.

DISCUSSION

Suspension cultures are initiated using calli maintained on semisolid media after at least for six to ten passages by which time the calli grow at a fairly constant rate indicating an adaptation to the medium. Dispersion is achieved by placing small sections of callus into liquid medium and shaking the flask on orbital shaker. Friable callus is easier to initiate suspensions. Sometimes flasks with indentations are used which physically break up the aggregates. The callus obtained in the present study after 25 passages was hard and hence was cut in pieces whilst inoculating the medium. Glass beads were used for dispersion of the callus in suspension. A uniform suspension was obtained after 4 weeks of shaking on rotary shaker. Suspension cultures are generally morphologically more homogeneous and hence offer advantages over callus cultures. Stability of culture is of great importance for reproducible experiments and regular subculture is essential to maintain a stable cell line. It is best to subculture cells at a defined stage of growth and this is usually performed towards the end of exponential growth phase. Subculturing is achieved by transferring a fixed volume of culture into fresh growth medium at constant time intervals.

The inoculum size varies depending on the research aims. For example 400 mg of callus was used by Chandler (1983) for solasodine production, while 0.7 g of free cells were used by

FIG. 4.3

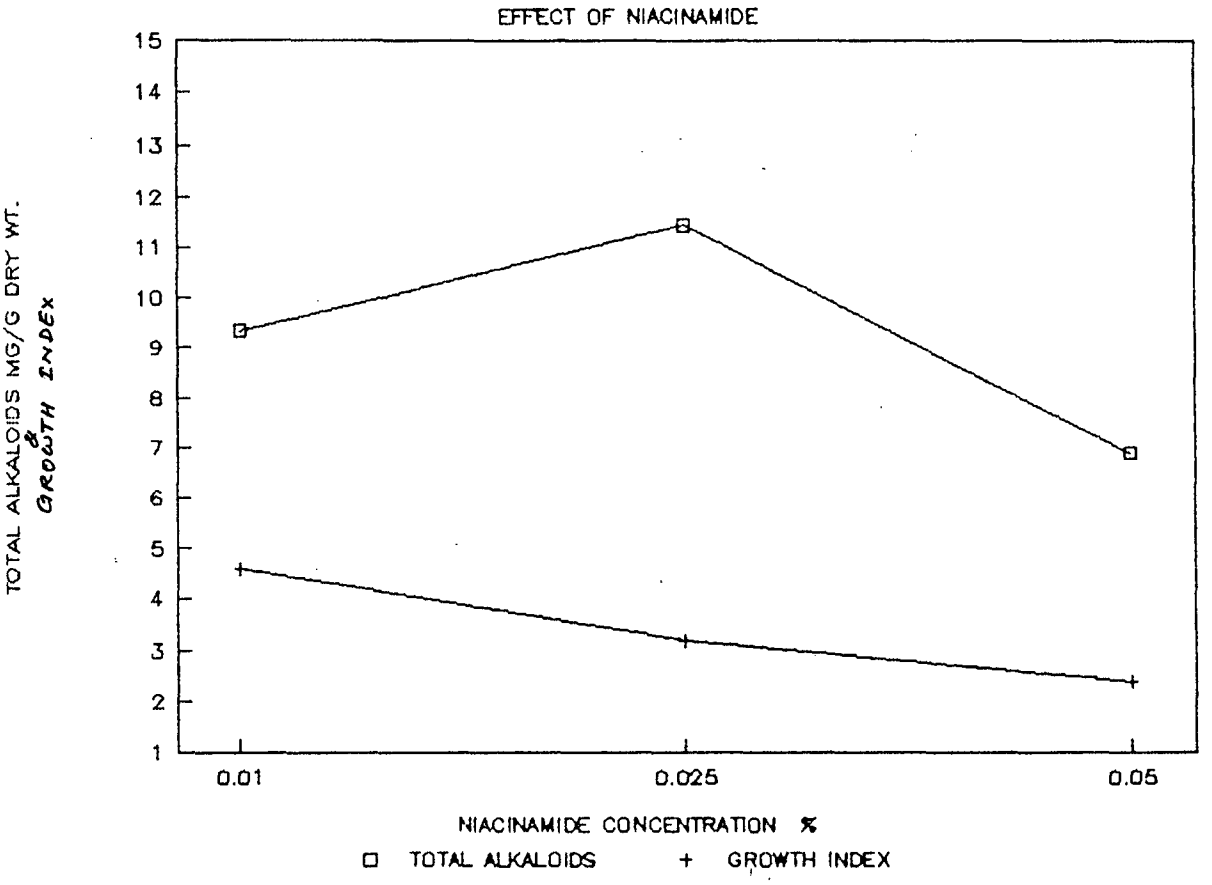

EFFECT OF NIACINAMIDE
TOTAL ALKALOIDS MG/G DRY WT.
&
GROWTH INDEX
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0.01
0.025
0.05
NIACINAMIDE CONCENTRATION %
TOTAL ALKALOIDS
GROWTH INDEX

FIG. 4.4

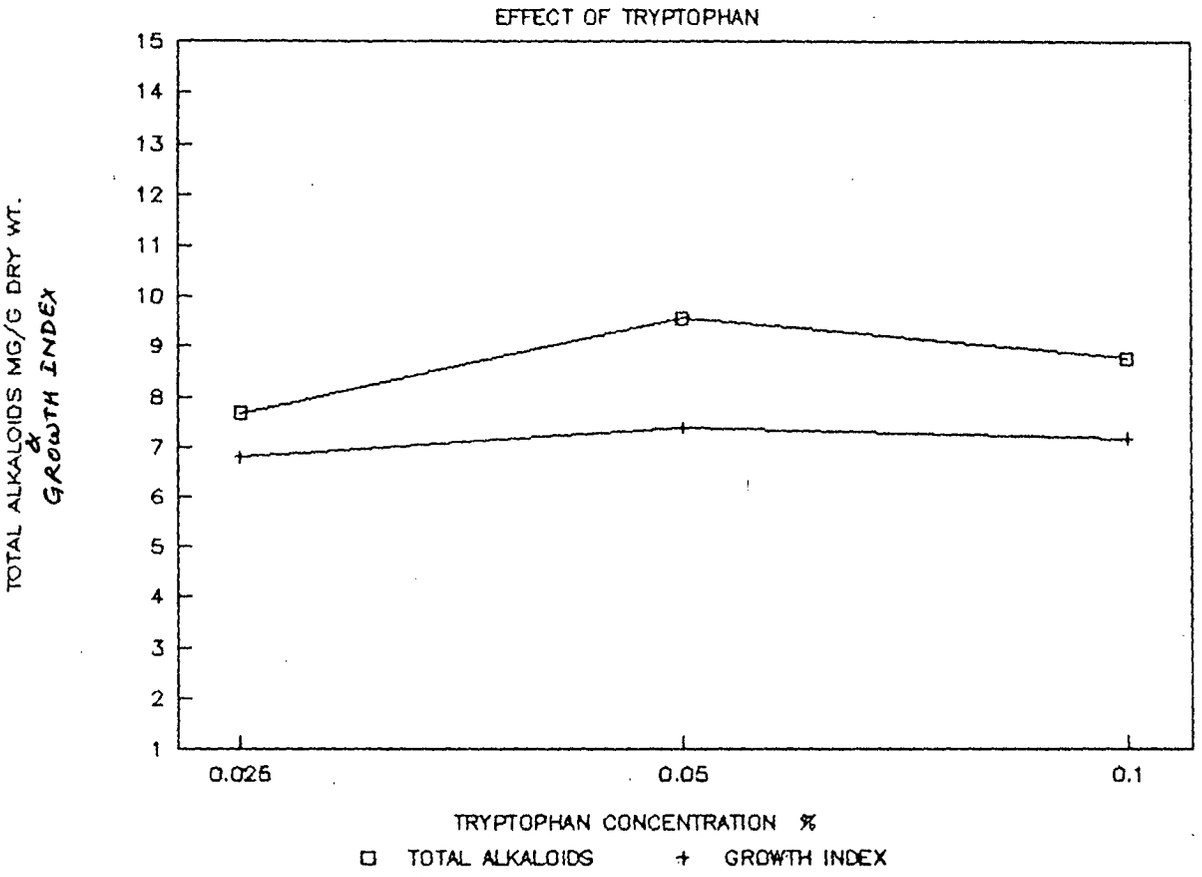
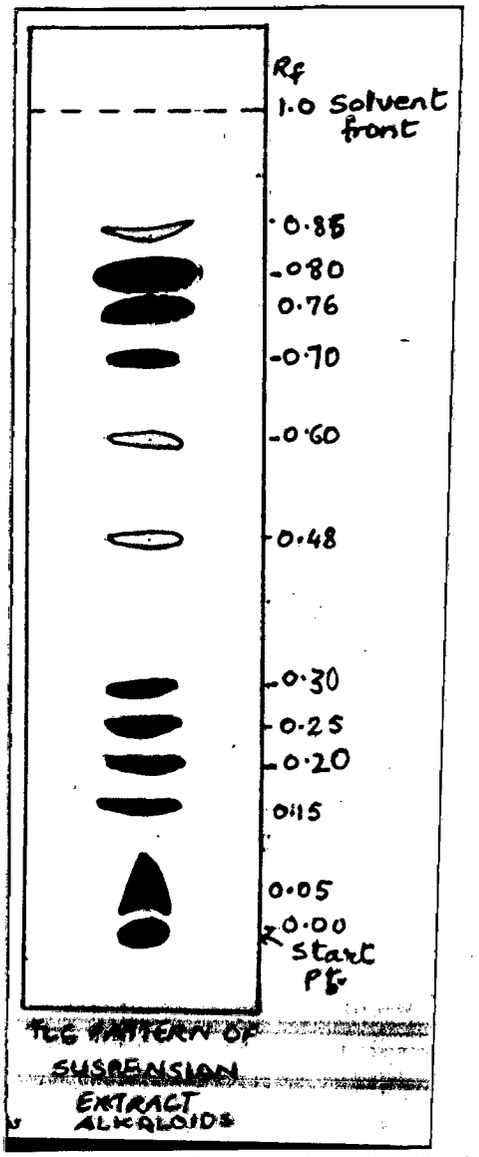

EFFECT OF TRYPTOPHAN
TOTAL ALKALOIDS MG/G DRY WT.
&
GROWTH INDEX
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0.025
0.05
0.1
TRYPTOPHAN CONCENTRATION %
TOTAL ALKALOIDS
GROWTH INDEX

Fig. 4.5



T. L. C. Pattern of total alkaloids of suspension culture.

Nakagawa (1984) for berberine production. Yamamato and Yamada (1986) studied the effect of inoculum on cell growth by inoculating the liquid medium with 0.6,1.0 and 1.4 g of fresh weight in case of reserpine optimisation. In this study the above parameters were maintained. The initial inoculum size was 500 mg of callus which was dispersed in the medium by using glass beads. The subsequent inoculations were done after a peak growth period of 4 weeks of incubation and shaking at 100 r.p.m. on a rotary shaker.

Plant cells are large, have rigid cell wall and usually become highly vacuolated during later stages of culture cycle and are sensitive to hydrodynamic stress (Allan, 1991). Fig. 4.2 shows the morphology of cells of H. tanjorense grown in suspension culture. When cell suspension cultures are agitated, slow shaker speeds usually 50-200 r.p.m. are used so that the cells are not damaged. These features of minimum inoculation should be considered during experimental work.

It can be concluded from the above discussion and the results obtained that 20 % inoculum size of stock suspension, a growth period of 4 weeks and a speed of 100 r.p.m. optimised the biomass formation. Also the alkaloid production increased in 4 weeks. Furthermore the growth substances niacinamide and tryptophan increased the alkaloidal yield suggesting that both the substances act as precursors in the biogenesis of alkaloids though the pathway of biogenesis remains to be investigated.

CHAPTER V
CHARACTERISATION OF ALKALOID A3 AND ANTIMICROBIAL ACTIVITY OF CALLUS EXTRACT OF *Heterostemma tanjorense*

Novel Secondary metabolites known for bioactivity are generally characterised for their chemical structure. The chemical structure is arrived at from elemental analysis and undertaking UV, IR, NMR and Mass spectroscopy. The purity of the compound is ascertained by TLC and melting point. Some of the prominent biological activities tested normally include antihistaminic, diuretic, antipyretic, antitumour, antimalarial, antihypertensive, antimicrobial action, etc.

In this chapter chemical and biological characterisation of alkaloids is presented. Alkaloid A3 with Rf 0.80 is obtained from the mixture of alkaloids as depicted in chapter III, by column chromatography and purified by recrystallisation from benzene. The antimicrobial activity of the alkaloidal mixture towards prominent bacteria and fungus is also presented.

MATERIALS AND METHODS

5.1. Physico-chemical characterisation

Physical properties such as appearance, solubility, melting point was obtained. Melting point was determined by Thiel's tube method. Studies on chemical properties involved Lassaigne's test (Campbell, 1962) for nitrogen, sulphur and halogens and tests specific for alkaloids using Wagner's, Mayer's and Dragendorff reagents. (Appendix 4). Reineckate derivative was prepared by taking sample equivalent to 2 mg each of

heterostemmine reference standard and alkaloid obtained from callus and dissolving in 2ml of dilute HCl. To this 5 ml of 4% ammonium reineckate solution in water was added with stirring. The mixture was kept in cold water for 15 minutes. The pink precipitate obtained was centrifuged and washed with water to free from excess reagent and dried. It was then dissolved in 5 ml of acetone and the absorbance of both the standard and sample was scanned from 450 to 550 nm on Shimadzu UV 160 spectrophotometer. HPLC was carried out using Perkin Elmer instrument with detector set at 254 nm and Waters μ Bonda pack CN column (150 mm x 4.6 mm. i.d.: 5μ particle size). Solvent system composed of acetonitrile : water (40:60) with a flow rate of 0.6 ml/min, injection volume 20 μl and run time 12 min.

5.2. Spectral Analysis

UV scanning of the standard heterostemmine and the isolated alkaloid was done as above. The solutions were prepared in ethanol, methanol and 0.1 N HCl and scanned from 200 to 350 nm. The concentration of the solutions was 0.005 mg/ml. λ max and absorbance was noted of each sample. IR spectra were obtained in KBr disc of both the standard and isolated alkaloid. The samples were scanned on Shimadzu FTIR 1800 spectrophotometer from 400 to 4600 nm. NMR and Mass spectra were also obtained.[1]

1.Courtesy Prof. S. K. Paknikar and Prof. R. K. Bates for information on NMR and Mass spectrum and measurement of 100 MHZ, 1HNMR of heterostemmine and useful suggestions. Heterostemmine standard was obtained from Prof. S.K. Paknikar.

5.3. Analysis of Bioactivity

Static tissues, (24 month old) were finely homogenised with 95% ethanol (1g of test material in 5 ml of solvent) in a mortar and pestle and the alcoholic extract obtained was filtered. The test organisms[2] used were Staphylococcus aureus, Escherechia coli, Bacillus pumilus and Aspergillus species. The growth medium was nutrient agar for S. aureus E. coli and B. pumilus while Sabourauds agar was used for Aspergillus species. Petri dishes containing 20 ml of the medium were seeded with 1ml of 18 to 24 hr old actively growing cultures of S. aureus and E. coli while, 1 ml of spore suspension of B. pumilus prepared as per IP (1985) was used for seeding. A four day old culture of Aspergillus was suspended in Sabourauds liquid medium and 1 ml of suspension was seeded in 20 ml medium. The medium and the test organisms were mixed thoroughly to ensure uniform distribution. Paper discs (6 mm diam.) of known antibiotic concentration were used for comparison. Blank paper discs which absorbed approx. 0.1 ml of solution were used for testing the extracts. Discs dipped in 95% alcohol were used as control. Crude alcoholic extract of callus, total alkaloids of callus and isolated alkaloid were dissolved in ethanol 95% and tested along with the total alkaloids obtained from the roots of H. tanjorense. A set of 3 plates were used for

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2.Test organisms S. aureus, E. coli and B. pumilus were obtained from Food and Drugs Laboratory, Maharashtra (Bombay). Aspergillus species were obtained from Microbiology Department, Goa University.

each test organism and mean zone of inhibition recorded. For the location of the alkaloid having antibacterial activity, the total alkaloids of the callus extract were subjected to preparative TLC on silica gel GF 254. Plates of 200 x 200 mm with 0.5 mm silica gel layer thickness were prepared and dried. The plates were then charged with the sample of the total alkaloids dissolved in chloroform and run in the same solvent as per section 2.16. The plates, were first viewed under UV light and then the edges were sprayed with Dragendorff reagent to serve as guideline for scrapping off the fractions. The spots visible under UV and those obtained after spraying with Dragendorff reagent were scrapped off the plate and eluted with 95% ethanol. The ethanol fractions were filtered and concentrated. A total number of 15 fractions were collected and tested for antibacterial activity as above. The spectral details of this alkaloid are given in section 5.6.

RESULTS

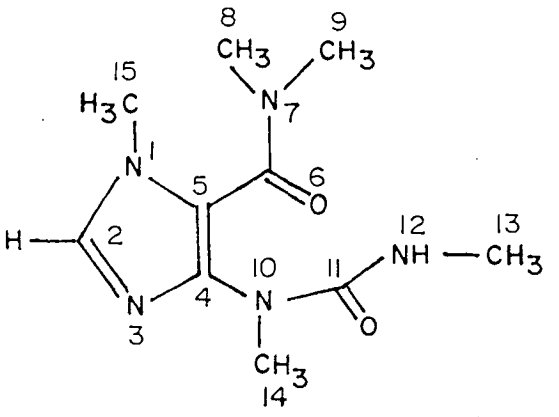
5.4. CHARACTERISATION OF THE ALKALOID A3

Physico-chemical tests indicated that the isolated alkaloid A3 had white, crystalline, needle shaped appearance. It was sparingly soluble in benzene, soluble in chloroform, methanol and acetone and sparingly soluble in water. Its melting point was 164 ^{0}C which was identical to heterostemmine. Also the mixed melting points of the isolated alkaloid A3 and heterostemmine indicated that the alkaloid was identical to heterostemmine. Lassaigne's test showed presence of nitrogen and absence of sulphur and halogens. Wagner's reagent gave brown flocculent precipitate,

Mayer's reagent gave a white precipitate while Dragendorff reagent gave an orange precipitate indicating positive test for alkaloids. The reineckate derivatives formed of the standard heterostemmine and the isolated alkaloid A3 was dissolved in 5 ml of acetone and scanned on spectrophotometer from 450 to 550 nm. Both derivatives showed identical λ max between 520 to 525 nm. Fig. 5.1. The melting point of both the reineckate derivatives was 146^0 C. The UV spectra in ethanol, methanol and 0.1 N HCl are given in Table 5.1 and represented by Fig. 5.2. The IR spectra of heterostemmine and the isolated alkaloid A3 (KBr) showed bands at ν 3370 (-NH), 1670 (>C=O), 1610 (>C=C<^{H}) cm^{-1} and both the spectra were superimposable as depicted in Fig 5.3. Thin layer chromatograms of isolated alkaloid A3 and heterostemmine also showed that the homogenous spots of both comigrated with each other Fig. 3.11. HPLC analysis indicated also that both the compounds had identical retention times (1.56 min. for pure heterostemmine and 1.53 min. for alkaloid A3). The results therefore confirm that both the alkaloids are identical compounds Fig. 5.4.

5.5. Structure Elucidation of Heterostemmine

The alkaloid heterostemmine was considered earlier to be a pyrimidine derivative (Bhonsle, 1973), but the spectral analysis reported herein shows unambiguously that it is a new imidazole derivative having structure as follows :



High resolution Mass spectrometry and elemental analysis proved heterostemmine to have molecular formula $C_{10}H_{17}N_3O_2$. The 70 eVEI mass spectrum Fig. 5.5 showed besides the molecular ion at m/z 239 (100%) other fragments at m/z 222 (22), 211 (23), 210 (53), 196(47), 195(44), 194(38), 181(19) and 42 (30).

Its UV spectrum λ_{max}^{EtOH} 219, 247.5 and 269 nm Fig. 5.6 was characteristic of substituted imidazoles. The IR spectrum of heterostemmine (KBr), Fig.5.7 showed sharp bands at ν 3370 (-NH), 1670 (>C=O), 1610 (>C=C<^{H}) cm^{-1}.

The structural features of heterostemmine were evident from its ^{1}H NMR spectrum (CDCl3) Fig. 5.8 which confirmed the presence of 17 hydrogens of which 15 belonged to 5 methyl groups, one as imidazole ring hydrogen and the remaining one as amidic proton. The singlets at δ 31.12 (6H) and 3.80 (3H) accounted for three tertiary methyl and three proton doublet at δ 2.93 (J = 5.0 Hz) could be assigned to a secondary methyl group. The fifth methyl appeared as a sharp doublet at δ2.94 but the J value of 0.8 Hz indicated it to be due to 4J long range coupling. The doublet

Table 5.1

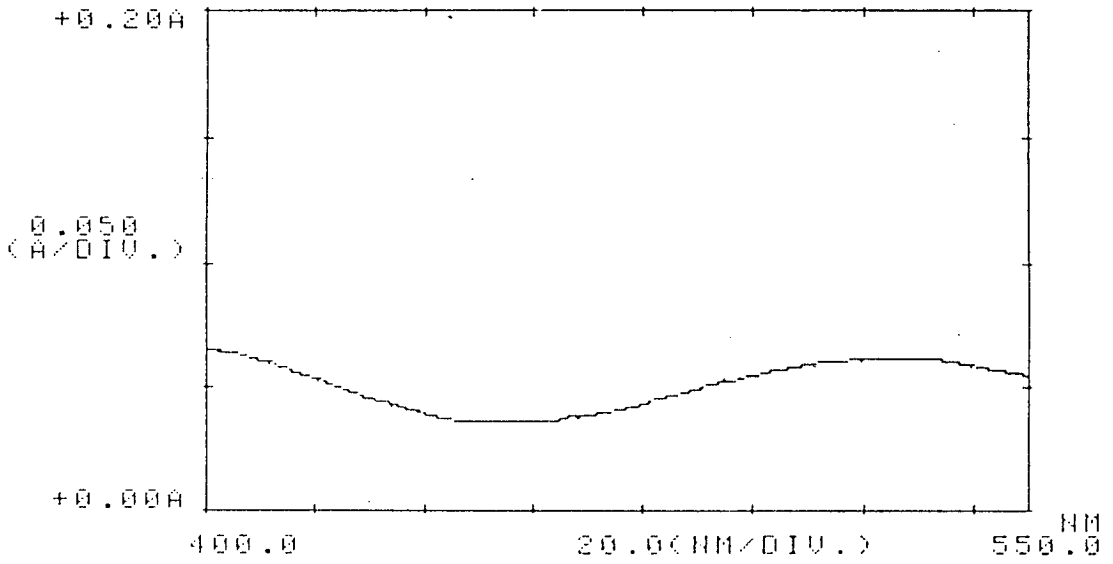
UV spectrum of Heterostemmine std. and callus isolated alkaloid A3

Sample concentration : 0.005 mg/ml in respective solvent.

Solvent	UV Spectrum			
	Heterostemmine		Callus isolated alkaloid	
	λmax	absorbance	λmax	absorbance
Ethanol	247.8 220.8 269.5	0.481 0.907 0.320	247.4 220.6 269.0	0.482 0.958 0.322
Methanol	247.6 220.0 268.5	0.481 0.867 0.336	248.0 220.6 267.5	0.489 0.904 0.330
0.1 N Hcl	295.0 242.2 204.0	0.485 0.467 0.849	295.0 241.4 204.2	0.486 0.455 0.871

FIG. 5.1

λMax of reineckate derivative of heterostemmine



λMax of reineckate derivative of alkaloid A3

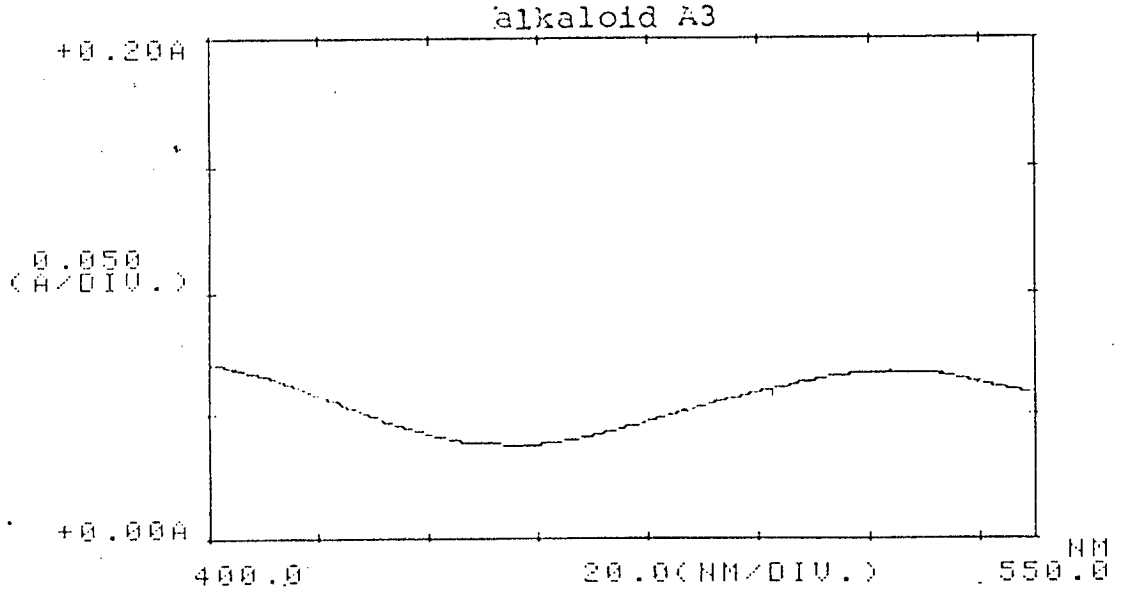
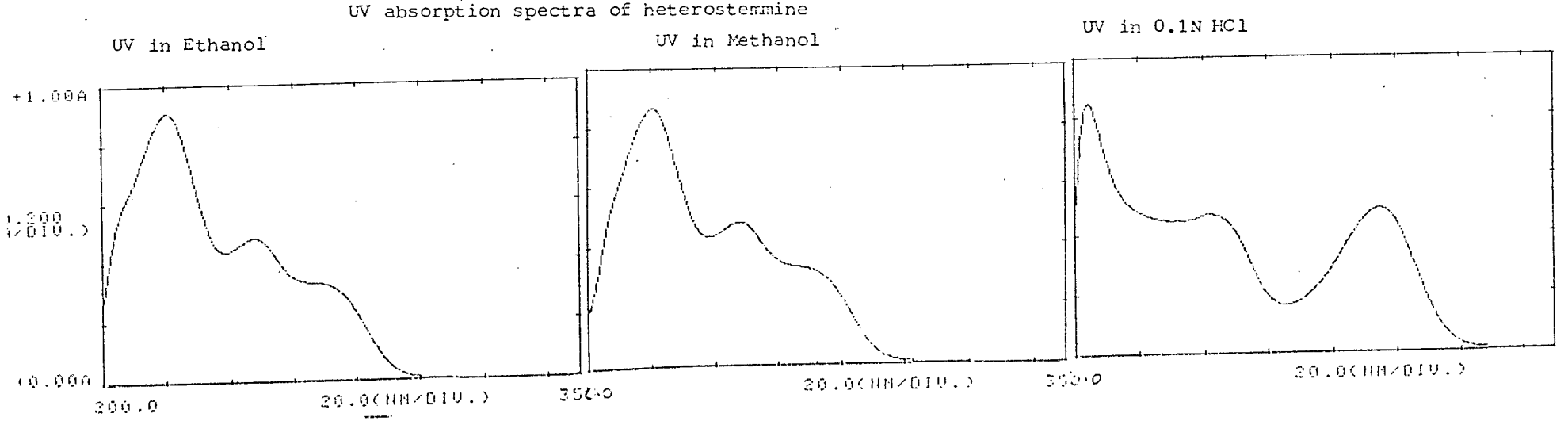


FIG. 5.2

UV absorption spectra of heterostemmine



UV absorption spectra of alkaloid A3

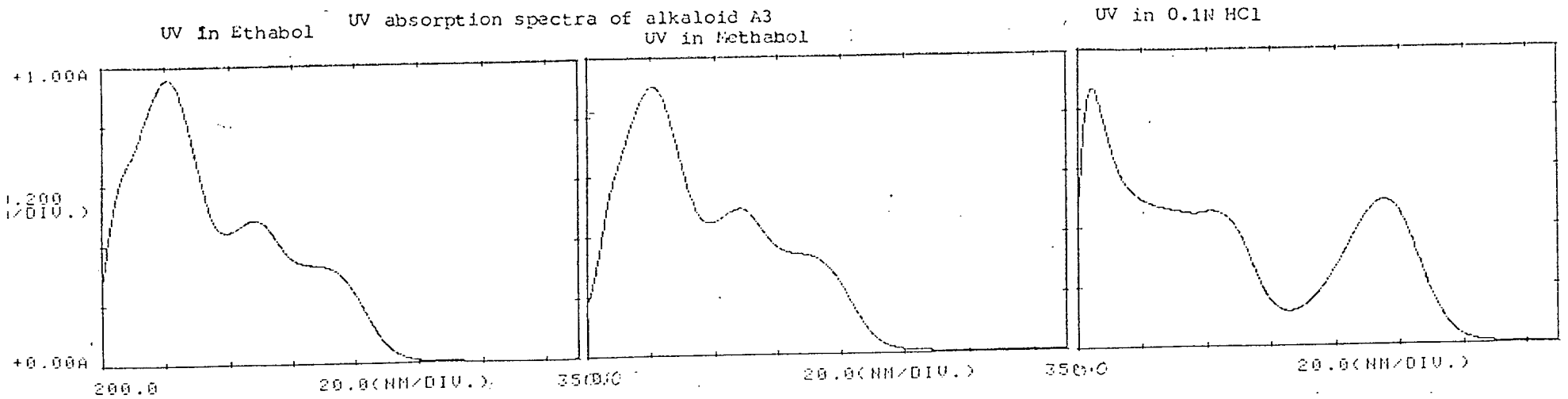


FIG. 5.3

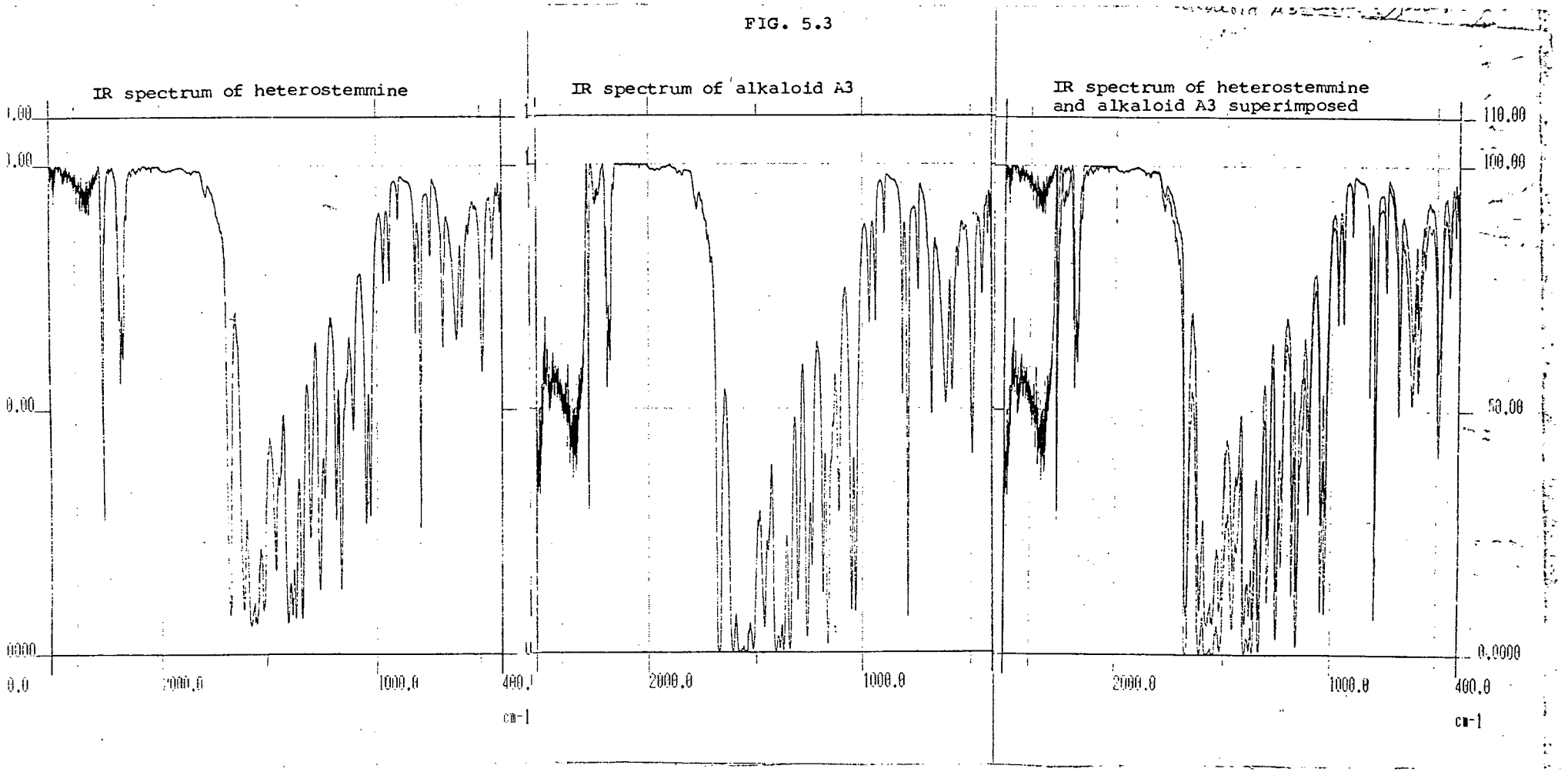

IR spectrum of heterostemmine
IR spectrum of alkaloid A3
IR spectrum of heterostemmine
and alkaloid A3 superimposed
110.00
100.00
50.00
0.0000
2000.0
1000.0
400.0
cm-1

FIG. 5.4

HPLC Profile

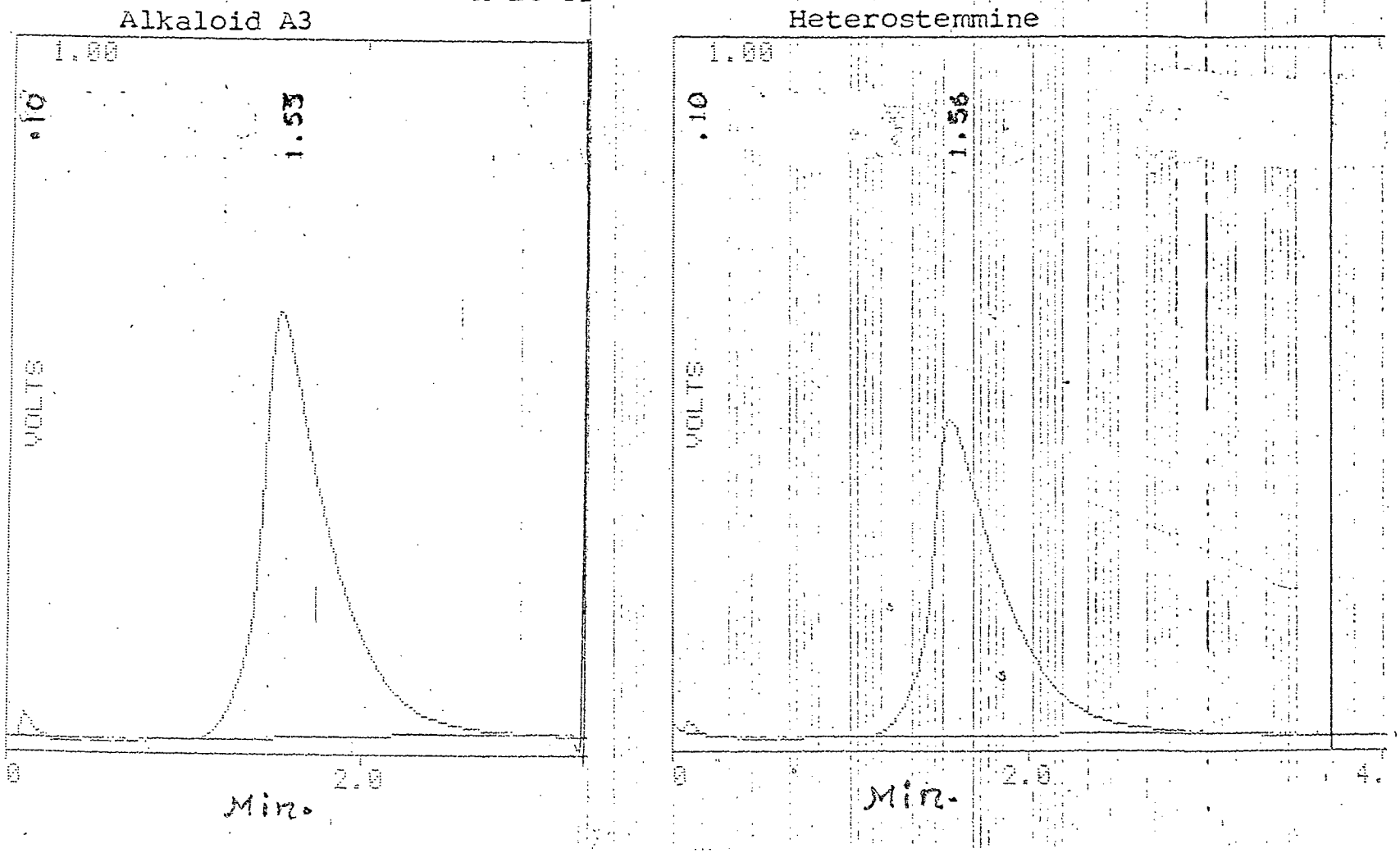


Fig. 5.5

Mass spectrum of heterostemmine

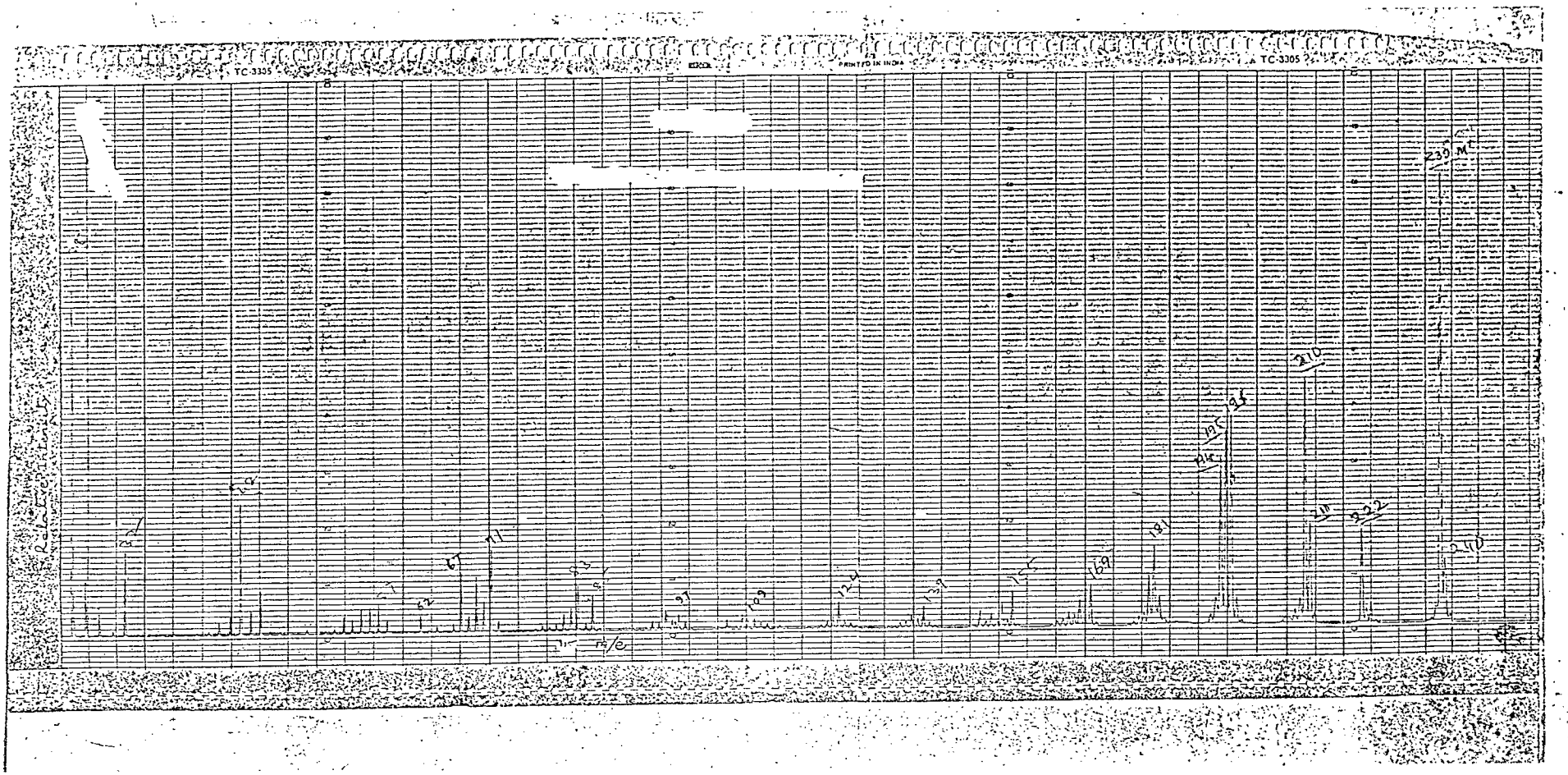


Fig. 5.6
UV Spectrum of heterostemmine

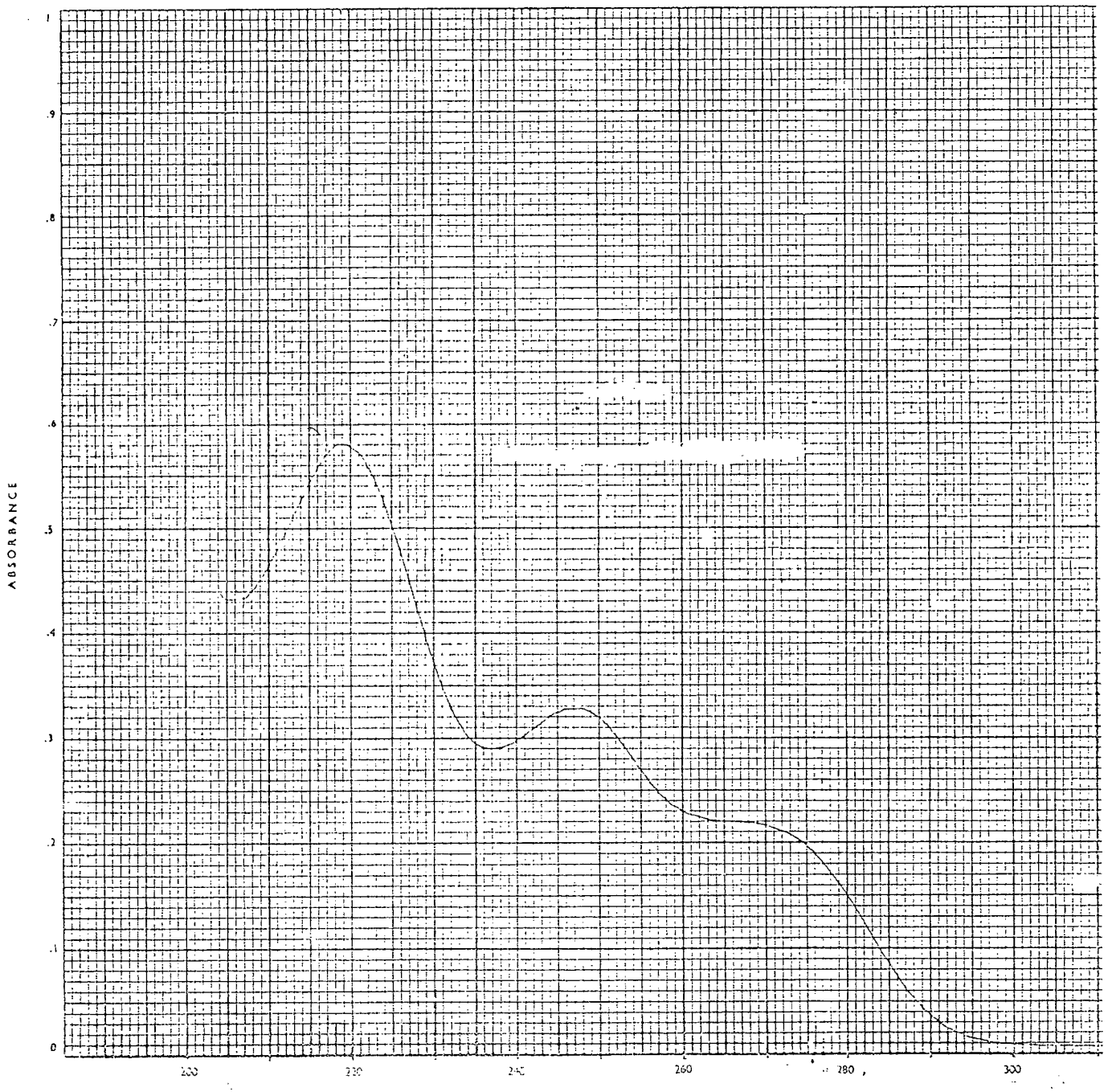
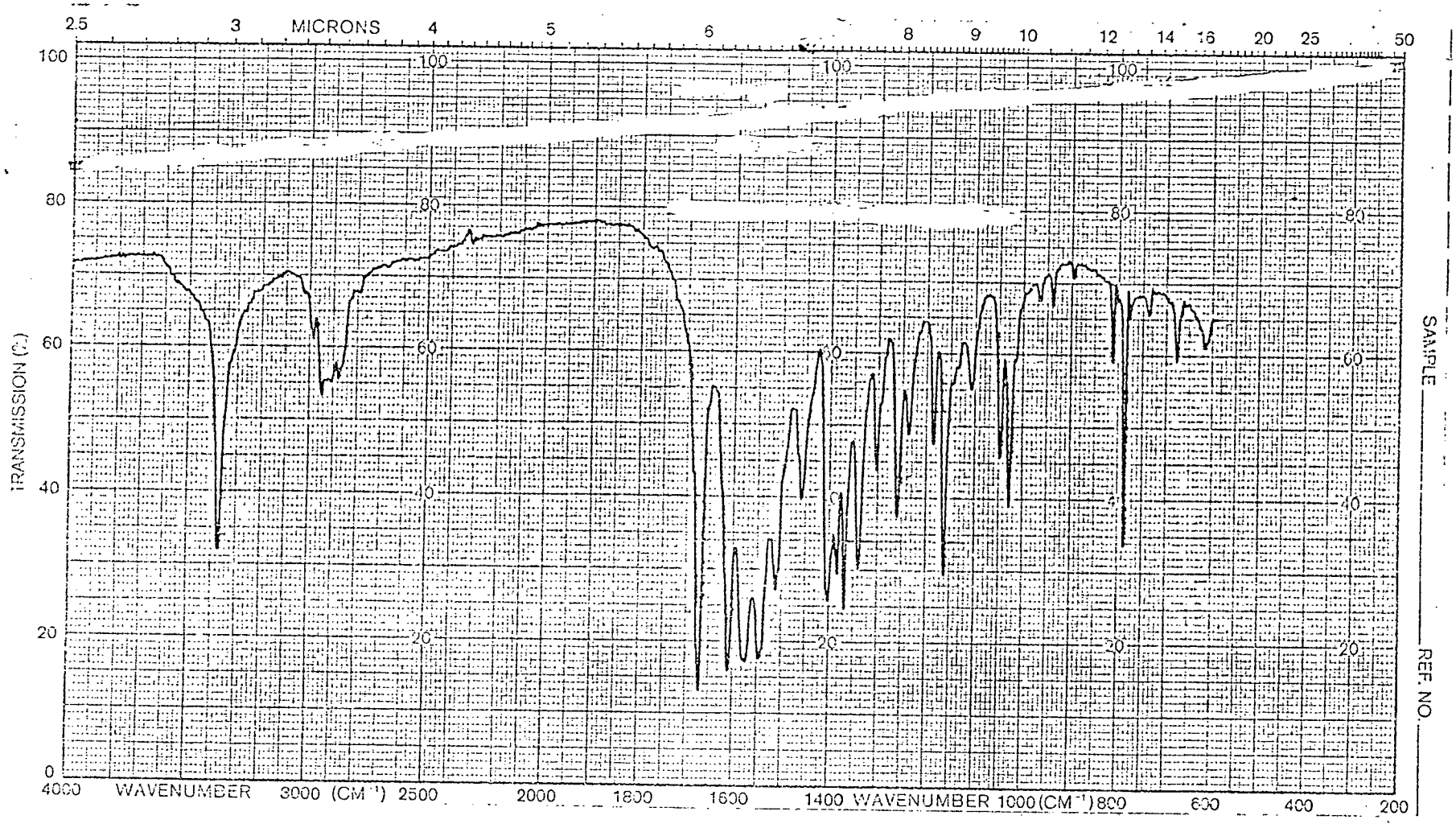


Fig. 5.7

IR Spectrum of heterostemmine



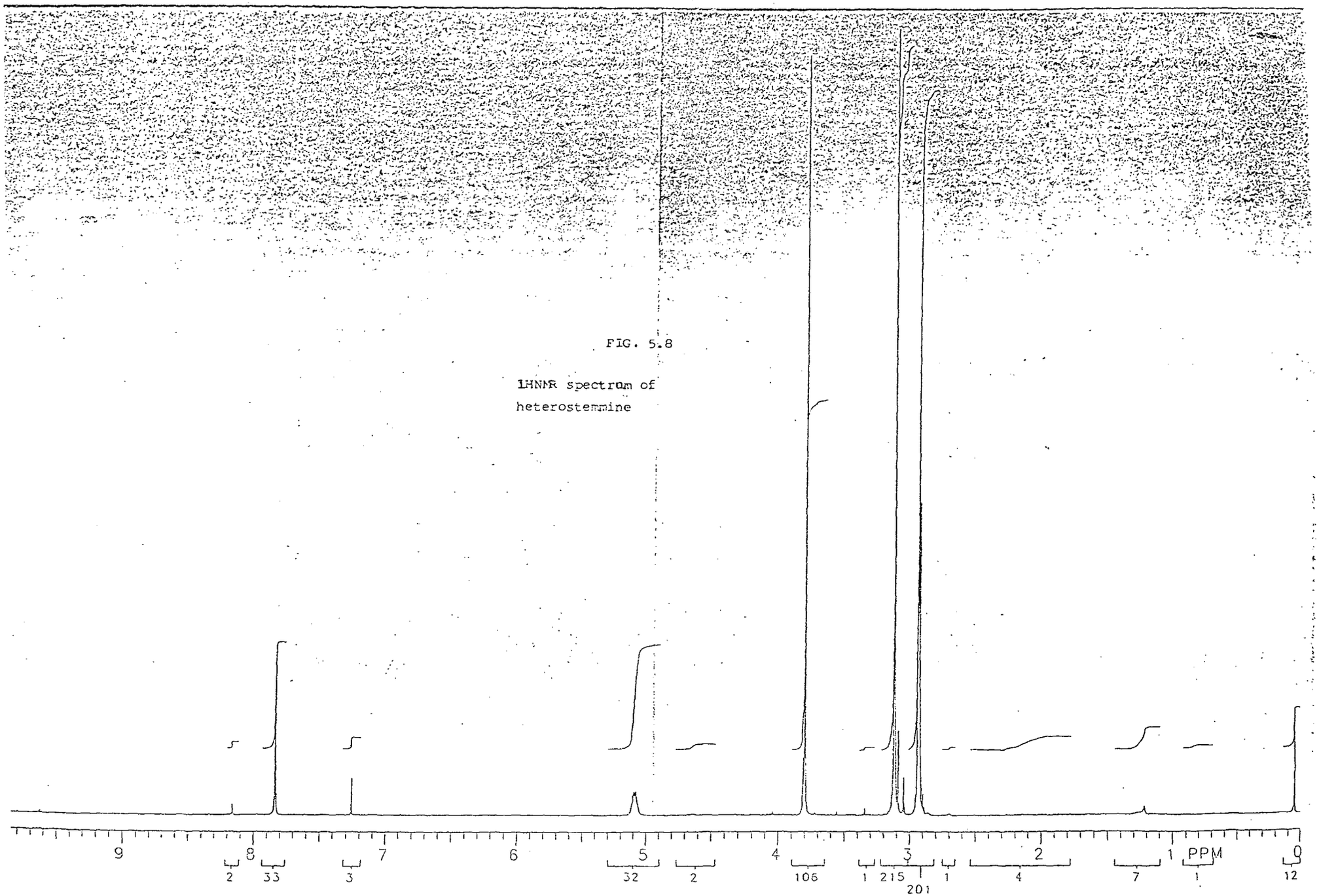
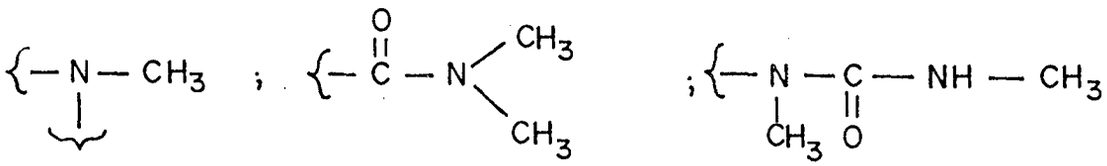


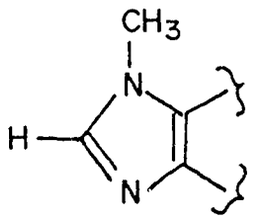
FIG. 5.8

^{1}HNMR spectrum of heterostemmine

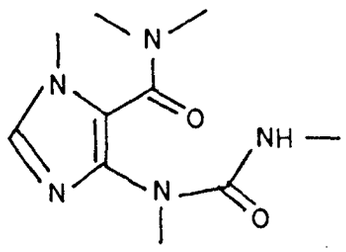
at δ 7.84 with the same coupling constant (J = 0.8 Hz) is due to H-2 of imidazole ring and indirectly suggested, the presence of a methyl group attached to one of the imidazole ring nitrogen. Finally, a three proton doublet atδ2.93 (J=5.0 Hz) and a one proton quartet (J=5.0 Hz) atδ5.09 was assigned to a -NH $-CH_3$ grouping. This was further confirmed by observing the disappearance of the signal atδ5.09 on equilibration with D_2O.

Considering the presence of 5 nitrogens in heterostemmine and also an imidazole ring together with the chemical shift of the methyl groups, all the five methyls are present as $-NCH_3$ groups. The above spectral data suggested the presence of following part structures :

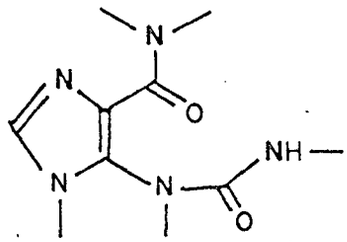




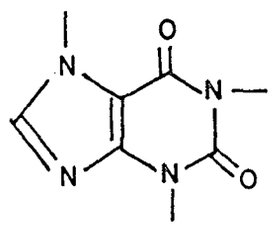
These part structures can be nicely accommodated in terms of structure 1 or 1a for heterostemmine.



1

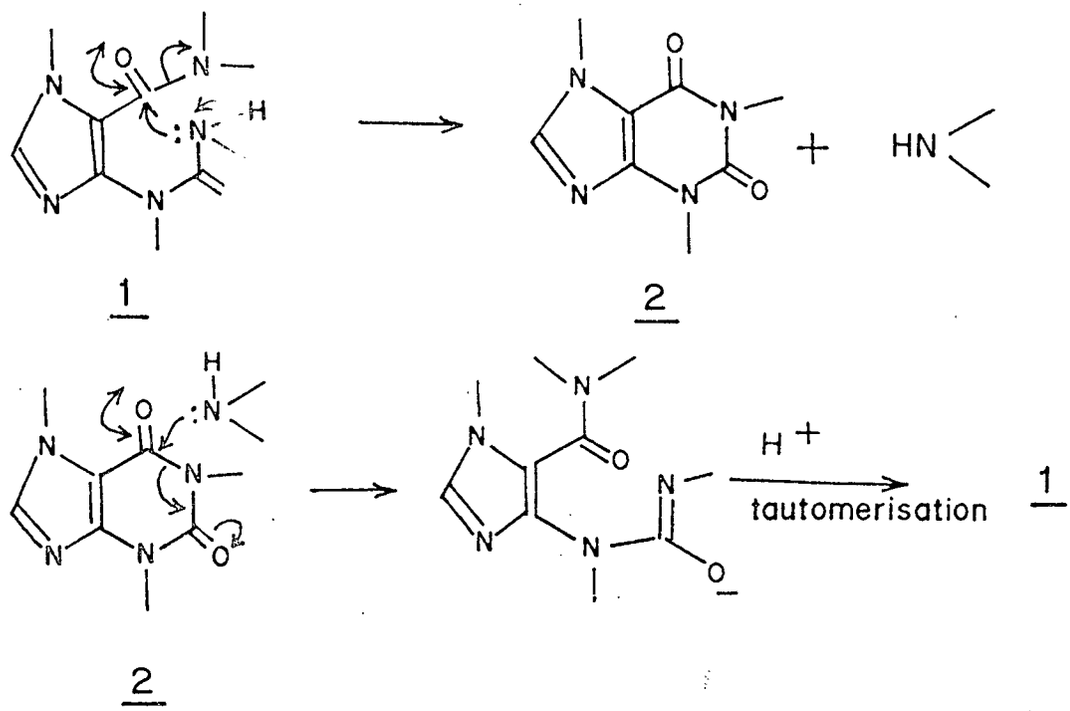


1a



2

The close structural relationship between 1 and the purine base caffeine 2 is immediately evident if one considers their chemical interconversion 1 -> 2 by intramolecular nucleophilic displacement of dimethyl amine or 2 -->1 by nucleophilic opening of caffeine ring system as shown in following equations (Scheme 1).



SCHEME I

In order to choose the correct structure between 1 and 1a for heterostemmine, it was of interest to attempt its conversion to caffeine 2. However, because of availability of heterostemmine in microquantities, we decided to start with caffeine 2 which is commercially available and try its conversion into heterostemmine or its structural isomer.

No reaction occurred when 2 was treated with dimethylamine or its hydrochloride at different temperatures and different time intervals. It is quite likely that the conversion may take place if the reaction is carried out in a sealed tube. It is also of interest to note that we failed to detect the presence of caffeine 2 in the alcoholic extract of the roots of H. tanjorense.

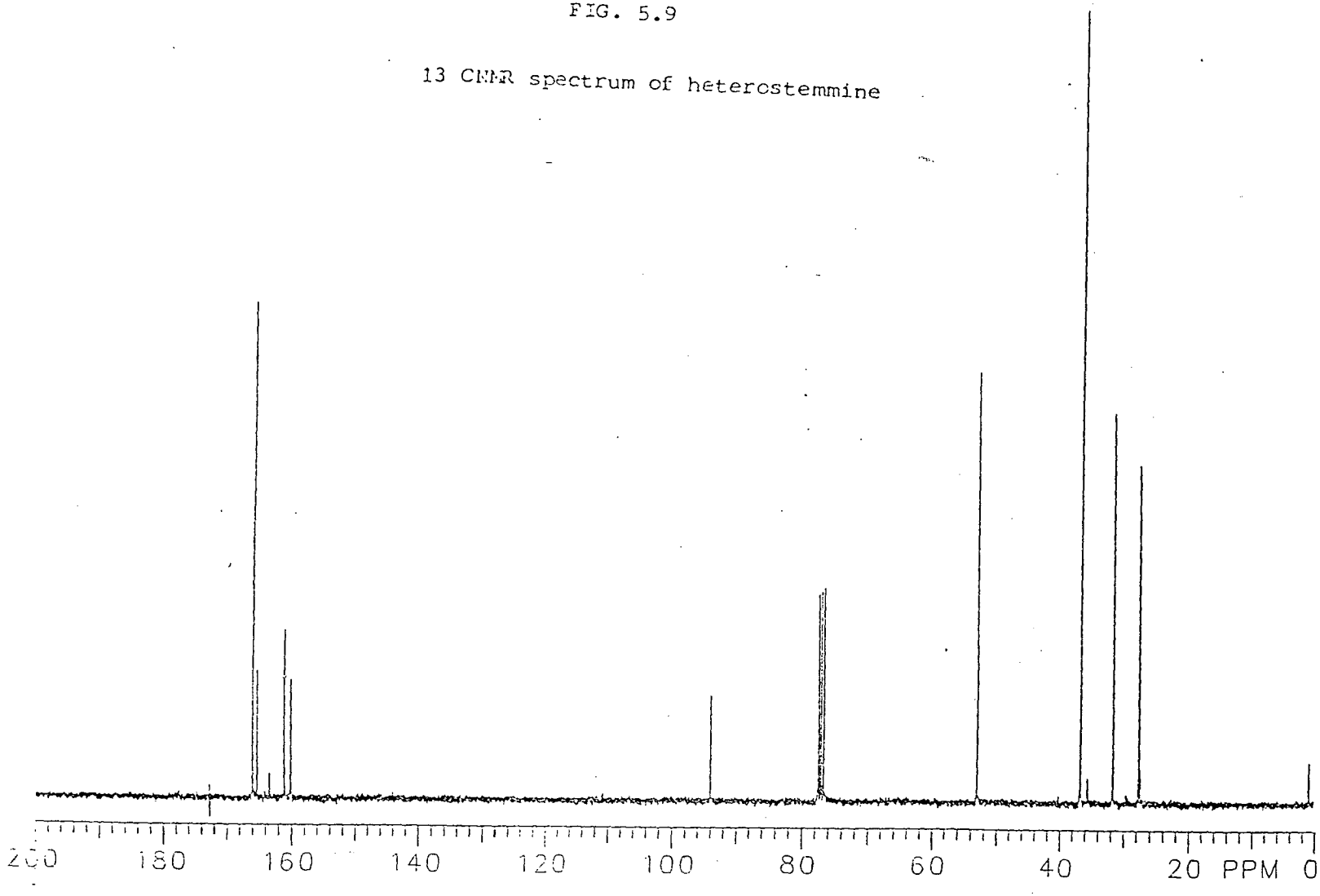
As mentioned earlier, the limited quantity of heterostemmine available precluded any degradative studies. The crystals of heterostemmine were also not suitable for x-ray analysis which would have given the final answer to the correct structure for heterostemmine. Finally, heterostemmine was shown to be correctly represented by structure 1 on the basis of extensive spectral analysis presented below.

The ^{13}C NMR spectrum Fig 5.9 showed the presence of four singlets at δ 93.90 ; 160.02, 161.0 and 165.18, one doublet at δ 165.90 and four quartets at δ 27.61; 31.60;36.67 and 52.92. The quartet at δ 36.67 is of double intensity and hence the ^{13}C NMR spectrum is fully consistent with the structure at either 1 or 1a.

The position of the doublet at δ 165.90 together with an extremely large one bond coupling constant of 196 Hz is typical of heteroaromatic SP^2 carbon between two nitrogen atoms typical of imidazole ring. Since there are only two quaternary SP^2 carbons besides two amidic carbonyl carbons, it follows that heterostemmine contains a 4,5 disubstituted imidazole ring system. The signal at δ 165.9 is actually observed as doublet of quartets indicating N-methylation at N-1. This is further supported by the

FIG. 5.9

13 CNMR spectrum of heterostemmine



observed ^{3}J splitting of ~4.0 Hz of the quartet at δ 31.60.

This consecutive splitting into a doublet is also observed for the quartet at δ 27.60 but with a typical small ^{2}J value of only 2.5 Hz clearly originating from a $-N-CH_3$ fragment. The quartet of double intensity shows further splitting into a quartet clearly showing the presence of a N-N-dimethyl amido grouping with a three bond interaction. The pure quartet at δ 52.92 is due to $-N-CH_3$ group between carbonyl and imidazole ring carbon with no long range interaction. C-4 and C-5 singlets with long range interactions at δ 165.2 and 93.9 respectively reflect the hetero and homosubstitution respectively.

The one-bond-Hetcor spectrum Table 5.2 displays crosspeaks for C-2 <--> H-2, C-14 <--> H-14, C-8/9 <--> H-8/9, C-15<--> H-15 and C-13 <--> H-13 and confirms the identification of carbon and proton signals derived from proton coupled carbon spectra and proton spectra respectively.

The final conclusion in favour of structure 1 for heterostemmine could be reached by recording the long range Hetcor spectra which showed crosspeaks for C-15 <--> H-2, C-5 <--> H-15, C-6 <--> H- 8/9, C-11 <--> H-13, C-4 <--> H-14, C-2 <--> H-15. The details are presented in Table 5.3

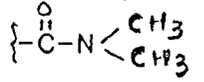
The most important feature is the three bond interaction between C-5 and the 15 $-CH_3$ protons which unambiguously established the attachment of  grouping to Cz5 which is vicinal to imidazole ring $-N-CH_3$ group. On the other hand there is no such interaction between C-4 and ring $N-CH_3$ group. The ^{1}H and ^{13}CNMR assignments now based on structure 1 for heterostemmine are

Table 5.2

Hetcor correlation of **1**

Carbon	δ (ppm)	Proton	δ (ppm)
C-2	165.90	H-2	7.84
C-14	52.92	H-14	3.80
C-8/9	36.67	H-8/9	3.12
C-15	31.60	H-15	2.94
C-13	27.61	H-3	2.93

Table 5.3

Hetcor - Longrange correlation of **1**

Carbon	δ (ppm)	Proton	δ (ppm)
C-2	165.90	H-15	2.94
C-4	165.18	H-14	3.80
C-11	161.0	H-13	2.93
C-6	160.02	H-8/9	3.12
C-5	93.89	H-15	2.94
C-15	31.60	H-2	7.84

presented in Tables 5.4 and 5.5.

The genesis of all the major fragments observed in the EI mass spectrum of heterostemmine is shown in scheme 1. The most unusual but interesting fragment ion is at m/z 222 (M^+ -17). This evidently is not due to loss of NH_3 but OH and a possible explanation is provided in the scheme 2.

5.6 Characterisation of alkaloid isolated from PTLC

The most polar alkaloid, melting point 129-130^{0}C was obtained in pure form and is present in relatively small amounts (0.1%). This substance has a very bitter taste, soluble in water which makes its isolation somewhat difficult. This compound exhibited antibacterial properties and formed a picrate, melting point 230^{0}C. Its IR spectrum Fig 5.10 showed a broad absorption centered at 3440 (- NH) and an intense band around 1630 cm^{-1} due to carbonyl grouping. Its UV spectrum is reproduced in Fig. 5.10 Its high resolution Mass spectrum showed the molecular ion peak at m/z 207.1119 corresponding to the molecular formula $C_9H_{13}N_5O$. It contains five nitrogen atoms as present in heterostemmine, but there is only one oxygen atom and this observation will be useful while making structure assignments. Because of its poor solubility in $CDCl_3$ and also availability in small quantity precluded any further studies at this stage. We could, however, measure its ^{1}Hnmr spectrum in deuterated DMSO which showed the presence of four

Table 5.4

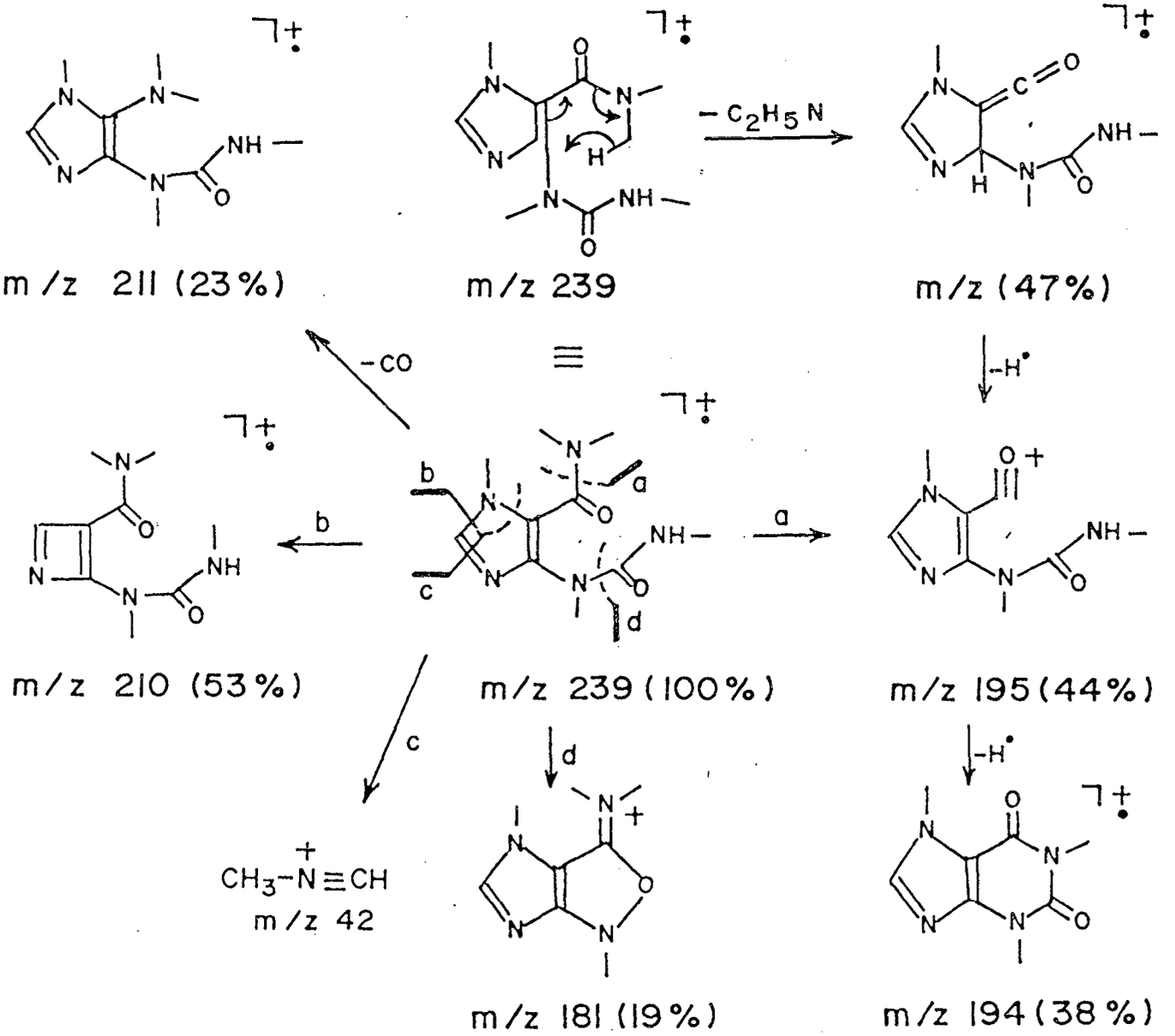
^{1}HNMR data of 1 ($CDCl_3$, TMS)

Proton	δ (ppm)	m	J(Hz)
2-H	7.84	q	0.8
13-NH	5.09	qq	5.0,0.8
14-CH_3 (N-10)	3.80	s	-
8-CH_3 (N-7)	3.12	s	- 9-CH_3
15-CH_3 (N-1)	2.94	d	0.8
13-CH_3 (NH-12)	2.93	d	5.0

Table 5.5

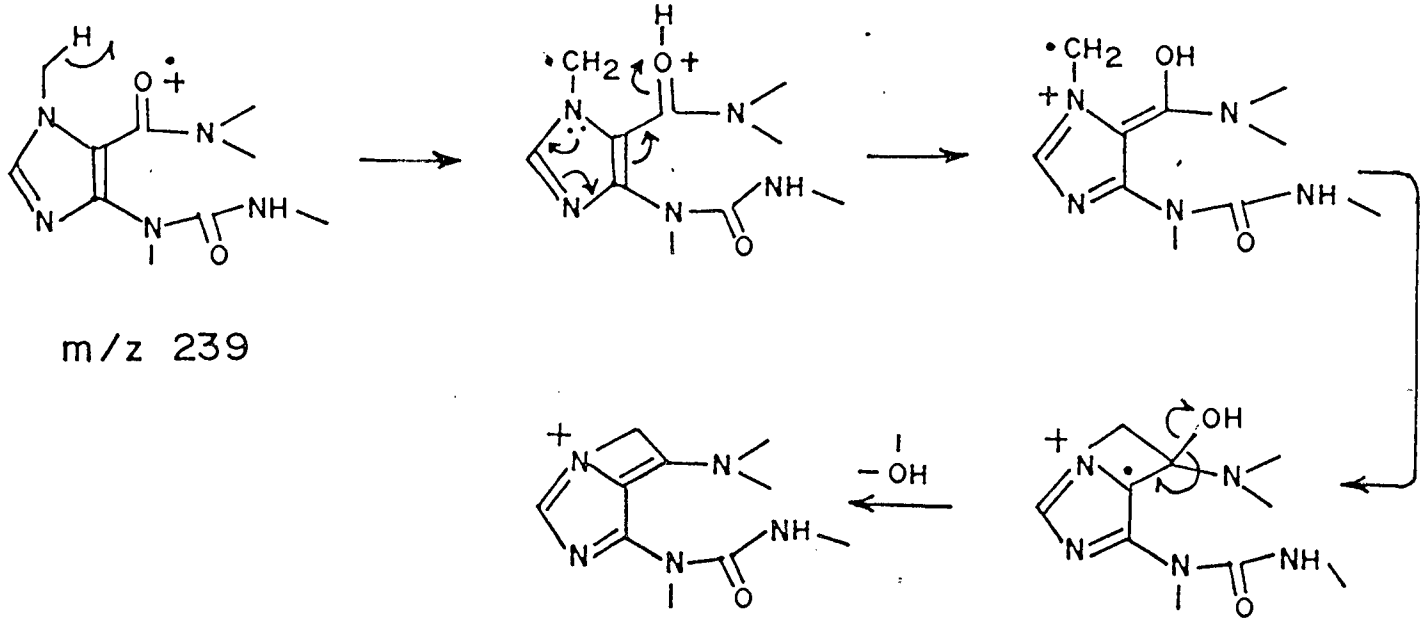
^{13}CNMR data of 1 ($CDCl_3$, δ TMS)

Carbon	δ (ppm)	m	^{1}Hz); $^{2.3}$J (Hz)
C-2	165.90	Dq	196.0 ; 2.6
C-4	165.18	Sq	— ; 3.8
11-C=O)	161.0	S quint	— ; 3.1
6-C=O	160.02	S mult	— ; 2
C-5	93.89	S quint	— ; 2.1
14-CH_3	52.92	Q	146.2; —
8/9 - CH_3	36.67	Qq	137.0 ; 3.3
15 - CH_3	31.60	Qd	139.4 ; 3.9
13 - CH_3	27.61	Qd	137.8 ; 2.4



Scheme - 2

Contd.



Scheme - 2

methyl groups attached to nitrogen and the imidazole ring (9.45). Though further studies are absolutely essential for correct structure assignment, the above spectral data suggests that this most polar alkaloid is definitely structurally related to heterostemmine.

Efforts are being made to isolate additional quantity of this polar alkaloid having antibacterial properties as well as other nitrogenous constituents of H. tanjorense.

5.7. Biological activity of the alkaloids of H. tanjorense

Pure heterostemmine does not show any antimicrobial activity which is confirmed from earlier studies (Bhonsle 1973). The alcoholic extract of the callus, the total alkaloids, and the alkaloid at the base level (R_f 0.05) showed antimicrobial activity against the tested organism Table 5.6 , Table 5.7 and Fig. 5.11. It can be observed from Table 5.6 that the total alkaloids of the callus had almost 50% activity as compared to standard antibiotics with tested organism S. aureus, E. coli and Aspergillus species. However inhibition zones obtained with B. pumillus were almost the same for callus extract and neomycin standard. Also PTLC isolated alkaloid showed antibacterial and antifungal activity in 1/10 th the concentration as compared with total alkaloids of callus.

DISCUSSION

Several workers have screened plants for their antimicrobial properties. The foregoing review gives an indication that plants and their parts in the form of extracts

Table 5.6

Antimicrobial Activity of the Alkaloids of *H. tanjorense*

Test material	Microorganism			
	S.aureus [1] TM /P	*E. coli* [1] TM / C	*B. pumilus* [1] TM / Ne	*Aspargillus* Sp. [1] TM / N
Alcoholic callus extract conc.10 mg/ml	10/35=0.28	8/32=0.25	15/25=0.6	negligible
Total alkaloids of callus conc. 5mg/ml	16/35=0.45	14/32=0.43	22.25=0.88	8/16=0.5
Total alkaloids of roots conc. 5mg/ml	20/35=0.57	16/32=0.5	25/25=1.0	10/16=0.62
Alkaloid with R_f 0.05 conc. 0.5 mg/ml	20/35=0.57	15/32=0.46	20/25=0.8	12/16=0.75
Heterostemmine conc. 0.5 mg/ml	N2	N2	N2	N2

1 Ratio of the diameters of the inhibition zone of the test material (TM) to the inhibition zone of the reference disc. concentration of the antibiotic discs.

Penicillin (P) = 10 units

Chloramphenicol (C) = 30 mcg

Neomycin (Ne) = 30 mcg

Nystatin (N) = 100 units.

2. All other alkaloids at different R_f did not show zone of inhibition. N2 - No zone of inhibition.

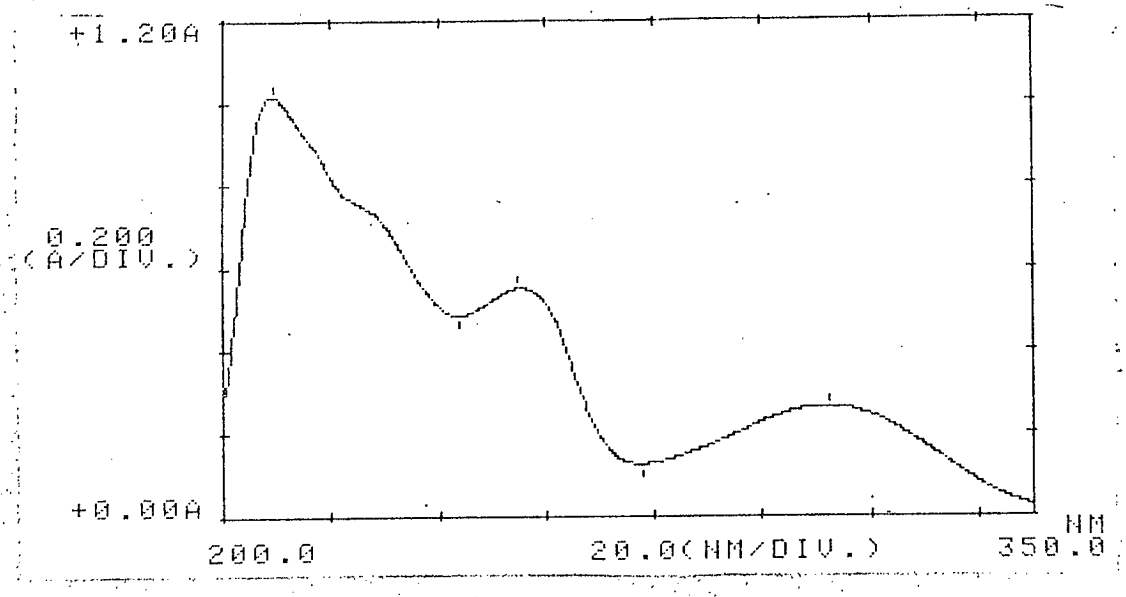
Table 5.7

Antibacterial activity of fractions from PTLC

Fraction No.	R_f of spots under UV	Alkaloid Reaction	Antibacterial Activity
1	0.92	-	-
2	0.85	+	-
3	0.80	+	-
4	0.76	+	-
5	0.70	+	-
6	0.60	-	-
7	0.48	-	-
8	0.40	+	-
9	0.30	+	-
10	0.25	+	-
11	0.20	+	-
12	0.15	+	-
13	0.12	-	-
14	0.08	+	+
15	0.00	+	-

FIG. 5.10

UV spectrum of PTLC isolated ackaloid



IR spectrum of PTLC isolated ackaloid

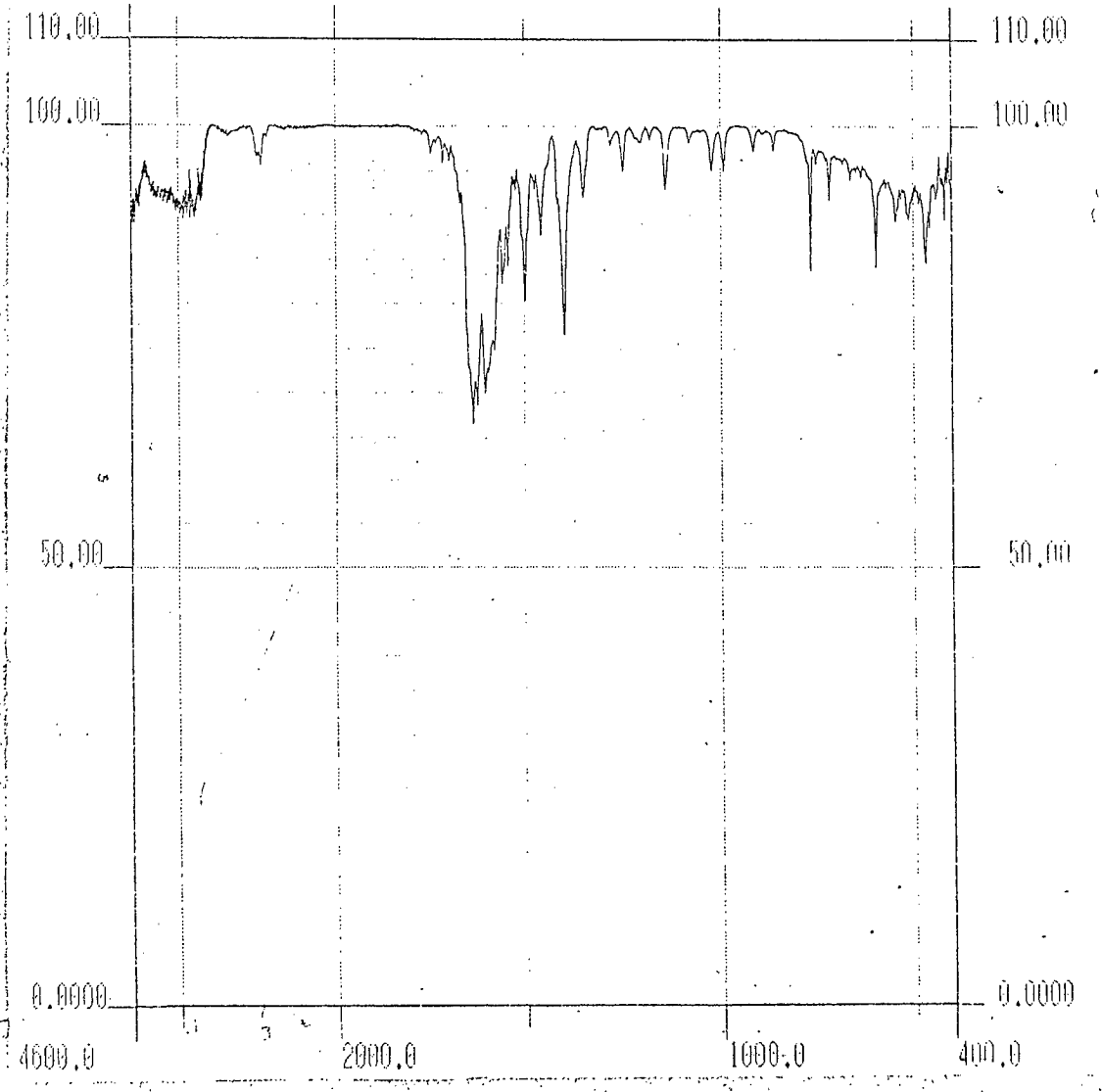
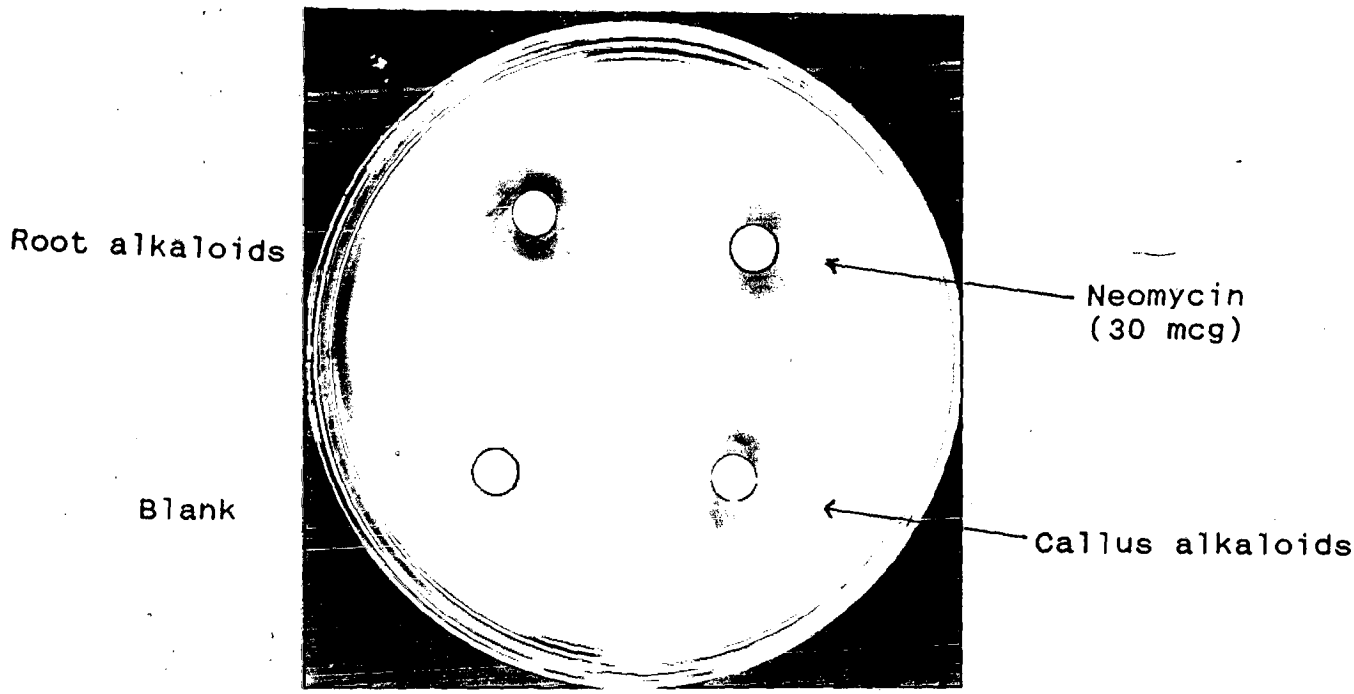
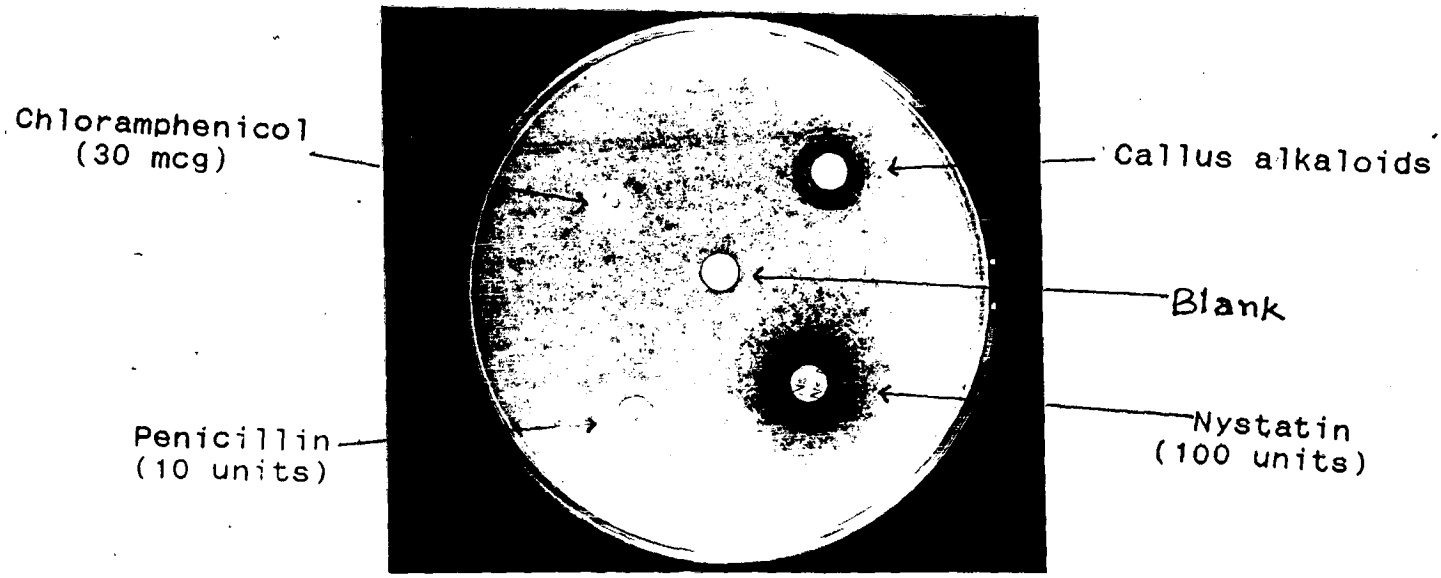


Fig. 5.11

Antibacterial activity of total alkaloids of *H. tanjorense*.



Antifungal activity of total alkaloids of *H. tanjorense*.



have been tested for antimicrobial action. George and Pandalai (1949) and Geroge et al. (1947), reported from their preliminary survey that many of the plants tested were antagonistic to S. aureus and E. coli. Joshi and Magar (1952), tested extracts of different 105 plants for antibacterial activity and found that a number of extracts inhibited either S. aureus or E. coli or both. Nakanishi et al. (1965), screened a wide number of Malaysian plants and found that many of the plant extracts inhibited S. aureus, E. Coli and P. vulgaris. Bhonsle (1973), tested extracts of 275 Goan medicinal plants against M. pyogenes, E. coli and B. subtilis and found that many plant extracts showed antibacterial activity. Other reviews on this subject include antifungal activity of Hyperium mysorense (Chandra et al., 1989), antimicrobial properties of Funarai hygrometrica (Ganaguru et al., 1992), Enterolobium saman (Annapurna et al., 1989), Cassia abbreviata a Zambian plant (Prakash et al., 1993), etc.

The alkaloids present in plant species are known to inhibit a large number of bacteria and fungi, some of which are; Chelidoine, chlerythine and Sanguinarine from Chelidonium majus (Stickle, 1928), solanine from Solanaceous plants (Fontaine et al., 1948), cepheranthine from Stephania cepherantha (Haines ,1958), chaksine from Cassia absus (Haque, 1951), vindoline from Vinca rosea (Kamat et al., 1958a), chakranine from Bragantia wallichii (Kamat et al., 1958b). Alam (1991), studied the antibacterial activity of alkaloidal fractions from Sida species. The bactericidal and fungicidal effects of Prosopsis juliflora alkaloidal extracts were studied by Kanthasamy et al. (1989).

The antimicrobial from plant tissue cultures have been studied by Klein (1960), Campbell et al. (1965), Mathes (1967), Khanna et al. (1968). Khanna et al. (1971) tested the antibiotic activity of the callus tissue extracts of ten plant species against S. aureus, E. coli, S. typhosa and Candida albicans and found that most of the extracts had inhibitory effect on either of the organisms tested.

The present study showed that the total alkaloids of callus extract of H. tanjorense had inhibitory effects on S. aureus, E. coli, B. pumilus and fungal species of Aspergillus which clearly indicates that the plant has antibiotic principle in alkaloid form. The isolation of this principle can lead to the discovery of a novel compound hitherto unknown to literature.

CHAPTER VI
SUMMARY AND CONCLUSION

In the course of the quest for bioactive substances from plants, several workers have investigated plants already known or supposed to possess useful medicinal properties. A large number of important medicinal plants have been investigated on the modern scientific lines, their constituents characterised and the pharmacological properties ascertained. The existence of such plants in Goa was assumed considering its rich flora. Generally, the plant products of commercial interest are the secondary metabolites. There are three categories of products mainly, essential oils, glycosides and alkaloids. Amongst these, are alkaloids, which are diverse group of naturally occurring compounds and most extensively investigated as they are physiologically active in humans and are of strong interest to pharmaceutical and medicinal fields.

The present study envisaged the need to explore the possibilities of producing secondary metabolites by tissue culture from the coastal hills of Goa facing the Arabian sea and plants of Asclepiadaceae family were chosen for preliminary investigation of callus induction and alkaloid production. These plants were Heterostemma tanjorense, Tylophora dalzellii, Cosmostigma racemosa, Hemidescus indicus, Marsdenia volubilis and Holostemma rheedianum. Callus induction was observed in all the plants tested. However,

only the callus from H. tanjorense produced secondary metabolites in the form of alkaloids.

Basal media with different composition were tried for callus optimisation along with varied nutritional and environmental conditions. It was observed that MS medium ½ strength, with 2% sucrose, 0.8 % agar supplemented with a phytohormone combination of 2,4-D (1mg/L), IBA (0.5 mg/L), and Kn (0.5 mg/L), fortified with 10 % coconut milk, a photoperiod of 12 hr light/12 hr dark, grown under 1500 lux intensity at a temperature of $25^0 \pm 2^0$ C and humidity of 60 to 80%, was conducive for callus production. The biomass and the alkaloid production was at the peak physiological state at the end of 6th week and gradually decreased at the end of 8th week. Also, the growth index was maximum at the end of the 6th week. To observe the alkaloid maximisation in the static callus cultures, different growth substances like amino acids, vitamins, natural organic nitrogen complexes etc., were fed in the medium. It was found that amongst all the amino acids added as precursors, tryptophan showed maximum production of alkaloids while amino acids like valine, leucine, lysine and adenine neither supported growth nor increased alkaloid production. Yeast extract in the form of organic nitrogen gave better yield of alkaloids so also niacinamide. But in both the cases growth index was inversely proportional to alkaloid production. Ascorbic acid helped to increase the growth, but did not have any effect on alkaloid production. Other precursors like cholesterol and caffeine suppressed the callus growth and alkaloid production. From these observations it can be inferred that

tryptophan and niacinamide may act as precursors in the pathway of biosynthesis of alkaloids of H. tanjorense.

A quantitative method for the estimation of total alkaloids was devised using bromothymol blue as the complexing agent. The yellow coloured complex formed gave an absorption maximum at 410 nm. The method was accurate and had the advantage that a wide number of samples could be tested at a time using minimum drug quantities. The method also obeyed Beer's law from 5 to 125 mcg/ml for pure heterostemmine.

The chromatographic profile of callus extract for pure heterostemmine showed the presence of 12 alkaloids. This pattern remained the same for in vitro callus extracts obtained from germinated seedling, leaf, stem and root. The pattern was also comparable with the total alkaloids of the roots of H. tanjorense. The results indicated that the alkaloidal pattern remained the same but differed only quantitatively in all the cases indicating that the concentration of alkaloids in vivo root extract was more than the callus extract and the intensity in callus extract decreased from seedling –> roots–> stem–> leaf. The estimation of the total alkaloids of callus and the roots showed that the concentration of total alkaloids in vivo roots was approx. 3 times more than in vitro callus extract calculated on dried basis.

Successful growth of callus cultures of H. tanjorense in suspension media was evident. The suspension culture displayed the production of all the alkaloids which were produced in static callus cultures.

One of the prominent alkaloid from callus extract was

isolated and purified to homogeneity using column chromatography. This alkaloid had R_f value of 0.80 in solvent system chloroform : methanol (95:5) with 2% NH3. The alkaloid was recrystallised from benzene.

Spectral analysis of the purified alkaloid revealed that besides comigrating along with the standard heterostemmine, it had identical point of 164^0 C, same retention time on HPLC and had superimposable UV and IR spectral characters.

From the above data it was confirmed that the alkaloid isolated from the callus was similar in all respects to standard heterostemmine having molecular weight 239 and molecular formula $C_{10}H_{17}N_5O_2$.

The elucidation of the structure for heterostemmine was done based on spectral characteristics and it was found to be of imidazole group.

The callus extract, the total alkaloids, and the individual alkaloids purified by preparative TLC were tested for their antibacterial action against test organisms S. aureus, E. coli, and B. pumilus. The antifungal activity was tested against Aspergillus species. It was observed that the alkaloids of the callus extract and the root extract of H. tanjorense showed antibacterial and antifungal activity against the tested organisms. The antimicrobial activity increased as the callus extract passed through stages of purification from crude alcoholic extract to total alkaloids. Although purified heterostemmine did not show any antimicrobial activity, the sample of the alkaloid isolated from PTLC had distinct

antimicrobial and antifungal activity. The UV, IR and some of the spectral data of this alkaloid reveal that it is structurally related to heterostemmine and has molecular formula $C_9H_{13}N_5O_2$.

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APPENDICES

Appendix 1

Preparation of solutions, sterilization and storage of plant growth regulators, vitamins and amino acids.

Compound	Solution preparation	Sterilization	Storage
2, 4-D	50% alcohol	co-autoclave	5° C
IAA	1N NaOH & 50% alcohol	Prefilter sterilize	0° C
IBA	50% alcohol	Prefilter sterilize	0° C
NAA	50% alcohol	co-autoclave	5° C
Kn	1N NaOH & 50% alcohol	co-autoclave	5° C
BAP	50% alcohol	co-autoclave	5° C
GA_3	50% alcohol	Prefilter sterilize	0° C
ABA	1N NaOH + 50% alcohol	"	0° C
B_1	Dist. water	co-autoclave	5° C
B_2	1NaOH & Dist. water	"	5° C
		"	5° C
B_6	Dist. water	"	5° C
Niacinamide	Dist. water	"	5° C
Folic acid	1NNaOH & Dist. water	"	5° C
		co-autoclave	5° C
Alanine	Dist. water	Prefilter sterilize	0° C
Arginine	Dist. water	"	0° C
Aspargine	Dist. water	"	0° C
Cysteine Hcl	Dist. water	"	0° C
Glutamic acid	Dist. hot water	"	0° C
Histidine	Dist. water	"	0° C
Leucine	1NHCl+Dist. water	"	0° C
L-lysine Hcl	Dist. water	"	0° C
Methionine	1NHcl+Dist. water	"	0° C
Ornithine	Dist. water	"	0° C
Phenylalanine	1NHcl+Dist. water	"	0° C
Proline	Dist. water	"	0° C
Threonine	Dist. hot water	"	0° C
Tryptophan	1NHCl+Dist. water	"	0° C
Tyrosine	1NHCl+Dist. water	"	0° C
Valine	Dist. hot water	Prefilter sterilize	0° C
Cholesterol	Hot Ethanol	add after sterilization	0° C
Caffeine	1NHCl+Dist.water	co-autoclave	0° C
Ascorbic acid	Dist. water	co-autoclave	0° C

Appendix 2

Sterilization procedure for different vegetative explants

Tissue	Presterilization	Sterilization	Post sterilization
Leaf Primordia	Submerge in 1% Twean 80 solution for 5 mins and wash with dist. water	Immerse for 3 mins in 0.1% W/V Hgcl2 solution	wash repreately 3 times with sterile water
Leaf	—— do ——	Immerse for 5 mins in 0.1% W/V HgCl2 solution	—— do ——
Stem	—— do ——	—— do ——	—— do ——
Flower buds		Immerse for 3 mins. in 0.1% w/v HgCl2 solution	
Anther	—— do ——	Immerse for 1 min. in 0.1% w/v HgCl2 solution	—— do ——
Roots	Scrub in running tap water. Immerse in 1% Tween 80 solution and rinse with alcohol.	Submerge for 20 mins. in 2% w/v sodium hypochlorite solution	—— do ——
Seeds	wash with water and rinse in alcohol.	Immerse for 15 mins in 2% w/v sodium hypochlorite solution	wash with sterile water & place 5 seeds in each 25x150 mm test tubes containing paper boats and MS medium ¼ strength.

Note : Leaf discs 10 mm diameter were cut by means of sterile cork borer from surface sterilized leaves.

Appendix 3

REAGENTS AND SOLUTIONS

Dragendorff Reagent : (According to Munier), (Stahl, 1969b).

Solution A : 1.7 g. of basic bismuth nitrate and 20 g of tartaric acid are dissolved in 80 ml of water.

Solution B : 16 g. of KI are dissolved in 40 ml of water.

Stock solution : 1:1(V/V) mixture of A & B is prepared and stored in refrigerator.

Spray reagent : 5 ml of stock solution are added to a solution of 10 g of tartaric acid in 50 ml of water.

Wagner's reagent : (Cromwell, 1955). 1.27 g. of Iodine and 2 g. of KI are dissolved in 5 ml of distilled water and the solution diluted to 100 ml. This reagent gives brown flocculent precipitate for the presence of alkaloids.

Mayer's reagent : (Cromwell, 1955). 1.36 g. of Hgcl2 are dissolved in 60 ml of distilled water and 5 g. of KI in 10 ml of water. The two solutions are mixed and diluted to 100 ml with distilled water.

Ammonium Reineckate : (Cromwell, 1955). A saturated aqueous solution (approx. 4%) slightly acidified with HCl gives with most alkaloids a pink flocculent precipitate. The precipitate is soluble in 50% acetone solution from which the alkaloid reineckate can be crystallized. The reiniculates are useful for characterization purpose.

Bromothymol blue solution : 250 mg of bromothymol were dissolved in 3 ml of 0.1 N NaOH. Volume was made to 250 ml with distilled

water, the solution was stirred for ½ hr. and filtered through Whatman No. 1 filter paper.

Citrate-phosphate buffer :(Colowick, 1955) 0.1 M citric acid (solution A) was prepared by dissolving 19.21 g. of citric acid in 1000 ml of distilled water. 0.2 M Dibasic sodium phosphate (solution B) was prepared by dissolving 53.65 g. of $Na_2HPO_4 . 7H_2O$ in 1000 ml distilled water.

Buffer solution of pH 3.5 was prepared by mixing 34.9 ml of solution A and 15.1 ml of solution B. Final adjustment was done either with solution A or solution B.

Buffer solution of varying pH from 2.5 to 5.5 were prepared by mixing X ml of solution A and Y ml of solution B. Final adjustment of desired pH was done either with solution A or solution B.

pH	x ml solution A	y ml solution B
2.5	45.8	4.2
3.0	39.8	10.2
3.5	34.9	15.1
4.0	30.7	19.3
4.5	27.2	22.8
5.0	24.3	25.7
5.5	21.6	28.4

Reagent buffer mixture :

Bromothymol blue solution and buffer solution pH 3.5 were mixed

in equal quantities. The resulting solution was extracted with chloroform (50ml) and chloroform layer was rejected. Whenever required quantities of reagent and buffer solution were varied to suit the experimental conditions.

Appendix 4

Methods of detection/estimation of alkaloids obtained from tissue culture

Alkaloids	Method of Estimation	Reference
1. Alkaloids from *Datura* species	TLC,GLC vitali-Moren	Sairam et al.(1971).
		Hiraoka et al.(1974).
2. Alkaloids from papaveraceae	TLC,GC,IR,NMR	Furuya et al.(1972).
		Ikuta et al.(1974).
3. Alkaloids from *scopalia parviflora*	TLC & Vitali-Moren	Tabata et al.(1972).
4. Alkaloids from *Ruta graveolens*	GC	Steck et al.(1973).
5. Solasodine from *solanaceous* species	TLC,PC Birner	Khanna et al.(1976d).
6.Atropine from *Atropa belladonna*	Prep. TLC, Spectrophotometry	Khanna et al.(1976e).
7.Caffeine from *Coffea arabica*	Speotrophotometry	Waller, G. R. (1983).
8.Cinchona alkaloids	TLC, Fluorimetry	Koblitz et al.(1983).
9.Berberine from *Thalicrum minus*	HPLC	Nakagawa et al.(1984).
		suzuki et al.(1987).
10. Reserpine from *Rauwolfia serpentina*	Spectrophotometry	Yamamoto et al.(1986).
11.Alkaloids from *Catharanthus roseus.*	Spectrophotometry	Parr et al.(1988).
12.Trigonelline from *Abrus precatorius* & *Dolichos lablab*	TLC,GLC, IR.	Khanna et al.(1988).
		Kaushik et al.(1990).
13. Emetine from *C.epecacuanha*	HPLC	Veeresham et al.(1991a)
14. Capsaicin from *Capsicum annum*	TLC,HPLC	Veeresham et al.(1991b)

Appendix 5

Legend to Figures

Fig.1.1. *Heterostemma tanjorense* potted plant and the seed.

Fig.2.1 *H. tanjorense* flowering plant; flower bud callus and germinated seedling callus.

Fig.2.2 *Tylophora dalzellii* flowering plant and leaf callus.

Fig.2.3 *Cosmostigma racemosa* flowering plant and leaf primordia callus.

Fig.2.4 *Hemidesmus indicus* plant and leaf primordia callus.

Fig.2.5 *Marsdenia volubilis* flowering plant and leaf disc callus.

Fig.2.6 *Holostemma rheedianum* flowering plant and stem callus.

Fig.2.7 *H. tanjorense* germinated seedling.

Fig.2.8 *H. tanjorense* callus at epicotyledonary and cotyledonary region.

Fig.2.9 TLC of *H. tanjorense* callus extract after spraying with Dragendorff reagent.

Fig.3.1a Growth pattern of callus of *H. tanjorense* (growth index/ age in weeks).

Fig.3.1b Growth pattern of callus of *H. tanjorense* (dry wt. mg/ T/ age in weeks).

Fig.3.2 Spectral absorbance curve of total alkaloids of callus of *H. tanjorense*.

Fig.3.3 Effect of pH on spectral absorbance in total alkaloids of callus of *H. tanjorense*.

Fig.3.4 Effect of volume of dye solution on spectral absorbance in total alkaloids of callus of *H. tanjorense*.

Fig.3.5 Effect of volume of buffer on spectral absorbance in total alkaloids of callus of H. tanjorense.

Fig.3.6 Standard curve of heterostemmine standard.

Fig.3.7 Standard curve of total alkaloids of callus.

Fig.3.8 Standard curve of total alkaloids of roots.

Fig.3.9 Total alkaloid contents of callus of H. tanjorense/ growth index and age in weeks.

Fig.3.10 TLC of total alkaloids of callus of H. tanjorense (observation under UV light and after spraying with Dragendorff reagent).

Fig.3.11 Co-Chromatography of heterostemmine and alkaloid A3 after spraying with Dragendorff reagent.

Fig.3.12 TLC of total alkaloids of callus extract and total alkaloids of roots of H. tanjorense.

Fig.3.13 Organogenesis in callus cultures of H. tanjorense, (shoot and root formation).

Fig.3.14 Callus of root, stem and leaf of H. tanjorense.

Fig.4.1 Growth index/ age in weeks of suspension culture of H. tanjorense. Total alkaloids/age in weeks of suspension culture of H. tanjorense.

Fig.4.2 Microscopic profile of cells in suspension culture. Wet mount (vacuolated cells) and cells stained with safranine.

Fig.4.3 % concentration of niacinamide/ growth index.
% concentration of niacinamide/ total alkaloids in H. tanjorense.

Fig.4.4 % concentration of tryptophan/ growth index.
% concentration of tryptophan/ total alkaloids in H. tanjorense.

Fig.4.5 TLC of total alkaloids of suspension culture of H. tanjorense.

Fig.5.1 max. of reineckate derivative of heterostemmine and alkaloid A3.

Fig.5.2 UV absorption spectra of heterostemmine and alkaloid A3.

Fig.5.3 IR spectrum of heterostemmine and alkaloid A3 (superimposed).

Fig.5.4 HPLC profile of heterostemmine and alkaloid A3.

Fig.5.5 Mass spectrum of heterostemmine.

Fig.5.6 UV spectrum of heterostemmine.

Fig.5.7 IR spectrum of heterostemmine.

Fig.5.8 ^{1}HNMR spectrum of heterostemmine.

Fig.5.9 ^{13}CNMR spectrum of heterostemmine.

Fig.5.10 UV and IR spectrum of PTLC isolated alkaloid.

Fig.5.11 Antibacterial and antifungal activity of H. tanjorense callus extract and comparison with standard antibiotics.