Bioprospecting *Strychnos* L. (Strychnaceae)

Thesis submitted to the University of Hyderabad

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DOCTOR OF PHILOSOPHY

By

Pasupuleti Sreenivasa Rao

School of Life Sciences

Department of Plant Sciences

University of Hyderabad

Hyderabad 500 046, India

Enrolment No. 01LPPH03

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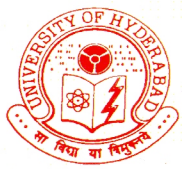
Dedicated
To
My parents

Contents



University of Hyderabad

School of Life Sciences

Department of Plant Sciences

Declaration

I hereby declare that the work presented in the thesis entitled **"Bioprospecting *Strychnos* L. (Strychnaceae)" has been carried out by me under the supervision of Prof. M.N.V. Prasad, Department of Plant Sciences**, School of Life Sciences, University of Hyderabad, Hyderabad 500046. Further, I declare that this work has not been submitted for any other degree or diploma to any University or Institute. This includes the typescript in its entirety or any part thereof.

P. Sreenivasa Rao

Pasupuleti Sreenivasa Rao
Candidate
Enrolment No. 01LPPH03

University of Hyderabad

School of Life Sciences

Department of Plant Sciences

Certificate

This is to certify that the research work in the thesis entitled **"Bioprospecting *Strychnos* L. (Strychnaceae)"** has been carried out by **Mr. Pasupuleti Sreenivasa Rao** under my supervision for the full period prescribed under the Ph.D ordinance of this University and that this work has not been submitted for any other degree or diploma to any University or Institute.

M.N.V. Prasad
Professor and Supervisor

Professor M. N. V. PRASAD
Dept. Plant Sciences
University of Hyderabad
Central University P.O.
HYDERABAD-500 046. INDIA

Head 19/6/07
Department of Plant Sciences

HEAD
Dept. of Plant Sciences
School of Life Sciences
University of Hyderabad
HYDERABAD-500 046, INDIA

Dean I/c 20/06/07
School of Life Sciences

DEAN
School of Life Sciences
University of Hyderabad
Hyderabad-500 046

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Hyderabad, June 2007 **Pasupuleti Sreenivasa Rao**

Abbreviations

BAP	6-Benzylaminopurine
BCIP	5-bromo-4-chloro-3-indolyl phosphate
CBD	Convention on biological diversity
^{13}C-NMR	Carbon nuclear magnetic resonance
DAPI	4, 6-Di amidino-2-2-phenylindole
DMSO	Di methyl sulfoxide.
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Florescence activated cell sorter
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter.
FBS	Fetal bovine serum
HEPES	N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid
IAA	Indole 3-acetic acid
IBA	Indole 3-butyric acid
IR	Infra red
Ig	Immunoglobulin
KDa	Kilodalton
KN	Kinetin (6-furfuryl aminopurine)
LC-MS	Liquid Chromatography and mass spectra
MTT	3-[4, 5-dimethylthiazole-2-yl]-2,5 diphenyl tetrazolium bromide
MS	Murashige and Skoog's medium
MM	Multiple myeloma
NAA	α-Naphthalene acetic acid
NBT	Nitroblue tetrazolium
NSAIDs	Non-steroidal anti-inflammatory drugs
PAGE	Polyacrylamide gel electrophoresis
PKC	Protein Kinase C
PVPP	Polyvinyl poly pyrrolidone
PI	Propidium iodide
^{1}H-NMR	Proton nuclear magnetic resonance
SDS	Sodium dodecyl sulphate
SSC	Side scatter
RP-HPLC	Reverse phase high performance liquid chromatography
TEMED	N,N,N', N'-tetramethylene diamine
TRIS	Tris-(hydroxymethyl) aminoethane
TBE	Tris borate EDTA buffer
TDZ	Thiadiazuron (N-phenyl-N'-1, 2,3-thiadiazol-5-ylurea)
TLC	Thin layer chromatography

1 Introduction

1.1 Bioprospecting and brief phytography of chosen species of *Strychnos*

Bioprospecting is the study of plant, microbe and animals resources that may be of commercial value. It is a rapidly developing field wherein biologists, chemists and other researchers are involved in building data base of the commercially potential bioresources (Figure 1). Several bioprospecting strategies are being initiated at global level in evaluating the bio resources for the benefit of human welfare. India has huge bioresource potential. Bioprospection is intended for economic purposes (e.g., new drugs, crops, industrial products). Bioprospection of under explored/ unexplored plant resources have relevance for the improvement of the rural/tribal community and economy. Before 1992, biological resources were considered a common heritage of humankind, as scientists could take samples from anywhere in the world without any specific permission.

The Convention on Biological Diversity (CBD, 1992) establishes sovereign national rights over biological resources. Though not granted property upon natural resources, biodiversity-rich countries are committed to : i) conserve their biodiversity ii) develop it for sustainable use and iii) share the benefits resulting from their use. In short, bioprospection has to be allowed by the biodiversity-rich country and must bring as much benefit to it (and to the communities that traditionally use these resources) as to

corporations (usually from developed countries) and universities collecting the bioresource. The scientific leads for new plant-based pharmaceuticals have been generated through:

a) High-throughput screening programs (Hunter, 2001) and
b) Ethno botanical studies (Houghton, 1995).

In recent times, the drug discovery research has been shifted to high-throughput screening coupled with computational and molecular modeling. Ethno botanical information is invaluable for the identification of potential compounds for novel pharmaceuticals. However, this important source for potential medicines (the knowledge of indigenous healers) is being lost at an alarming rate (Cox, 2000). The plant resources that the traditional healers rely upon are vanishing. *Strychnos* is one among these (Table 1).

Medicinal plants generate substantial income for the rural people in developing countries through the sale of wild-harvested material. The collectors are often the economically weaker sections of the population such as tribes, landless people and woman. In central part of Nepal, 50-100 % of households depend on medicinal plants and materials being traded to the wholesale markets (Olsen, 2005). The money received represents 15-30% of the total income of poorer households. In India also the same phenomenon occurs and majority of tribes depend on this plants.

Table 1: Brief phytography of the research material investigated

Parts	***Strychnos potatorum***	***Strychnos nuxvomica***	***S. wallichiana***
Plant	A medium sized deciduous tree having height upto 12 meters.	A medium sized deciduous tree having height up to 20 meters	It is a woody climbing shrub
Bark	Cracked and scaly bark.	Dark grey or yellowish grey bark.	Dark grey or yellowish grey bark
Trunk	Irregularly fluted.	Fairly straight.	Irregularly fluted.
Leaves	Simple, opposite, elliptic, acute, 15x6.25 cm, glabrous, shining.	Broadly elliptic, coriaceous, glabrous.	Simple, opposite, Oval to oblong , Bluntly acuminate.
Flowers	White fragrant, axillary cymes	Numerous, greenish white in terminal cymes.	Small greenish yellow, Compound axillary cymes
Fruits	Ovoid or globose, glabrous berries, black when ripe.	Globose, orange-red when ripe.	Berry as large as an orange.
Seeds	One or two; yellow, circular, not much compressed.		Discoid

The *Strychnos* is widely distributed in India especially in Andhra Pradesh. The phtographical features of *S. nux-vomica* and *S. potatorum* and *S. wallichiana* were demonstrated (Table-1). These species has huge economic importance and as well as widely used in traditional medicine (Table-2, 3 and 4). In Andhra Pradesh *S. nux-vomica* and *S. potatorum* have been contributing significantly to the village economy. *S. nuxvomica* and *S. potatorum* fruits are harvested during November-March. Girijan Co-operative Corporation Limited is organising collection via a network of gatherers, looking into fair-pricing of the produce and benefit sharing among the local communities (Tables 2). *Strychnos potatorum,* is widespread in the villages of Salugu Panchayat of Vishakapatnam district. The predominant tribal groups are Bagatas, Valmikis, Nookadoras, Malis, and Kutias. Seeds of *S. potatorum* are source of a coagulant and tribes use it as a simple method for the treatment of drinking water (Figures 2-5)

Table 2: Traditional uses of ***Strychnos potatorum***

Part	Importance in traditional medicine.
Whole plant	Ayurveda, Siddha, Unani, Folk, Modern, Tibetan and Homeopathy Systems of Medicine.
Root	Fever, Epilepsy.
Leaves	Used for ulcer.
Bark	Powdered bark mixed with lime juice is given during cholera.
Seeds	Local application in case of eye diseases. A paste of seeds is reported to be consumed internally along with little tender coconut milk against urinary disorders and retention of urine. The ripe seeds are used for clearing muddy water.

Table 3: Traditional uses of ***Strychnos nux-vomica***

Part	Importance in traditional medicine.
Whole plant	Ayurveda, Siddha, Unani, Folk, Modern, Tibetan and Homeopathy Systems of Medicine. Powerful poison in large doses produces titanic convulsions and eventually death, while smaller doses it causes mental derangement. Effective as animal poison, used as insecticide to kill vermin, it is also effectively used in paralysis, as it acts upon the spinal marrow with out affecting the brain.
Fruits	Fruit pulp used in treating paralytic affections of paws and foot.
Seeds	Diabetes, gonorrhea, anemia, asthma, bronchitis, constipation, intermittent and malarial fevers, insomnia, cardiopalmus, skin diseases, paralysis and weakness of limbs. in south east asia tribes use the seeds in the preparation of arrow and dot poisons
Leaves	Applied as poultice in treating chronic wounds, used for ulcer, leaf decoction used in paralytic complaints.
Bark	Powdered bark mixed with lime juice is given during cholera.
Root	Antidote for venomous serpents and snakes, leprosy, paralysis. The root bark is used in cholera.

Table 4: Traditional uses of ***Strychnos wallichiana***

Part	Importance in traditional medicine.
Whole plant	Ayurveda, Siddha, Unani, Folk, Modern, Tibetan and Homeopathy Systems of Medicine. The plant is used in intermittent fevers , antihelminthic,and externally in cutaneous diseases, especially for alleviating the pain, attending the swelling in the confluent smallpox, obstinate malarial fevers cachexia and dyspepsia.
Fruits	Fruit pulp used in paralysis.
Seeds	Intermittent fevers, malaria.
Leaves	Intermittent fevers, treating chronic wounds, leaf decotion used in paralytic complaints.
Bark	Intermittent fevers, cutaneous diseases.
Root	Roots used as antivenom in case of snake bite and this species yields the real, or at least one sort of lignum colubrinum.

Strychnine and brucine salts are extracted from *S. nux-vomica.* These salts are being used in the preparation of strychnine sulphate/chloride tablets for dog poison. The Girijan Cooperative Corporation Ltd. (Visakhapatnam) developed a product from seeds of *S. potatorum* which has been used the removal of turbidity from potable waters.

1.2 Ethnopharmacological importance

Ethno botany deals with the study and relationship between humans and nature. Ethnic people are highly knowledgeable about the plants and their medicinal values. This knowledge is passed through oral communication from generation to generation. Over the last century, ethno botany has evolved into a specialized discipline that looks at the people-plant relationship in a multidisciplinary manner, such as ecology, economic botany, pharmacology, public health and other disciplines as needed (Balick and Cox, 1996). Ethno botany also deals with studies among the tribal and rural people for recording their unique knowledge about plant wealth and for discovering new resources of herbal drugs, edible plants and other economic aspects of plants (Anonymous, 1983). The predominant tribes in Andhra Pradesh are: Koya, Jathapu, Valmiki, Kondareddy, Nokareddy, Gonds, Kodulu, Chenchu, Eruka, Enadi etc and these tribal populations provide considerable information about the use of many plants and their parts. There are considerable economic benefits in the development of indigenous medicines and in the use of medicinal plants for the treatment of various diseases. Plants have been used since ancient times for the treatment of various ailments. The traditional system of medicine together with folklore continues to

serve a large portion of the population, particularly in rural areas, in spite of considerable advancement in modern medicines.

In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants. In a report recently published by the World Bank, (Lambert *et al*., 1997) pointed out that preserving and enhancing the plant knowledge and use was equivalent to "rescuing a global heritage". Traditional medicinal practices are important part of the primary healthcare system in the developing world (Sheldon *et al.*, 1997). Herbal medicines are comparatively safer than synthetic drugs. About 25% of drugs prescribed worldwide come from plants, 121 of such active compounds being in current use. Of the 252 drugs considered basic and essential by the WHO, 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors. According to WHO as many as 80 % of the world's population depends on traditional medicine for their primary health care needs (Azaizeh *et al*., 2003). The medicinal plant *Strychnos* has long been studied for its pharmacological properties, including analgesic and antipyretic. It is used in the treatment of Parkinson's disease and as an anti-diabetic. Some of the important research findings on *S. potatorum* (Figure 6), *S. nux-vomica* (Figure 7) and *S. wallichiana* were documented in Tables 5-7 respectively .

1.3 Review of literature on phytochemistry and economic importance of selected species of *Strychnos*

Table 5: Selected works on ***S. potatorum***

Research findings	Reference
Reports on the mechanism of the clarification of muddy water by seeds.	Subbaramiah and Sanjiva Rao,1937
A pilot study on the use of nirmali seed as a coagulant aid.	Bulusu and Sharma 1965
Diaboline is the major alkaloid found in root, stem, leaves and seeds.	Singh, 1974
Nirmali seed –as an efficient naturally occurring coagulant.	Tripathi *et al.,* 1976
Reported the presence of isomotiol, sitosterol, stigmasterol and campesterol from the leaves and the bark mixtures.	Singh *et al.,*1978
Reports on the chemical constituents of the seeds, and seed oil and as well as pharmacological studies on the alkaloid content. Synthesis and anti hypotensive activity of diaboline.	Singh *et al.,* 1973; 1976; 1977; 1980; Kapoor,1988
Reports on seed consists of mannogalactan is composed of mannose and galactose; tested for antihypercholesterolemic activity on rats and their results suggested that manaogalactan showed inhibition on cholesterol and triglycerides.	Rao *et al.,*1990
Twenty-four compounds have been isolated and identified in the root bark.	Massiot *et al.,*1992

Reports on the composition of the coagulant polysaccharide fraction from seeds and found that this fraction comprises a 1:1.7 mixture of a galactomannan and a galactan.	Adinolfi *et al.,* 1994
Reported that a seed offers nuclear waste treatment, capable of binding heavy metals.	Jayaram,1993 ; Puvvada and Chandrasekhar 1997
The antidiarrhoeal activity of the methanol extract of the dried seeds has been evaluated out in rats using different models, castor oil-induced diarrhoea, effects on gastrointestinal motility and on PGE2-induced gastric enteropooling.	Biswas *et al.,* 2002
Posses strong anti diabetic activity.	Mandal, *et al.,* 2002
The methanolic seed extract exhibited strong anti diuretic activity in Wistar albino rats and concluded that this evidence further supports the use of the seeds as a diuretic in folk remedies.	Biswas *et al.,* 2001
Seeds are used in the Indian traditional system of medicine for the treatment of hepatopathy, nephropathy, gonorrhea, leucorrhoea, gastropathy, bronchitis, chronic diarrhoea, strangury, renal and vesicle calculi, diabetes and eye diseases.	Sanmugapriya *et al.,* (2006)
Seeds exhibit the hepatoprotective and antioxidant activities.	

Table 6**:** Selected works on ***S. nux-vomica***

Research findings	**Reference**
Strychnine possesses the strychnan group, which was isolated for the first time in 1818–1819 by Pelletier and Caventou. Brucine was also discovered by them in 1819.	Pelletier and Caventou 1818a ; 1819
The major components are strychnine and brucine, both existing in mixture with *igasuric acid*, a tannic principle identical with caffeo-tannic acid. Igasurin was aslo reported.	Desnoix, 1854; Sander, 1897.
The effect of seed storage on the alkaloidal content.	Puntambekar,1947
Reports on the Ayurvedic process of purification of seeds.	Srivastava *et al.,*1953
Reports on the morphology and histology of the seeds	Turner *et al.,*1963
Reports on the separation of minor alkaloids.	Monache 1968
Reports on the phytochemical and pharmacological importance of African *Strychnos* species and comparative study on the convulsant effects of *Strychnos* alkaloids.	Sandberg *et al.,*1969 ;1970
Reports on the biosynthesis of strychnine	Heimberger and Scott, 1973
Reports on the effect of purification (shodhan) on the alkaloid concentration of kuchila seeds.	Agrawal and Joshi,1977
Alkaloids of Sri Lankan *S. nux-vomica*; identified 22 alkaloids have been isolated from root bark and leaves.	Baser and Bisset, 1982

The presence of different alkaloids in *S. nux-vomica*. Strychnine and brucine were well characterized has been previously reported in other *Strychnos* species e.g. *S. nux-vomica, S.lucida* R. Br., *S.ignatti* Berg., (Asia), *S.icaja* Bail (Africa) and *S.panamensis* Seem (Central America).	Bisset, 1970,1972,1974,1976; Bisset and Phillipson,1971, 1973; Bisset and Leewenberg ,1968 :Bisset and Choudhury 1974,1974 b; Bisset *et al.*,1974; Marini-Bettolo *et al.,* 1972; Bisset and Choudhary 1974; Galeffi 1972; Baser *et al.*,1979, 1982.
Methoxy-3 icajine.	Rodriguez *et al.*,1979
Reports on the *kupilu satva*, the seeds treated with milk were less toxic than those treated with ghee or aloes and ginger, and crude samples; their lethal dose was 12 times higher than that of the crude samples	Chandra Kant,1982
Traditional uses and ethnobotany of "Kuchila"	Sen *et al.,* (1983).
TLC and spectroscopic analysis of strychnine and brucine	Gaitonde and Joshi, 1985
The screening of 46 alkaloids isolated from various *Strychnos* species and tested on several cell lines (i.e. B16 melanoma, Flow 2002, DB A/2 Mouse ascites tumor, He La cells)	Leclercq *et al.,* 1986
Reports on the comparative pharmacognosy of *S. nux vomica* and *S. potatorum* based on the stem barks	Chakraborti, *et al.*,1988

Reports on the relationship between alkaloids in semen strychni and its processing.	Sha *et al.,*1989.
In the treatment of dyspepsia, diseases of the nervous system, chronic rheumatism, in incontinence of urine in children, in sexual impotence and for rejuvenation	Kapoor, 1990
Reports on different chromatographic methods including HPLC for the effective separation of alkaloids	Verpoorte and Svendsen 1975; Egloff *et al.,* 1982; De and Bisset 1991
Different chromatographic methods including HPLC for the effective separation of alkaloids	Verpoorte and Svendsen 1975; Egloff *et al.,* 1982; De and Bisset 1991
Anti inflamatory properties have been described for *Strychnos* alkaloids or extracts	Tits *et al.,* 1991
Reports on the effect of brucine lethality in mice	Marvin *et al.,* 1991
Analysis of spectral data for ^{13}C NMR of sixteen *Strychnos* alkaloids	Cai *et al.,* 1994
Effect of processing on the alkaloids in *S. nux-vomica*	Wu *et al.,* 1994
Determination of strychnine and brucine by capillary zone electrophoresis	Zong, and Che,1995
Presence of vomicine in callus cultures of *S. nux-vomica.*	Bandyopadhyay *et al.,* 1995
Polysaccharides from seeds of *Strychnos* species	Corsaro *et al*., 1995

A new HPLC method for the assay of alkaloids in *S. nux-vomica* and *Strychnos ignatii*	Biala *et al.,* 1996.
Strychnine is powerful spinal cord convulsant, but the functions of the brain cortex and subcortical centres are not influenced	Neuwinger, 1996
Seasonal variation of strychnine and brucine in vegetative parts	Bandopadhyay and De, 1997
The effect of *S. nux-vomica* alcohol extract on lipid peroxidation in rat liver.	Tripathi and Chaurasia, 1997
Reported different isolated alkaloids (more than 40 types) that are composed of mainly monomeric tertiary indole alkaloids, dimeric teriatiary alkaloid and a few quartenary alkaloids.	De, 1997
The determination of strychnine and brucine in the seeds, root, stem and leaves of *Strychnos* species by HPLC.	Gu *et al.,* 1997
Reports on several traditional drug-processing methods on the alkaloid content, their composition, acute toxicity of the processed drug and cytotoxic assessment of alkaloids from the processed seeds on the tumor cell lines	Cai *et al.,*1990; 1993; 1994; 1995; 1996; 1998
Reports on the influence of processing methods on alkaloid content, toxicity and effect of *S. nux-vomica*	Chen *et al.,* 1998
The separation and purification of strychnine from crude extract by high-speed countercurrent chromatography	Miao *et al.,* 1998
Influence of processing methods on alkaloid, toxicity and effect	Chen *et al,* 1998

Separation and purification of strychnine from crude extract of *S. nux-vomica* L. by high-speed countercurrent chromatography	Miao *et al.,* 1998
Occurrence of free radicals in Ayurveda and revealed that Rasayana drugs are similar to antioxidants and could be used to manage diseases mediated by the free radicals and concluded that *S. nux-vomica* having the potential antioxidant property along with other plants	Tripathi, 1998
Loganin, isolated from *S. nux-vomica,* had a significant effect in the prevention of galactosamine-induced hepatic damage in rats	Visen *et al.,* 1998
The chemical structure of strychnine was elucidated in 1947 owing to the major contributions of H. Leuchs and Sir Robert Robinson. Its absolute configuration was determined by X-ray crystallography in 1956 and total synthesis was achieved in 1963 by Woodward *et al.*	Saxton, 1983; Simon, 1999
Toxic effects of alkaloid extracts on the behaviour of chromatophores and mucous glands of Indian minor carp *Labeo bata* (Ham.)	Sinha *et al.,* 1999
S. *nux-vomica* (detoxified) assessed for sexual activity in rats and found that significant effect in sexual activity	Ali *et al.,*1999

The *Nux vomica* x V30, prepared with 90% ethanol from the ethanolic seed extracts has an effect of reducing sleeping time in the male adult albino mice	Sukul *et al.,* 1999
The use of *S. nux vomica* as an anti-ageing substance could be related to the anti-lipid peroxidation property of strychnine	Tripathi and Chaurasia, 2000
HPLC method for determination of brucine in seed dressing agents	Zhu *et al.,*2000
Methanolic plant extracts were more effective and significantly reduce induction time of diarrhoea and total weight of the faeces than aqueous plant extracts against the castor-oil induced diarrhoea	Shoba and Thomas, 2001
Anti alcoholic effect on toads	Sukul *et al.,* 2001
HPLC method which is accurate, simple and reliable for determination of strychnine and brucine in semen *strychni* and its processed products	Jiang *et al.,* 2002
Reports on the analgesic and anti-inflammatory activities of brucine and brucine N-oxide extracted from *S. nux-vomica* for evaluating pharmacological profiles	Yin *et al.,* 2003
Reports on the presence of iridiod glycosides	Zhang *et al.,* 2003a
A new method for the analysis of herbal Chinese medicines by capillary electrophoresis coupled to mass spectrometry	Feng *et al.,* 2003

A method to study the pharmacokinetic process in rat plasma of the alkaloids from processed seeds with RP-HPLC and concluded that this method is a good reference for pharmacokinetics in human bodies	Xu *et al.,* 2003
A method by capillary electrophoresis with field-enhanced stacking for rapid and sensitive determination of strychnine and brucine	Zhang *et al.,* 2003
The structure and composition of strychnine and brucine which were quite complex and elucidated based on the IR, ^{1}H-NMR, ^{13}C-NMR and MS the spectral data. Strychnine chemical formulae is $C_{21}H_{22}N_2O_2$ with molecular weight of 334.42, while composition in brucine is $C_{23}H_{26}N_2O_4$, with molecular weight 394.47	Verpoorte *et al.,* 1977; Verpoorte 1980; Cai *et al.,* 1994; Wang *et al.*,, 2004 Duverneuial *et al.,* 2004.
The analysis of strychnine from detoxified seeds using liquid chromatography-electrospray mass spectrometry	Choi *et al.*,2004
An efficient method for the determination of strychnine in blood by liquid chromatography	Duverneuial *et al.*,2004
The identification and quantification of the toxic alkaloids, strychnine and brucine, from postmortem specimens	Wang *et al.,* 2004

The ^{1}H NMR spectrometry and multivariate analysis techniques were applied for the metabolic profiling of three *Strychnos* species: *S. nux-vomica* (seeds, stem bark, root bark), *Strychnos ignatii* (seeds), and *Strychnos icaja* (leaves, stem bark, root bark, collar bark)	Frederich *et al.,*2004
An enantioselective total synthesis of (-)-strychnine was accomplished through Michael reaction and described that chemistry paves the way for the synthesis of more advanced *Strychnos* alkaloids	Ohshima *et al.,* 2004
The potent anti snake venom activity of the ethanolic seed extract of *S. nux-vomica* and the neutralizing effects of extracts against Daboia russelii and Naja kaouthia venom	Chatterjee *et al.,*(2004)
BHUx, a polyherbal formulation, is used to prevent atherosclerosis and has anti-inflammatory property which also consists of *S. nux-vomica* as one of the major ingredient with other plants	Tripati *et al.,* 2004
Some species of *Strychnos* have been employed mainly in hunting and fishing, as an adjunct to weapons and as well as in antiplasmodial activity.	Philippe *et al.,* 2004; 2005; Neuwinger, 1998 ; 2004
Concentration and determination of strychnine alkaloid in biological fluids.	Zhang *et al.,* 2005

Reported an easy, rapid method for simultaneous separation and determination of strychnine and brucine and its preparation by nonaqueous capillary electrophoresis	Li *et al.*,2006
A simple, fast and sensitive method for the quantitative determination of strychnine residues in urine	Van Eenoo *et al.*, 2006
A rapid method for separation of strychnine and brucine on a dynamically modified poly (dimethylsiloxane) microchip, followed by electrochemical detection	Zhang *et al.*, 2006
The methanolic extract along with other plant extracts were showed antihypouricaemic activity with 50% inhibition against potassium oxonate-induced hyperuricaemia in mice.	Umamaheswari *et al.*,2006
The strychnine and brucine showed pharmacological effects on several neurotransmitter receptors, including some members of the super family of ligand-gated ion channels	Jensen *et al.*, 2006
A new method for the enrichment of *Strychnos* alkaloids in biological samples via liquid-phase micro-extraction based on porous polypropylene hollow fibers combined with on-line sweeping in micellar electrokinetic chromatography	Wang *et al.*, 2006
The anti-tumor effects of the four alkaloids: brucine,strychnine, brucine N-oxide and isostrychnine from the seed, on the human hepatocellular carcinoma cell lines (SMMC-7721 and HepG2)	Deng *et al.*, 2006; 2006a ; Yin *et al.*, 2007

Table 7: Selected works on ***S. wallichiana***

Reports on the presence of indole alkaloids from the leaves of *S. wallichiana* Steud. ex DC which was collected from Bangladesh and found that icajine and novacine are the major alkaloids, while strychnine, brucine, pseudostrychnine, *N*-methyl-*sec*.-pseudo-β-coubrine, 14-hydroxyicajine, strychnine-*N*-oxide and brucine-*N*-oxide	Bisset and Choudhury, 1974
S. wallichiana seeds used as an adulterant of *S. nux-vomica*. It is one of the most endangered species according to IUCN red listed category	Anonymous, 2001

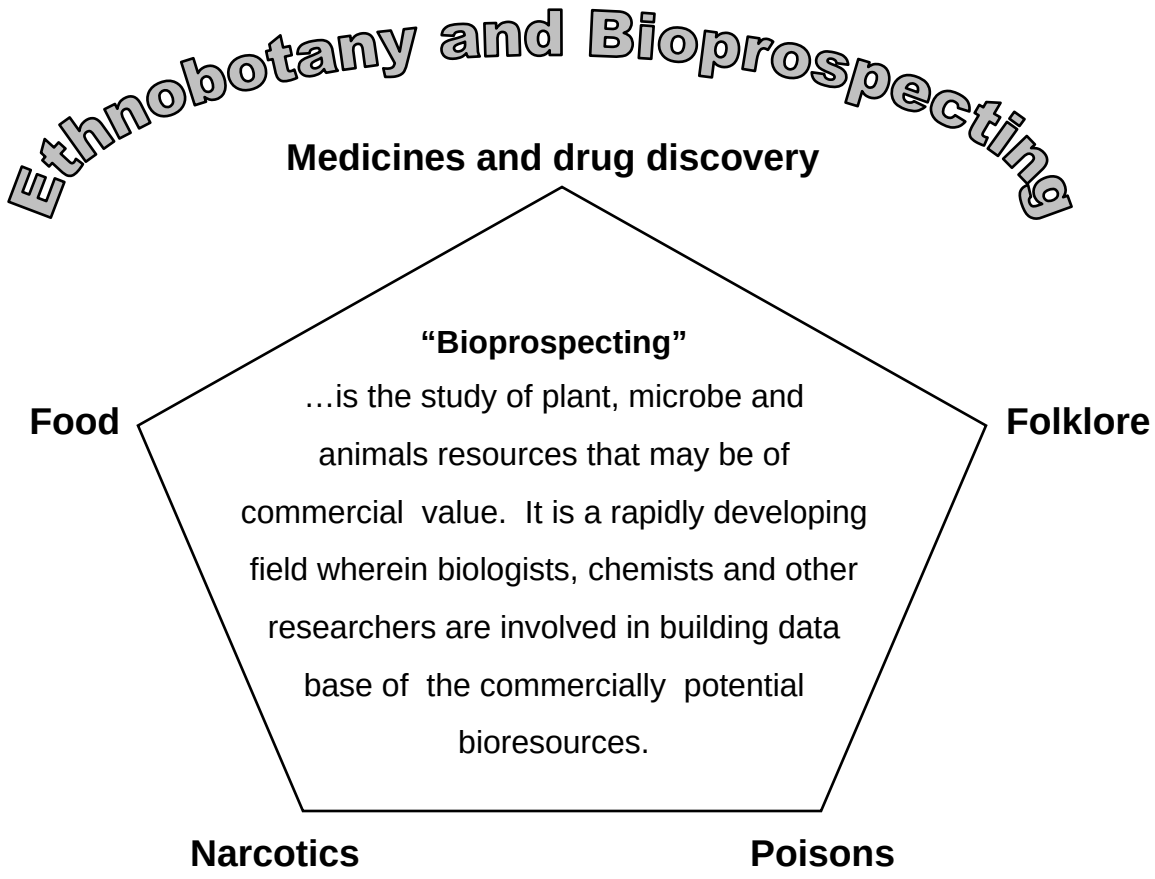


Figure 1: The scientific leads for the discovery of plant-based pharmaceuticals have been generated through: a) High-throughput screening and b) Ethnobotany Ethnobotany deals with studies among the tribal and rural people for recording their unique knowledge about plant wealth and for search of new resources of herbal drugs, edible plants and other economic aspects of plants.

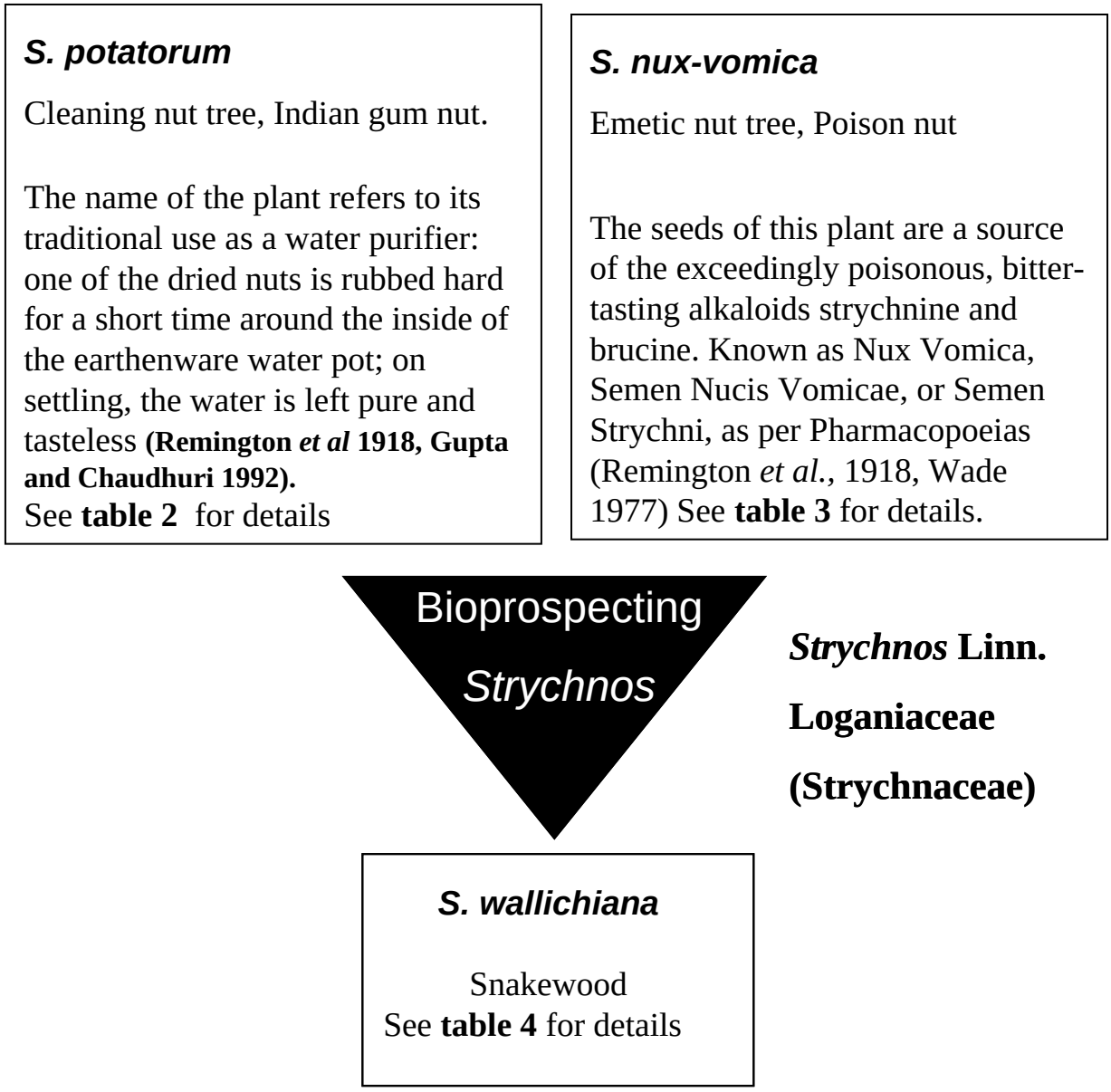


Figure 2: Bioprospecting *Strychnos potarorum, S. nux-vomica* and *S. wallichiana.* Lakshminarasimhan (2003) drew attention to the confusion over a period of 250 years regarding the identity of *Strychnos colubrina,* formally proposing that the name be rejected in favour of *Strychnos wallichiana*.

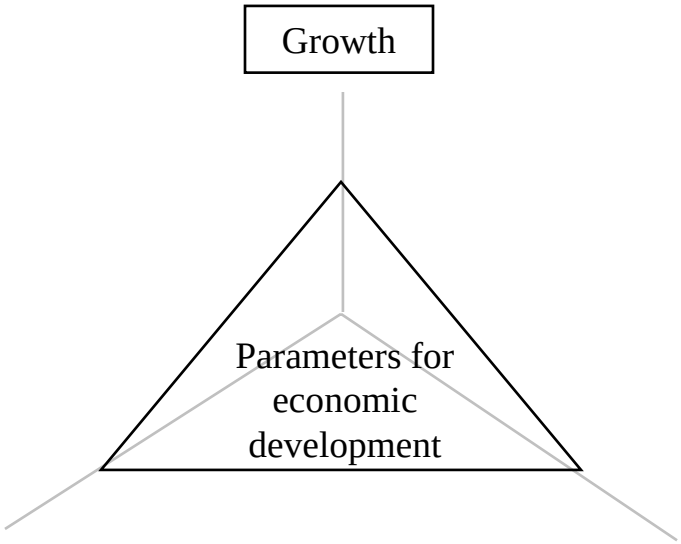


Figure 3: Girijan Cooperative Corporation Ltd (GCCL), Andhra Pradesh plays an crucial role in the socio-economics of the rural/tribal community. GCCL procures and sells minor forest produce for e.g. seeds, nuts, gums, resins, barks, roots etc. Bioprospection of under explored/ unexplored plant resources have relevance for the improvement of the the rural/tribal community economy

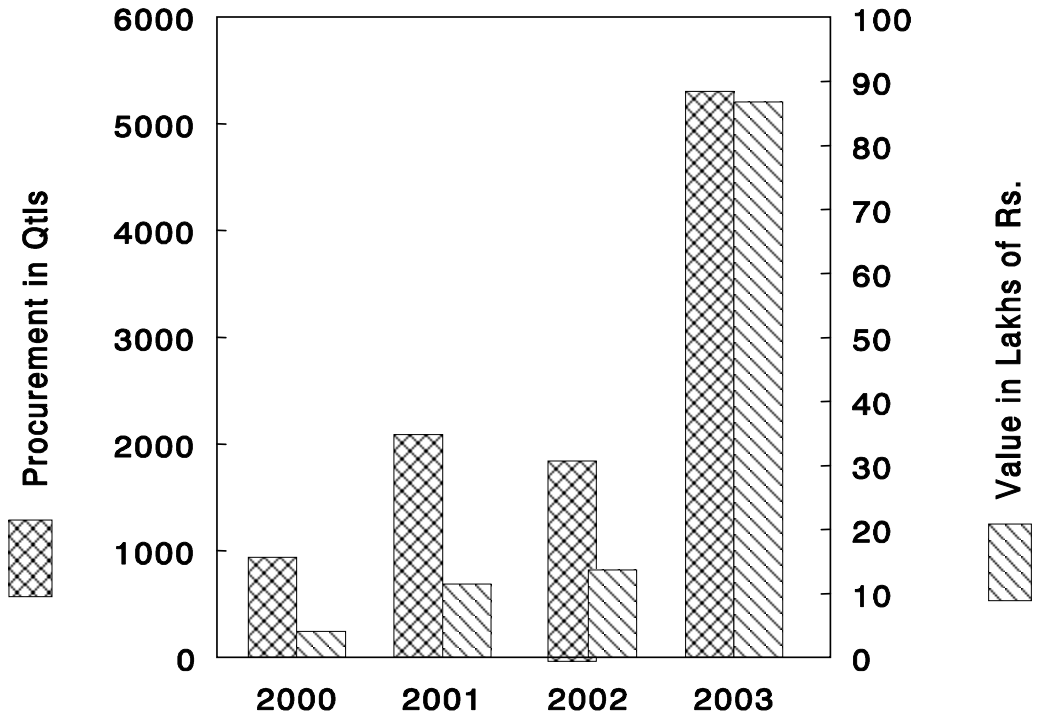


Figure 4: Graph demonstrating statistics of *Strychnos nux-vomica* seed procurement and value.

Source : Girijan Co-operative Corporation Limited, Visakhapatnam, Government of A.P.

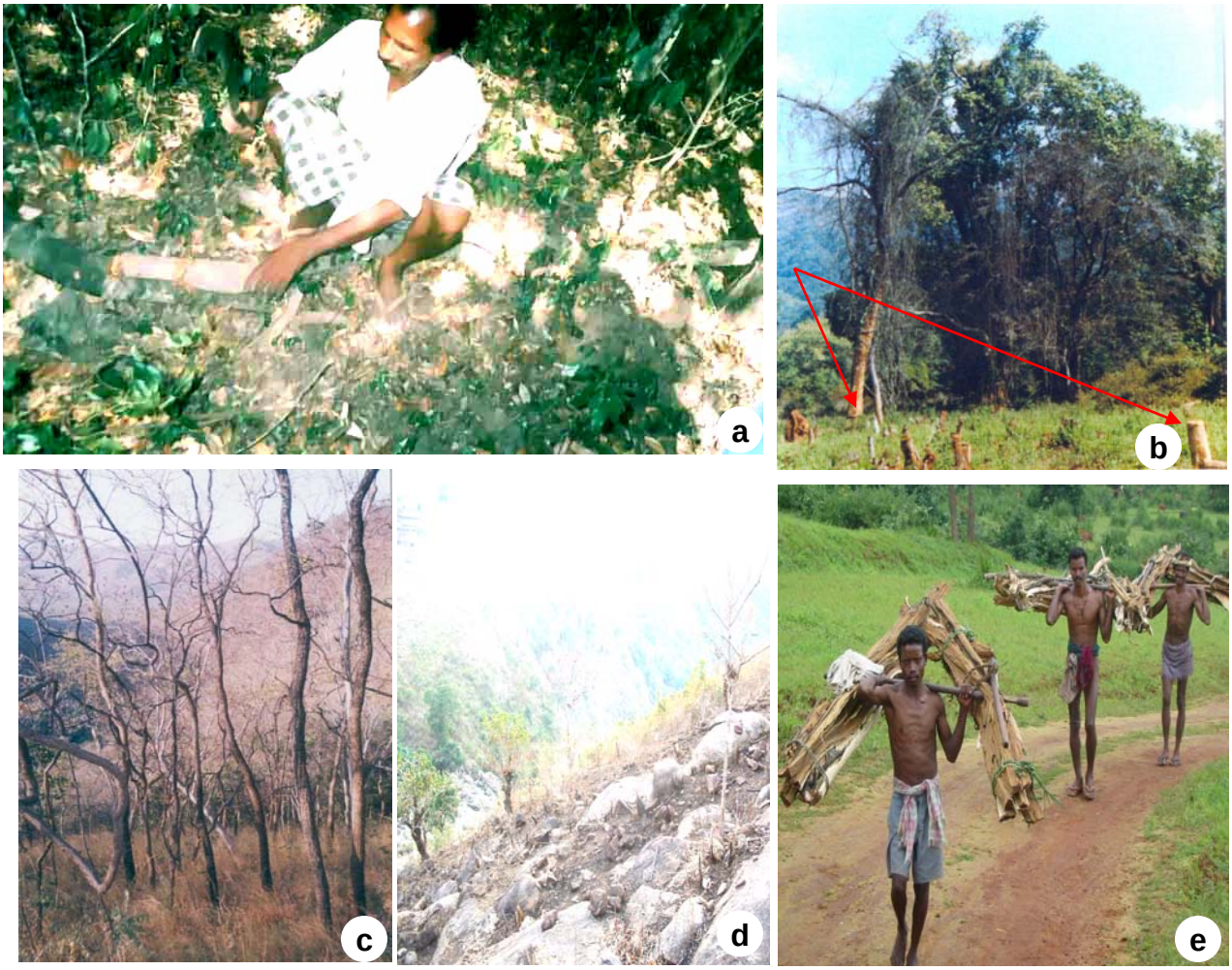


Figure 5: Yeruka and yanadi tribes exploiting *Strychnos* sp. in Andhra Pradesh

a. *S. wallichiana* - root collection by local tribe at kutlamari valley (Rapur-veligonda range)

b. Destruction of *S. nux-vomica* in natural population

c. Natural deciduous forest

d. Forest clearance

e. *S. nux-vomica* wood collection by local tribes

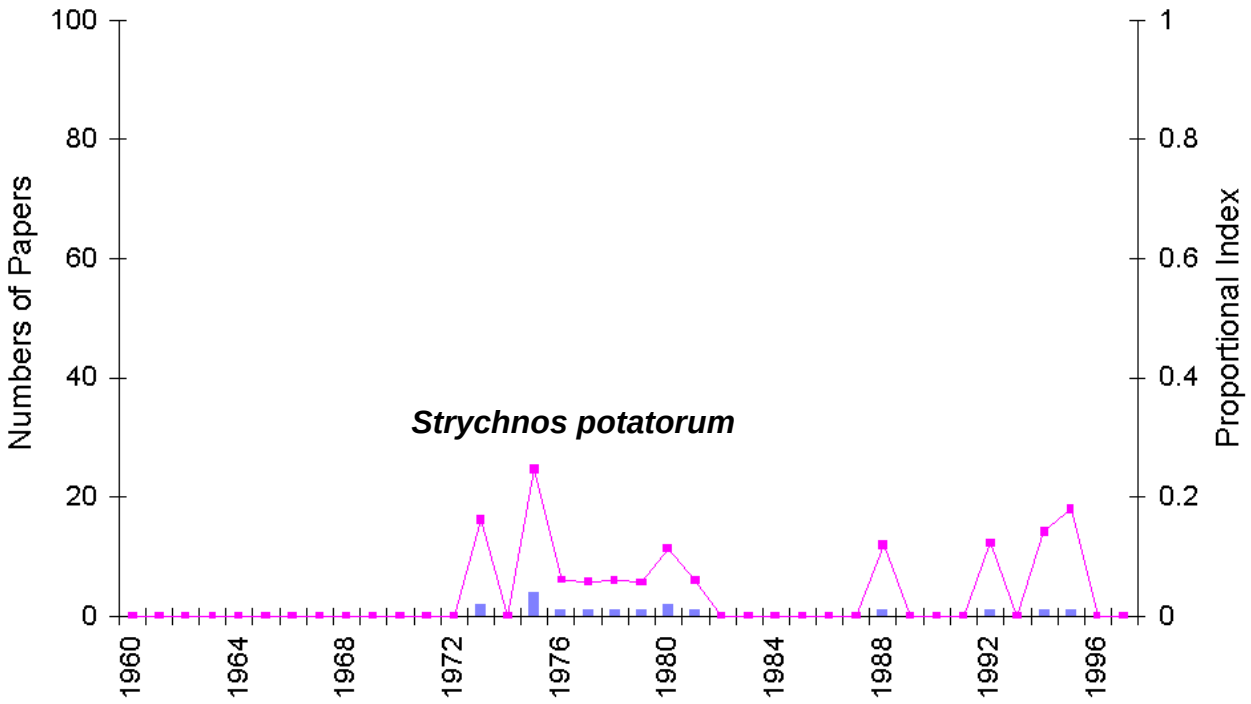


Figure 6: ***Strychnos potatorum*** - Analysis of numbers of papers/mentions over time. Source: AGRICOLA database (1970-1996)

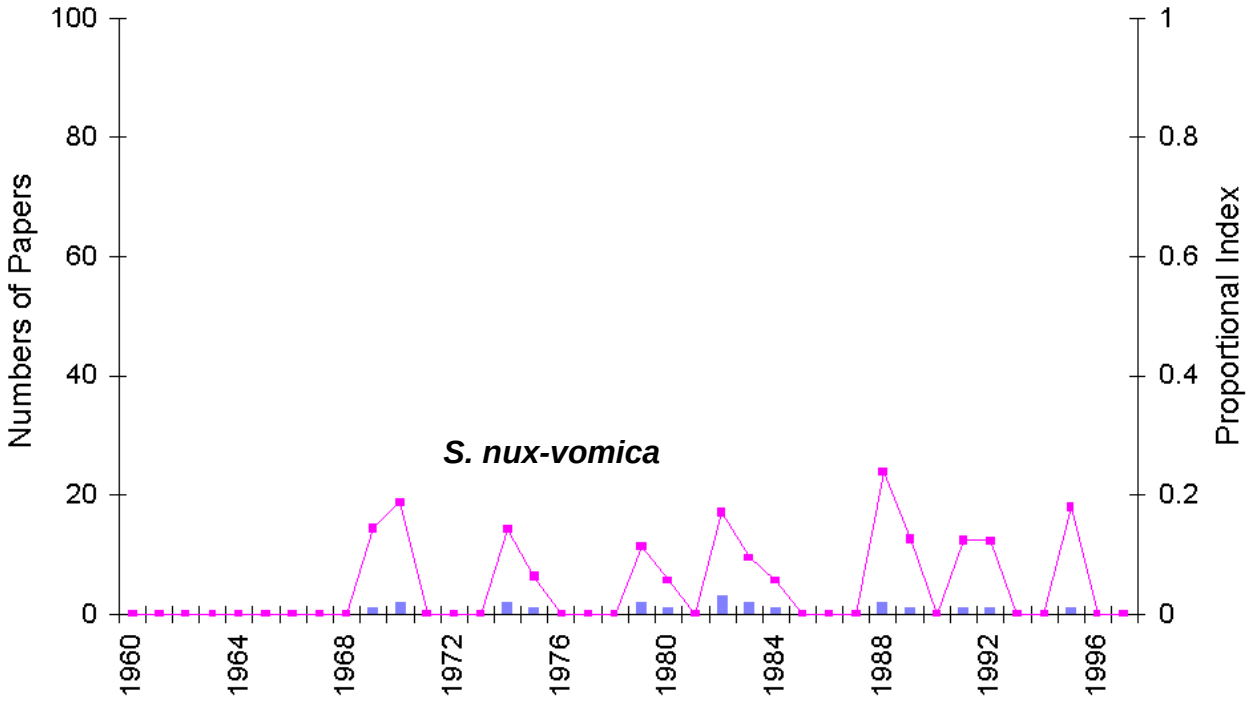


Figure 7: *S. nux-vomica* - Analysis of numbers of papers/mentions over time

Source: AGRICOLA database (1970-1996)

2 Problems and prospects

2.1 Ecological and commercial implications

Strychnos species is in cosmopolitan distribution and occurs in tropical dry deciduous forests. Extensive field survey has been done in the major parts of Deccan eco-region and collected elite germplasam from the following districts. They are Adilabad, Anantapur, Chittor, East Godavari, kadapah, Karimnagar, Khammam, Mahaboobnagar, Nellore, Prakasam, Srikakulam, Visakhapatnam, Vizayanagaram, Warangal and West Godavari districts of Andhra Pradesh which are rich in traditional knowledge and ethnobotany.

The crucial step for conservation of plant genetic resources is collection and establishment of an organized database. Coordination is required to collect reasonable samples of elite germplasm and to establish field gene banks. The sampling scheme should be based on the level and distribution of genetic diversity within a species and its partitioning within and among individuals and populations. The genetic structure of some species is homogeneous across its wide range, and its detectable genetic variation is nearly zero. Here few collections might be sufficient for genetic conservation and few locations might be sampled as a precaution against missing genetic variants. Some species have high levels of genetic diversity, and extreme populations may differ extensively in their genetic resources. In such cases, larger samples should be collected to capture the species genetic resources. The germplasm samples should be stored in core and active collections. Seeds from each individual plant should be maintained separately in active collections. Core or base collections are intended for use only in extreme cases

with the hope that they will never be needed for active breeding or reforestation. Active collections provide materials needed to enable testing or evaluation of the resource, facilitate breeding programmes, and to provide materials that can be multiplied to reintroduce key populations to the forest in the event of catastrophes (Ledig, 1993).

These plants are under threat due to high commercial value. The natural population is dwindling due to several factors such as, over exploitation for medicinal properties, habitat conversion, urbanization, spread of alien species, and consequent abiotic (environmental) changes, biotic disturbances, environmental catastrophes, dwindling forests is also one of the causes for its depletion and resulting loss of genetic variation. Even human interference has been more often accountable for the depletion of plant resources and subsequent decline of this species to the very risk of extinction. Majority of traders collect wild medicinal plants through untrained and unskilled laborers or local tribes, which is generally in the form of exploitation of the entire plant material, seeds, bark, roots, before seed set, making low regeneration of the species in its natural habitat. Earlier these plants were used only by the local people in small quantities but commercialization of these species in recent years has increased their demands and consequent exploitation resulted in depletion from the wild. Though the species were well established previously, now it has come under threat category in some areas due to some of the above enumerated factors. *S. potatorum* is also in threatened state and Govt. of India prohibited export of this plant, plant derivatives and products from this plant [Under negative list of Exports, of the Export and Import Policy, 1997-2002]. *S. nux-vomica* is in more threatened in Western Ghats when compared to Eastren ghats.

S. nux-vomica is renowned for drug value of its poisonous alkaloids, namely strychnine and brucine and hence, commonly called as "*nux-vomica* of commerce" (Troup, 1921). *S. wallichiana* is woody climber, its distribution is very limited, restricted to confined regions and at present it occurs only in Kutlamari-Veligonda range Eastern Ghats. The present status of the plant is critically endangered. After, Suryanarayana and Sreenivas Rao (2001) this is the only report.

2.2 Factors affecting germination

In natural habitat *S. nux-vomica* seeds complete germination in 70-120 days (Krishnamurthy, 1993; Rao, 2001). *S. potatorum* is similar to *S.nux-vomica* but viability of the seeds are very poor (Kirthikar *et al*., 1935). The present status of the plant is critically endangered. The distance between two plants is about of 20 kilometers and the viability of the seeds is very poor. Plant propagation is by vegetative means and also seeds but viability of the seeds is very poor (Anonymous, 1976). It has been observed that *S. potatorum* grows naturally and regeneration through seeds is negligible and recruitment of seedlings almost absent. Kumar and Bhanja (1992) observed only 10% seed germination of this species in Andhra Pradesh (India), indicating its poor germinability. Whitish pulp covering the seeds attracts a number of storage fungi which hamper the germination of the seeds both in nursery and forest. The seed exhibits dormancy because of hard seed coat. These problems attracted attention to find out the causes of poor regeneration of this species through seeds.

2.3 Conservation Implications

It is obvious that trade in medicinal plant has become serious implication on the survival of the plant species. If one considers the plant material exported to the developed countries and the material collected for indigenous use, certainly the survival of many species may well be under threat given the increasing demand for medicinal plants. There are number of reasons as to why the trade in wild harvested material has been left to reach such a critical point, one is that the legislation that exists to control harvesting and trade of medicinal plants is inadequate and ineffective in its current form. New policies and easier mechanisms to control the trade are needed. Lack of awareness among many of the end users, as to the extend to which wild harvested materials are used; indeed, it is only the last five to ten years that wild harvesting has become a subject of concern. In order to control the market, the traders will be given virtually no information on the extent of wild-harvesting. It has become absolutely essential to apply process of sustainable harvesting methodologies immediately to these species to reduce the risk factor for their existence.

The biodiversity loss is not only a threat to ecology of the planet but also immediate threat to the livelihood security of the rural communities. Thus, the developing countries are put under tremendous pressure to adopt intellectual property rights (IPR) regimes that accord with the standards of North America, Europe, and Japan. It is well understood that bio-technology not only support the present need but also protects the endangered plant species in their natural environment; and establishment of botanical garden, gene-bank to conserve the genes and techniques of sustainable harvesting will

certainly help to conserve the natural resources. Since the beginning of this century more than half of the worlds tropical forest area has been destroyed and today more than 11 ha of tropical forest are lost every day, and the experts estimate that only 5-10% of all plants in the world have been systematically investigated for their pharmacological activity and many of them are uninvestigated and are threatened in the tropical forest. Strong strategies in terms of conservation through biotechnology and legal support need to be evolved.

Conventional methods of multiplication are an age old practice in India and other South East Asian countries. It is one of the fast, simple and economical methods for the mass multiplication of species and for production of uniform planting materials of superior quality (Hartmann *et al.,* 1990). Micropropagation is gaining increasing attention as a tool for tree improvement and establishment of clonal plantations. Use of this technique can make investment in cultivation of plants more attractive by increasing yields and quality, and by shortening rotation age. Micropropagation is another alternative method for mass multiplication of plant species. Propagation of plant species by *in vivo* and *in vitro* methods using different explants (*viz*., seeds, nodes, internodes, shoot tips and leaves etc.) and different media with different combinations of hormones is generally effective means of establishing a wide variety of plants within a short span of time. The key advantages are pathogen free and without any environmental disturbances (Withers and Engelmann, 1997).

2.4 Importance of *Strychnos* in cancer therapy

Strychnos has been widely used in traditional medicine from ancient times (Table 2, 3 and 4) reported in Chinese medicine for the treatment of liver cancer (Dengu *et al*., 2006). The Multiple myeloma is an incurable malignancy, tumor of hematopoietic system preceded by an age-dependent premaligant disease. It gained resistance towards the available anti-cancer drugs and has also been demonstrated severe side effects. The use of medicinal plant extracts to cure cancer with minimal side effects is gaining importance. No substantial research has been carried out in India on the efficacy of active principles of *Strychnos* for the treatment of the MM. Therefore, the present work has been undertaken to evaluate the effect of active principles of root extracts of *Strychnos* on the MM Cell lines (RPMI 8226 and U266B1) for bioprospection targeting towards identification of lead molecules.

2.5 Objectives

1. To assess the traditional uses, ethnobotany and commercial potential of *Strychnos potatorum, S. nux-vomica* and *S. wallichiana.*
2. To study the seed germination as a function for the dwindling population of *Strychnos* sps.
3. To evaluate the i*n vitro* differentiation for multiplication of *Strychnos potatorum S. nux-vomica* and *S. wallichiana.*
4. Extraction, purification, and characterization of Indole alkaloids from *Strychnos wallichiana.*
5. To investigate anti-proliferative and cytotoxic properties of *Strychnos* extracts and its active principles on multiple myeloma cell lines (RPMI 8226 and U266B1).

3 Plant material and road map to work

3.1 Study area

Andhra Pradesh is rich in traditional knowledge districts namely Adilabad, Anantapur, Chittor, Cuddapah, Nellore, Prakasam, Kurnool, East Godavari, Karimnagar, Khammam, Mahaboobnagar, Srikakulam, Visakhapatnam, Vizayanagaram, Warangal and West Godavari, were chosen for field study (Figure 8). Plant genetic resources are rich in these areas together with folklore and traditional knowledge. Pullaiah and Chennaiah (1998) recorded 2531 species in the State of Andhra Pradesh, of which about 400 are endemic. Globalization and market economies are partly responsible to the loss of plant genetic resources (Tables 3 and 4). The following areas (district in bold face followed by locality) have been surveyed and *Strychnos nux-vomica, S. potatorum* and *S. wallichiana* (Figures 9-11) germplasm was collected: Srikakulam (Mahendhragiri hills), Khammam (Taliperu, Kothur), Warangal (Pakala, Tandwai, Mallur, Atmukur), Medak (Narasapur), Ranga Reddy (Gachibowli), Nalgonda (Mannanur, Vatavaripally), Kurnool (Srisailam, Dornal), Kadapa (Balpally Siddhavotam, Sanipaya, Gadela, Rayachoti, Rajampet, Kodur), Chittoor (Talakona, Tirupathi, Thirumala, (Sri Venkataeswara University Campus, Bakrapeta), Nellore (Rapur, Penchalakona, Tegacherla, Somasila, Kutlamarri, Mallemadugu, Veligonda Hills), Prakasam (Chinnaruntla). Outlines of work carried out were shown in figure 12.

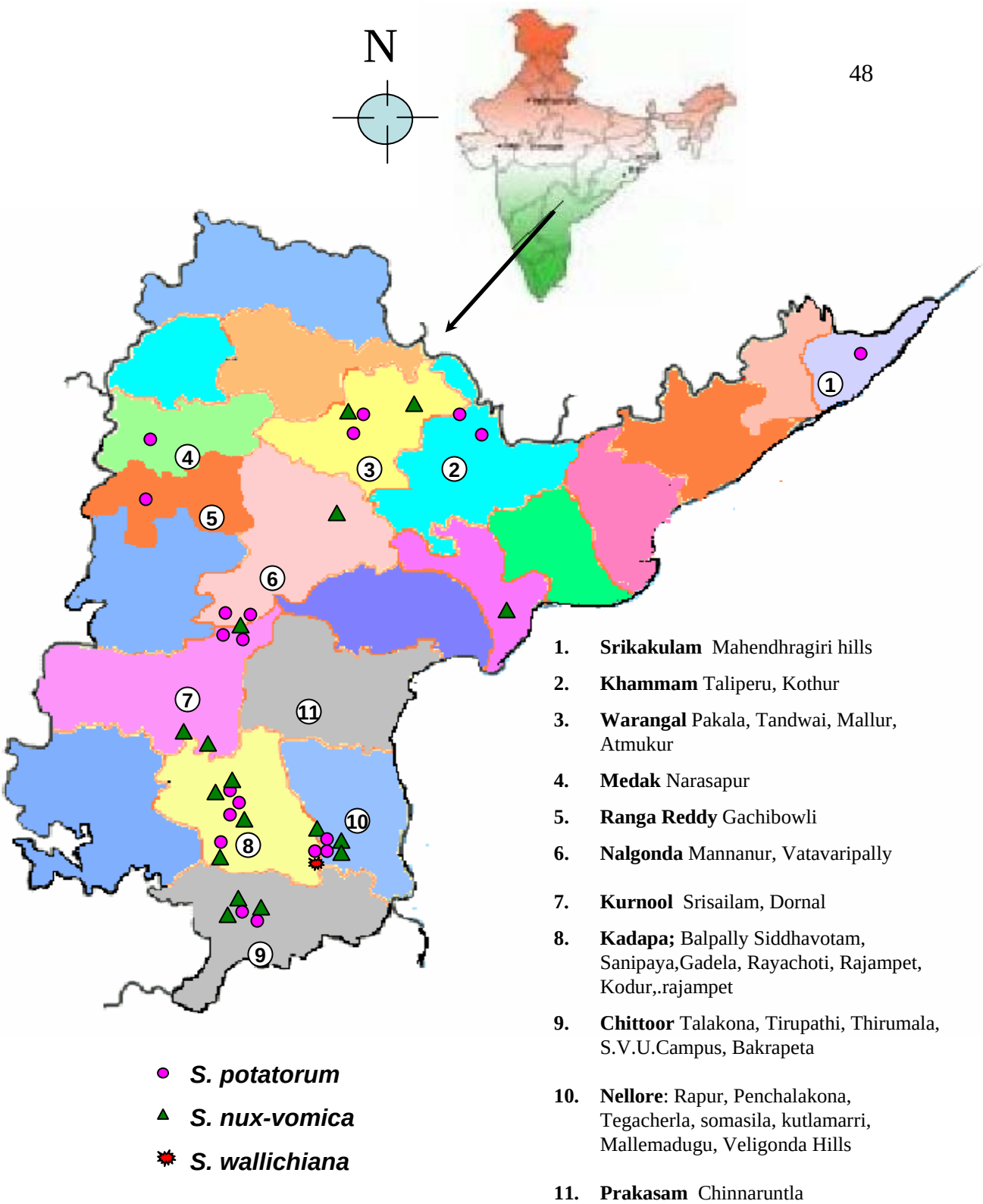


Figure 8: Collection of *Strychnos nux-vomica*, *S. potatorum* and *S. wallichiana* germplasm from different localities of Andhra Pradesh

Figure 9: Natural habitat, floral and morphological characters of *Strychnos potatorum*

a. Naturally grown *S. potatorum* in University of Hyderabad campus; Ranga Reddy Dist.

b. Fruit setting in the month of November

c. Flowering in the month of September

d. Sun dried seeds

e. Mature fruits

Figure 10: Natural habitat, floral and morphological characters of *Strychnos nux-vomica*

a. Naturally grown *S. nux-vomica* in vetavarupally Kurnool Dist.

b. Fruit setting in the month of March.

c. Flowering in the month of January

d. Sun dried seeds.

e. *Mature fruits.*

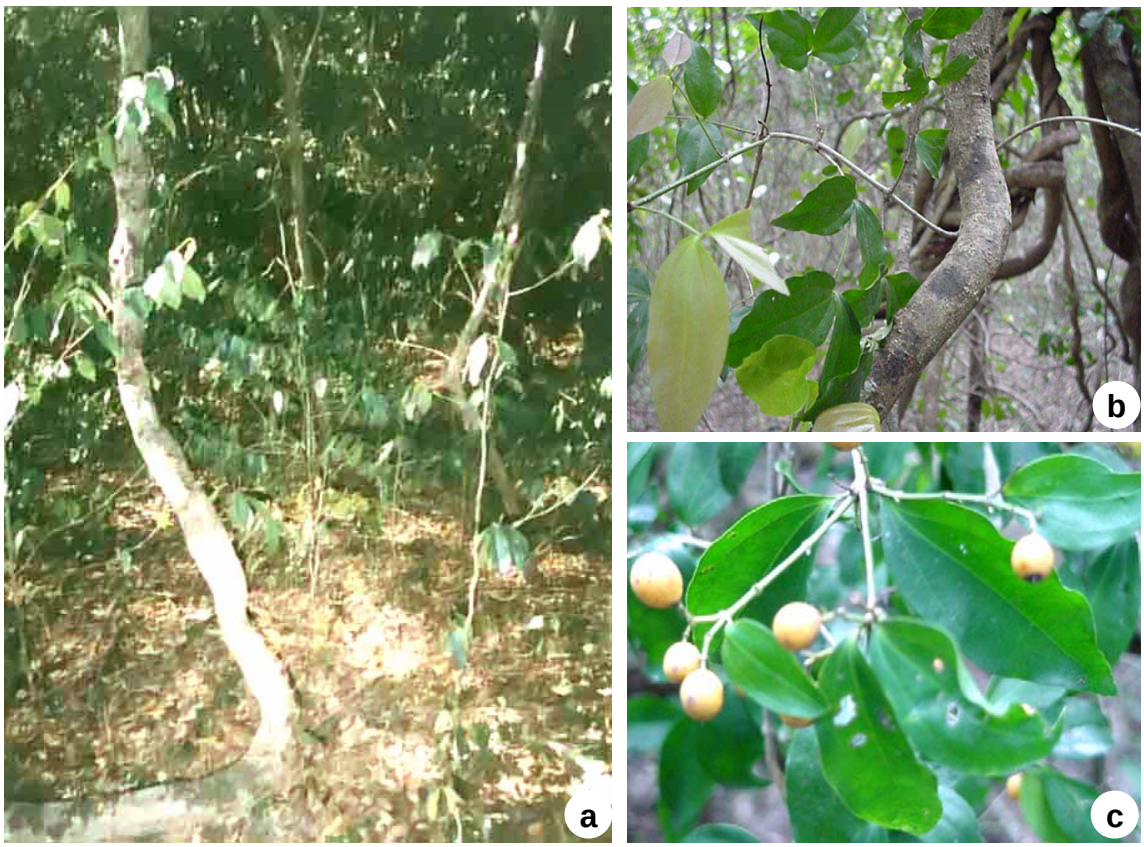


Figure 11: Natural habitat, floral and morphological characters of *Strychnos wallichiana* *a.& b.* Habit of *S. wallichiana* in Kutlamarr valley, Rapur veligonda range, Nellore Dist. *c*. Fruit setting in the month of April

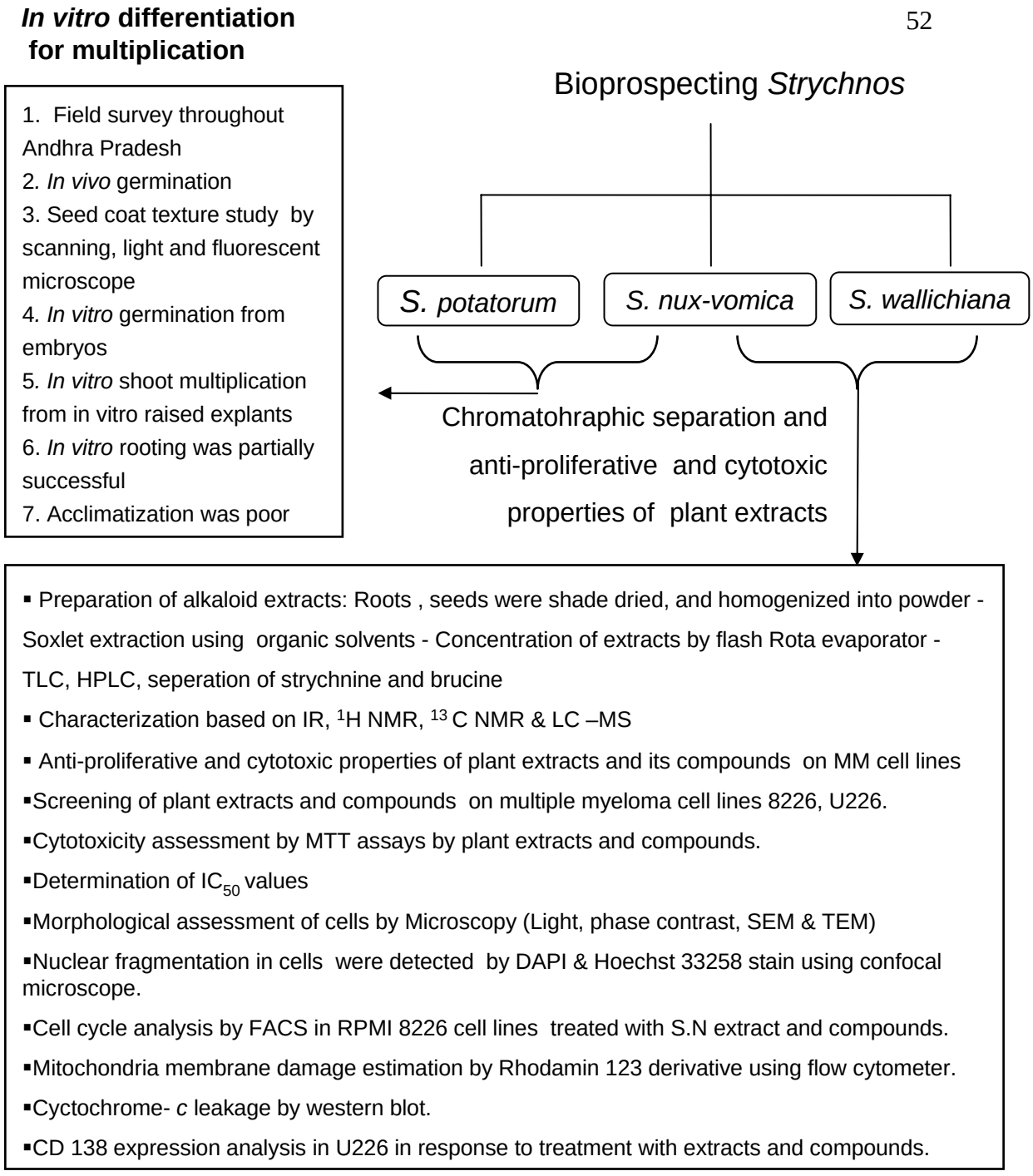


Figure 12: Outlines of work carried out

4 *In vitro* differentiation for multiplication of *Strychnos potatorum S. nux-vomica* and *S. wallichiana.*

4.0 Introduction

Tropical forests, the most complex of all the terrestrial ecosystems and major repository of biodiversity, are undergoing rapid fragmentation and degradation all over the world. The conversion of tropical forests into land for agriculture is a major threat to biodiversity (Dobson *et al.,* 1997). Large areas are brought back under forest cover in order to reverse the current trend of deforestation and to conserve biodiversity. Dry forests are more threatened and less protected than moist and wet forest (Gerhard, 1993). Also, the original extent of dry forest will never be known, since many of them were converted to grasslands, open secondary forest, savanna, or lodged, burned and converted in to cattle ranches or agricultural lands over hundred of years. The status of dry forest is just as critical, or worse for regions of Australia, South-East Asia, Africa and major parts of South America (Janzen, 1988). In India *S. potatorum*, *S. nux-vomica* and *S. wallichiana* is distributed in dry deciduous forests which are highly depleted at alarming rate. Many flora and fauna were eliminated and among them *S. potatorum* is also facing threat. In recent years much attention has been given to

medicinal plants as a source of curare compounds for severs aliments. Phytochemical compounds have been isolated from this species during the past few years, including alkaloids produced from root, stem bark fruits, and seeds exhibiting anti microbial and antifungicidial activities.

Due to well known traditional uses, there is an increasing demand for fruits/seeds, bark and wood of this species by the Indian pharmaceutical industries (Tewari, 2000). A recent estimate puts the demand for *S. nux-vomica* fruits in the Maharashtra state of India alone as 300kg per year (Khadiwale, 2000). Pharmaceutical companies depend largely upon material procured from natural stands that are being quickly depleted. Medicinal plants are living resources that are exhaustible if overused, but sustainable if properly used. Modern practices of harvesting are indefensible, and many studies have highlighted depletion of the reserve base. To meet the prospect requirements of raw materials for pharmaceutical industries and local medicinal consumption, urgent attention should be paid to farming of medicinal plants. The development of rapid large scale propagation systems for rare medicinal plants is a necessity in order to meet pharmaceutical needs and also prevent the plants from becoming endangered or extinct.

As propagation by seed is the most frequently used and cheapest method, information of seed management is a prerequisite for growing this species successfully. In spite of its massive industrial potential and the declining status of

its natural population, little or no attention has been given for the conservation and establishment of huge range plantations of *S. potatorum,S. nux-vomica* and *S. wallichiana.* The natural regeneration of the species in its environment is heavily constrained by massive collection of fruits for industries or local medicinal practices. This reduces the availability of propagules in the forest floor for the establishment of new seedlings. In addition, it has been shown that freshly harvested seeds of *S. nux-vomica* germination is poor and take about 70-120 days (Krishnamurthy, 1993; Rao, 2001).

The seeds of *S potatorum, S. nux-vomica* and *S. wallichiana* are rich source of carbohydrates and the endosperm is covered with a layer of hard radially elongated cells, termed as macrosclereids, which were compactly arranged and interspersed making the seed coat very hard. The rich endosperm serves as a reserve food for the developing embryo. Plant propagation is by vegetative means and also, seeds but viability of the seeds is very poor (Anonymous, 1976). It has been observed that wherever *S. potatorum* grows naturally, its regeneration through seed is negligible and recruitment of seedlings almost absent. Kumar and Bhanja (1992) observed only 10% seed germination of this species in Andhra Pradesh, indicating its poor germinability. Whitish pulp covering the seeds attracts a number of storage fungi namely *Alternaria alternata, Fusaarium oxysporum, Aspergillus flavus, Aspergillus niger* and *Rhizopus*

Species (Anonymous, 2003) which hamper the germination of the seeds both in nursery and forest.

Seeds that fail to germinate under favorable environmental conditions are considered to be dormant. The seed exhibits dormancy because of hard seed coat. The different types of dormancy, and their underlying causes and methods to break them, are described in Baskin and Baskin (1998) and Bewley and Black (1994). There is currently no information on whether seeds of *S. potatorum, S. nux-vomica* and *S wallichiana* possess dormancy, and if so, what type of dormancy it possesses and how can it be broken. For *ex-situ* conservation purposes, the storage behavior of seeds, i.e., desiccation sensitivity, storage temperature and moisture content of the seeds, and the longevity of the seeds in storage, should be known. Such information is scanty or not available for *S. potatorum*, *S. nux-vomica* and *S. wallichiana*.

At present, natural forests are the source of *Strychnos* genus. It is not cultivable variety; the plants set seeds natural regeneration and conventional propagation through cuttings are poor and insufficient for conservation. Conventional methods of multiplication such as cutting, budding, grafting and air layering are not available in this species and seed germination is very poor. Therefore a method to multiply this plant using modern methods is needed.

Medicinal plants are of vast concern to the researchers in the area of biotechnology not only for quick propagation but also for the production of

secondary metabolites (Vander Heijden, 1989). Micro-propagation has been advocated as one of the most potential biotechnological tools for *ex-situ* conservation of germplasm. Moreover plant *in vitro* regeneration is a biotechnological tool that provides a means of the plants which offers a potential solution to the plant resources which are being depleted at alarming rate as a result of over exploitation. As propagation via stem cuttings is cumbersome, micro propagation is an effective for meeting the need of clones of *Strychnos* plants within reasonable time frame without affecting the wild bioresources. Propagation through seeds via *in vitro* germination and axillary shoot bud multiplication is an easy and safe process for obtaining homogeny, and it also assures the reliable production of true-type plants with in a short span of time. Many medicinal plants have been propagated through micropropagation.

Non-availability of the plants in other parts of the India and rapid depletion due to over exploitation of the natural stands has forced the researchers to look for alternatives. New propagation methods would be beneficial in accelerating large scale multiplication, improvement and conservation. Limited tissue culture work has been done in *Strychnos* species; to date only one study has been reported on micropropagation of *S. nux-vomica* through hypoctyles (Kumar and Datta, 1989). However rate achieved by this investigation was very low. In case of *S. potatroum* and *S. wallichiana* the lack of viable seed, limits the opportunities for ex-*situ* germplasm storage. These problems attracted attention to

find out the causes of poor regeneration of this species through seeds and to establish alternatives.

Rationale for *in vitro* multiplication of *Strychnos potatorum, S. nux-vomica* and *S. wallichiana* :

a) Population is dwindling as seed germination is poor, perhaps due to hard seed coat: Morphological observations were performed directly on seeds of *S. potatorum S. nux-vomica* and *S. wallichiana* using scanning electron microscopy (SEM). The seeds for SEM were washed and dried, and then mounted without further drying on aluminum stubs using double-sided conductive tape. The seeds were sputter coated with 20nm layer of gold-palladium, and examined under Philips-XL ESEM microscope, at 20mm working distance and a consistent spot size. The resulting images were captured and stored in the computer attached to the microscope (Figures 13, 14, 15, and 16)
b) Over exploitation – Commercial and medicinal value
c) Seeds exhibit prolonged dormancy due to hard seed coat
d) Very poor viability of seeds
e) The germination period is lengthy (70-120 days)

4.1 Experimental

4.2 Seed collection

Collection of *S. potatorum S. nux-vomica* and *S. wallichiana* seeds were collected from local forests of Andhra Pradesh and were deposited in our field gene bank located at the University of Hyderabad.

4.3 In *vivo* germination of seeds

A nursery plot was selected and soil was dug to a depth of 10 cms and mixed with 1:1 ratio of sand and FYM. Four beds in the sub plots each measuring 50 x 50 cm were prepared and the medium containing soil mixture was reduced to fine tilth to facilitate seed germination and radicle growth. Line sowing method was followed for sowing the seeds, for which lines are prepared by means of a stick, with line to line distance of 5cms on either side. 5 lines were prepared in each bed to facilitate sowing of 50 seeds per bed (Bahuguna *et al.*, 1987). All seeds were sown at a depth of 1cm. First plot was used as a control and remaining plots were used for treatments. 50 seeds were sown. This was repeated with another batch of seeds. Seeds were subjected to pretreatments (Mild hot water + 48hr soaking, Mechanical scarification and Sulfuric acid scarification) in order to obtain rapid and uniform germination (Aliero, 2004). Observations were made on daily basis to check germination of seeds. Emergence of radicle was considered as the criterion of germination (Sadhu and Kaul, 1989; Salisbury and Ross, 1993).

Germinated seeds were scored out and the data was transferred into the tabular column. The temperature at the department nursery ranged between 24.6^0C [min] and 33.8^0C [max].

4.4 *In vitro* germination of zygotic embryos

Methodology feasible for *in vitro* propagation of *S. potatorum S. nux-vomica* and *S. wallichiana* is detailed in Figure 17. Seeds were soaked in water for 48 hrs and thoroughly washed in running tap water 2-3 times. Then the seeds were washed with distilled water and kept in orbital shaker at room temperature of 28^0C at 175rpm for 24hrs. Later seeds were surface sterilized with 70% Ethanol for 15 minutes and disinfected in 0.1% $HgCl_2$ for 15 minutes, seeds were washed three times with sterile distilled water. Seeds were air dried in a laminar flow hood for 10 minutes prior to dissection and embryo excision (Rai and Comb, 2002). Each *S. potatorum* seed consists of a mature embryo with two cotyledons, a short plumule and a thick radicle. Polyembryony seeds were avoided for culture. The mature zygotic embryos were removed with a surgical scalpel and were placed horizontally on MS (Murashige and Skoog, 1962) medium (induction medium) alone, supplemented with different concentrations of Cytokinin BAP. Media was dispensed in 500ml screw –capped glass jars containing 50 ml per jar.

The induction medium was MS basal medium supplemented with BAP at various concentrations and 3% (w/v) sucrose. The media was solidified with 0.8% agar (Hi-media, India) and the pH was adjusted to 5.8 with 1N NaOH or 1N HCl

before autoclaving at 121^0C for 20 min. Each treatment consisted of 20 explants and the experiment was repeated thrice. All the cultures were maintained under 16/8 hrs [light/dark] photoperiod at a temperature of 25 ± 1^0C

Turning of embryos in to green color and emergence of radical can be accounted as germination. *In vitro*-raised seedlings are used as explant source for shoot multiplication *i.e.* hypocotyl, epicotyl, nodal segments, and shoot meristem. Nodal segments responded in giving rise to multiple shoots. Primary shoots formed *in vitro* were sectioned into 3-4 node pieces, after removing the leaves. The nodal segments containing the dormant axillary buds were cultured on MS media supplemented with BAP at various concentrations and 3% (w/v) sucrose. The media was solidified with 0.2% Phytagel (Sigma) and the pH was adjusted to 5.8 with 1N NaOH or 1N HCl before autoclaving at 121^0C for 20 min. *In vitro* rooting was carried out in MS supplemented with Auxins.

Twenty explants were used per treatment and each experiment was repeated atleast three times. The data pertaining to mean percentage of cultures showing zygotic embryos germinated, mean number of multliple shoots per culture and percentage of response were statistically analyzed by ANOVA and Duncan multiple range test (Harter, 1960).

4.5. Results of *Strychnos potatorum* - Dormancy and Seed germination

SEM studies of *S. potatorum* seed suggests that the seed is a rich source for carbohydrates and endosperm is covered with a layer of hard radially elongated cells, which are paliside but devoid of intercellular spaces, may be termed as malphigian cells or macro sclereids. The normal view of the seed surface (Figure 13 a) with interwoven sclereids are responsible for hard seed coat (Figure 13 b and c). The internal structure of seed (Figure 13 d) shows the presence of multicellular hairs originating from a thick layer of cuticle, below compressed epidermis. The epidermal hairs are seen bending towards one side and have a bulbous base. These are the idioblasts below the cuticle/epidermis containing raphides. There is an extensive zone of sclereids which make up the albumin and the embryo (Figure 13 e and f). The special tissue is composed of macro sclereids which are isolated and are long and tapering towards both the ends with a narrow cell lumen (Figure 13 g). They vary in size and both length and width (Figure 13 h). Such sclereids are interspersed and compactly arranged in making the seed coat very hard.

Free hand sections of water soaked seeds reconfirmed the presence of unicellular hairs originating from thick layer of cuticle, below which compressed epidermis exists. Light microscope images of *S. potatorum* dissected seed revealed that sclerenchymatous tissue consists of extensive layer of

macrosclereids which vary in size (Figure 14 a and b) and exhibited auto florescence due to the presence of bioactive compounds (Figure 14 c and d).

4.6 *In vivo* response

Seed germination in *S. potatorum* is epigeal and germination period was lengthy (Figure 18 a). The opening of cotyledonary leaves takes place after 45 days. Treatment with H_2SO_4 and mechanical scarification was ineffective in breaking the seed dormancy and none of the seeds germinated. However mild hot water treatment showed maximum percentage of germination was 24% after the end of 50 days, where as in control only 9% germination was recorded (Table 8).

4.7 *In vitro* response

The methodology for *in vitro* propagation was described in the flow chart (Figure 15). Embryos separated from the mechanically scarified seeds were transferred to MS basal salts, vitamins and 3% (w/v) sucrose, with and without growth regulators. Turning of embryos into green color and emergence of radical can be accounted as germination. 33% of embryos germinated into complete plantlets on the MS media devoid of growth regulators with well-developed roots without secondary callusing of the embryos. However, the rate of germination inclined to 78% on MS media containing cytokinin BAP and plantlets had a well-developed root system. The growth rate of *in vitro* germinated embryos was faster (Figure 18 b and c). Maximum 78% of germination was observed after one week

in MS+1mg L^{-1} BAP (Figure 18 c), where as only 70% of germination was observed in 0.7, 0.8 and 0.9 mg L^{-1} BAP respectively (Table 9).

Table 8: *S. potatorum in vivo* germination of seeds

Treatments	Germination of Seeds (Mean ± SE)	% of response
Control	4.50 ± 0.5	9
Mild hot water + 48 hr soaking	12.0 ± 1.0	24
Mechanical Scarification	0	0
Sulfuric acid Scarification	0	0

Table 9: *S. potatorum in vitro* germination of embryos

Medium/Hormone ($BAPmgL^{-1}$)	No of embryos responded (Mean ± SE)	% of response
MS Basal	6.33 ± 0.8	31.65
0.5	7.33 ± 0.6	36.65
1.0	15.70 ± 0.5	78.50
1.5	11.16 ± 0.6	55.80
2.0	10.83 ± 0.4	54.15

4.8 Effect of BAP on multiple shoot induction

Phenol exudation was major impediment in the establishment phase of culture of *S. potatorum* on the MS. The medium turned brown within 24 hrs due to release of brown exudates. To avoid the injurious effect of exudates on explant establishment various possible protectants were tried, among which PVP, activated charcoal, prior to inoculation was found to be ineffective and still media browning percentage was quite high which lead to growth retardation of the explant. Frequent subculturing and use of 0.2% phytagel instead of 0.8% agar as a solidifying agent found to be significant in reducing the browning to a considerable extent and no effect on retardation of growth. Out of different hormonal combinations tried for *in vitro* shoot regeneration from nodal explants of *S. potatorum*, BAP worked well for effective shoot multiplication. BAP is the most widely used cytokinin for multliple shoot generation. However at lower concentration of BAP one or both axillary buds developed well from the nodal explants. The nodal segments cultured on MS, supplemented with varying concentrations of BAP produced multiple shoots after 3-4 weeks (Figure 18 d and e). 16 multiple shoots were produced at the end of 3-4 weeks in MS+2.5 mg L^{-1} BAP (Table 10). Increasing concentrations of BAP up to 4 mgL^{-1} (Figure 19 f) resulted in shoot elongation and as well as callus formation from the nodal segments was observed.

4.9 Shoot induction in combination

Axillary bud proliferation from the nodal explants was observed in the TDZ and kinetin (0.1-5 mg L^{-1}) individually and as well as in combination, while multiple shoot regeneration was completely absent. All concentrations of BAP / TDZ (0.1-5mgL^{-1}) in combination or TDZ alone were ineffective at lower concentrations, however higher concentrations led to callus initiation from the nodal explants. The synergistic effect of BAP + Kinetin at lower concentration was found to be insignificant, while effective shoot multiplication and callus induction from the base of nodal explants was noticed in 2.5 mg L^{-1} BAP+ 2.5 mg L^{-1}Kinetin after 4 weeks (Figure 19 c). Similarly the combinations of BAP + 2, 4- D (0.1-5mgL^{-1}) was not effective for shoot proliferation from both nodal and shoot tip explants. The other two auxins 2, 4-D and NAA (0.1-5 mgL^{-1}) were also not effective in combinations with Kinetin for shoot proliferation. Shoot induction from the explants also occurred in combinations of BAP and NAA. Shoot multiplication was noticed on media containing 2.5mgL^{-1} BAP+ 1mg L^{-1} NAA (Figure 19 d) whereas along with multiple shoot regeneration along with shoot elongation and profuse callus induction was recorded from the base of nodal explants in 3 mg L^{-1}BAP+ 1 mg L^{-1} NAA after 4 weeks (Figure 19 e).

Table 10: *S. potatorum, in vitro* shoot multiplication from nodal explants

MS+BAP (mgL^{-1})	Multiple shoots (mean) ± SE	% of response	Calli response
Control	0	0	-
2.2	1.60 ± 0.08	3.2	-
2.3	2.10 ± 0.13	6.4	-
2.4	6.40 ± 0.43	12.8	-
2.5	16.4 ± 0.41	32.88	+
2.6	15.0 ± 0.58	30.18	+
2.7	14.4 ± 0.52	29.0	+
2.8	11.6 ± 0.59	23.36	++
2.9	11.0 ± 0.58	22.0	+++
3.0	9.90 ± 0.58	20.0	++++

Calli response +Very low, ++ normal, +++ high, +++very high

Table 11: *S. potatorum* callus induction from leaf explants.

BAP and 2,4-D (mgL^{-1})	Calli response	Results
1 BAP + 0.5 2,4-D	+	Low callus initiation
1 BAP +1 2,4-D	++	Moderate callus initiation
1 BAP + 1.5 2,4-D	+++	White creamy callus observed

Calli response + normal, +++ high, +++very high

4.10 *In vitro* rooting

In vitro rooting was induced by auxins. Initially *in vitro* regenerated shoots were cultured on solidified MS media without growth regulators. After four weeks of incubation in MS media, shoots 1.5 cm long were harvested for rooting experiment. A two-set of experimental procedure was adopted for standardizing most suitable growth regulator type and concentration for root induction. In the first experiment, shoots of 1.5cm were excised from incubated media and inoculated on full strength MS containing various concentrations of IBA, IAA and NAA (0.1-0.5 mg L^{-1}) and as well as in combinations. Shoots showing 5 mm long roots were considered as rooted. Rooting was recorded after 45 days of incubation on rooting medium. Well-rooted shoots were removed from culture bottles after rooting.

In the second experiment, shoots 1.5 cm were excised and were inoculated on 1/4, 1/2 MS supplemented with different concentrations of auxins. The first experiment gave positive results, while second experiment failed to induce roots. NAA and IAA did not respond towards rooting at concentrations ranging from 0.1 to 5 mgL^{-1}, but resulted in shoot proliferation and callus at the base of the excised shoots (Figure 20 a and b). Rooting was observed in 1.5 mg L^{-1}IBA on full strength MS (Figure 20 c) and further increase in concentration led to formation of callus from the base of the shoots. However, the percentage of rooting was as low as 10 %. The combination of NAA with IBA, NAA with IAA and IAA with

IBA did not initiate root induction. Incorporation of BAP or kinetin in combination with these auxins also did not show any response except callusing from the base of excised shoots. Full strength, ½ and ¼ MS with or without plant growth regulators failed to induce rooting.

4.11 Acclimatization

Rooted shoots were washed thoroughly with tap water to remove the adhering medium and transferred to majenta box containing 120g (w/v) Topsoil with sand and supplemented with FYM in 1:1:1 ratio. Transparent polyethylene bag was inverted over each plantlet to maintain high humidity, and watered daily. Plantlets were monitored under the same environmental conditions as *in vitro* cultures for two weeks and thereafter transferred to polyhouse for further growth. The transparent polyethylene bag was removed permanently upon new leaf appearance. Survival rate of these plants was very poor.

4.12 Leaf explants for callus development

In order to induce organogenesis leaves from *in vitro* raised seedlings of *S. potatorum* were tried for embryogenic callus regeneration with growth regulators. Leaf explants enlarged and developed callus at the cut surfaces in the MS media within 12-14 days of inoculation; the calli subsequently covered the entire surface of the explants. There was no sign of callus formation when explants were cultured in media without auxin or cytokinin. White creamy callus developed on the surface of the leaf explants within 5-6 weeks after culture initiation on the MS

basal medium supplemented with varying concentrations of 2, 4-D and constant levels of BAP. The combination of MS with 1 mg L^{-1} BAP and.5 mg L^{-1} 2,4-D resulted in low callus generation (Figure 20 d) while increasing concentration of 2,4-D up to 1.5 mg L^{-1} (Figure 20 e) induced more callus growth. The medium containing 1.0 mg L^{-1}BAP and 1.5 mg L^{-1} 2, 4-D promoted rapid callus growth from leaf explants which subsequently turned brown within 8 weeks of culture (Table 11). Callus in cultures containing BAP and 2, 4-D were soft and granular. The rate of growth of callus increased from the second week of culture initiation until the eighth week, after which the rate of callusing declined. After 4 weeks of callus induction media (MS+1mg L^{-1} BAP + 1.5 mg L^{-1} 2, 4- D), the calli were subcultured on fresh media containing different concentrations of BAP and 2,4-D or NAA with 3% (w/v) sucrose for the induction of somatic embryogenesis. Callus did not respond to somatic embryogenesis.

4.13 Results of *Strychnos nux-vomica* - Dormancy and Seed germination

SEM studies suggests that *S. nux-vomica* seed is rich source of carbohydrates and the endosperm is covered with a layer of hard radially elongated cells, which are paliside but devoid of intercellular spaces may be termed as malphigian cells or macro sclereids. (Figure 15). In addition to macro sclereids, astero sclereids are also present with radiating arms (Figure 15 a). The internal structure of seed showed the presence of multicellular hairs originating from a thick layer of cuticle, below it compressed epidermis exists. The epidermal hairs are seen bending towards one side and have a bulbous base. These are the idioblasts below the cuticle/epidermis containing raphides which are needle like. There is an extensive zone of sclereids which make up the albumin and the embryo**.** Both these tissues are very hard. The special tissue is composed of macro sclereids. Isolated macro sclereids are long and tapering towards both the ends with a narrow cell lumen. They vary in size (both length and width) and such sclereids are interspersed which are compactly arranged making the seed very hard (Figure 15 b, c and d)

4.14 *In vitro* response

Embryos separated from the mechanically scarified seeds of *S. nux-vomica* were transferred to the MS basal salts, vitamins and 3% (w/v) sucrose, with and without growth regulators. Turning of embryos in to green color and emergence of radical can be accounted as germination. 50% of embryos germinated into

complete plantlets on the MS media devoid of growth regulators with well-developed roots without secondary callusing of the embryos (Figure 21 b). However, the rate of germination inclined to 91% on the MS media containing cytokinins and plantlets had a well developed root system. The growth rate of *in vitro* germinated embryos was faster (Figure 21 c) and upto 91% of germination was observed after one week in MS in addition of 1mg L^{-1} BAP (Figure 21 d) where as 78% of germination was observed in 0.7 mgL^{-1} BAP While 60% germination was recorded in 0.8 and 56% was observed in 0.9 mg L^{-1}BAP (Table 12).

4.15 Effect of BAP on direct multiple shoots induction

Phenol exudation was major impediment in the establishment phase of culture of *S. nux-vomica* on the MS. The medium turned brown within 24 hrs due to release of brown exudates. To avoid the injurious effect of exudates on explant establishment, various possible protectants were tried, among which PVP,activated charcoal prior to inoculation was found to be ineffective and still media browning percentage was quite high which led to growth retardation of the explant. Frequent sub culturing and use of 0.2% phytagel instead of 0.8% agar as a solidifying agent found to be significant in reducing the browning to a considerable extent and no effect on retardation of growth. *In vitro*-raised seedlings were used as an explant source for shoot multiplication *i.e.* hypocotyl, epicotyl, nodal segments, and shoot meristem. But none of them responded for

multiple shoot regeneration. The nodal explants supplemented with BAP did not facilitate the multiple shoot induction; however the lower concentrations regulated the proliferation of single shoots, while increasing concentrations up to 5 mg L^{-1} resulted in callus formation from the nodal segments. Further experiments were carried out for direct shoot multiplication from *in vitro* germinated embryos. Out of different hormonal combinations tried for *in vitro* direct shoot regeneration from, *in vitro* germinated embryos of *S. nux-vomica* BAP worked well for effective shoot multiplication (Table 13). BAP is the most widely used cytokinin for multliple shoot formation. However in only MS devoid of growth regulators, single shoot proliferated well from *in vitro* germinated embryos, while supplementation with BAP initiated multiple shoot regeneration. Maximum number of shoots were observed in the optimal concentration of 0.6 mgL^{-1} BAP (Figure 22 a and b) and mean number of multiple shoots produced at the end of four subcultures was 7-8. Increasing concentrations of BAP up to 1mg L^{-1} (Figure 22 c and d) did not show any impact.

4.16 Shoot induction in combinations

Individual shoot proliferation was recorded in TDZ and kinetin (0.1-5mgL^{-1}) while multiple shoot initiation was completely absent. TDZ and kinetin were found ineffective in combination at low or high concentrations. The combination of BAP+ kinetin, BAP+TDZ (0.1-5mgL^{-1}) were also found to be

insignificant at low concentrations, while at high concentrations it led to callus initiation from the nodal explants.

4.17 Leaf explants for callus regeneration

In order to induce organogenesis, leaves from *in vitro*-raised seedlings of *S. nux-vomica* were tried for embryonic callus regeneration with growth regulators. Leaf explants enlarged and developed callus at the cut surfaces in MS media within 12-14 days of inoculation; the calli subsequently covered the entire surface of the explants. There was no sign of callus formation when explants were cultured in media without auxin or cytokinin. Friable calli developed on the surface of the explants 5-6 weeks after culture initiation on MS basal medium supplemented with varying concentrations of BAP and 2,4-D. The combination of MS+1mg L^{-1} BAP + 0.5 mg L^{-1} 2,4 D resulted in low callus generation while increasing concentration of 2,4 D up to 1.5 mg L^{-1} induced more callus growth. The medium containing 1.0 mg L^{-1} BAP and 1.5 mg L^{-1} 2,4-D promoted rapid callus growth from leaf explants which subsequently turned brown within 8 weeks of culture (Table14). Callus in cultures containing BAP and 2,4-D were soft and granular. The rate of growth of callus increased from the second week of culture initiation until the eighth week, after which the rate of callusing declined. After 4 weeks on callus induction media (MS+1mg L^{-1} BAP + 1.5 mg L^{-1} 2,4 D), the calli were subcultured on fresh media containing different concentrations of

BAP, and 2,4-D or NAA with 3% (w/v) sucrose for induction of somatic embryogenesis. Callus did not respond to somatic embryogenesis.

Table 12: *S. nux-vomica in vitro* germination of embryos

Medium/ Hormone ($BAPmgL^{-1}$)	No of embryos responded (Mean ± SE)	% of response
MS basal	10.0 ± 0.5	50.
0.5	10.0 ± 0.6	50
0.6	**18.3 ± 0.5**	**92**
0.7	15.6 ± 0.8	78
0.8	12.6 ± 0.8	64
0.9	12.3 ± 0.8	62
1.0	11.3 ± 1.4	57

Table 13: *S. nux-vomica in vitro* shoot multiplication from embryos

Medium/ Hormone ($BAPmgL^{-1}$)	Multiple shoots (mean) ± SE	% of response
Control	-	-
0.5	3.85 ± 0.31	19.2
0.6	**8.05 ± 0.32**	**40.2**
0.7	7.15 ± 0.22	35.7
0.8	6.40 ± 0.48	32.8
0.9	5.90 ± 0.52	29.5
1.0	5.50 ± 0.52	27.5

Table 14: *S. nux-vomica* callus induction from leaf explants.

BAP and 2,4-D (mgL^{-1})	Calli response	Results
1 BAP + 0.5 2,4-D	+	Low callus initiation
1 BAP +1 2,4-D	++	Moderate callus initiation
1 BAP + 1.5 2,4-D	+++	White creamy callus observed

Calli response + normal, +++ high, +++very high

4.18 Results of *Strychnos wallichiana* - Dormancy and Seed germination

SEM studies suggests that *S. wallichiana* seed is rich source in carbohydrates and the endosperm is covered with a layer of hard radially elongated cells, which are paliside but devoid of intercellular spaces may be termed as malphigian cells or macro sclereids (Figure 16). The internal structure of seed showed the presence of multicellular hairs originating from a thick layer of cuticle, below compressed epidermis exists. The epidermal hairs are seen bending towards one side and have a bulbous base. These are the idioblasts below the cuticle/epidermis containing raphides which are needle like. There is an extensive zone of sclereids which make up the albumin and the embryo. Both these tissues are very hard. The special tissue is composed of macro sclereids. Isolated macro sclereids are long and tapering towards both the ends with a narrow cell lumen. They vary in size (both length and width). Such sclereids are interspersed and compactly arranged in making the seed very hard (Figure 16 b, c and d)

4.19 *In vivo* and *In vitro* response

The seeds of *S. wallichiana* failed to germinate and did not respond to various treatments and as well as control. Treatment with H_2SO_4 and mechanical scarification were found ineffective in breaking the seed dormancy rendering failure in germination. In natural population seed germination in *S. wallichiana* was found to be epigeal, however the viability of the seeds was very poor**.** Embryos separated from the mechanically scarified seeds and transferred to MS basal salts, vitamins and 3% (w/v) sucrose, with and without growth regulators also failed to germinate. Field grown plants were used as

an explant source for shoot multiplication *i.e.* leaf explants, nodal segments, and shoot meristem. Out of different hormonal combinations tried for *in vitro* shoot regeneration, none of them responded for multiple shoot regeneration. Further experimentation is required for the effective propagation of *S. wallichiana*

4.20 Discussion

Andhra Pradesh is wealthy in plant genetic resources that need to be conserved. *Strychnos* genus is well distributed in this region but in some places they are under severe threat. During present investigation, it was noticed that the habitats of these taxa were getting exploited, resulting in thrashing of their genetic population. The harm to their habitats could be attributed to their commercially viability and a good strategy is required to defend their habitats. To ensure their continued existence, habitats and protection management should be given maximum precedence. This will facilitate in rejuvenation of these taxa and to restore their populations in their natural habitats and for long-term preservation. Breakup of populations into tiny subpopulations include reduced their colony size and augment threat to their inherent diversity. Therefore use of small nature assets is a viable method for long-term conservation (Tansley, 1998).

Propagation of plants is defined as the knowledge of duplication of plants by either sexual or asexual reproduction. Among the variety of methods of vegetative propagation, sexual reproduction plays crucial role in most of plants for their existence through seed germination. Therefore information regarding seed germination and seedling establishment is essential, due to a mounting demand for huge quantities of seeds and to meet the growing demand for these seedlings due to their therapeutic usage and a forestation programme.

SEM and Light microscope studies revealed that the seeds of *S. potatorum S. nux-vomica* and *S.wallichiana* are rich source of carbohydrates and the endosperm is covered

with a layer of hard radially elongated cells, termed as macrosclereids, which were compactly arranged and interspersed allowing the seed coat very hard. The asterosclereids with radiating arms are also present in *S. nux-vomica* along with macrosclereids and well interspersed enabling the seed coat very hard (Figure 13, 14 15and 16). Seed germination studies were undertaken to break dormancy and improve the germination of viable seeds of *S. potatorum* by using physico-chemical treatments.

The seed germination of *S. potatorum* and *S. nux-vomica* were found to be epigeal (Figure 18.a and 21 a) and germination period was lengthy. The opening of cotyledonary leaves takes place after 45-60 days in *S. potatorum* while 70-120 days in case of *S. nux-vomica* (Krishnamurthy, 1993; Rao, 2001). In *S. potatorum*, mild boiling water (subsequent soaking in water for 48 hrs) treatment was found to induce seed germination. Sudden dip of dry seeds in boiling water may lead to rupture of seed coat allowing water to permeable to seed tissue causing physiological changes and subsequent germination of the embryo which was reported in other woody plants (Agboola *et al.,* 1991; 1998). The rich endosperm serves as a reserve food for the developing embryo.

In natural population seed germination in *S. wallichiana* was found to be epigeal, how ever the viability of the seeds was very poor**.** The seeds of *S. wallichiana* failed to germinate and did not respond to various treatments and as well as control. Treatment with H_2 SO_4 and mechanical scarification was ineffective in breaking the seed dormancy and none of the seeds germinated.

Breaking seed dormancy with acid treatment has been successful for many tropical and temperate species, but the time required for effective treatment differed between species and appeared to be related with seed coat thickness (Bonner *et al.*, 1974). In *S. potatorum,* acid scarification treatments resulted in poor germination (Table 6). While the mechanical scarification and sulfuric acid treatments were found ineffective in seed germination,the seeds attracts a number of storage fungi namely *Alternaria alternata, Fusaarium oxysporum, Aspergillus flavus, Aspergillus niger* and *Rhizopus* sp (Anonymous, 2003) which hamper the germination of the seeds both in nursery and forest. Acid treatment some times adversely effected seed germination (Bimlendra and Toky, 1993). Todaria and Negi (1992) reported similar findings with acid treatment in *Cassia* species and suggested that reduced seed germination might be due to some deleterious effect on the embryo through thin seed coat.

Scarification in nature often occurs by fall seeding. Freezing temperatures or microbial activities modify the seed coat during the winter. Scarification can also occur as seed pass through the digestive tract of various animals. In several plant families, the seed coat plays a role in the control of water absorption, and shows impact on germination, often representing a temporarily impermeable barrier. Seed dormancy in *S. potatorum* was probably associated with the hard seed coat, while disrupting the coat there is expose of the lumens of the macro sclereids, permitting imbibition of water (Nikoleave, 1977), which triggers germination. The results showed that an increasing severity of treatment promotes germination up to critical point beyond which a decline in

final germination takes place. The decline in germination percentage of seeds is attributed to the deteriorative changes in physical, physiological and biochemical conditions (Roberts, 1972; Abdul-Baki and Anderson, 1972). Some seeds showed endogenous or physiological type of dormancy, while seeds imbibed in water but did not germinate, there by suggesting a physiological inhibitory mechanism of the embryo that prevents radicle emergence. The mild hot water and 48 hr soaked seeds showed good response when compared to other mechanical and acid scarification treatments (Table 6.)

The moisture content of *S. potatorum* seed is 8% (Anonymous, 1976). The activities of seed storage fungi are ultimately more influenced by the relative humidity of the inter seed atmosphere than by the moisture content of the seeds themselves. Seed storage fungi exhibit some deleterious effects on seeds which lead to loss of viability, discoloration, production of mycotoxins *etc*. Survival of the seeds in the soil does, however, depend on the maintenance of viability. Loss of viability is the final stage in seed deterioration (Fenner, 1992). Prior to death ageing results in a decline in many aspects of seeds potential performance such as the rate of germination.

S. potatorum S. nux-vomica and *S. wallichiana* seeds exhibit recalcitrant nature and they loose viability very quickly (Anonymous, 1976). The proportion of viable seeds might depend on the time of seed collection. Many tropical woody plants behave in similar way. Seeds of *Shorea robusta* which are common in both moist and dry tropical forests remain viable for only 7-10 days. This sort of viability is due to very high rate of moisture loss from the seeds and also increased leachate conductivity and decreased fatty

acid content due to ageing, resulting in loss of viability thus leading to decline in germination percentage (Thapliyal and Connor, 1977) In *Dalibergia sissso*, maximum number of viable seeds are yielded during the period November to July (Bangarwa *et al.*, 1996). Many dry forest tree seeds lose viability very quickly under ordinary conditions and some times even in cool dry storage conditions (Ray and Brown, 1995). The inability to store seeds of recalcitrant seeds is a serious problem and can only be solved by trial and error methods which are tedious, time consuming and extensive approach. The retention of a viable seed stock is desirable in order to preserve maximum genetic diversity; unfortunately, methods of storage other than drying may be detrimental to recalcitrant seeds.

S. potatorum and *S. nux-vomica* are difficult to propagate using *in vitro* techniques. Since traditional propagation methods are slow, inefficient and have been reported in many woody plants (Chapman, 1984). However, there could be many advantages in clonal propagation. One of the problems in the micropropagation of *S. potatorum* and *S. nux-vomica* is exudation of polyphenols into the medium. Another problem is that field grown material used as a source of known genetic material is heavily contaminated with organisms, which are difficult to remove prior to culture. Both of these problems are frequently encountered with several hardwood species and it is well documented. Pretreatment with PVP was found to be effective in controlling phenol exudation from mature explants of guava (Amin and Jaiswal, 1988). To avoid phenol exudation from the injurious portion of explant various possible protectants were tried,

among which PVP and activated charcoal for 2-3 hr prior to inoculation was found to be ineffective and media browning percentage was quite high. The usual approach is to develop the experimental culture techniques using young embryos from seeds germinated *in vitro*. This technique has an advantage of providing very clean material as well as producing mature embryos which usually respond well in culture.

Similaraly Kantharajah *et al.*,(1992) attempted to develop methods for rescuing and culturing immature embryos from seeds of lychee *(Litchi chinensis* Sonn.) and achieved multiplication through induction of adventitious buds from the embryonic shoots. One of the problems encountered by them was browning of very young tissues. Lychee provides one method of clonal propagation and if successfully applied, could produce up to 15 plants from a single embryo (Puchooa, 2004).

In *S. potatorum* and *S. nux-vomica*, *in vitro* zygotic embryo germination, resulted in reduction of germination time, increase in germination percentage upto 78% and 91% respectively and enhancement in the seedling growth rate (Table 6 and 9). Embryos germinated after one week and complete seedlings were observed after 15-20 days. In case of *S. wallichiana* embryos separated from the mechanically scarified seeds, with and without growth regulators failed to germinate. *In vitro* germination of embryos has become a powerful tool to overcome the problems derived from the low ability of the seeds to germinate in the field (Gracina *et al.,* 1994). Germination of excised embryos is regarded as a more exact and reliable test than the commonly used staining methods for seed viability (Barton, 1961). Embryo culture is helpful in studying the basic aspects of

embryogenesis *i.e*. impact of phytohormones, role of nutrients and other physico-chemical factors involved in embryonic growth and differentiation were well documented in other species (Monnier, 1990; Liu *et al.*, 1993b).

There is a great deal of variability between plants and the growth regulators necessary for morphogenesis, and the growth regulator(s) requirement of each type of explant should be determined (Hussey G 1975; Hughes, 1981). Cytokinins are known to reduce the dominance of apical meristems and induce axillary as well as adventious shoot formation from meristamatic explants (Cronauer-Mitra and Kirikorian, 1984). According to Buising *et al.*, (1994) a single exposure to a low concentration of BAP re-programs the development throughout the shoot apex and causes cells that would normally remain quiescent to instead divide repeatedly and give rise to supernumerary vegetative buds. Although BAP stimulates multiple shoot formation, it interferes with development and elongation of shoots (Wright *et al.*, 1986). The phenol exudation was clearly observed and the medium turned brown within 24 hrs due to release of brown exudates. To avoid the injurious effect of exudates on explant establishment frequent subculturing and use of 0.2% phytagel instead of 0.8% agar as a solidifying agent found to be significant in reducing the browning to a considerable extent and no effect on retardation of growth.

In vitro raised seedlings of *S. potatorum* is used as explant source for shoot multiplication *i.e.* hypocotyl, epicotyl, nodal segments, and shoot meristem. Nodal segments gave rise to multiple shoots. At the optimal concentration of BAP an average of 16 multiple shoots produced per nodal explant was recorded. The maximum number of

shoots was achieved in 2.5 mg L^{-1} BAP (Figure 18 f ;Table-10). However, at low concentrations significant results were not observed, while increasing concentrations resulted in reduced shoot regeneration efficiency. Field grown plants used as explant source for shoot multiplication of *S. wallichiana i.e.,* leaf explants, nodal segments, and shoot meristem. Out of different hormonal combinations tried for *in vitro* shoot regeneration none of them resulted in multiple shoot regeneration. In case of *S. nux-vomica*, multiple shoot regeneration from hypocotyl, epicotyl and nodal segments were not observed. Frequent subculturing resulted in direct shoot multiplication of embryos from the axially bud, 7-8 multiple shoots were produced at the optimal concentration of 0.6 mg L^{-1} BAP (Figure 22.a; Table-13). However the treatment with other hormones i.e. Kinetin, TDZ failed to induce multiple shoot regeneration in both plants. These hormonal combinations also resulted in callus regeneration from the explants. Similar results were reported in several woody plants.

Auxins influence the cell enlargement, root initiation and suppress lateral buds (Jarret *et al.,* 1985). In some woody species, rooting may require a high concentration of auxin. Prolonged exposure to high auxin levels however has some undesirable effects, such as callusing, leaf chlorolosis, inhibition of root elongation and quiescence or dormancy in the shoot tip which is difficult to overcome in the acclimatization stage (Maynard, 1991). It is often hard for woody species to express their rooting capacity, *in vitro*, under suitable conditions via direct or indirect rhizogenesis. Rhizogenesis is a complex phenomenon involving interactions between external factors, endogenous

growth regulators levels and nutrition. However, the auxin related factors found to play a pivotal role in rhizogenesis. The percentage of root induction and number of roots per shoot were also highly influenced by the concentration and types of auxins. No rooting was found on auxin omitted media. Various concentrations of IBA, IAA and NAA (0.1-0.5mg L^{-1}) were tried in which IBA responded well. IBA has been preferred auxin for root initiation in many species (Amin *et al.*, 2001). Hutchninson (1981) found IBA as superior auxin to IAA or NAA for *in vitro* rooting of apple shoots, while Amin and Jaiswal (1987) observed its superiority for rooting in guava and jack fruit micro cuttings. Shoots showing 5 mm long roots were considered as rooted.

Rooting was recorded after 45 days on rooting medium when shoots were transferred to rooting media, supplemented with 1.5 mg L^{-1} IBA, it improved the rooting frequency and root quality (Figure 20.c). While In case of *S. nux-vomica* the *in vitro* raised multiple shoots failed to induce roots. MS media with or without various growth regulators were tried, but none of them responded neither individually nor in combinations. After rooting the *in vitro* plants were transplanted into majenta box containing 120 g (w/v) Topsoil +sand +FYM (1:1:1). Transparent polyethylene bag was inverted over each plantlet to maintain high humidity, and watered daily. Plantlets were monitored under the same environmental conditions as *in vitro* cultures for four weeks and thereafter transferred to polyhouse for further growth. The transparent polyethylene bag was removed permanently upon new leaf appearance. Survival rate of these plants is very poor.

Biotechnology offers a break to exploit the cell, tissue, organ or entire organism by growing them *in vitro* and to genetically manipulate them to produce desired compounds. Plant cell cultures are an attractive alternative source to whole plant for the production of commercially important secondary metabolites (Ravishankar *et al.,* 1999). It is desirable to grow callus from leaf explants to produce secondary metabolites, for example, the generation of indole alkaloids in cell culture as of *Catharanthus roseus*. Similar results were obtained in a separate study for the production of betacyanins in callus cultures of *Beta vulgaris* (Bohm and Rink, 1988). Growth regulator concentration is often an essential factor in secondary product accumulation (Deus and Zenk, 1982; Di Cosmo and Towers, 1984). The type and concentration of auxin or cytokinin or the auxin/cytokinin ratio alters significantly, both the growth and the product formation in cultured plant cells (Mantell and Smith, 1984).

Cytokinins have different effects depending on the type of metabolite and species concerned. Thus, kinetin stimulated the production of anthocyanin in *Haplopappus gracilus,* but inhibited the formation of anthocyanins in *Populus* cell cultures (Seitz and Hinderer, 1988). 2,4-D is used as effective auxin for callus regeneration. Stimulation by 2,4-D has been observed in carotenoid biosynthesis in suspensions of *D. carota* (Mok *et al.,* 1976) and in anthocyanin production in callus cultures of *Oxalis linearis* (Meyer and van Staden, 1995), however in some cases 2, 4-D has shown to inhibit the production of secondary metabolites in a large number of cases. In such cases, elimination of 2, 4-D or replacement of 2, 4-D by NAA or IAA has been shown to enhance the production of

anthocyanins in suspensions of *Populus* and *D. carota*, of betacyanins in suspensions of *Portulaca*, of nicotine in suspensions of *N. tabacum*, of shikonin in suspensions of *L. erythrorhizon* and of anthraquinones in *M. citrifolia* (Rajendran *et al.*, 1992; Seitz and Hinderer, 1988; Tabata, 1988; Sahai and Shuler, 1984; Bohm and Rink, 1988; Zenk *et al.*, 1975). However, in the present study in order to induce organogenesis, leaves from *in vitro*-raised seedlings of *S. potatorum* and *S. nux-vomica* were tried for embryonic callus regeneration with growth regulators. Leaf segments did not demonstrate organogenic capability for all the media tested. However, leaf explants enlarged and developed callus at the cut surfaces in MS media within 12-14 days of inoculation; the calli subsequently covered the entire surface of the explants. White creamy callus has developed from the cut ends of the leaf explants 5-6 weeks after culture initiation on the MS basal medium, supplemented with constant concentration of BAP with varying concentrations of 2,4-D. Similar results were reported in *Acacia salicina*, that the leaf explants generated excessive callus generation with 2,4 D in combination with cytokinin BAP (Zhao *et al.*, 1990). The combination of MS+1mg L^{-1} BAP + 0.5 mg L^{-1} 2,4 D resulted in low callus generation while increasing concentration of 2,4 D up to 1.5 mg L^{-1} induced more callus growth (Table 11 and 14). The rate of growth of callus increased from the second week of culture initiation until the eighth week, after that the rate has declined. After 4 weeks on callus induction media (MS+1mg L^{-1} BAP + 1.5 mg L^{-1} 2,4 D), the calli were subcultured on fresh media containing different concentrations of BAP, and 2,4-D or NAA with 3% (w/v) sucrose for the induction of somatic embryogenesis. Callus did not

result in somatic embryogenesis. Moreover, replacement of 2, 4 D with NAA a weak auxin, failed to induce somatic embryogenesis. Phenolic accumulation may also arrest the formation organogenic callus. Similar results reported in *Ecualyptus* species (Nugent *et al.,* 2001, Pinto *et al.,* 2002). The callus generated failed to induce somatic embryogenesis how ever the callus regeneration may be useful for the production of commercially viable secondary metabolites.

4.21 Conclusions

Strychnos is commonly propagated via seeds. Vegetative propagation through conventional methods is inefficient as it is difficult to induce adventious roots from cuttings. A reduced span of viability and the low germination rate of *Strychnos* genus seeds restrict propagation through seeds. Commercial exploitation for production and conventional propagation is hampered due to its poor seed viability, low rate of germination and poor rooting ability of vegetative cuttings. Consequently micropropagation techniques are the most suitable alternative for rapid mass production of clones.

In *vitro* propagation of *S. potatorum* and *S. nux-vomica* through zygotic embryos may be the alternative way for effective propagation of these plants. By culturing the embryos from dissected seeds of *S. potatorum* and *S. nux-vomica,* germination rate was increased and decrease in time duration was achieved through *in vitro* propagation. The best cytokinin for shoot multiplication was BAP. How ever the seeds of *S. wallichiana* failed to germinate and did not respond to in *vivo* and in *vitro* propagation methods.

In vitro raised seedlings of *S. potatorum* is used as explant source for shoot multiplication *i.e.* hypocotyl, epicotyl, nodal segments, and shoot meristem. Nodal segments gave rise to multiple shoots. At the optimal concentration of BAP an average of 16 multliple shoots produced per nodal explant was recorded. The maximum number of shoots was achieved in 2.5 mg L^{-1}BAP. In case of *S. nux-vomica* multiple shoot regeneration with hypocotyl, epicotyl and nodal segments was not observed. Frequent

subculturing resulted in direct shoot multiplication from the axially bud of the embryos. 7-8 multliple shoots were produced at the optimal concentration of 0.6 mg L^{-1}BAP.

No rooting was found on auxin omitted media with *S. potatorum in vitro* raised multiple shoots. Various concentrations of IBA, IAA and NAA (0.1-0.5mg L^{-1}) were tried among which IBA was found effective. Shoots showing 5 mm long roots were considered as rooted. Rooting was recorded after 45 days on rooting medium, when shoots were transferred to rooting media supplemented with 1.5 mg L^{-1} IBA, it improved the rooting frequency and root quality. Transparent polyethylene bag was inverted over each plantlet to maintain high humidity, and watered daily. Plantlets were monitored under the same environmental conditions as *in vitro* cultures for four weeks and thereafter transferred to polyhouse for further growth. The transparent polyethylene bag was removed permanently upon new leaf appearance. Survival rate of these plants is very poor. In order to ensure higher *ex vitro* survival rate, experiments are being carried out to standardize rooting, acclimatization and hardening system.

While in case of *S. nux-vomica*, the *in vitro* raised multiple shoots failed to induce roots. Out of various growth regulators tried, none of them either individually or in combinations proved efficient. Further experimentation is required. *S. wallichiana* failed to germinate by both in *vivo* or in *vitro* methods. The proportion of viable seeds might depend on the time of seed collection. Many tropical woody plants behave in similar way and seed collection time is standardized where as further experimentation is required for effective propagation of this plant.

Micro propagation protocol, detailed above is highly effective to produce large number of plants in short duration through *in vitro* culturing of the embryos. It is an ideal technique for cloning dioecious woody plants such as *S. potatorum* and *S. nux-vomica* trees are preferred due to higher economic importance.

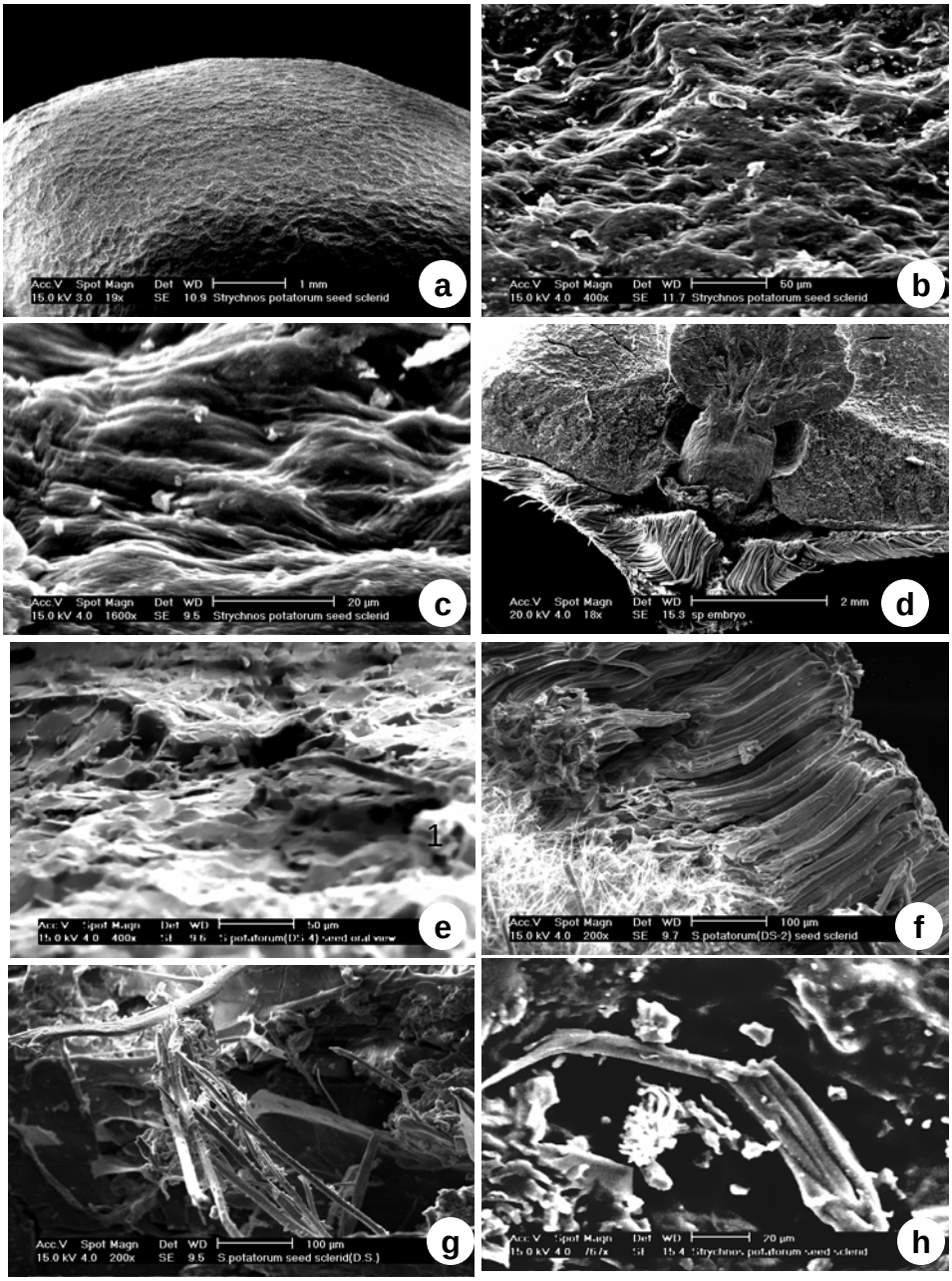


Figure 13 : Scanning electron microscope studies of *S. potatorum* seed

a) Normal view of the seed surface.

b) and **c)** Interwoven sclereids are responsible for hard seed coat.

d) Embryo encovered by cluster of sclereids.

e) Starch embedded with sclereids .

f) Outer layer of the seed showing sclereids

g) Separated sclereids. **h)** Individual sclereid.

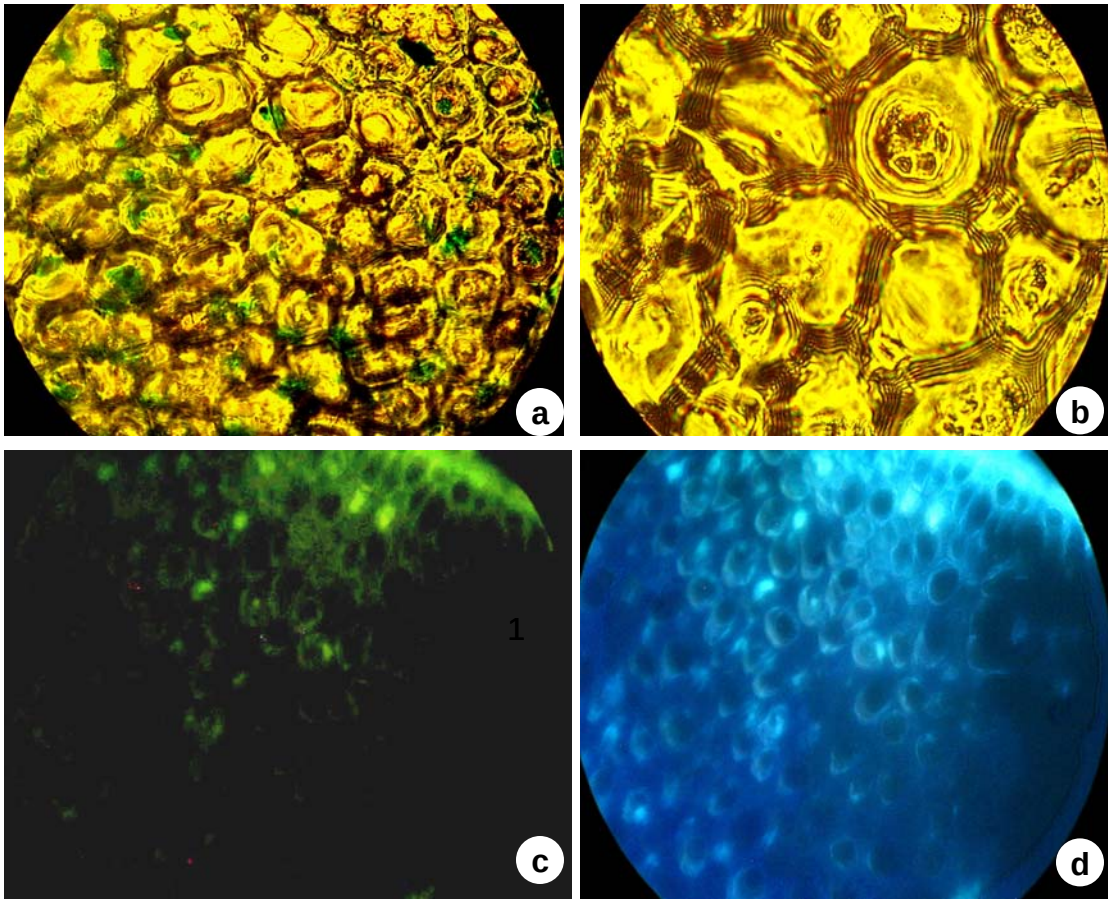


Figure 14 : Light microscope images of *S. potatorum* dissected seed.

a) Dissected seed showing sclerenchymatous tissue (magnification *40x*)

b) Magnification (*100x*) of sclerenchymatous tissue

c) and **d)** Sclerenchymatous tissue showing auto florescence due to bioactive compounds

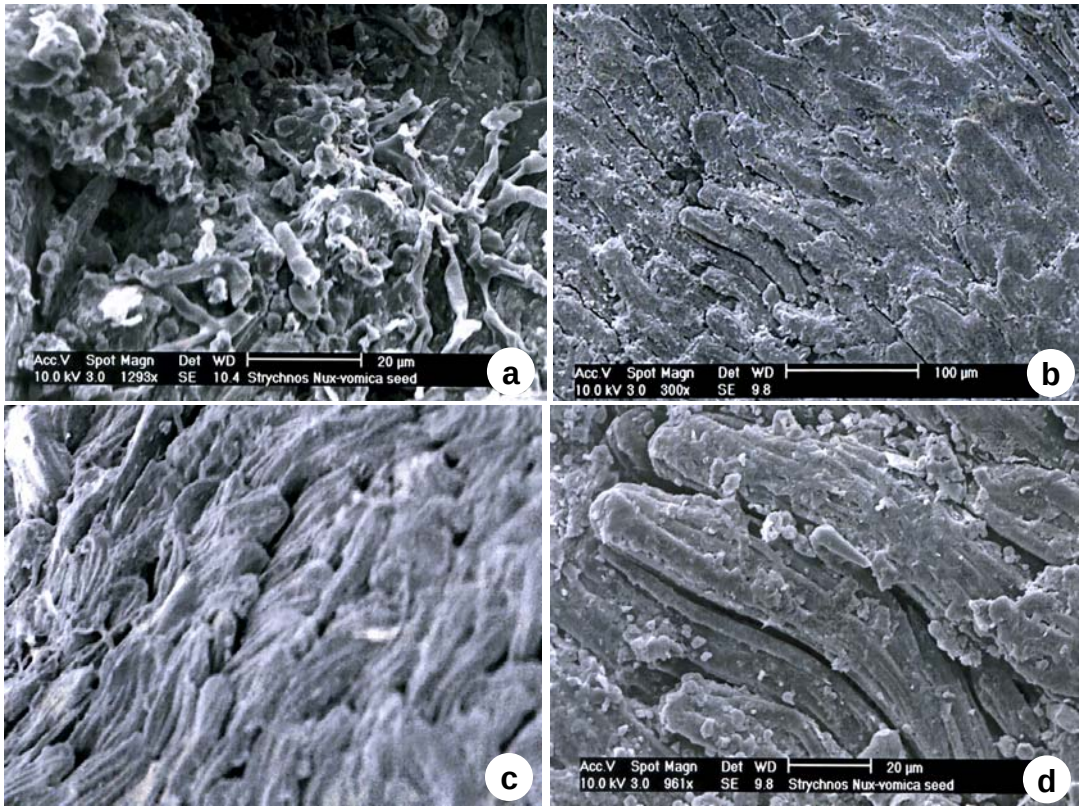


Figure 15 : Scanning electron microscope studies of *S. nux-vomica* seed.

a) Astero sclereids are also present and the radiating arms varies 8-20.

b) , c) and **d)** Interwoven sclereids are responsible for hard seed coat.

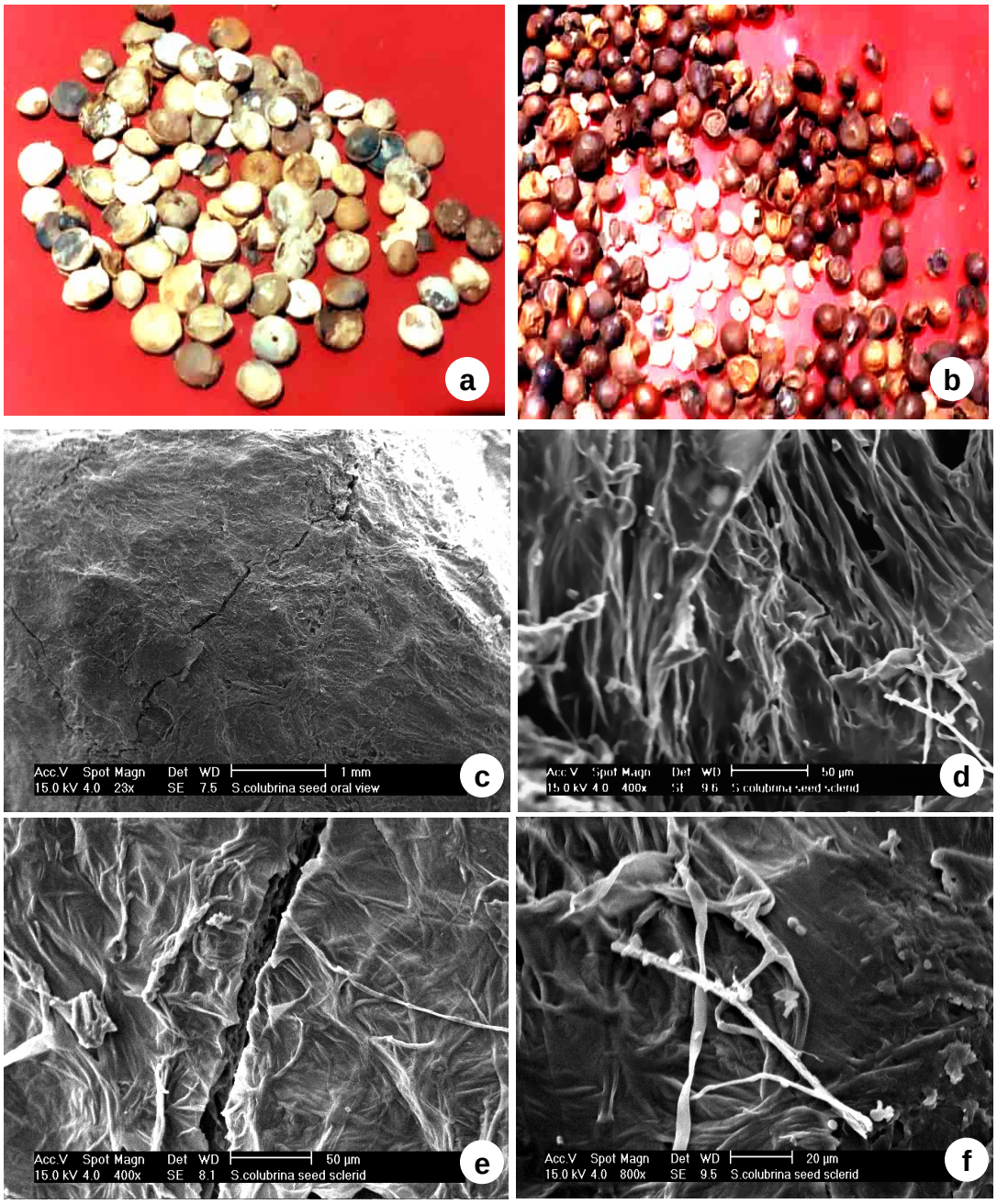


Figure 16: *S. wallichiana* seed and seed coat structure

a. Sun dried seeds.

b. Mature fruits with seeds

c. Scanning electron microscopy of seed surface

d. Interwoven sclereids showing.

e-f Individual macro sclereid.

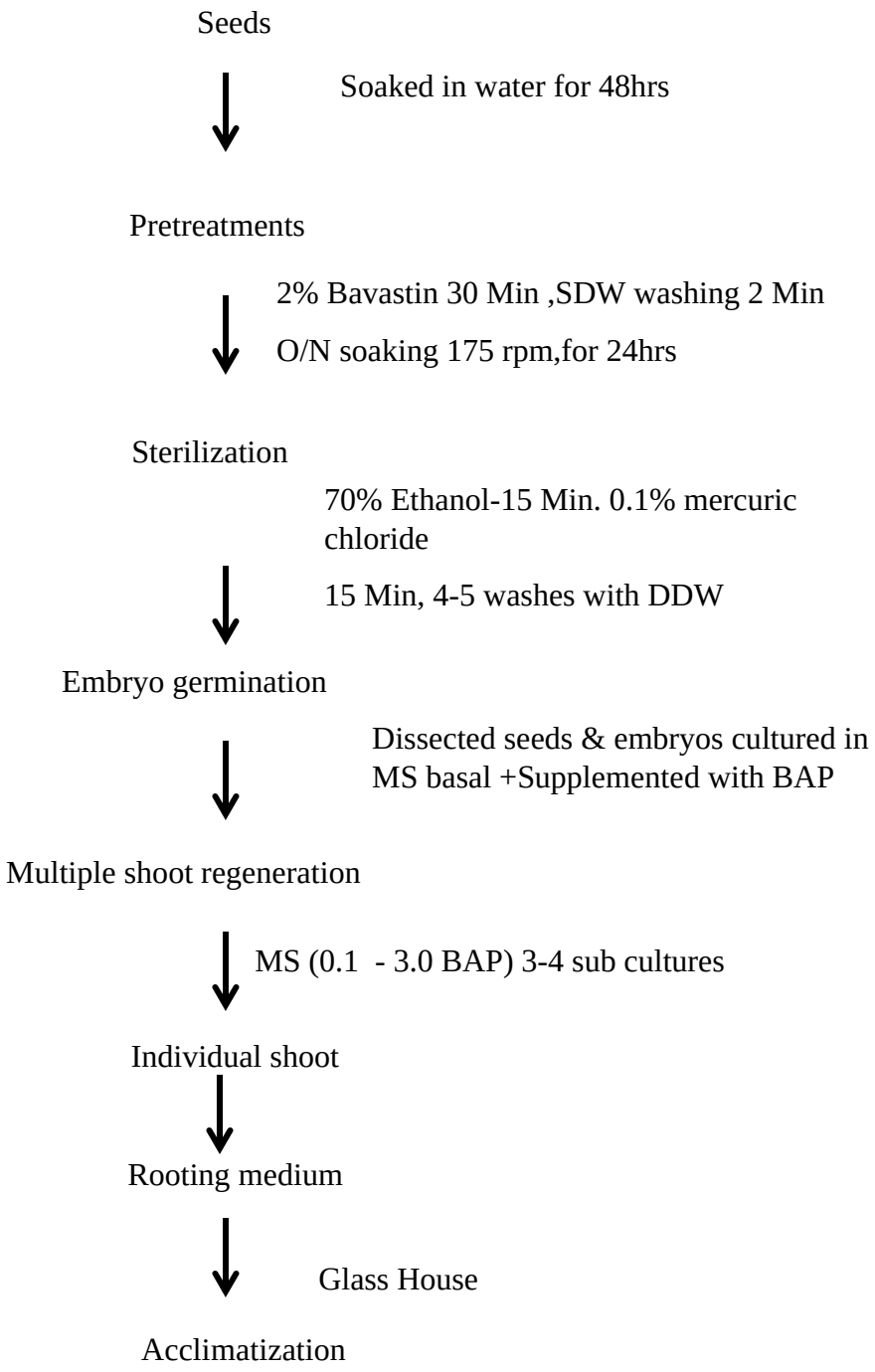


Figure 17 : Flow chart representing the methodology for *in vitro* propagation of ***S. potatorum*** and ***S. nux-vomica.***

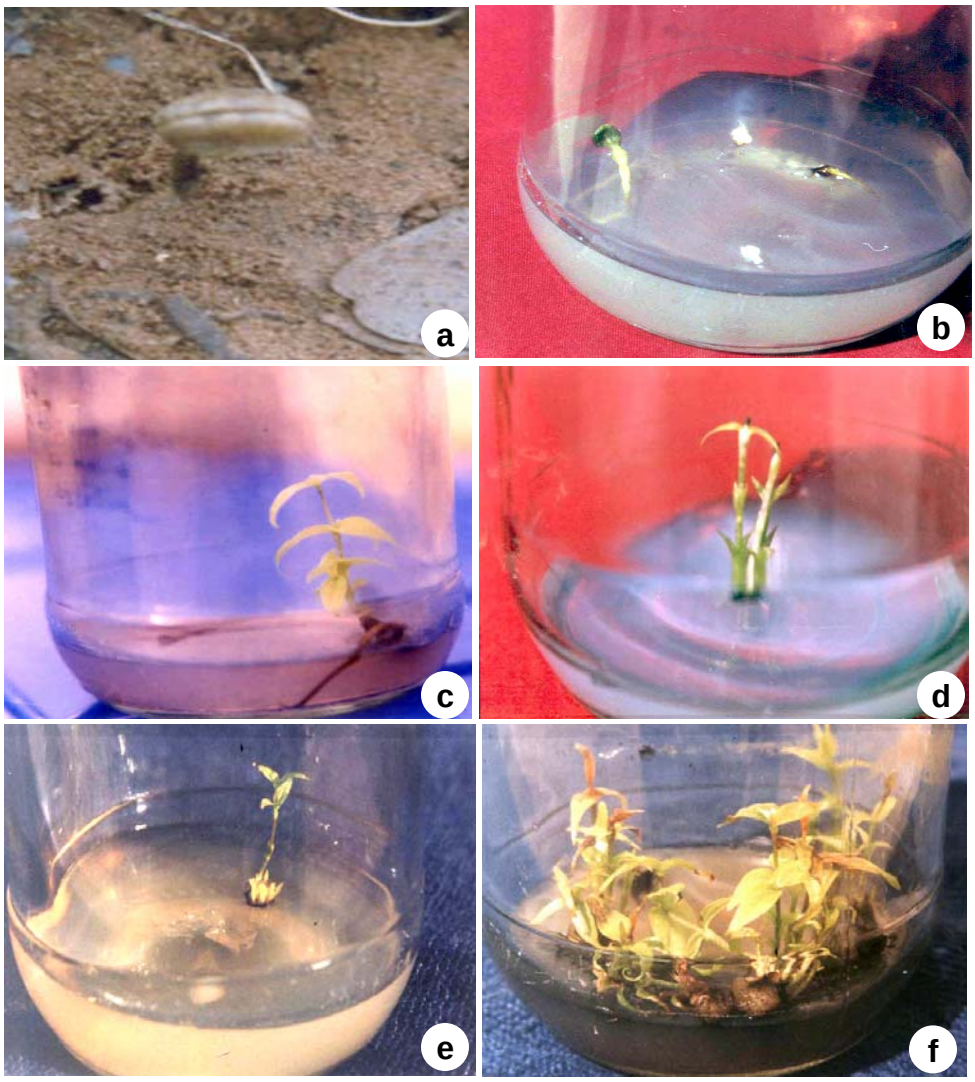


Figure 18 : *In vivo* germination and *in vitro* germination of *Strychnos potatorum.*

a) In *vivo* germination of seeds in the experimental site. **b)** In *vitro* germination of embryos from dissected seeds in MS medium supplemented with 1.0 mg L^{-1} BAP after 2 weeks **c)** *In vitro* germination of embryo in to seedling in MS medium supplemented with 1.0 mg L^{-1} BAP after 5 weeks **d)** and **e)** Multiple shoot regeneration initiation from nodal explants on MS medium with 2.5 mg L^{-1}BAP after 3- 4 weeks. **f)** Multiple shoot regeneration on MS medium with 2.5 mg L^{-1} BAP after 3 weeks

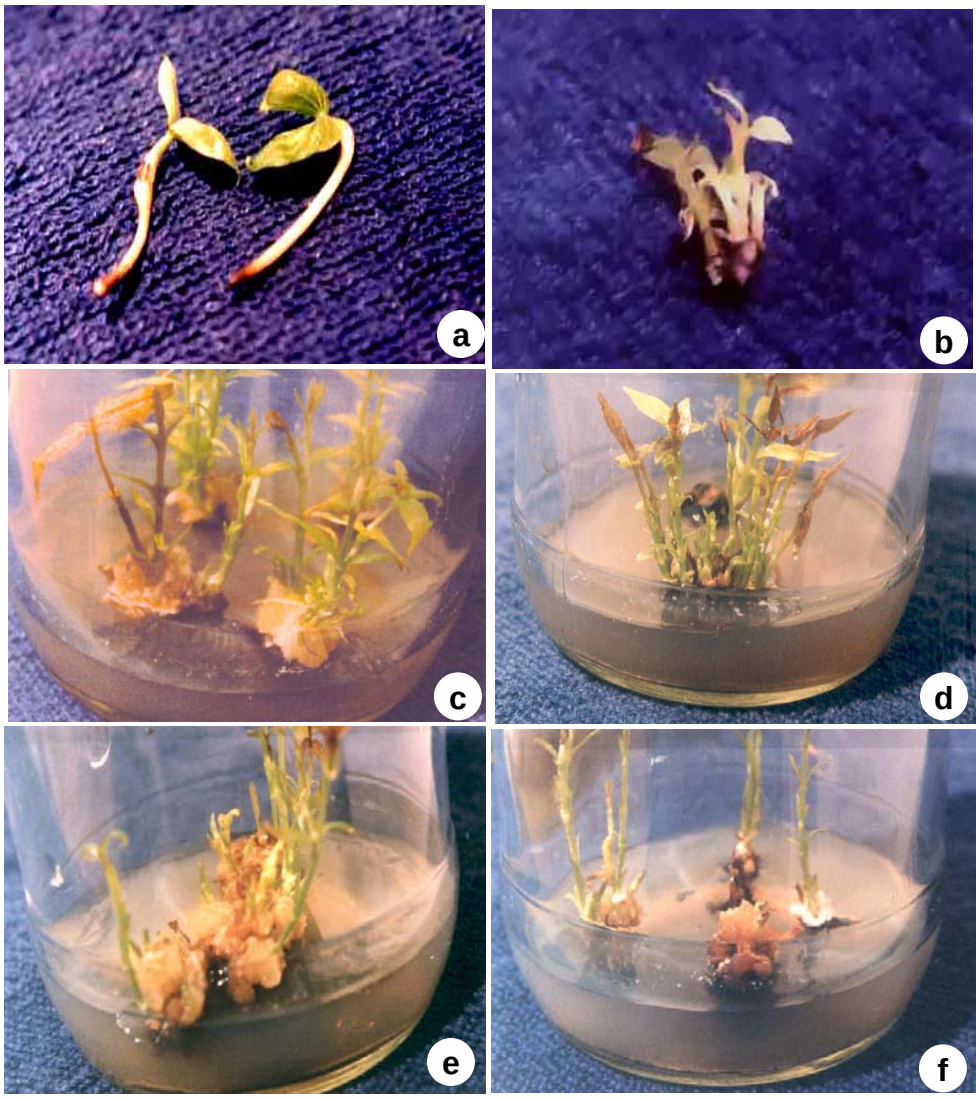


Figure 19 : *In vitro* differentiation of nodal explants of. *S. potatorum.*
a) In *vitro* germination of embryos from dissected seeds in to full fledged seedlings in MS medium supplemented with 1.0 mg L^{-1} BAP after 4 weeks **b)** *In vitro* raised seedlings used as explants for effective shoot multiplication in 2.5mg L^{-1} BAP after 3-4 weeks **c)** Shoot multiplication and callus induction from the base of nodal explants in 2.5 mg L^{-1} BAP+ 2.5mg L^{-1} Kinetin after 4 weeks **d)** Shoot multiplication from nodal explants in 2.5 mg L^{-1} BAP+ 1 mg L^{-1} NAA after 4 weeks **e)** Multiple shoot regeneration, shoot elongation and callus induction from the base of nodal explants in 3 mg L^{-1}BAP+ 1 mg L^{-1} NAA after 4 weeks *f.* Multiplication and elongation of shoots and callus induction from the base of nodal explants in 4 mg L^{-1} BAP after 5 weeks

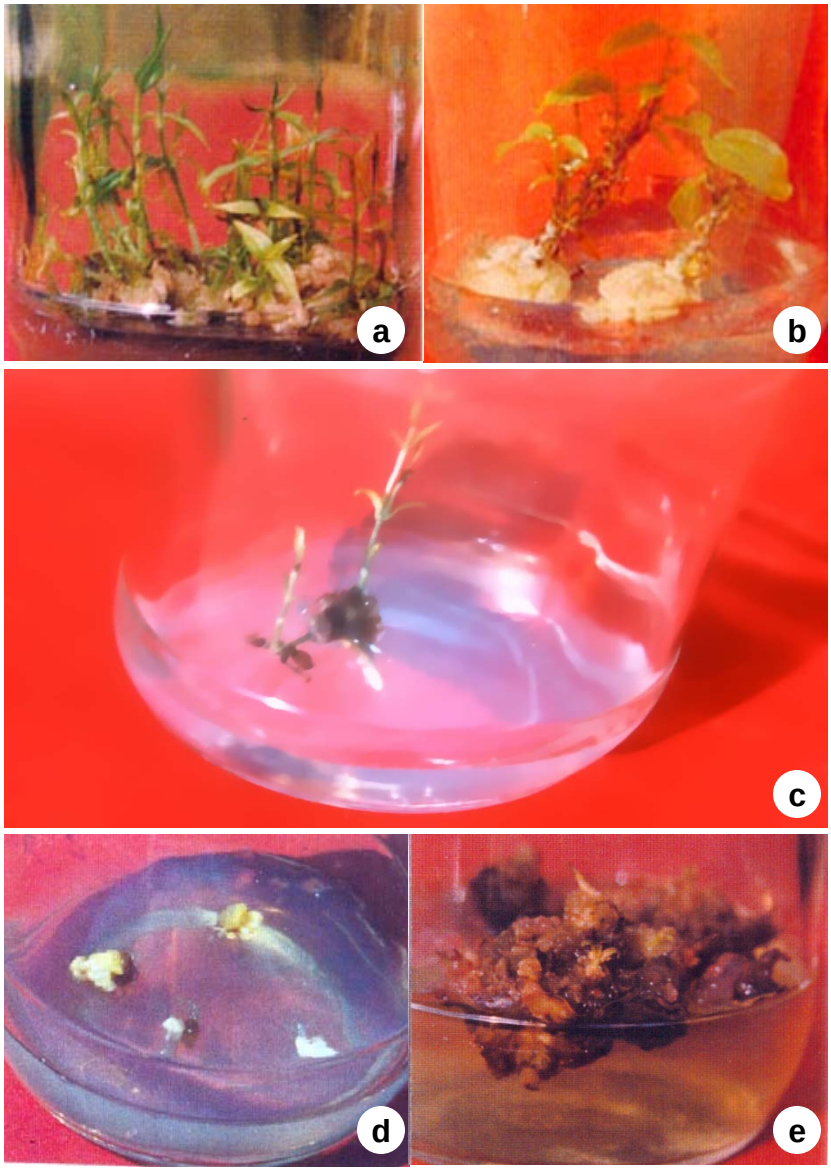


Figure 20: *In vitro* rooting ,callus induction from leaf explants of *Strychnos potatorum.*
a) Callus formation at the base of *in vitro* regenerated shoots from the nodal explants on MS medium supplemented with 2.5 mg L^{-1} IAA
b) Callus formation at the base of *in vitro* regenerated shoots from the nodal explants on MS medium supplemented with 2.5 mg L^{-1}NAA
c) Rooting of *in vitro* regenerated shoots from the nodal explants on MS medium supplemented with 1.5 mg L^{-1} IBA.
d) Callus development from leaves on MS medium supplemented with 0.5 mg L^{-1} 2, 4D
e) Callus development from leaves on MS medium supplemented with 1.5 mg L^{-1} 2, 4D

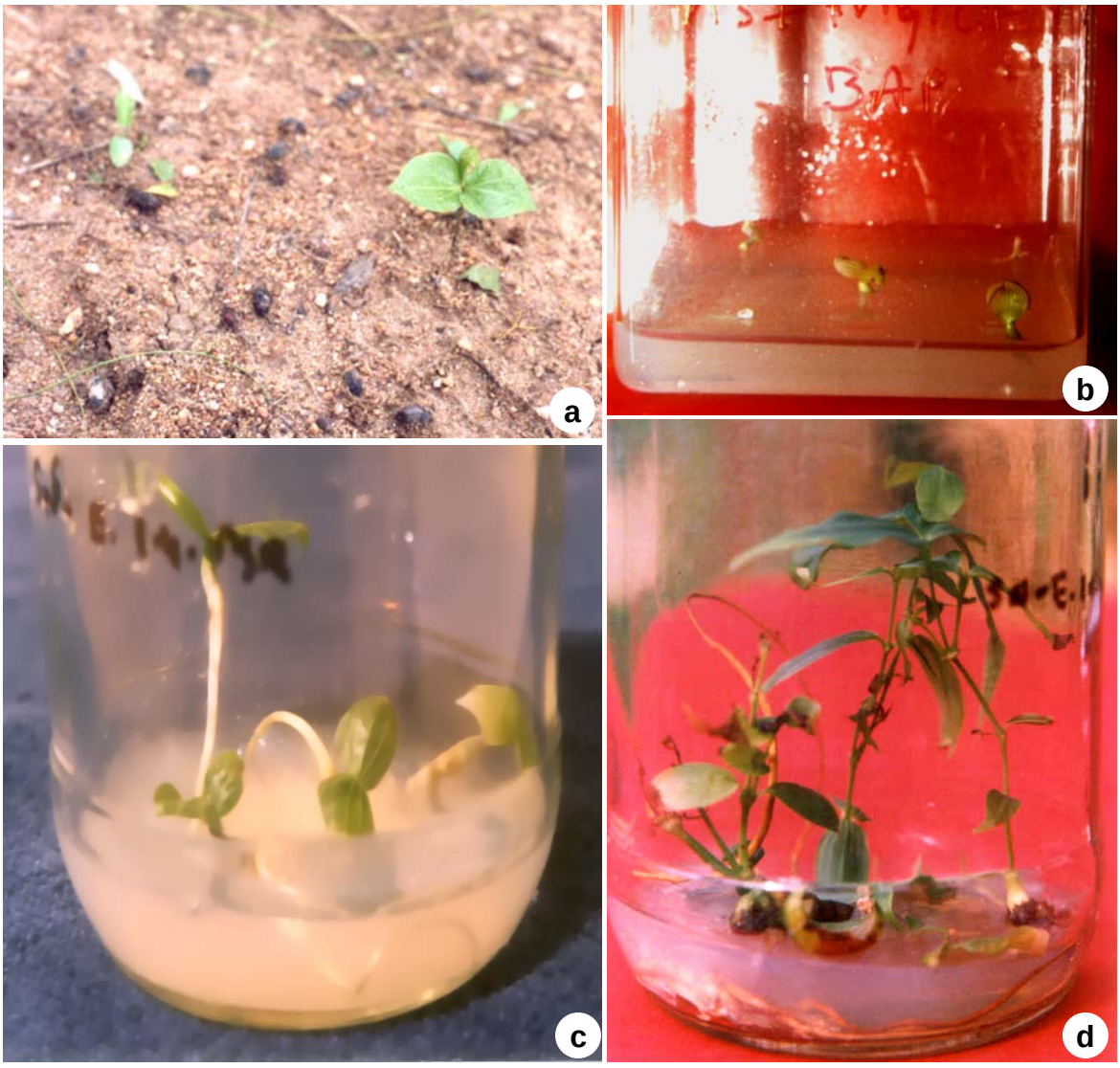


Figure 21 : *In vivo* germination and *in vitro* germination of *Strychnos nux-vomica.*

a) *In vivo* germination of seeds in the experimental site.

b) In *vitro* germination of embryos from dissected seeds in MS medium supplemented with 0.6 mg L^{-1} BAP after 1 week.

c) *In vitro* germination of embryos in to seedlings in MS medium supplemented with 0.6 mg L^{-1} BAP after 3 weeks

d) In *vitro* germination of embryo in to seedling in MS medium supplemented with 0.6 mg L^{-1} BAP after 8 weeks

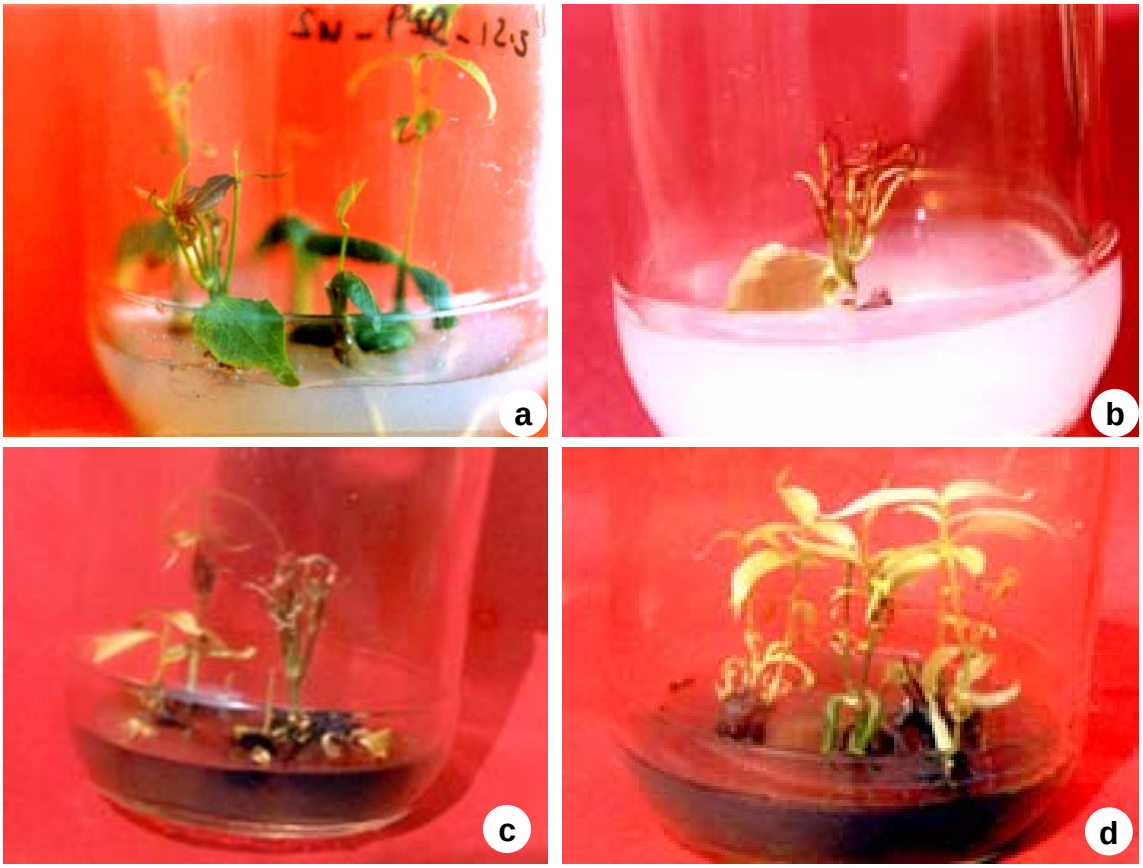


Figure 22 : *In vitro* axillary shoot bud multiplication. of ***Strychnos nux-vomica***
a) and **b)** Direct *In vitro* axillary shoot bud multiplication from *in vitro* germinated embryos on MS medium supplemented with 0.6 mg L^{-1} BAP after 8 weeks
c) Direct *In vitro* axillary shoot bud multiplication from *in vitro* germinated embryos on MS medium supplemented with 0.7 mg L^{-1} BAP after 8 weeks.
d) Direct *In vitro* axillary shoot bud multiplication from *in vitro* germinated embryos on MS medium supplemented with 1 mg L^{-1} BAP after 8 weeks.

5 Extraction, purification and characterization of Indole alkaloids from *S. wallichiana*

5.1 Introduction

During the ancient times 130 million years, flowering plants have colonized practically each habitat on earth, from arid deserts, boggy meadows and windswept alpine summits, to sun-baked grasslands, lush rain forests and wave-battered rocky shores. They have replaced the majority of the olden ferns and seed plants that dinosaurs subsisted on, and developed a complex and fascinating relationship with insects and mammals. During these innumerable centuries of time, flowering plants have steadily evolved all sorts of ingenious protective devices to discourage hungry herbivorous animals. Leaves and stems have developed a variety of vicious spines and stinging hairs (trichomes). In various plants, the intense covering of silvery hairs may also provide extra ecological advantages such as solar reflection and insulation in arid environments. But mechanical defenses, such as spines and trichomes, are of limited value and probably would not prevent all hungry herbivores, particularly the chewing and sucking insects. Therefore, plants have developed a "chemical warfare," a defense strategy based on a vast arsenal of chemicals which are toxic or distasteful to animals. The "predatory pressure" has resulted in the progression of an endless array of complex plant molecules, from gums and terpenes to alkaloids and phenolic compounds (Singer *et al.,* 2003).

5.2 Alkaloids

One of the major groups of chemical arsenals produced by plants are the

alkaloids, typically contain nitrogen and have complex ring structures. They naturally occur in seed-bearing plants and are found in berries, bark, fruit, roots, and leaves. Often, they are bases that have some physiological effect. Many of these metabolic by-products are derived from amino acids and include an enormous number of bitter, nitrogenous compounds. According to Raffauf (1996), more than 10,000 different alkaloids have been discovered in species from over 300 plant families. Evidence suggests that alkaloids have been used by people for thousands of years. The first civilizations to use them were perhaps the ancient Sumarians and Egyptians. However, it was not until the early nineteenth century that these compounds were reproducibly isolated and analyzed. Advances in analytical separation techniques, such as chromatography and mass spectroscopy, led to the elucidation of the chemical structure of alkaloids. The term for these compounds is thought to have originated from the fact that the alkaloid, morphine, had similar properties to basic salts derived from the alkali ashes of plants; thus, it was called a vegetable alkali or alkaloid. Since the first alkaloids were isolated, thousands more have been identified and classified. Although numerous alkaloids exist, they have similar properties when separated. In general, they are colorless, crystalline solids that are basic, have a ring structure, and have definite melting points. They are also derived from plants and have a bitter taste. However, some exceptions are known. For instance, some alkaloids are not basic and others are brightly colored or liquid. Other alkaloids are produced synthetically. Most are chiral molecules, meaning they have non super imposable mirror images. This results in isomers that have different chemical properties.

For *eg*. one isomer may have a physiological function while the other does not. It is mostly unknown why plants produce alkaloids. Various theories have been proposed to explain their existence. Some suggest that they are byproducts of normal plant metabolism. It is also thought that alkaloids may provide a means of defense against insects and animals (Luijendijk *et al.*, 1996; Baldwin, 1999; Kessler and Baldwin, 2002) and may be a reservoir for molecules that plants often use. It is likely that all of these theories are correct to some extent. Alkaloids are a large and diverse group of nitrogenous secondary metabolites found in 20% of plant species. Although plant alkaloids are most often derived from certain amino acids, metabolic pathways leading to different alkaloid types are generally unrelated in terms of both biosynthesis and phylogeny. A complex and diversified relationship between cellular differentiation and alkaloid metabolism has emerged (Samanani *et al.*, 2005).

Alkaloids regularly contain one or more rings of carbon atoms, frequently with a nitrogen atom in the ring. The location of the nitrogen atom in the carbon ring varies with different alkaloids and with different plant families. In some alkaloids, such as mescaline, the nitrogen atom is not within a carbon ring. In fact, it is the precise position of the nitrogen atom that affects the properties of these alkaloids. Although they undoubtedly existed long before humans, some alkaloids have outstanding structural similarities with neurotransmitters in the central nervous system of humans, including dopamine, serotonin and acetylcholine. The amazing effect of these alkaloids on humans has led to the development of powerful pain-killer medications, spiritual drugs,and serious

addictions by people who are ignorant of the properties of these powerful chemicals.

Plant alkaloids are chemotherapy treatments derived made from certain types of plants. The vinca alkaloids are made from the periwinkle plant (*Catharanthus rosea*). The taxanes are made from the bark of the Pacific Yew tree (Sato *et al.,* 2001). The vinca alkaloids and taxanes are also known as antimicrotubule agents. The podophyllotoxins are derived from the May apple plant. Camptothecan analogs are derived from the Asian "Happy Tree" (*Camptotheca acuminata*). Podophyllotoxins and camptothecan analogs are also known as topoisomerase inhibitors, which are used in certain types of chemotherapy (Kitajima, 2002; Memelink *et al.,* 2001). The plant alkaloids are cell-cycle specific. This means they attack the cells during various phases of division. Nicotine is an alkaloid found in the tobacco plant. It is concentrated in this plant's leaves and is derived from pyridine molecules. In addition to being a component of cigars and cigarettes, it is a poison that is used as an insecticide (Baldwin, 1999; Rao and Ravishankar, 2002).The opium poppy contains a variety of alkaloids. Morphine, which gets its name from the Greek god of dreams Morpheus, is a powerful painkiller. It is often given to terminally ill patients. Codeine, similar in structure to morphine, is also obtained from the poppy. It functions much like morphine but is less potent. Heroin is a synthetic derivative of morphine that is highly addictive. The muscle relaxer papavarine is also derived from the opium poppy. The majority of opiates are produced in India. Some alkaloids are based on chemical structures called indole rings. Strychnine is an example of this type of compound. It is a powerful central nervous system stimulant. Lysergic

acid, which is produced by a fungus that grows on rye, is another example. A synthetic variation of this compound called lysergic acid diethylamide is a powerful hallucinogen called LSD. Another class of alkaloids are based on structures called piperidine rings. These include compounds such as cocaine and atropine. Cocaine is a powerful stimulant that can be addictive. Atropine is an important medicine that is used to dilate the pupils of the eye, or act as a smooth muscle relaxer. Other important alkaloids are caffeine, ricinine, and quinine (Facchini, 2001).To produce commercial quantities of alkaloids, manufacturers begin by drying large quantities of the plants in which they occur. Since most alkaloids are basic, they can then be separated from their biomass sources by extraction with a dilute mineral acid. Using HPLC, the alkaloids can be purified and crystallized. In this way, large amounts of physiologically active compounds can be obtained. The physiological effects of alkaloids have made them important compounds in medicine such as painkillers, stimulants, muscle relaxers, tranquilizers, and anesthetics. The four types of alkaloids that have the most important economic impact include opiates, cocaine, caffeine, and nicotine (Rao and Ravishankar, 2002).

There are three main types of alkaloids. True alkaloids, Proto alkaloids, and pseudo alkaloids. Pseudo-alkaloids can be derived from; Terpenoids or Purines. Colchicine is an example of a proto-alkaloid. The basic units in the biogenesis of the true alkaloids are amino acids. The non-nitrogen containing rings or side chains are derived from terpene units and / or acetate, while methionine is responsible for the addition of

methyl groups to nitrogen atoms. Alkaloids are highly reactive substances with biological activity in low doses.

5.3 Definition

Contains nitrogen - usually derived from an amino acid. Bitter tasting, generally white solids (exception - nicotine is a brown liquid). They give a precipitate with heavy metal iodides. Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer's reagent (potassiomercuric iodide solution).Cream colored precipitate. Dragendorff's reagent (solution of potassium bismuth iodide) gives orange colored precipitate with alkaloids. Caffeine, a purine derivative, does not precipitate like most alkaloids (Harborne, 1984).

Alkaloids are basic - they form water soluble salts. Most alkaloids are well-defined crystalline substances which unite with acids to form salts. In plants, they may exist in the Free State, as salts or as N-oxides. Occur in a limited number of plants. Nucleic acid exists in all plants, whereas, morphine exists in only one plant species. Alkaloids can be classified; in terms of their biological activity, chemical structure (nucleus containing nitrogen), biosynthetic pathway (the way they are produced in the plant).

5.4 Pathways execution and biosynthesis of terpenoid indole alkaloids

Various pathways execute for the biosynthesis of secondary metabolites. Chorismate is a key intermediate in the biosynthesis of the shikimic acid pathway, leading to the formation of the aromatic amino acids, phenylalanine, tyrosine and

tryptophan, as well as a variety of other compounds including the anthraquinones, ubiquinones and folate. These compounds all occur through five distinct biochemical paths, which branch from the point of chorismate, and for this reason, chorismate is often considered a limiting factor in the formation of tryptophan, with much of the compound being utilized for a variety of other metabolites (Weaver and Herrmann, 1997).The primary reactions in plants leading to the formation of secondary metabolites from aromatic amino acids usually involve either deamination or decarboxylation. The formation of the indole alkaloids is no exception to this generalization, the first committed step in their formation being the decarboxylation of the primary metabolite, tryptophan to the secondary metabolite, tryptamine by tryptophan decarboxylase (Facchini *et al.,* 2000). This enzyme has been characterized in cucumber hypocotyls, tomato shoots, barley shoots and *Phalaris tuberosa* as well as in the terpenoid indole alkaloid (TIA) producing plants, *Catharanthus roseus* and *Camptotheca acuminata* (Facchini, 2001) This enzyme, is not found in all plant species, although it does occur in all indole alkaloid producing species and is one of the possible pathways leading to the formation of the essential plant hormone, IAA. As with all other decarboxylases, tryptophan decarboxylase shows an absolute requirement for pyridoxal 5'-phosphate (PLP) and the mechanism is similar with other types, however the only difference is being loss of the carboxyl group from the carbon, rather than the hydrogen and replacement of tryptophan for serine. In *Camptotheca acuminata,* tryptophan decarboxylase was sequenced and a Pro-His-Lys series of residues was discovered at

position 317, this series of residues occurring in all decarboxylases, the lysine residue being the Schiff base forming activating amino acid.

This enzyme is also able to decarboxylate 5-hydroxytryptophan to 5-hydroxytryptamine, but it has no action on D-tryptophan, tyrosine, phenylalanine or 3,4-dihydroxyphenylalanine (DOPA).Due to its importance as the first committed step in the formation of secondary products based around the indole nucleus, the regulation of tryptophan decarboxylase has been the focus of many studies. This includes its relevance in the production of TIAs, and N,N-dimethyltryptamine (DMT) in *Phalaris tuberosa.* The studies focusing on tryptophan decarboxylases role in the formation of simple indole alkaloids discovered a fairly large number of compounds which caused inhibition at some level, most of them representing some form of final product: Similar studies directed at the TIA producing plants focused on hormonal and genetic controls in an attempt to raise cell cultures with higher alkaloidal contents. Generally, increased auxin concentrations in the medium used to raise the cell cultures resulted in decreased expression of tryptophan decarboxylase transcripts (TDC). Omitting NAA from the medium resulted in a quick rise in TDC mRNA, while addition of extra NAA, IAA or 2, 4-D resulted in a rapid down-regulation of TDC transcript levels (2,4-D being the only one to cause non-transient reductions). In many plant species tryptophan decarboxylase expression is strongly regulated by developmental factors, it often occurs in apexes, young stems, young bark and newly germinated shoots. The formation of strictosidine is the first step in TIA production, representing an important combination of terpenoid and indole paths

(Connor and Maresh, 2006). It occurs through a cyclization starting with formation of a Schiff base between the primary amino group of tryptamine and the formyl group of secologanin. After the formation of this Schiff base, the C-2 of the indole nucleus of tryptamine, which is nucleophilic due to the adjacent nitrogen, then attacks the imine carbon in a Pictet-Spengler type reaction (Pictet and Spengler, 1911; Cox and Cook, 1995) to cyclize, followed by loss of the hydrogen at the C-2 position to regain its aromatic status.

All of the monoTIAs are derived from the product of this step, strictosidine, which represents a comparatively large portion of the indole alkaloids, with over 1800 characterized so far, many of them having important medicinal properties (Kutchan, 1995; Memelink *et al.,* 2001), One of the main reasons that this particular path has been studied is for similar reasons to that of tryptophan decarboxylase; it may represent an important bottleneck in the formation of useful indole alkaloids, such as Corynanthe, Yohimbe, *Strychnos*, Aspidosperma, Iboga and Hunteria type indole alkaloids (Connor and Maresh, 2006). Other examples of combinations of indole moieties with other pathways come from the phenyl propanoids, isoprenoids and polyketides, with a large variety of alkaloids. The TIAs are found in a number of plant species belonging to the plant families Apocynaceae, Loganiaceae, Rubiaceae and Nyssaceae. TIAs form a large group of structurally diverse molecules comprises a family of 3000 compounds with pharmaceutically interesting activities (Connor and Maresh, 2006). Several TIAs are used in modern medicine, such as the anti-neoplastic agents vincristine and vinblastine

(Beckers and Mahboobi, 2003; Islam and Iskander, 2004) the anti-hypertensive drugs reserpine and ajmalicine, the anti-arrhythmic drug ajmaline, the antimalarial drug quinine, the rat poison strychnine, and some play in defence mechanisma against herbivory. (Facchini, 2001; Rolf et *al.,* 2003). TIAs consist of an indole moiety provided by tryptamine and a terpenoid component derived from the iridoid glucoside secologanin. Tryptophan is converted to tryptamine by tryptophan decarboxylase, which is encoded by a single gene in *Catharanthus roseus* (De Luca *et al.,* 1989; Goddijn *et al.,* 1992) and by two autonomously regulated genes in *Camptotheca acuminate* (Lopez-Meyer and Nessler, 1997).The differential regulation of *TDC* genes in *C. acuminata* suggests that one participates in a developmentally controlled defense pathway, while the other is involved in an inducible defense mechanism. The first committed step in secologanin biosynthesis is the hydroxylation of geraniol to 10-hydroxygeraniol. The enzyme geraniol 10-hydroxylase (G10H) was characterized as a P450 monooxygenase because it is membrane bound, dependent on NADPH and O_2, and displays light-reversible CO inhibition (Meehan and Coscia, 1973). G10H is specific for the C-10 position and exhibits similar affinity for geraniol and nerol, the *cis*-isomer of geraniol. The conversion of loganin to secologanin represents the last step in the pathway and is also catalyzed by a P450-dependent enzyme (Yamamoto *et al.,* 2000). Central to the biosynthesis of all TIAs is the intermediate strictosidine formed by coupling of tryptamine and secologanin, which are derived directly from the amino acid tryptophan and via several steps from (10-hydroxy-) geraniol, respectively. G10H was suggested to have a regulatory effect on

alkaloid production (Collu *et al.,* 2001). The production of terpenoid precursors might play a regulatory role in TIA biosynthesis since the addition of secologanin or loganin to *C. roseus* cell cultures increases alkaloid accumulation (Merillon *et al.,* 1989; Moreno *et al.,* 1993). The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), which is involved in the biosynthesis of mevalonate and methyl jasmonate (MeJA) was suggested to contribute to the regulation of TIA biosynthesis. Several studies using *Catharanthus roseus* indicate that then formation of secologanin can be a limiting factor in TIA production (Whitmer *et al.,* 1998; Vander fits and Meme link, 2000). However, secologanin was recently shown to be derived from the triose phosphate/pyruvate pathway (Contin *et al.,* 1998). While Dagnino *et al.,* (1995) have revealed that the ability to produce terpenoid precursors limits the biosynthesis of terpenoid indole alkaloids, the biosynthetic pathway to secologanin and the method for its production in high yield by plant cell suspension cultures has not yet been established. Tryptamine and secologanin are condensed by strictosidine synthase to form strictosidine, the common precursor to all TIAs. Strictosidine is deglucosylated by strictosidine β-D-glucosidase (Luijendijk *et al.,* 1998). Deglucosylated strictosidine is converted via several unstable intermediates to 4, 21-dehydrogeissoschizine. Strictosidine is the branch point from which biosynthesis routes diverge towards the various alkaloids found in different TIA-producing plant species (Veeporte *et al.,* 1997; Kutchnan 1998; Meme link *et al.,* 2001).

Indole alkaloids such as reserpine, ajmaline, ajmalicine, vincristine and vinblastine are biosynthesized from tryptamine and secologanin, a secoiridoid gluco-side

(Cordell, 1974). These alkloids are distributed in the Apocynaceae, Rubiaceae, Loganiaceae and Nyssaceae, and many are of importance in medicine manufacture (Mann *et al.,* 1994).

5.5 Chemical composition in *Strychnos* genus

The majority of *Strychnos* genus was related in their chemical composition and several of them consists of the indole alkaloids. The major components of *S. nux vomica* are strychnine and brucine, both existing in mixture with *igasuric acid*, a tannic principle identical with caffeo-tannic acid (Sander, 1897). A crystallizable glucosid (loganin, $C_{25}H_{34}O_{14}$) was discovered by Dunstan and Short (1884), in the pulp surrounding the seeds, the dried pulp containing between 4 and 5 per cent. Loganin was also found in the seeds in small amounts. When tenderly heated with a few drops of strong sulphuric acid, a striking red color is developed, changing to purple on standing. When boiled with weak acids, it splits into glucose and loganetin. Loganin is readily dissolved by alcohol or water, but is less soluble in ether, chloroform, and benzene. A supposed third alkaloid, igasurin (Desnoix, 1854), according to Shenstone (1881) is probably nothing but impure brucine. The seeds also contain a fatty substance (3 to 4 per cent), yellow coloring matter, nitrogenous matter (11 per cent), gum, sugar, and about 1.5 per cent of ash. The amount of total alkaloids in the seeds, usually containing strychnine and brucine in about equal proportions, has been found to vary from about 2 to 5 per cent. Dunstan and Short (1884) found specimens of Ceylon *S. nux vomica* especially rich in alkaloids, the latter

amounting on an average to 1.7 per cent of strychnine and 3.2 per cent of brucine; the total amount in one instance was 5.34 per cent.

Strychnine is an indolomonoterpenic alkaloid possessing the strychnan group, which was isolated for the first time in 1818–1819 by Pelletier and Caventou (Pelletier and Caventou, 1818a, b), with brucine (2), its dimethoxylated analog, from the seeds of *S. nux vomica* L. Brucine was also discovered by Pelletier and Caventou in 1819. It exists in the bark and seeds of *S.nux vomica*, and in St. Ignatius' bean. It was obtained by its discoverers from false angustura bark (formerly thought to be the bark of *Brucea antidysenterica*, Miller hence the term brucine), but is now obtained as a by-product in the preparation of strychnine from *S. nux vomica.* The chemical structure of strychnine was elucidated in 1947 owing to the major contributions of H. Leuchs and Sir Robert Robinson. Its absolute configuration was determined by X-ray crystallography in 1956 and its total synthesis was achieved in 1963 by Woodward *et al.,* (see Saxton, 1983; Simon, 1999). Since then, strychnine has been shown to be present in other Asian *Strychnos* species, e.g. *S. ignatii* (Bisset, 1972; Bratati and Bisset, 1990), *S. wallichiana* (Bisset, 1972; Bisset and Choudhury, 1974), and in *S. lucida* (Bisset, 1972; Shaw and Lande, 1948) from Australia. To date, strychnine has been found in only one African species: it was isolated from nearly all parts of *S. icaja* (Sandberg *et al.,* 1968; 1969b). In American *Strychnos*, this alkaloid was isolated from *S. panamensis* and to be present in *S. tabascana* (Marini-Bettolo *et al.,* 1972). The strong convulsivant strychnine is always accompanied by other, generally minor alkaloids of the strychnine series [e.g. 12-

hydroxystrychnine, the colubrines, brucine], of the pseudo series [e.g. pseudo strychnine], and of the N-methyl-sec-pseudo series [icajine, vomicine and novacine]. Experiments investigating the strychnine-like properties of these alkaloids will be related hereafter. Strychnine and related alkaloids could be present in other species, but the complete chemical composition of many *Strychnos* species is still unknown. Note that the seco-alkaloid diaboline, found in different species such as *S. ignatii, S. henningsii* and *S. potatorum*, is not as 'diabolical' as its name suggests: the opening of the lactam of the basic strychnine skeleton and the introduction of a hydroxyl group at the 17- position produce a marked decrease in toxicity—high doses even have a weak curare effect (Neuwinger, 1998).

5.6 Rationale for chromatographic separation of major alkaloids

S. wallichiana is one of most endangered species, rare according to IUCN red listed category (Anonymous, 2001). No reports on the alkaloid composition from the Indian *S. wallichiana* while Bisset and Choudhury (1974) showed the presence of indole alkaloids from the leaves of *S. wallichiana* Steud. ex DC which was collected from Bangladesh. They reported that icajine and novacine are the major alkaloids, while strychnine, brucine, pseudostrychnine, *N*-methyl-*sec*.-pseudo-β-coubrine, 14-hydroxyicajine, strychnine-*N*-oxide and brucine-*N*-oxide.Based on this the present investigation is carried out in an aim to evaluate the alkaloid profile from the Indian *S. wallichiana* with the specific objectives.

- Identification of this Plant from the South Indian local forests

- Studying the alkaloid profiles from the roots and seeds of south Indian *S. wallichiana*.
- Characterization and structural elucidation of HPLC purified alkaloids based on IR, ^{1}H NMR, ^{13}C NMR & LC –MS spectral analysis.

5.7 Experimental

Experimental plant material were collected in August 2002 from Eastren Ghats (Veligonda hills) in Kutlamarri valley in Nellore district of Andhra Pradesh in Southern India and deposited in our field laboratory at University of Hyderabad.

5.7.1 Extraction of alkaloids and sample preparation

The detailed methodology was described in (Figure 23) for the extraction, purification and characterization of alkaloids from the roots and seeds of *S. wallichiana*. The general alkaloid extraction method was followed.5 g of seeds and roots were shade dried, finely powdered, moistened with 25 % NH_4OH and extracted in Soxhlet apparatus with $CHCl_3$ for 24 hrs. The $CHCl_3$ was extracted with 2% aqueous H_2SO_4 (x 3). The combined acid extracts were alkalized with 25%NH_4OH solution to pH 10 and then extracted with $CHCl_3$ (x 3). The combined organic layers were dried over anhydrous sodium sulfate to dryness and concentrated with flash rota evaporator. The dried plant extract was dissolved in 2 ml $CHCl_3$ and filtered through 0.2 mm pore size syringe filter and subjected to TLC and HPLC analysis.

5.7.2 Chemicals

Strychnine, brucine purchased from Fluka. HPLC grade Acetonitrile Chloroform,

1-Heptanesulfonic acid sodium salt, Glacial Acetic acid from Qualigens.0.45 and 0.2 filters from sortorius, Disposable 2 ml Syringes, TLC Silica gel aluminum sheets F_{254} 20 X 20 cm from Merck.

5.7.3 Apparatus

The HPLC analysis performed using a Waters Model 1525 pumping system, a Waters Dual lambda 2487 Absorbance detector fitted with a Zorbax ODS C18 RP analytical column F-35993 [4.6 mm x 25cm] packed with 5-µ particles was used. A20 - µl injection loop was used. Breeze software was used to analyze the data.

5.7.4 Standard solution and mobile phase

Approximately 5 mg of each alkaloid were precisely weighed and dissolved in 5ml of Chloroform and stored at 4^0C. The mobile phase was aqueous1-Heptanesulfonic acid sodium salt + Acetonitrile: water (1:1). Initially 1.1014 gm 1-Heptanesulfonic acid sodium salt was dissolved in 980 ml of Acetonitrile: water (1:1) and pH was adjusted to 3.5 with glacial acetic acid and diluted to 1L with Acetonitrile: water (1:1) and filtered through 0.45µ filters and refrigerated. The flow rate was 0.75 ml/min. The eluate was monitored at 254 nm.

5.7.5 Calibration

Stock solution was used for making serial dilutions. Calibration was done ranging from 0.2 to 3 µg. Regression equations for strychnine and brucine were obtained from peak area [y] and concentration [x] of authentic alkaloids.

5.7.6 Peaks identification from extracts

Identification of peaks from the crude plant extracts was established by

comparison of the UV spectra and retention time with that those of authentic alkaloids. The sample peak purity determination was based on the absorbance at 254 nm and compared to authentic standards. Some samples were analyzed as is or spiked with known amounts of standards.

5.7.7 Statistical analysis

Sigma plot was employed to establish means, standard deviation [S.D] of the distributions of compounds concentration in vegetative parts. The coefficient of variance or relative standard deviation (RSD) was calculated by expressing the standard deviation as a percentage of the revalent mean. The capacity factor K is defined as follows $K=(V_a-V_0)/V_0$ where V_a is the retention volume of the analyte A and V_0 is the void volume of the column. (Masuda, 2001) The selectivity factor ($\propto$) is the measure of the relative retention of the two compounds and denoted by $\propto = K_2 / K_1$ (De Beer, 1991)

5.7.8 Charactization and structural reconfirmation of HPLC purified strychnine and brucine based on IR, ^{1}H NMR, ^{13}C NMR & LC –MS spectral analysis.

IR (KBr) spectra were recorded on JASCO FT-IR spectrophotometer Model 5300. The neat IR spectra were recorded on JASCO FT-IR spectrophotometer Model 5300 and SHIMADZU FT-IR spectrophotometer Model 8300 with polystyrene as reference. ^{1}H-NMR (200 MHz), ^{13}C-NMR (50 MHz)) spectra were recorded on Bruker-AC-200 with chloroform-d as solvent and TMS (internal standard) as reference (δ = 0 ppm). The chemical shifts are expressed in δ downfield from the signal of internal TMS. Liquid Chromatography (LC) and mass analysis (LC-MS) were performed on

SHIMADZU-LCMS-2010A. The mass spectral analyses were carried out using Chemical Ionization (CI) or Electro Spray Ionization (ESI) techniques.

5.8 Results

5.8.1 Separation of standards

The Alkaloids extraction procedure was described for the extraction, purification and characterization of alkaloids from the roots and seeds of *S. wallichiana* (Figure 23). Strychnine composition was determined as $C_{21}H_{22}N_2O_2$ with a Mol. Wt. of 334.42, while composition in brucine determined as $C_{23}H_{26}N_2O_4$ with a Mol. Wt. of 394.47 which was reported in literature (Bisset). Initially alkaloids were separated from the crude plant extracts *i.e.* roots and seeds by TLC (E-Merck F-256 plates) using a solvent system (EtOAc: iso-PrOH: 25% NH_4OH= 80:15:5) and run once. Three bands were separated (Figure 24 a).The most polar band on further TLC (system EtOAc: iso-PrOH: 25% NH_4OH= 90:7:3) run once separated into strychnine and brucine. (Figure 24 b)The alkaloids were detected by spraying with Dragendorff's reagent. TLC analysis revealed the separation of alkaloids from the plant extracts was similar to that of standards. The R_f values of the commercially purchased strychnine and brucine gave 0.35 and 0.5 respectively. The crude extracts *i.e.* roots and seeds were analyzed along with the above standards gave an R_f values of 0.35 and 0.51. Analysis of the two alkaloids has been limited by lack of accuracy in terms of purity and quantification. All the authentic alkaloids were further studied and first characterized individually to record their UV spectrum. Optimal chromatographic conditions were obtained after testing different

mobile phases with a reverse-phase C_{18} column. Isocratic elusions resulted in good separation how ever the separation of strychnine and brucine was shown to be pH dependent. Accuracy of the pH was critical to achieve the separation. The analysis time is a key factor in analytical work and the run time should be reduced to a minimum in order to optimize equipment use and solvent consumption.

5.8.2 Qualitative analysis

The optimized HPLC fingerprinting method was developed for quick analysis of chloroform extract. The optimal conditions led to good separation of the peaks, which could be identified in the chromatogram, brucine (Rt = 4.3) and strychnine (Rt =5.1). They were identified with the comparison with the chromatograph of the two reference compounds (Figure 25 a) obtained under similar conditions. The eluted peaks of strychnine and brucine from root (Figure 25 a and b) and seed extracts (Figure 26 a and b) were matched with reference compounds by internal spiking (Figure 25c, 26 c) and overlaying (Figure 25 d, 26 d)Retention times were similar for the root and seed extracts.

5.8.3 Quantitative analysis

Initially authentic alkaloids were analyzed by increasing concentrations ranging from 0.2 to 3.0 μg. Concentrations of strychnine and brucine were calculated from peak area [y] and concentration [x] of known authentic standards with UV detection at 254nm. Calibration curves showed linearity in the concentration range used for the standards. The standard solutions were injected in triplicates and the resulting curves had a very good linear correlation coefficient.

Strychnine**:** showed regression equation y = 2548.01x + 888956.6, Correlation coefficient 0.999.

Brucine: showed regression equation y = 1382.78x +526350.5 Correlation coefficient 0.999.

Strychnine and brucine showed seasonal variation in vegetative parts examined. The values of the selectivity factor [α] and capacity factor [K] summarized (Table-15). In the roots the amount of strychnine and brucine was very high when compared to other vegetative parts. The amount of strychnine and brucine was 1.2 mg/5gm and 5.3 mg/5gm dry wt of root wood. In seeds strychnine content was 0.33 mg/5gm and brucine content 0.22 mg/5gm dry wt respectively. The results of the quantitative analysis are the average of the three samples and the data are presented (Table-16). The reproducibility of retention times of the strychnine and brucine were studied and RSD also calculated (Table-16).

5.8.4 Charactization and structural reconfirmation of HPLC purified strychnine and brucine based on IR, ^{1}H NMR, ^{13}C NMR & LC –MS spectral analysis

Strychnine and brucine were well characterized and reported in literature (Bisset *et al.,* 1971;1972;1974) Hence we tried to reconfirm and elucidate the structure and identity of the HPLC purified strychnine and brucine based on IR, ^{1}H-NMR, ^{13}C-NMR & LC–MS spectral analysis. Table – 15 lists the identified alkaloids from the roots and seeds of *S. wallichiana.* The major indole alkaloids isolated from *S. wallichiana* are strychnine and brucine.

Strychnine composition was determined as $C_{21}H_{22}N_2O_2$ with a Mol Wt of 334.42 the IR spectrum of strychnine (Figure 27, Table-17) represents the presence of amide

group (1670 cm^{-1}), phenyl ring (1597 cm^{-1}) and aliphatic group (2941-2816 cm^{-1}). ^{1}H-NMR Spectrum of Strychnine (Figure 28, Table-17) the phenyl ring protons were noticed in δ 8.10-7.10 ppm range, alkene proton in δ 5.85 ppm range and aliphatic protons in δ 4.40-1.10 ppm range respectively. In the ^{13}C-NMR spectrum of strychnine (Figure 29, Table-17), the peak corresponds to amide carbon was observed in δ 169.3 ppm, aromatic and alkenyl carbons in δ 142.3 -116.3 ppm range and aliphatic carbons in δ 64.6- 26.92 ppm range.

Brucine composition was determined as $C_{23}H_{26}N_2O_4$with a Mol Wt of 394.47 which was reported in literature. The IR spectrum of brucine (Figure 30, Table-15) represents the presence of amide group (1653 cm^{-1}), phenyl ring (1597 cm^{-1} merged with carbonyl peak) and aliphatic group (2928-2868 cm^{-1}). In the ^{1}H-NMR Spectrum of brucine (Figure 31, Table-18) , the phenyl ring protons were noticed in δ 7.82-6.70 ppm range, alkene proton in δ 6.05 ppm range and aliphatic protons in δ 1.0-4.4 ppm range respectively(Table-18). In the ^{13}C-NMR spectrum of brucine (Figure 32, Table-18) the peak corresponds to amide carbon was observed in δ 168.9 ppm, aromatic and alkenyl carbons in δ 149.0 -101.0 ppm range and aliphatic carbons in δ 64.0- 26.0 ppm range.

High resolution LC-MS is the method of choice for identifying the mass of the molecules. In addition L.C. Mass spectra data (Figure 33) displays that the presence of strychnine and brucine in the crude organic root extract of *S. wallichiana* (Figure 33 a). The molecular ion peak of strychnine was noticed at 335 MHz (Mol Wt: 334.42), while that of brucine at 395 MHz (Mol Wt: 394.47). The HPLC purified strychnine and brucine

also exhibited similar results. The mass spectrum of strychnine showed single peak (Figure 33 b; Table-17) at 335 MHz whereas, brucine (Figure 33 c; Table-15) at 395 MHz. The spectral data based on IR, ^{1}H-NMR, ^{13}C-NMR & LC–MS spectral analysis (Table -17 and 18) reconfirms the structure and identity of these alkaloids as strychnine and brucine.

5.9 Discussion

Strychnos were found to produce indole alkaloids which were widely used in anti cancer, anti tumor, anti viral, anti fungal and anti bacterial and the secondary metabolite pathway of indole alkaloids in *Strychnos* species has already been documented and it is quite complex (Heimberger *et al.,* 1973). In higher plants secondary metabolites were produced from the shikimate pathway (Weaver and Herrmann, 1997), where tryptophan is synthesized and converted to tryptamine, which is in turn condensed with secologanin to yield strictosidine, the common precursor of all indole alkaloids (Nagakura *et al.,* 1978). In this step, the enzyme strictosidine synthase catalyzes a stereoselective Pictet–Spengler condensation between tryptamine and secologanin to yield strictosidine. The biosythesis of strychnine from trytophan and geraniol has been demonstrated in full accord with the seco-iridoid pathway in *S. nux-vomica* (Heimberger *et al.,* 1973). Strictosidine is the branch point from which biosynthesis routes diverge towards the various alkaloids found in different indole alkaloid producing plants. (Verpporte, 1997; Kutchan, 1998). Tryptophan decarboxylase plays an important role in formation of simple indole alkaloids. It has been suggested that in Asian *Strychnos* Species (Bisset

1976) the root is the main site of alkaloid biosynthesis and later transported to other vegetative parts and the alkaloids of the normal series become converted via the N-oxides to the corresponding bases of psuedo-N-methyl-sec-pseudo series due to seasonal variations and environmental conditions. (Bandopadhyay, 1997) Secologanin can be a limiting factor in alkaloid production (Whitmer, 1998; Vander Fits, 2000) and Geraniol 10-hydroxylase, a cytrochrome P450 enzyme has a regulatory effect in alkaloid biosynthesis which was documented in *Catharanthus roseus.* (Collu.*et al.,* 2001). Strychnine and brucine were isolated and well characterized has been previously reported in other *Strychnos* species i.e., *S. nux-vomica, S. lucida*, *S. ignatti*, in Asia, *S. icaja* in Africa and *S. panamensis* in Central America. (Bisset, 1972; Bisset and Choudhury, 1974; Marini-Bettolo *et al.,* 1972).

The extraction from lyophilized plant is performed with methanol, ethyl acetate or chloroform. Soxhlet extraction can be used to dissolve the secondary metabolites in an organic phase (Bhadra, 1993). Hence the shade dried plant material was finely macerated and Soxhlet extracted with organic solvents. Finally crude alkaloid extract was separated. The alkaloids were separated from the crude plant extracts by TLC (solvent system EtOAc: iso-PrOH: 25% NH4OH= 80:15:5) and run once. Three bands were separated (Figure 24 a).The most polar band on further TLC (system EtOAc: iso-PrOH: 25% NH4OH= 90:7:3) run once separated into strychnine and brucine. (Figure 24 b)The alkaloids were detected by spraying with Dragendorff's reagent (Harborne, 1984). TLC analysis revealed the separation of alkaloids from the plant extracts was similar to that of

standards with the R_f values 0.35 (strychnine) and 0.5 (brucine) respectively. Analysis of the two alkaloids has been limited by lack of accuracy in terms of purity and quantification. Hence further investigation is carried out and HPLC analysis performed. The authentic alkaloids were further studied individually to record their UV spectrum and optimal chromatographic conditions were obtained with a reverse-phase C_{18} column (Wang *et al.,* 2004). The present investigation reports the separation of strychnine and brucine by RP-HPLC for the first time in *S. wallichiana.* The optimized HPLC fingerprinting method was developed for quick analysis of chloroform extract. The optimal conditions led to good separation of the peaks, which could be identified in the chromatograms brucine with retention time of 4.3 min and strychnine 5.1 min. respectively (Figure 25 and 26). The eluted peaks of strychnine and brucine from root and seed extracts were matched with reference compounds by internal spiking and overlaying (Figure 25 and 26). Retention times were similar for the root and seed extracts. Strychnine and brucine showed seasonal variation in vegetative parts examined. The values of the selectivity factor [α] and capacity factor [K] summarized in the (Table-15). In the roots the amount of strychnine and brucine was very high when compared to other vegetative parts. The amount of strychnine and brucine (Table-15) was 1.2 mg/5gm and 5.3 mg/5gm dry wt of root wood. In seeds strychnine content was 0.33 mg/5gm and brucine content 0.22 mg/5gm dry wt respectively. Strychnine and brucine were well known alkaloids, characterized and documented (Bisset 1971; 1972; 1974). Hence we tried to reconfirm and elucidate the structure and identity of the HPLC purified

strychnine and brucine from *S. wallichiana* based on IR, ^{1}H-NMR, ^{13}C-NMR & LC –MS spectral analysis. The structure and composition of strychnine and brucine which were quite complex and reported (Wang *et al.,* 2004). Strychnine identity determined as $C_{21}H_{22}N_2O_2$ with a Mol Wt of 334.42 while brucine $C_{23}H_{26}N_2O_4$with a Mol Wt of 394.47. IR spectroscopy is the ideal instrument for identifying the functional groups. An IR spectrum of strychnine and brucine (Figure 25 and 27) were similar to that of existing literature confirming the functional groups (Bisset *et al.,* 1971;1972; 1974).

The structural elucidation was established by the examination of its 200 MHz ^{1}H-NMR Spectrum (Figure 28 and 31; Table-17) and ^{13}C-NMR Spectrum (Figure 29 and 32, Table-17) which has been the subject of preliminary examination. The ^{1}H-NMR demonstrates the presence of hydrogen atoms while ^{13}C-NMR displays the presence of carbon atoms. Strychnine composition was determined as $C_{21}H_{22}N_2O_2$ with a Mol Wt of 334.42, while composition in brucine determined as $C_{23}H_{26}N_2O_4$with a Mol Wt of 394.47 which was reported in literature (Verpoorte *et al.,* 1977; Verpoorte, 1980; Duverneuial *et al.,* 2004; Wang *et al.,* 2004;). The ^{1}H-NMR and ^{13}C-NMR Spectral data analysis of strychnine and brucine suggests that the presence of in 22 hydrogen atoms and 21 carbon atoms in strychnine composition (Figure 28 and 31; Table-17) where as 26 hydrogen atoms and 21 carbon atoms in brucine composition (Figure 29 and 32; Table-18) which was proved again in the present study.

High resolution LC- mass spectroscopy is the method of choice for identifying the mass of the molecules. LC-Ms analysis of crude organic root extract of *S. wallichiana*

suggested the presence of strychnine and brucine. The ions of strychnine was noticed at 335 MHz (which was similar to its Mol Wt of 334.42) and while brucine ions at 395 MHz (which were similar to its Mol Wt of 394.47) respectively (Figure 33 a). The HPLC purified strychnine and brucine also exhibited similar results, showing strychnine peak at 335 MHz where as brucine peak at 395 MHz with out impurities (Figure 33 a and b;Table-17,18).The present spectral data correlated with existing data (Wang *et al.,* 2004) in convincing manner further reconfirming the identity of strychnine and brucine.

5.10 Conclusions

S. wallichiana is one of the rich sources of important bioactive alkaloids which is an alternative source for indole alkaloids such as strychnine and brucine. These alkaloids were isolated and characterized from other *Strychnos* genus. In the present study strychnine and brucine were isolated and characterized from the roots and seeds of *S. wallichiana.* Strychnine and brucine were separated by RP-HPLC. The described HPLC procedure could be useful for the qualitative and quantitative analysis of alkaloids of Loganiaceae family, in quality control of phyto preparations containing strychnine and brucine and as well as in chemosystematics. Strychnine and brucine were well characterized and documented, How ever the present investigation is carried out to reconfirm and elucidate the structure and identity of the HPLC purified strychnine and brucine based on IR, ^{1}H-NMR, ^{13}C-NMR and LC –MS spectral analysis. The spectral data correlates with the existing literature and reconfirms the presence of strychnine and brucine in roots and seeds of South Indian *S. wallichiana.*

Table 15: Parameter values and content of alkaloids in *S. wallichiana* by reverse-phase isocratic-elution chromatography.

Alkaloid	Capacity factor (K)	Selectivity factor (∝)	Root mg/ 5g dry wt	Seed mg/ 5g dry wt
Brucine	0.11	-	5.3	0.33
Strychnine	0.33	3	1.2	0.22

***N = 3 samples**

Table 16: *S. wallichiana*, reproducibility of retention times and RSD for brucine and strychnine by reverse-phase -HPLC.

Alkaloid	RT mean (min.)	RSD
Brucine	4.3	1.02%
Strychnine	5.1	3.6%

***N = 12 samples**

Table 17: Spectral details of strychnine

IR (KBr)	(cm-1) 2941,2816, 1670, 1597
^{1}H-NMR	(200MHz,CDCl3,δ ppm):1.10-4.40 (m,17H), 5.85 (s,1H), 7.15-8.10 (m,4H)
^{13}C-NMR	(50MHz,CDCl3, δ ppm) 26.9, 61.7, 42.5, 42.8, 48.2, 50.3, 52.0, 52.7, 60.1, 60.2, 64.6, 116.3, 122.2, 124.2, 127.4, 128.5, 132.7, 140.4, 142.2,169.3
LC-MS	MHz 335 (M+)

Table 18: Spectral details of brucine

IR (KBr)	(cm-1), 2928, 2868, 1653
^{1}H-NMR	(200MHz,CDCl3, δ ppm): 1.10-4.40 (m,23H), 6.70 (s, 1H), 7.82 (s,1H)
^{13}C-NMR	(50MHz, CDCl3, δ ppm) 26.8, 31.6, 42.4, 48.3, 50.2, 51.9, 52.7, 56.2, 56.5, 60.0, 60.4, 64.6, 101.2, 105.7, 123.4, 127.4, 136.6, 140.4, 146.3, 149.3, 168.9.
LC-MS	MHz 395 (M+)

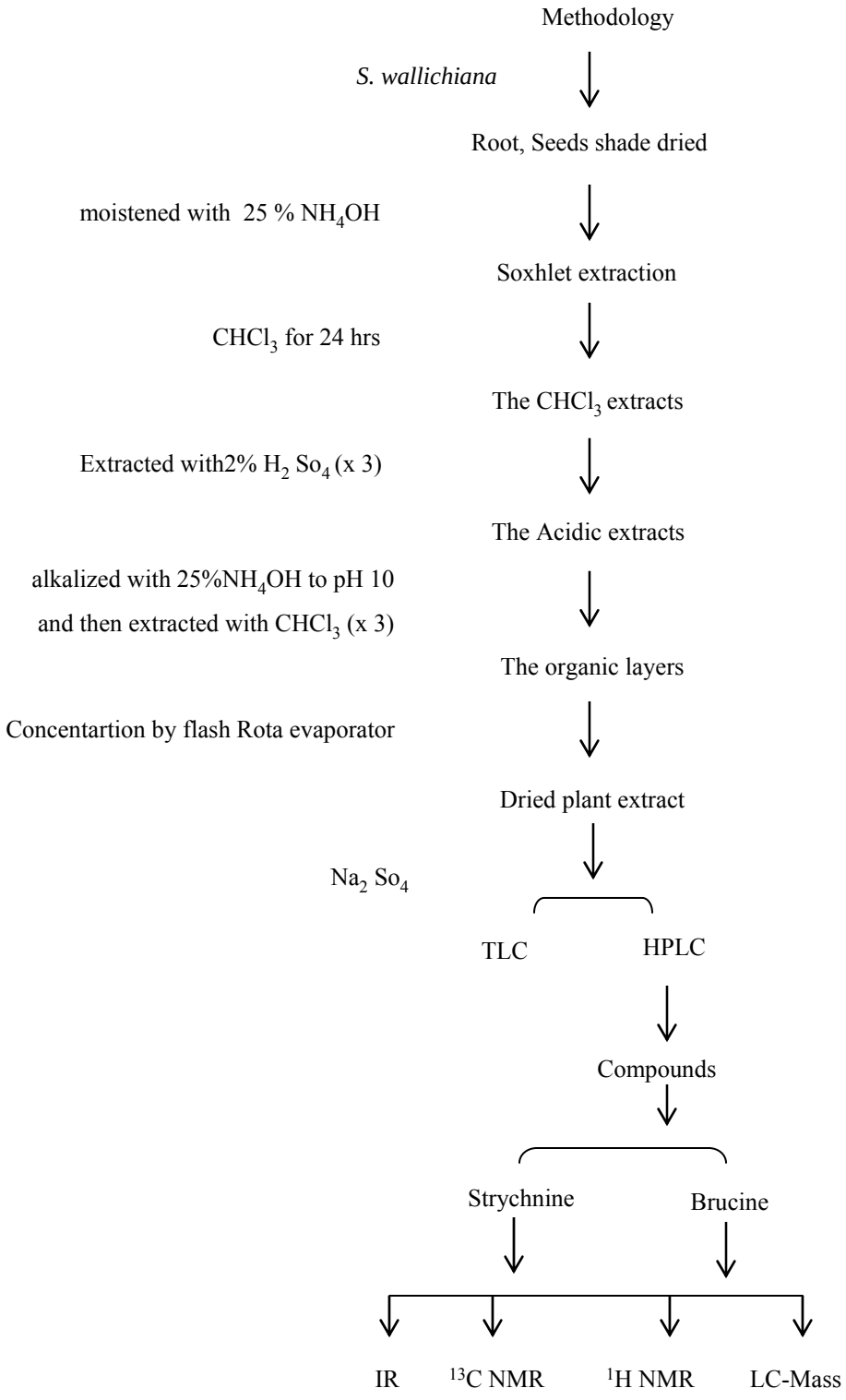
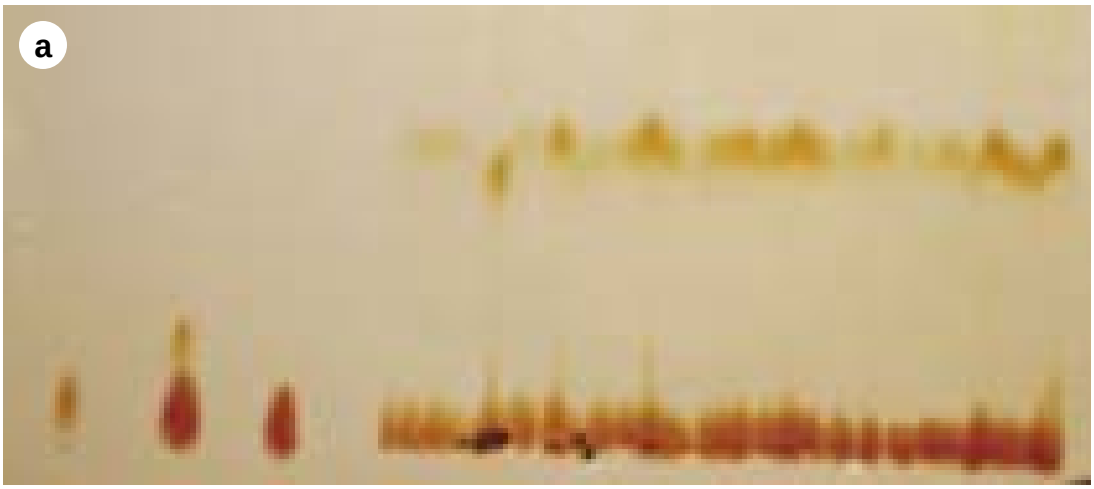


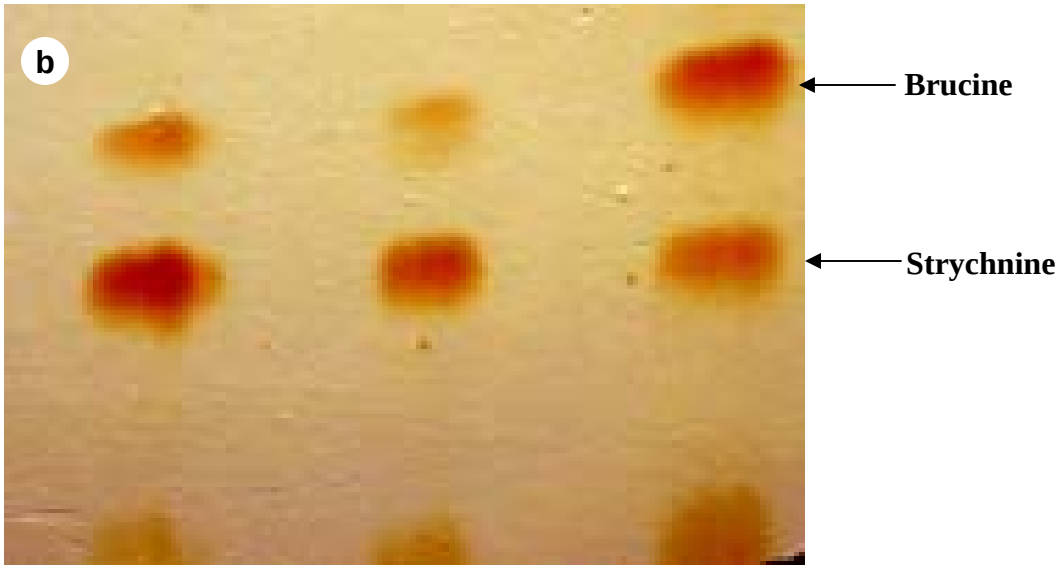
Figure 23: Schematic representation of Identification, isolation, purification & characterization of the strychnine and brucine from *S. wallichiana.*



Standards **Seed extract** **Root extracts**

Alkaloids of the crude extracts were separated by TLC

(solvent system EtOAc: iso-PrOH: 25% NH4OH= 100:2:1)



Standards **Seed extract** **Root extracts**

The most polar band on further TLC run once separated into strychnine and brucine

(solvent system EtOAc: iso-PrOH: 25% NH4OH= 80:15:5)

Figure 24 : Separation of alkaloids by TLC(Thin layer chromatography)

a) Alkaloids of the crude extracts were separated by TLC

b)The most polar band on further TLC run once separated into strychnine and brucine

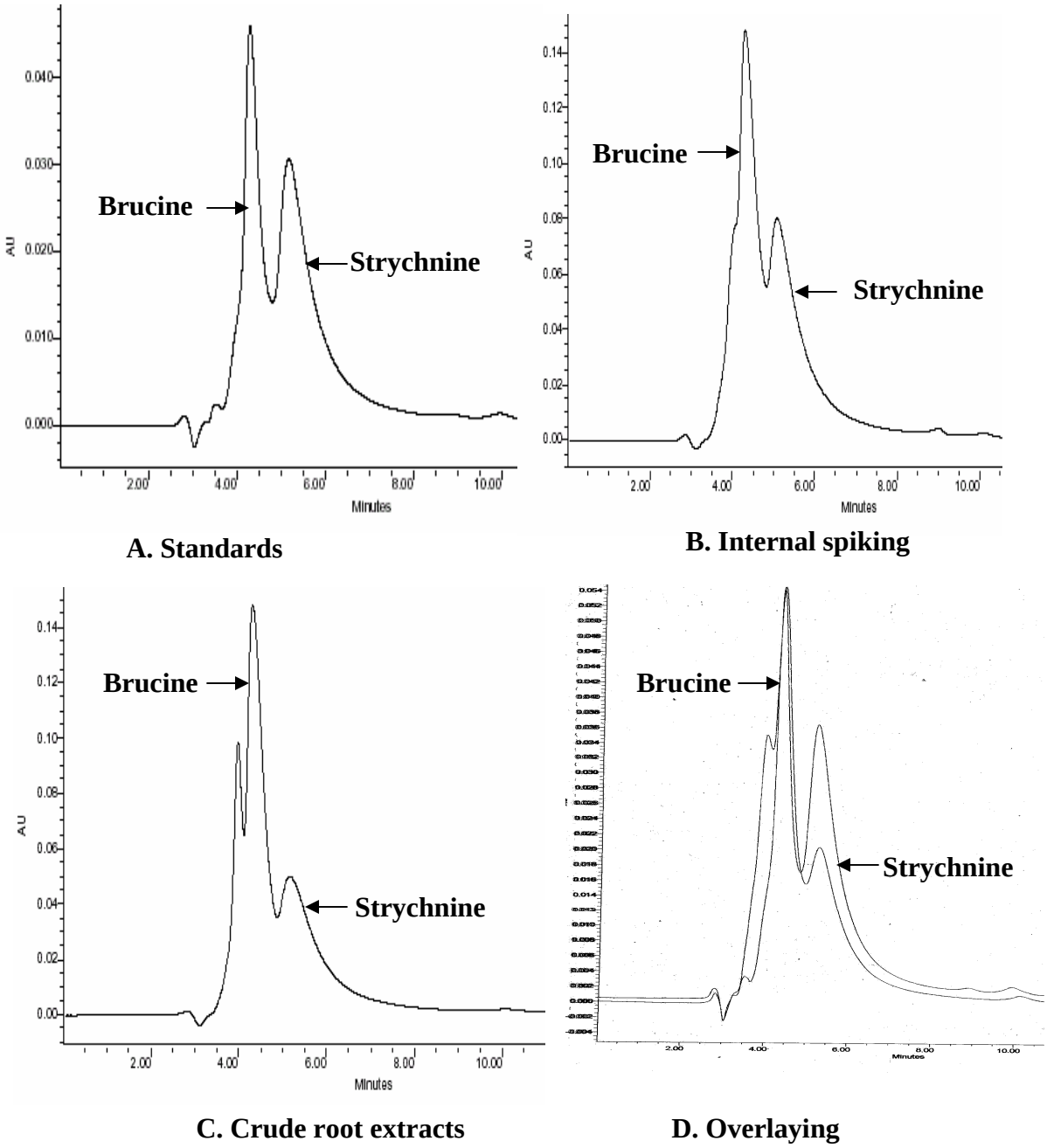


Figure 25 : RP-HPLC (Reverse phase high performance liquid Chromatography) Chromatograms of root extracts of *S. wallichiana.*

a) Authentic samples of brucine and strychnine.

b) Chloroform extract of *S. wallichiana* from roots

c) Chloroform extract of seeds with known amounts of standards by internal spiking.

d) Chromatograms of authentic samples along with root extracts by overlaying.

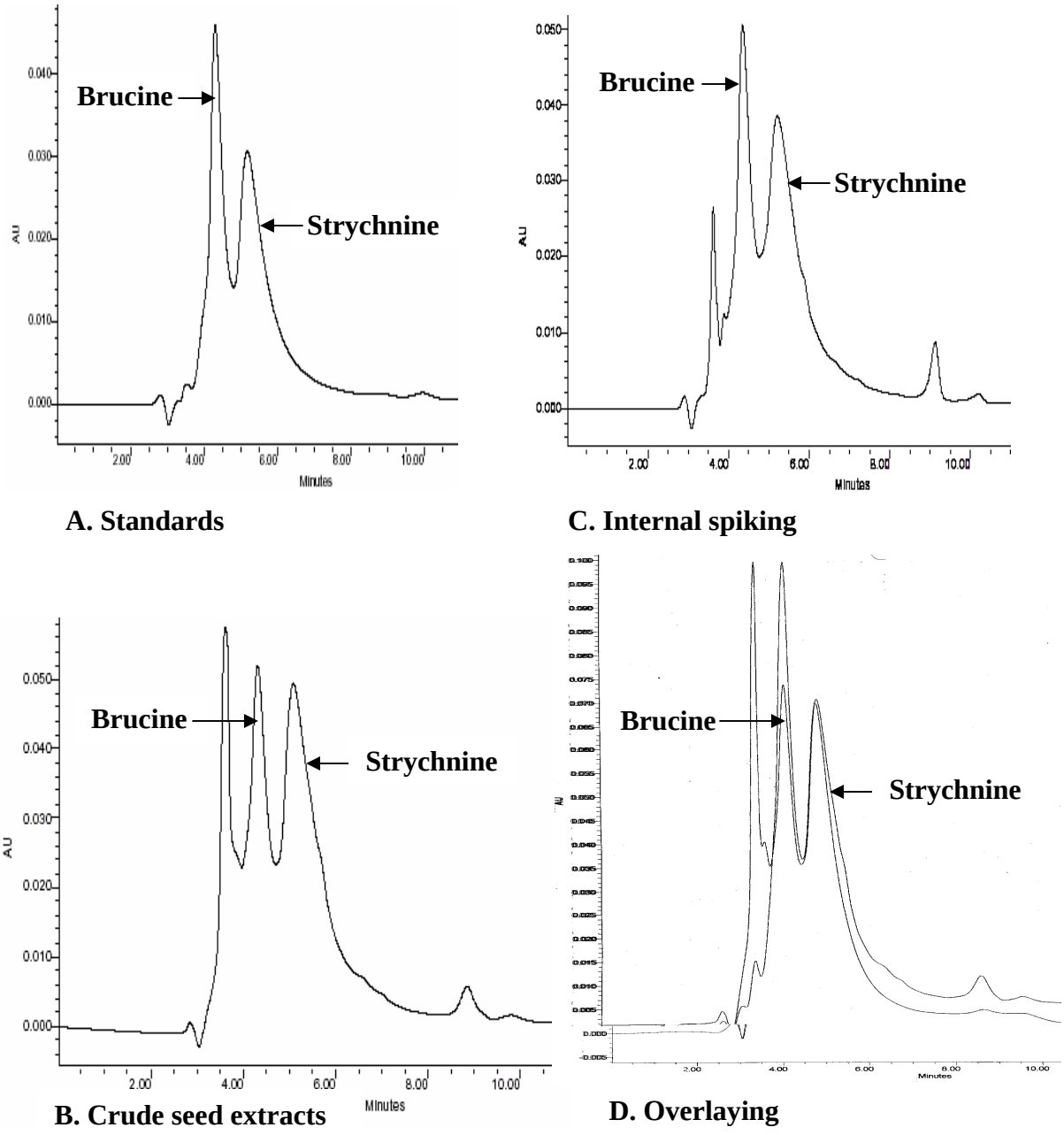


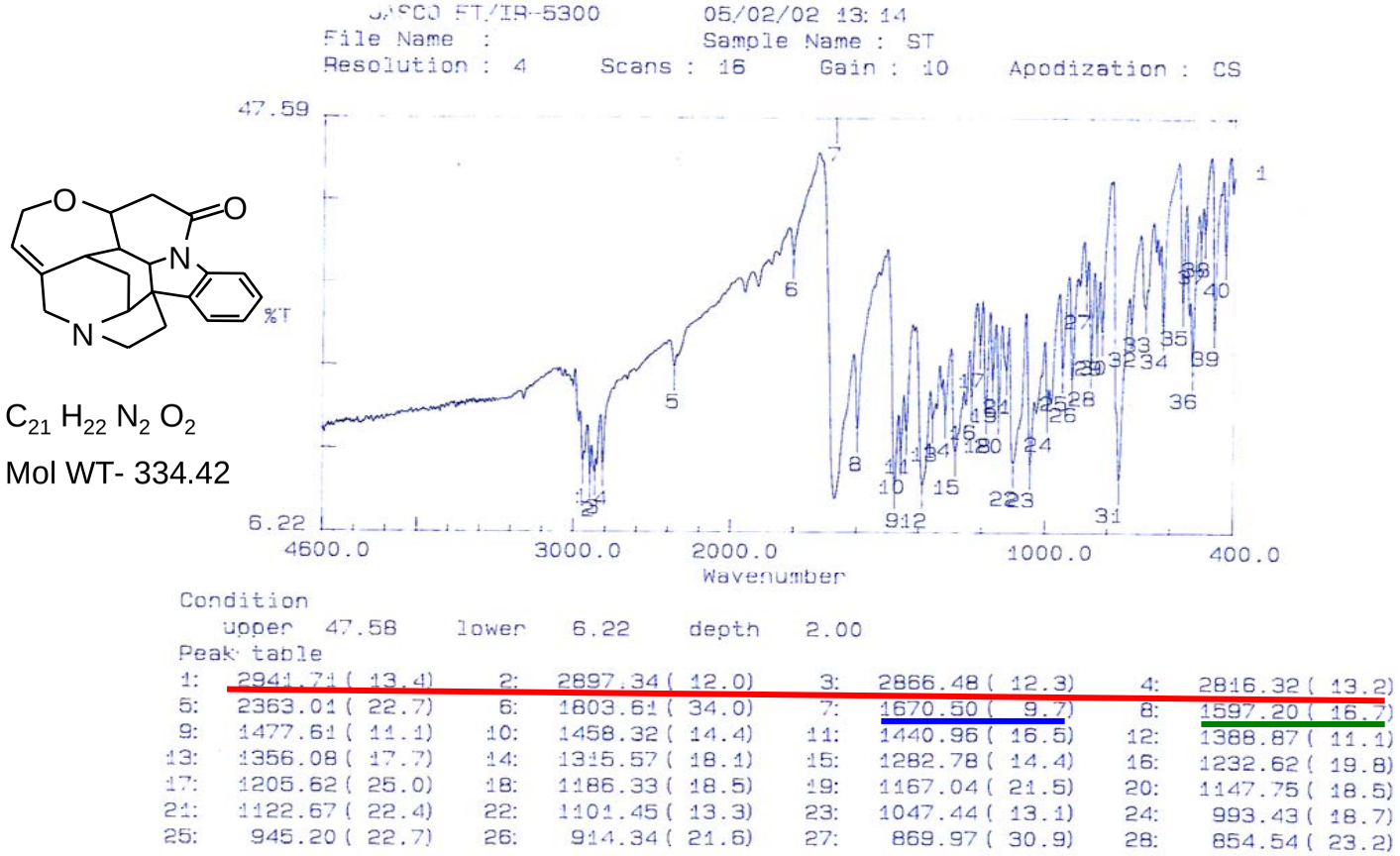
Figure 26 : RP- HPLC Chromatograms of seed extracts of *S. wallichiana.*

a) Authentic samples of brucine and strychnine.

b) Chloroform extract of *S. wallichiana* from seeds

c) Chloroform extract of seeds with known amounts of standards by internal spiking.

d) Chromatograms of authentic samples along with seed extracts by overlaying.



$C_{21}H_{22}N_2O_2$

Mol WT- 334.42

Figure 27 : The IR spectrum of strychnine represents the presence of amide group (1670 cm^{-1}), phenyl ring (1597 cm^{-1}) and aliphatic group (2941-2816 cm^{-1}).

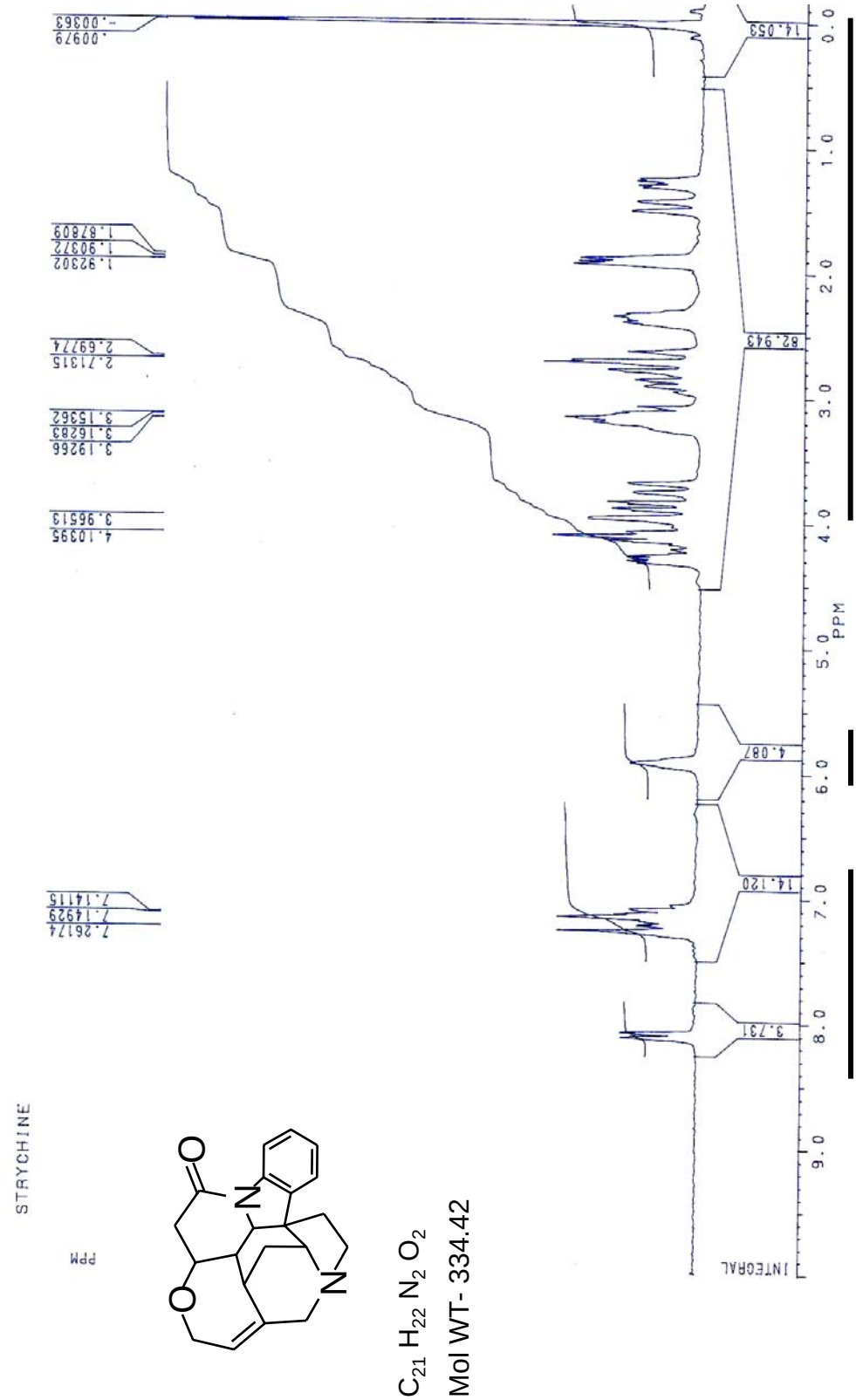
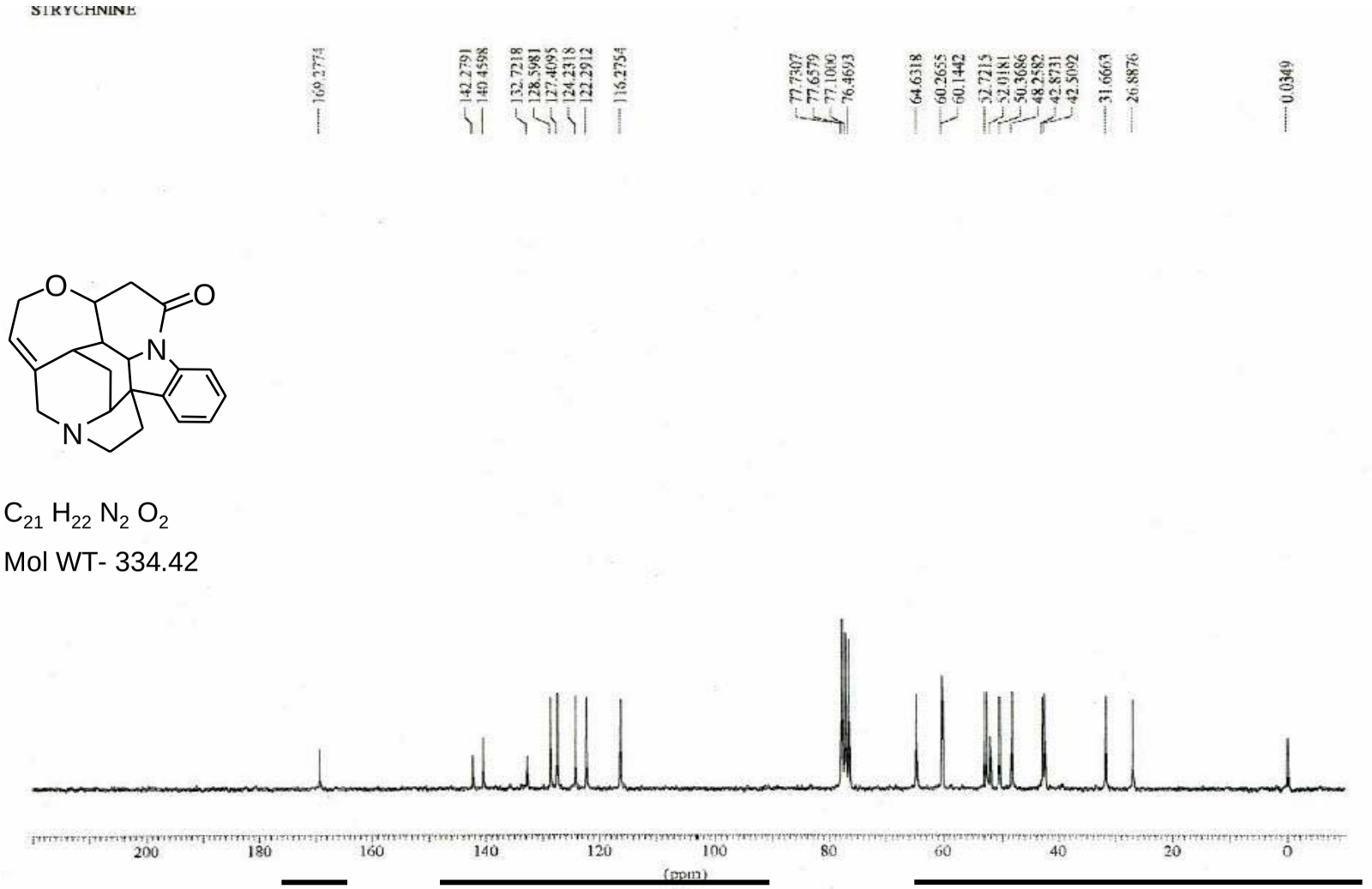


Figure 28: The 1H-NMR Spectrum of strychnine defines the phenyl ring protons in δ 8.10-7.10 ppm range, alkene proton in δ 5.85 ppm range and aliphatic protons in δ 4.40-1.10 ppm range respectively.



C_{21} H_{22} N_2 O_2

Mol WT- 334.42

Figure 29: The ^{13}C-NMR spectrum of strychnine reveals the peaks corresponds to amide carbon was observed in δ 169.3 ppm, aromatic and alkenyl carbons in δ 142.3 -116.3 ppm range and aliphatic carbons in δ 64.6- 26.92 ppm range.

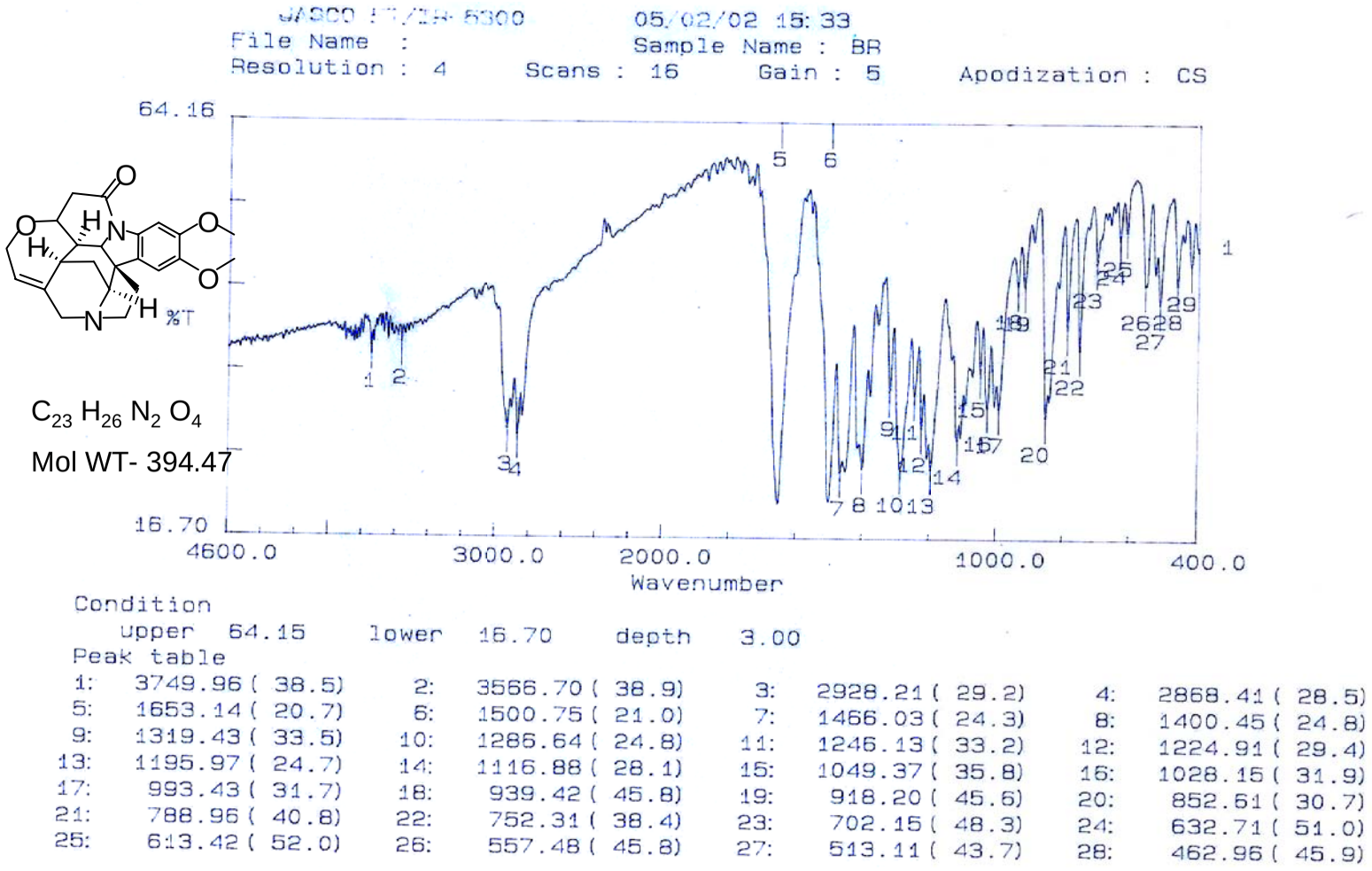


Figure 30 :. The IR spectrum of brucine represents the presence of amide group (1653 cm^{-1}), phenyl ring (1597 cm^{-1} merged with carbonyl peak) and aliphatic group (2928-2868 cm^{-1}).

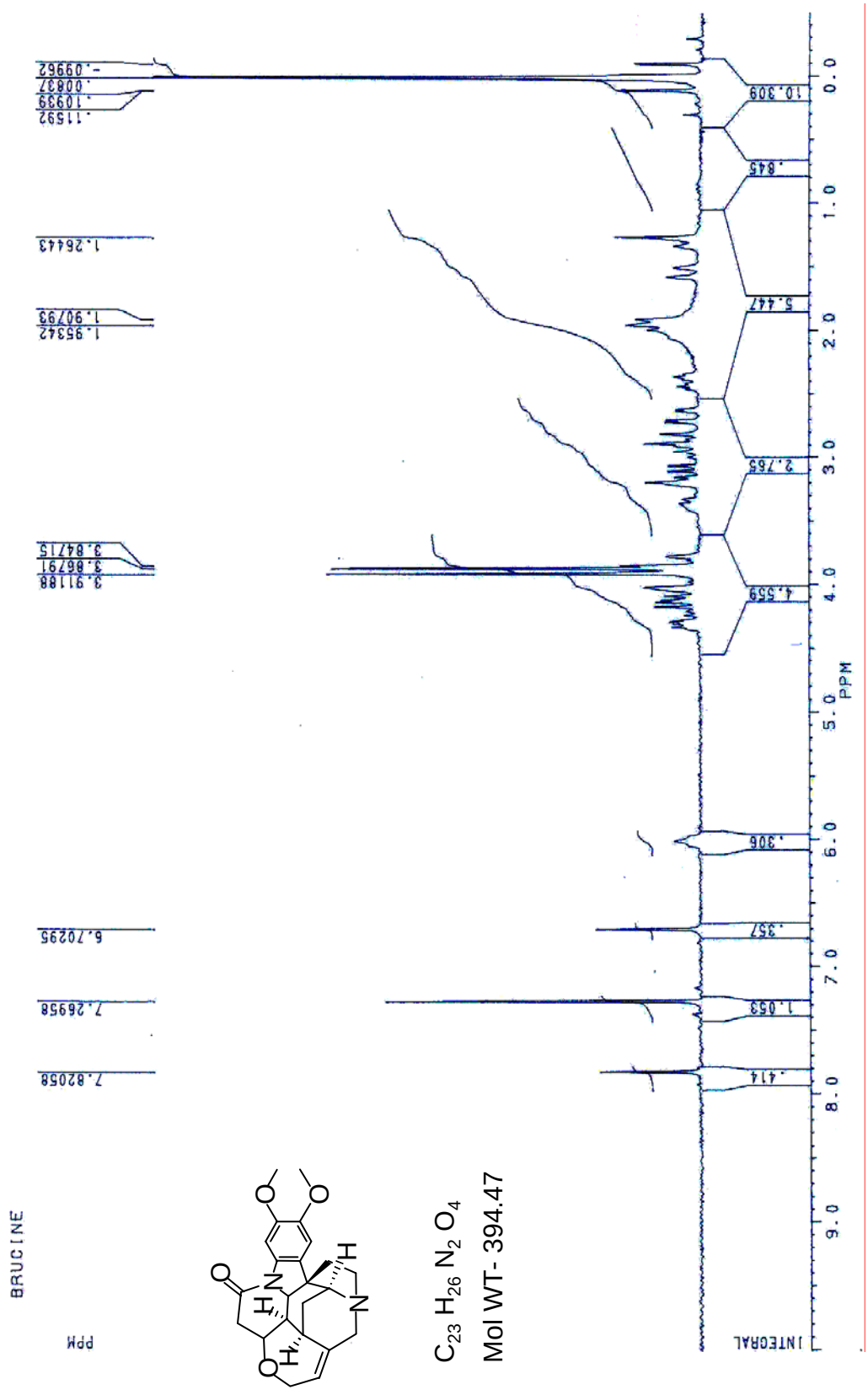


Figure 31 : The 1H-NMR Spectrum of brucine defines the presence of the phenyl ring protons in δ 7.82-6.70 ppm range, alkene proton in δ 6.05 ppm range and aliphatic protons in δ 1.0-4.4 ppm range respectively.

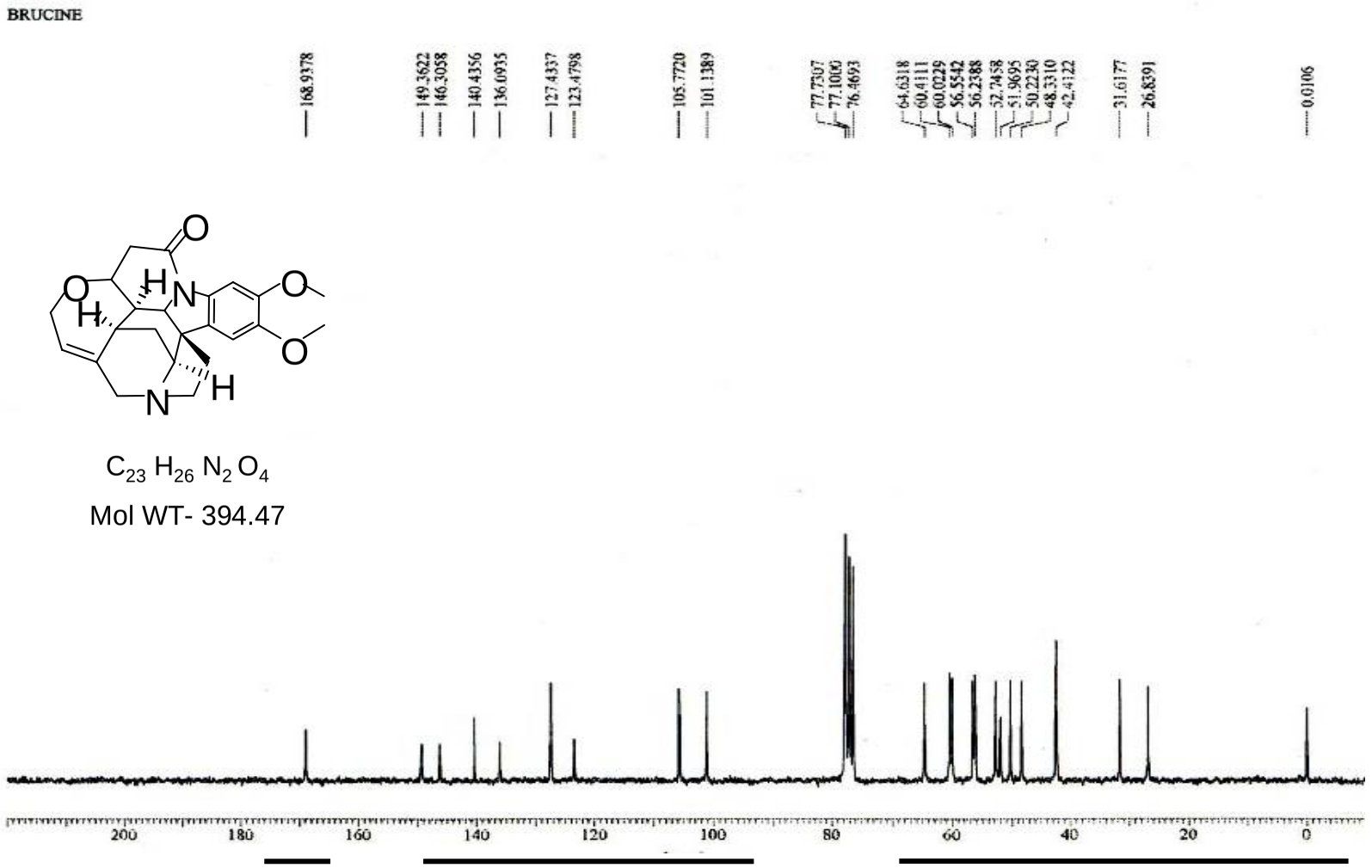


Figure 32: The ^{13}C-NMR spectrum of brucine reveals the peak corresponds to amide carbon was observed in δ 168.9 ppm, aromatic and alkenyl carbons in δ 149.0 -101.0 ppm range and aliphatic carbons in δ 64.0- 26.0 ppm range.

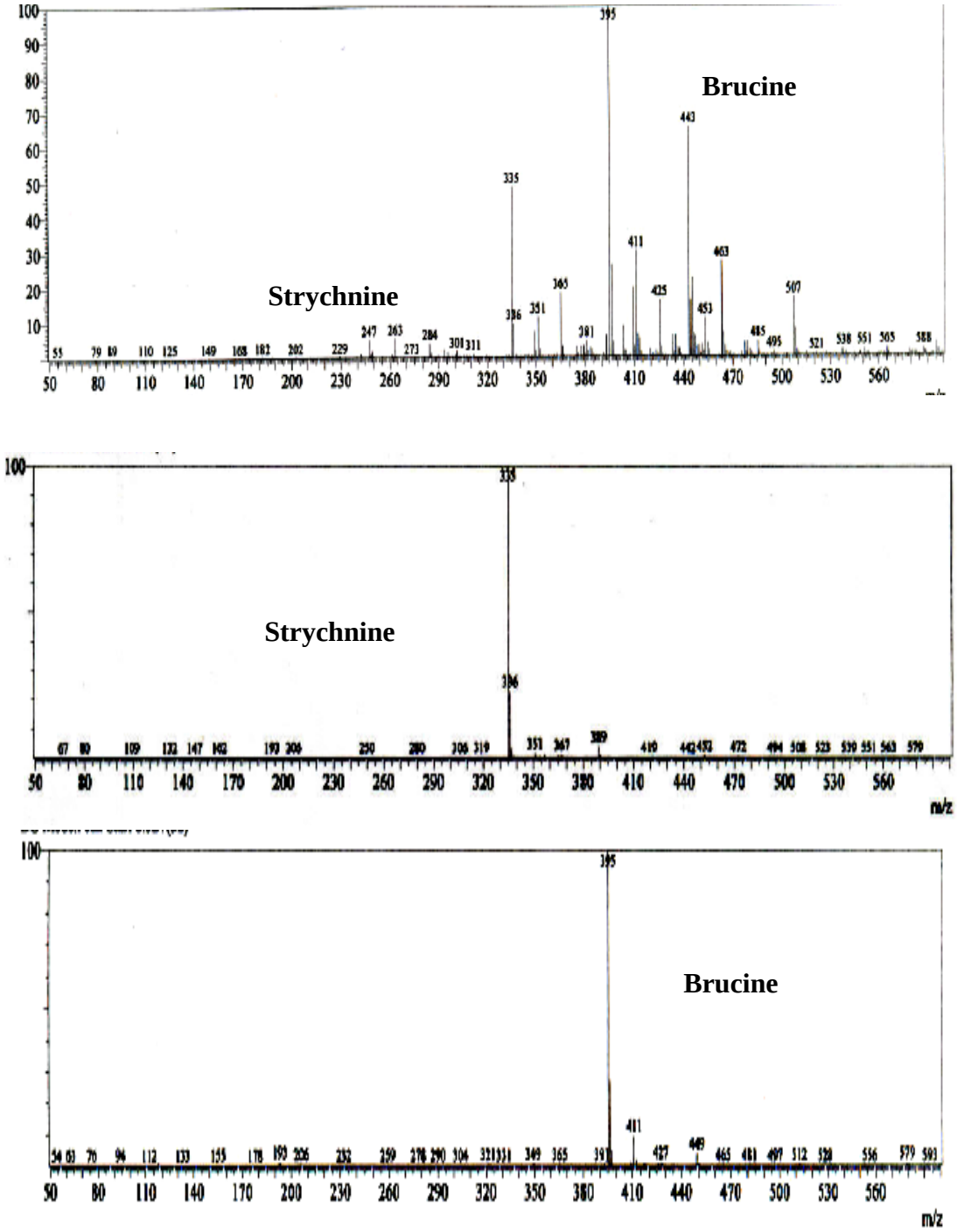


Figure 33 : L.C. Mass spectral analysis

a) L.C. Mass analysis of crude organic root extract of *S. wallichiana* showing molecular ion peak of strychnine 335 MHz and brucine peak at 395 MHz **b)** L.C. Mass analysis of HPLC purified strychnine showing molecular ion peak at 335 MHz **c)** L.C. Mass analysis of HPLC purified brucine showing molecular ion peak at 395 MHz

6 Anti-proliferative and cytotoxic properties of plant extracts and its active molecules on multiple myeloma cell lines

6.1 Introduction

Numerous medicinal plants classified as "Rasayana" in Ayurveda are assumed to be useful in strengthening the immune system of an individual (Patwardhan *et al.,* 1990). Ayurveda (with particular reference to plants) may play a part an essential role in modern health care, principally where satisfactory treatment is not available. Traditional medicines are still very commonly used in India for numerous diseases. Some plants with known immunomodulatory activities are *Acorus calamus*, *Pongamia glabra*, *Strychnos nux-vomica Viscum album, Withania somnifera, Panax ginseng, Tinospora cordifolia, Asparagus racemosus etc* (Nadkarni, 1954). Components such as polysaccharides, lectins, proteins and peptides present in plants have been shown to stimulate the immune system. There is a need to evaluate the potential of ayurvedic remedies as adjuvant to counteract side effects of modern therapy and compare the cost effectiveness of certain therapies vis-à-vis modern therapeutic schedules (Dahanukar and Thatte, 1997). Synthetic chemotherapeutic agents produced today are immunosuppressant, cytotoxic, and exert wide series of side effects. Cytoprotective agents should reduce or prevent these toxicities. These agents should ideally be selective for normal cells versus cancer cells, be effective in reducing or preventing toxicity, should have no negative impact on anticancer therapy, and have minimal adverse effects. None of the agents currently under development fulfils these

criteria completely (Hoekman *et al.,* 1999). Various chemical agents are used as chemoprotectants for conventional cancer chemotherapy and/or radiation therapy. However, their effect is loco-regional and is dependent on dose and time of administration in context to anticancer drugs (Pfeifle *et al.,*1985).Tumor invasion and metastasis still remains at the major cause of treatment failure in malignancy. Metastatic cells have to perform a series of events to reach a distant site for establishing a new colony (Fidler and Hart, 1982; Schirmacher, 1985). If any agent can intercept any of the steps in metastatic cascade, the probability for its clinical trails will be promising. Metastasis may also be controlled by the immuno surveillance and the process succeeds when immune defenses were partially or completely abrogated (Train, 1995). A number of medicinal plant extracts that are used in indigenous system of medicine are known to boost the immune system (Davis and Kuttam, 2002). Plant extracts used in traditional therapy are being reviewed for their chemo protective and immunomodulatory activities. Immunomodulators are biological response modifiers; exert their antitumor effects by improving host defense mechanisms against the tumor. They have a direct anti-proliferative effect on tumor cells and also enhance the ability of the host to tolerate damage by toxic chemicals that may be used to destroy the cancer. Certain agents have been shown to possess activity to normalize or modulate path physiological processes and are hence called immunomodulatory agents (Wagner, 1983).

6.2 Cancer

Cancer is one of the major human diseases causing suffering and economic loss worldwide. It is the second leading cause of death. Today, millions of people are living with cancer or have had cancer. The risk of developing most types of cancer can be reduced by changes in a person's lifestyle, for example, by quitting smoking and eating a better diet. The sooner a cancer is found and treatment begins, the better are the chances for living for many years. A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli, which evoked the change. All tumors can be benign and malignant have two basic components. A) Proliferating neoplastic cells that constitute their parenchyma. B) Supportive stroma made up of connective tissue and blood vessels. Tumors can occur in both mesenchymal cells and epithelial cells. Next to the development of metastases, invasiveness is the most reliable feature that differentiates malignant from benign tumors. Cancer cells develop because of damage to DNA. Most of the time when DNA becomes damaged the body is able to repair it. In cancer cells, the damaged DNA is not repaired. People can inherit damaged DNA, which accounts for inherited cancers. Non-lethal genetic damage lies at the heart carcinogenesis and such genetic damage or mutation can be acquired by the action of environmental agents, such as chemicals, radiation, or viruses, or it may be inherited in the germ line. A disability in the DNA repair genes can predispose to mutations in the genome and hence to neoplastic transformation. The DNA repair genes affect cell proliferation or survival indirectly by

influencing the ability of the organism to repair non-lethal damage in other genes, including proto-oncogene, tumor-suppressor genes and genes that regulate apoptosis. At the molecular level, tumor progression in carcinogenesis results from accumulation of genetic lesions that in some instances are favored by defects in DNA repair (Hong and Sporn, 1997). Anti-cancer drugs presently used have been demonstrated to have severe side effects. Alternative system of medicine comprising the use of medicinal plant extracts to cure cancer with minimal side effects is gaining importance.

6.3 Multiple Myeloma

Multiple myeloma (MM) is a malignancy of plasma cells. MM is a progressive hematologic (blood) disease. It is a cancer of the plasma cell which produces immunoglobulins (antibodies) to help fight infection and disease (Hallek *et al*., 1998). MM is an incurable malignancy primarily localized in the bone marrow, characterized by the expansion of monoclonal Ig secreting cells with low proliferative activity affecting the immune system and causing progressive bone lesions. Although the MM cells are sensitive to chemotherapy, in some patients development of drug resistance occurs. Epidemiologically, MM is the second most frequent hematological malignancy. It has almost a world-wide occurrence. New therapeutic modalities are desirable for this disease. Normal plasma cells are differentiated B lymphocytes which secrete antibodies actively and are important in humoral immunity. MM is characterized by excessive numbers of abnormal plasma cells in the bone marrow and overproduction of intact monoclonal immunoglobulin (IgG, IgA, IgD, or IgE) or Bence-Jones protein. Hypercalcemia, anemia,

renal damage, increased susceptibility to bacterial infection, and impaired production of normal immunoglobulin are common clinical manifestations of MM. It is often also characterized by diffuse osteoporosis, usually in the pelvis, spine, ribs, and skull. Immunoglobulins are made up of protein chains, two long chains called heavy chains and two shorter chains known as light chains. Myeloma is often referred to by the particular type of immunoglobulin or light chain (kappa or lambda type) produced by the cancerous plasma cell. There are five major classes of immunoglobulins. Each class has a unique type of heavy chain that is defined by use of a Greek letter: gamma (IgG), alpha (IgA), mu (IgM), epsilon (IgE), or delta (IgD). Each type has a slightly different function in the body. Normally, a plasma cell makes one of these five major classes of immunoglobulin which normally present in the largest amounts in blood is IgG, followed by IgA and IgM. IgD and IgE in small amounts. Chemical messengers called cytokines are produced by both MM cells and stromal cells. These cytokines, such as interleukin 6 (IL-6), receptor for activation of NF-κB (RANK) ligand, and tumor necrosis factor (TNF), stimulate the growth of MM cells and inhibit (prevent) natural cell death (apoptosis), leading to proliferation of MM cells. MM cells also produce growth factors that promote angiogenesis, the creation of new blood vessels. These new blood vessels provide the oxygen and nutrients that promote tumor growth. A growth factor called vascular endothelial growth factor (VEGF) plays a key role in angiogenesis. Angiogenesis encourages reproduction of MM cells. As tumors grow, they invade the hard outer part of the bone, the solid tissue. In most cases, the MM cells spread into the cavities of all the

large bones of the body, forming multiple small lesions. This is why the disease is known as "multiple" myeloma. There are three distinct classifications of myeloma. They are monoclonal gammophathy of undertermined significance, asymptomatic MM and symptomatic MM (Chauhann *et al.,* 2001).

6.3.1 Mode of cell death

Cell death can go after two distinct pathways, apoptosis or necrosis. Apoptosis and necrosis are two forms of cell death, through evidently distinguishing morphological and biochemical features (Wyllie, 1980) However, these two types of termination can occur simultaneously in tissues or cell cultures exposed to the same stimulus (Ankarcrona *et al.*, 1995; Leist *et al.*,1996; Shimizu *et al.*,1996). Often, the intensity of the same initial insult decides the prevalence of either apoptosis or necrosis (Bonfoco *et al.,* 1995; Dypbukt *et al.*, 1994).This suggests that while some early events may be common to both types of cell death, a downstream controller may be required to direct cells towards the organized execution of apoptosis.

6.3.2 Necrosis

Necrosis appears to be the result of acute cellular dysfunction in response to severe stress conditions or after exposure to toxic agents, and is a relatively passive process associated with rapid cellular ATP depletion. Morphologically, necrosis is characterized by a dramatic increase in cell volume and rupture of the plasma membrane, with spilling of the cellular contents into the intercellular milieu (Gores *et al.,* 1990). This release of the dying cells' contents into the extra cellular space can cause further tissue damage by

affecting neighboring cells or by attracting proinflammatory cells to the lesion (Haslett, 1992).

6.3.3 Apoptosis

Apoptosis is termed as programmed cell death or a form of cell death that occurs during several pathological situations in multicellular organisms and constitutes a common mechanism of cell replacement, tissue remodeling and removal of damaged cells (Delong, 1998).

6.3.4 Morphological and Biochemical Features of Apoptosis

In the majority of cells, a multitude of changes occur during the apoptotic cascade of events. These morphological and biochemical events are seen as hallmarks of apoptosis and suggest the presence of an underlying conserved cell death pathway (Cohen, 1997; Wyllie *et al.,* 1980). The general morphological changes of apoptosis include cytoskeletal disruption, cell shrinkage, and membrane blebbing (Squier *et al.,* 1995; Thompson, 1995). Apoptosis is also characterized by condensation and fragmentation of nuclear chromatin, compaction of cytoplasmic organelles, dilation of the endoplasmic reticulum and a decrease in cell volume (Cohen, 1997). These morphological modifications distinguish apoptotic cells from cells undergoing pathological and necrotic cell death. There are a number of biochemical events that serve as markers of apoptosis in the majority of cells. Loss of mitochondrial function is a common event that occurs in response to extra cellular cues and internal insults such as DNA damage (Green and Kroemer, 1998). During the apoptotic process, the mitochondrial inner transmembrane potential is frequently disrupted.

The change in transmembrane potential leads to a physical disruption of the outer mitochondrial membrane (Green and Reed, 1998; Green and Kroemer, 1998). This process contributes to apoptosis in many cells by allowing the release of cytochrome c (Li *et al.,* 1997; Chen *et al.,* 2000) and SMAC/DIABLO (Chauhann *et al.,* 2001). Thus, loss of mitochondrial membrane potential propagates downstream apoptotic signaling. However cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation (Kerr *et al.,* 1972; Wyllie *et al.,* 1980) and formation of ‘‘apoptotic bodies’’ are all characteristic features of apoptosis. Several protease families are implicated in apoptosis, the most prominent being caspases. Caspases are aspartic acid-specific cysteine proteases, which exist as zymogens in the soluble cytoplasm, mitochondrial intermembrane space, and nuclear matrix of virtually all cells (Nicholson and Thornberry, 1997). At least three models for caspase activation have been proposed. Apoptosis induced by ligation of cell surface receptors like the Fas or TNF-R, dubbed ‘‘death receptors,’’ indicating a pathway, referred as “extrinsic death pathway”, almost entirely controlled by caspases. In these scenarios, ligand binding of the receptor causes the assembly of a series of proteins called the death-inducing signaling complex (DISC), which then activates an apical caspase, procaspase- 8 (Peter and Krammer, 1998). The ensuing events represent the strongest evidence that caspases act in cascades, with caspase-8 causing activation of caspase-3, which can activate other caspases and ultimately cleave a variety of cellular proteins. One of these proteins is a caspase-dependent endonuclease, which is freed from its inhibitor by caspase-3, and subsequently cuts DNA into oligonucleosomal (180 bp) fragments (Liu *et*

al., 1998; Sakahira *et al.,* 1998). A different model for caspase activation has been proposed for the numerous agents that trigger apoptosis without involving cell surface receptors. This pathway, "named intrinsic death pathway", focuses on mitochondria and contends that mitochondrial dysfunction occurs during apoptosis and causes the release of cytochrome c from mitochondria into the cytosol, where it binds to apoptotic protease activating factor 1 (Apaf-1), a mammalian homologue of the pro-apoptotic nematode protein, ced-4 (Zou *et al.,*1997;1999). Apoptotic protease activating factor 1 (Apaf-1) contains binding sites for cytochrome c and dATP and oligomerizes with other Apaf molecules. This complex, termed the apoptosome, recruits and binds pro-caspase-9 by using the caspase recruitment domain (CARD) of Apaf-1 (Saleh *et al.,* 1999). Mature caspase-9 then activates the more distal caspases-3 and -7. Finally, a third pathway, which is capable of activating the caspase cascade, is initiated by cytotoxic cells (Yang *et al.,* 1998). Perforin and granzyme B cooperate to induce apoptosis in tumor cells and in cells infected with intracellular pathogens. Perforin permeabilizes cells, allowing granzyme into the cytosol where it activates caspase-3, either by direct cleavage or via recruitment of the mitochondrial pathway of caspase activation. Regardless of the mechanism, upon activation, caspases cleave numerous cellular proteins including poly (ADP-ribose) polymerase (PARP) and fodrin (Nicholson and Thornberry, 1997). In fact, more than a hundred cellular proteins have now been identified as potential caspase substrates during apoptosis, and most events in apoptosis appear to require a caspase mediated proteolytic step. Although caspase activation via death receptors or granzyme B has been reported to

proceed without mitochondrial participation, recent reports suggest that amplification of these pathways does involve mitochondria. The common partaker is Bid, a proapoptotic Bcl-2 family member, which is cleaved by both caspase-8 (Lou *et al.,* 1998) and granzyme B (Alimonti *et al.,* 2001). The truncated version of Bid (tBid) translocates to mitochondria and causes cytochrome c release and caspase-9 activation.

6.3.5 Cell Cycle

This term collectively refers to the different discrete successive stages that can be discerned in mitotically active eukaryotic cells. The well organized sequence of events yields two identical daughter cells.

This cell cycle can in general be categorized into four distinct phases, viz., G1-, the pre synthetic phase, S- the synthetic phase, G2- the post synthetic phase and M- the mitotic phase. In the pre-synthetic G1 phase (Gap-1), the cell prepares itself for the subsequent DNA synthesis. Enzymes and proteins required for initiating and carrying out DNA synthesis are synthesized late in G1 phase and in early S phase. The G1 phase is followed by the S phase (the synthetic phase) in which the cell replicates its DNA. The post synthetic G2 phase (Gap-2) is the phase after completion of DNA synthesis in which the cell division by synthesizing molecules required in mitotic operation.

6.3.6 The CD138

Heparan sulfate proteoglycans are multifunctional molecules that regulate cell behavior by fine tuning the function of regulatory proteins, helps in both cell-cell and cell-extra cellular matrix adhesion, proliferation, maturation, migration and also play important

roles in regulating disease processes including cancer. They can also promote tumor growth and metastasis.

CD 138 or syndecan-1 is a glycoprotein of 150-250, 80-150, 220-300, 80-150 kDa expressed on simple epithelial, stratified epithelia, mesenchymal cells and lymphocytes. The core protein is composed of 251(including signal peptide), 34 and 25 residues for the extra cellular, transmembrane and intracellular domains respectively. The protein bears heparin sulfates and sometime chondroitin sulfates. The ectodomain contains five putative glycosaminoglycan (GAG) attachment sites, and a putative protease cleavage site at its C-terminus. Comparison between syndecan-1 sequences of different species reveals that the extra cellular domain diverges as much as that of rapidly evolving proteins, although the position of the potential GAG attachment and cleavage sites remain constant, and the transmembrane and intracellular domains are conserved (Wijdenes *et al*., 2002). Syndecan-1 also named CD138; the dominant heparan sulfate proteoglycan expressed on the surface of myeloma cells, and also on the surface of other tumor cells of various origins including Hodgkin's disease, and certain human immunodeficiency virus (HIV) associated lymphomas. Syndecan-1 is also widely used as a standard marker for identification of tumor cells which is anchored in the plasma membrane by a transmembrane domain, acts to bind myeloma cells to extracellular matrix, to adjacent cells, to (fibroblast) growth factors and to potent angiogenic growth factors. Functional studies in myeloma reveal that syndecan-1 may act as a multifunctional regulator of cell behavior in the tumor microenvironment; it mediates cell-cell adhesion, binding of myeloma cells to type I

collagen, and inhibits tumor cell invasion into collagen gels. In addition, syndecan-1 is released from the surface of myeloma cells and this shed form of the molecule inhibits growth and induces apoptosis of myeloma cells and may modulate myeloma bone disease by inhibiting osteoclast formation and promoting osteoblast formation. Sydecan-1 is also expressed by human plasma cells while its expression is lost which undergo apoptosis. In the bone marrow it is expressed on precursor B cells and lost immediately before maturation and release of B lymphocytes in to the circulation. Re expression of sydecan-1 on B lymphocytes was found immobilized plasma cells, how ever in recent years the presence of malignant B cells in peripheral blood of MM patients has been reported (Wijidenes *et al.,* 2002). In view of its effects on tumor cell growth, survival, adhesion and invasion and on bone cell differentiation, syndecan-1 may be an important potentially beneficial regulator of myeloma pathobiology. The soluble form of the syndecan-1 heparan sulfate proteoglycan acts as a tumor suppressor molecule that inhibits growth and induces apoptosis of some cancer cell lines *in vitro* (Dhodapkar and Sanderson 1999). The present study is undertaken to check the CD138 expression levels against the plant extracts and compounds in U226 cell line.

6.4 Traditional use of *Strychnos* in the treatment of cancer

The genus *Strychnos* belongs to the family Loganiaceae and comprises of species, all of which are commonly distributed in India (Anonymous, 1976). They are found naturally growing in the native evergreen and deciduous forests. *Strychnos nux-vomica* is renowned for drug value of its poisonous alkaloids, namely strychnine and brucine and

hence, commonly called as *nux-vomica* of commerce (Troup, 1921). *S. nux-vomica* is widely used in all kinds of systems of medicine i.e., Ayurveda, Siddha, Unani, Folk, Modern, Tibetan and Homeopathy (FRHLT report). *S. nux-vomica* L. (ma-qian-zi in Chinese) has been used in traditional Chinese medicine as an anti-inflammatory and analgesic agent to relieve arthritic and traumatic pains. It has been demonstrated that strychnine and brucine, which are central nervous system stimulants and with anti-inflammatory properties. The South African *S. icaja* is also used in the treatment of Cancer and the active principles from the root i.e. Sungicine, isosungicine induced apoptosis in Human cancer cells (HL-60, HeLa cells, HCT-116 Colon cancer cells) (Lansuix *et al*., 2002, Frederich *et al*., 2003). *S. nux-vomica* has long been one of the primary components in traditional Chinese herbal medicine such as "Ping-xiao" capsule, "Ci-dan" capsule or "Ma-quian-zi" which is used for the treatment of Liver cancer (Xu *et al*., 2003). The alkaloid fractions of *S. nux-vomica* potentially exhibited cytotoxicity towards cell proliferation of HeLa and K562 cell lines (Cai *et al*., 1998).

6.5 Rationale for undertaking this work

- MM is incurable malignancy and most of the available drugs fall of short in treatment, whereas some patients have developed drug resistance, while some demonstrated to have severe side effects.
- Alternative system of medicine comprising the use of medicinal plant extracts to cure cancer with minimal side effects is gaining importance.
- *Strychnos* genus has significant role in Chinese medicine for the treatment of liver cancer however the detailed mechanism of action is still unknown.

Based on this the present investigation is carried out in an aim to evaluate the antiproliferation properties of Indian *S. nux-vomica* and *S. wallichiana* and its active principles on MM cell lines with the specific objectives.

- Cytotoxicity assessment and evaluation of ethanol root extracts (*S. nux-vomica* and *S. wallichiana)* on MM cell lines (RPMI 8226, U226 B1)
- Studying the mode of cell death and identifying mechanism for antiproliferative activity.
- Evaluation of ethanol root extracts (*S. nux-vomica* and *S. wallichiana)* for identifying the bio-active principles.
- Cytotoxicity assessment and evaluation of bio-active principles (*S. nux-vomica* and *S. wallichiana)* on MM cell lines (RPMI 8226, U226 B1)
- Studying the mode of cell death and identifying mechanism for antiproliferative activity.

6.6. Experimental

Germplasm of *Strychnos nux-vomica* and *Strychnos wallichiana* collected from local forests of Deccan eco- region (Andhra Pradesh) of southern India. Voucher specimens and live collections were deposited in our field gene bank laboratory at University of Hyderabad.

6.6.1 Preparation of plant extracts

The general ethanol extraction method was followed. 5 g of roots were shade dried, finely powdered, and extracted in soxhlet apparatus with ethanol for 48 hrs. Then, it was filtered through filter paper and the filtrate was concentrated in a thermostatic flash evaporator at 60^0C. The final residue was dissolved in 5% DMSO, sterile filtered and kept refrigerated until use.

6.6.2 Estimation of the dry weight of the extracts

The detailed procedure for preparation of ethanolic extracts from shade dried roots of *S. nux-vomica* and *S. wallichiana* was documented in **(Figure 34).** A clean and 1.5ml appdendorf was taken and weighed. The empty weight of the 1.5ml appdendorf was noted and 1ml of the plant extract added. Later it was subject vacuum freeze drying. The 1.5ml appdendorf was weighed again. The dry weight of the plant residue present in 1ml of the extract was obtained by deducing the initial weight of the empty appdendorf from the final weight. Concentration of the plant extract was calculated from the amount found to be present in 1ml.

6.6.3 Spectrophotometric scanning of extracts

The plant extracts were diluted 1:1000 distilled water and a scan was taken in the range of 200 – 400 nm.

6.6.4 Cell lines used for the study: Obtained from NCCS, Pune.

RPMI 8226: It is a light chain secreting myeloma cell line established from the peripheral blood of a 61-year old man with multliple myeloma.

U 266B1: It is an IgE secreting myeloma cell line established from the peripheral blood.

6.6.5 Chemicals:

RPMI- 1640 medium (with 2mM L-Glutamine and 25mM HEPES buffer) Heparin, p-nitro phenyl phosphate sodium salt, Fetal bovine serum, all the chemicals used was of analytical grade. 96 well flat bottom plates, 15ml and 50ml tubes were purchased from the Orange Scientific.

6.6.5 Medium preparation

Powdered RPMI- 1640 medium was dissolved in ultra pure water. 2g of sodium carbonate was added, pH was adjusted to 7.2 and then the volume made up to 1L with ultra pure water. This was sterile filtered through 0.22μ membrane filter using Sartorius filtration unit.

6.6.7 Determination of cell viability

Principle: Dead cells take up the dye trypan blue while the live cells exclude it, thereby viable cells could be distinguished from nonviable dead cells, which are stained.

Procedure**:** A small volume of the cell suspension was diluted approximately in Trypan blue solution (0.2%w/v in 0.9% NaCl). Minimum of 200 cells were counted microscopically using Haemocytometer. The percentage of viable cells was calculated using the formula.

% Viability = (number of unstained cells/ Total number of cells) x 100

6.6.8 Maintainance of Multiple myeloma cell lines (RPMI 8226 and U266B1)

The human MM cell lines obtained from the National Center for Cell Sciences, Pune. The growth medium used for the propagation of the cell line was 90% RPMI-1640 with 10% FBS, 100 UI /ml penicillin and 100µg/ml streptomycin respectively. The cell lines were maintained in humidified atmosphere with 5% CO_2 at 37°C. The seeding ratio of the cells was 0.2 X 10^6 cells/ml of the medium. When the cells reached a density of 1 X 10^6 cells/ml they were sub cultured. Doubling time of the cells was observed to be 55 hrs. Medium was renewed 2 to 3 times per week

MTT assay principle**:** 3-[4, 5-dimethylthiazole-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) is reduced by mitochondrial dehydrogenase enzymes of the living cells to Formazan (purple compound). The absorbance of Formazan is measured in a micro plate ELISA reader equipped with a 570 and 630nm filter and is proportional to the number of viable cells.

Reagents: 1.5mg/ml MTT in PBS, 2.0.01 N HCl in 10% SDS

6.6.9 Methodology for cell viability and cytotoxicity

The pant extracts and HPLC purified compounds induced growth inhibitory effects were assessed using the MTT assay (Mosmann, 1983). For MTT assay 30 x 10^3 exponentially growing cells were plated in 100 ml of the growth medium in the presence or absence the pant extracts and HPLC purified compounds in 96 well plates and cultured at 37 °C in 5 % CO_2 for 12 -72 hrs. Cultures in 96 well microtitre plates were centrifuged at 1000 rpm for 10 min at room temperature and 100µl of supernatant was discarded and 20µl of MTT was added to each well. The plate was incubated for 4 hrs at 37^0C and 100µl of acidified SDS was added to wells. The plates were incubated overnight to solubilize the Formazan compound and the absorbance measured at 570nm single wavelength mode.Each concentration was tested in three independent experiments run in four replicates. Standard errors of means were calculated and data were presented as the % growth vs control. The concentrations of the plant extracts and compounds that inhibited cell growth by 50% (IC_{50}) were determined from these cell survival plots.

6.6.10 Cell morphology assessment

Light and Phase contrast microscopy**:** RPMI 8226 and U266B1 cells were treated with IC_{50} value of *S. nux-vomica* extract incubated for 48 and 72hrs were examined under the Phase and light microscope.The HPLC purified compounds were also exposed to cells (RPMI 8226 and U266B1) for 24 and 48hrs in a similar fashion and same procedure followed in further experiments.

Scanning electron microscope**:** Cells (1 X $10^{6)}$ were treated with IC_{50} Concentration of *S. nux-vomica* root extract and as well as control cells for 48hrs. After the treatments the cells were harvested, rinsed with PBS. 1µL of this suspension was place on to a plastic cover slip that was previously coated with Poly-L- Lysine .Cells were fixed with glutaradehyde for 1hr and post fixed with 1% osmium tetrxoide for 1hr Cells were dehydrated by passing through graded alcohols and dried by the critical-point technique. After trimming, mounting and coating with Gold-platinum the specimens were observed on SEM (Model: JSM- 5600, JEOL. Co)

Transmission electron microscope**:** Cells (1 X $10^{6)}$ were treated with IC_{50} Concentration of *S. nux-vomica* root extract and as well as control cells for 48 hrs and harvested, rinsed with PBS and fixed for 12hrs at 4^{o}C with 2% vol/vol Glutaradehyde in 0.1% sodium cacodylate buffer, pH 7.4. Cells were post fixed with a solution by 3-5 min rinses with 0.1% sodium cacodylate buffer, pH 7.4 cells were post fixed with a solution containing 0.1% Osmium tetroxide and 2% k_4 $Fe(CN)_6$ solution both wt /vol , stained with uranyl acetate, and pelleted down in 2% agar. Pellets were dehydrated in a series of graded ethanols and embedded in spur resin. Ultra thin sections (60nm nominal) sections were cut on Reichert Ultra cut microtome on rhodanium 400-mesh grids, post-stained with urynyl acetate followed by lead citrate, and rinsed with water. Examination was carried out in Philips CM-12 electron microscope at 80 KV.

6.6. 11 Nuclear staining assay

a) DAPI Staining: Cells (1 X $10^{6)}$ were treated with IC_{50} Concentration of *S.nux-vomica* root extract for 48hrs and harvested, washed with ice cold PBS and fixed in a solution of methanol: acetic acid (3:1) for 30 min. fixed cells were placed on slides and stained with 1µg / ml DAPI for 15min. Nuclear morphology of the cells were assessed by fluorescent confocal laser microscope (Model: LIECA –TCS SP2 AOSBS). The HPLC purified compounds were also exposed to cells (RPMI 8226 and U266B1) in a similar approach and same procedure followed in further experiments.

b) Hoechst 33258 staining: Like DAPI, Hoechst 33258 is a DNA binding fluorescent probe used for detecting apoptosis or necrosis. Cells (1 X $10^{6)}$ U266B1 cells were treated with IC_{50} Concentration of HPLC purified compounds for 48hrs and harvested, washed with ice cold PBS and fixed in a solution of methanol: acetic acid (3:1) for 30min. fixed cells were placed on slides and stained with 1µg/ ml Hoechst 33258 for 15min. Nuclear morphology of the cells was assessed by the fluorescent confocal laser microscope.

6.6.12 Mitochondrial Membrane Potential Assay by rhodhamine derivatives

Rhodamine123 (a fluorescent dye) was used to estimate the membrane potential. Cells (2 X $10^{6)}$ were treated with or without plant extracts (0. 5.5, 11, 22, 44 mg/ ml) for 24hrs, were harvested, washed with PBS and incubated with fluorescent dye Rhodamine123 for 30min with a concentration of 100µM. Flow cytometric analysis were performed using a Becton- Dick- inson FACS (Fluorescence activated cell sorter).

Similary the HPLC purified compounds were also exposed to MM cells (RPMI 8226) in a similar fashion with the IC_{50} concentration (Strychnine 0, 4mM; brucine 0, 316μm) for 24hrs and same procedure followed in further experimentation.

6.6.13 Propidium iodide staining and analysis of cellular DNA content by FACS

Cells were seeded at 3.6X 10^4 cells in 6 well culture plates, cultured in 10% FBS with or without plant extracts (22, 44 mg/ ml) for, 24, 48, and 72 hrs. After treatment cells were harvested, washed with PBS, and the viability was determined by trypan blue dye exclusion method. For DNA content analysis, ($1x10^6$) cells were fixed in 70% ethanol, washed with PBS, incubated with 0.1mg/l RNase-A and stained with PI (final concentration: 50μg /ml). FACS analyses were performed.Similarly the HPLC purified compounds were also exposed to MM cells (RPMI 8226) in a similar fashion (with strychnine 0, 1.86, 3.73, 5.59, 7.47mM; brucine 0, 158.4, 316.8, 474.5, 633.7μm) at various concentrations for 48hrs and the same procedure followed in further experimentation.

6.6.14 Preparation of whole cell extracts and immunoblot analysis

To prepare the whole cell extract, cells were washed with PBS and suspended in a lysis buffer (20mM Tris, 1mM EDTA, 150mM NaCl, 1% NP 40, 0.5% deoxycholic acid, 1mM beta-glycerophosphaste, 1mM Sodium orthovanadate, 1mM PMSF, 10μg/ml leupeptin, 20 ug/ml aprotinin). After 30min of shaking at 4^oC, the mixtures were centrifuged (10, 000 x g) for 10min and the supernatants were collected as the whole cell extracts. The protein content was determined according to the Lowry method. An equal

amount of total cell lysate was resolved on 15% SDS-PAGE gels along with protein molecular weight standards and then transferred onto nitrocellulose membranes. The membranes were blocked with 5% (w/v) nonfat dry milk and then incubated with the primary antibodies (cyctochrome *c*) in 10ml of antibody- diluted buffer (1X Tris – buffered saline and 0.05% Tween with 5% milk) with gentle shaking at 4^{o}C for 8-12h and then incubated with ALP conjugated secondary antibodies. Signals were detected by using a BCIP-NBT substrate.

6.6.15 CD138 expression analysis by flowcytomer

A flow cytometer analysis was performed using anti-CD138 FITC labeled antibody for the detection of Sydencan-1 expression in U266B1 cell lines. The control and treated cells were harvested after 48hrs and the CD138 expression was analyzed using a gating protocol for measuring mean FITC florescence intensity of labeled cells.

Treatment with *S. nux-vomica*, *S. wallichiana* root extracts and HPLC purified brucine and strychnine

The MM cell line U266B1 was cultured in T25 flasks in RPMI-1640 medium supplemented with 10% FBS and treated with or without plant extracts and as well as with HPLC purified compounds. The treatment of cells with extracts and compounds with the following concentrations, *S. wallichiana* ethanolic root extract (0.5 and 2, mg / ml), *S. nux-vomica* root extract (5.5 mg / ml 22 mg / ml), brucine (63µM and 316 µM) and strychnine (747µM and 3.7 mM) and incubated for 48hrs.

Treated cells and as well as untreated cells were harvested after 48hrs, following by centrifuging at 200x g for 10min and washed with wash buffer (PBS without calcium, magnesium, or phenol red, pH 7.2 containing 0.1% sodium azide filter sterilized). The cells were resuspended in staining buffer (PBS containing 0.1% sodium azide and 25% FBS) cell count was adjusted to 1 X 10^6 cells / 50µl. To this suspension an appropriate volume (20 µl) of anti-CD138 FITC labeled antibody was added and incubated for 45 minutes in on ice. Later the suspension was centrifuged at 200 x g for 10 minutes at 4^0C and supernatant was removed and the pellet was washed twice with 100 µl of ice cold wash buffer. The pellet was resuspended in 0.5ml of PBS and stored at 4^0C and until analyzed. The expression of CD138 was assessed by flow cytometer using a gating protocol set for measuring the fluorescence intensity of labeled cells. Ten thousand events were counted and the data is presented as light scatter plots. Appropriate isotypes controls were prepared and processed similarly.

6.7 Results

The general ethanol extraction method was followed (Figure 34). The final residue was dissolved in 5% DMSO made up to 15ml, sterile filtered and kept refrigerated until use. 1ml filter sterilized extract was taken in an appendorf, subjected to Freeze vacuum drying and weighed. Prior to this the weight of the empty appendorf was noted. Based on the difference between initial and final weights the dry weight of the extract was calculated from the 1ml. The plant extracts were diluted 1:1000 distilled water and absorption spectra were taken in the range of 200 – 400 nm. The yield of plant extracts and absorption spectral data were presented (Figure 35). The yield of *S. nux-vomica* root extract wad found to be 450mg/ml while *S. wallichiana* root extract showed 42 mg/ml (Table 19)

Table 19: Five grams (wet weight) of root material was processed using ethanol extraction. The dry weight of the extract is presented

Plant /Root	Dry weight (mg/g weight) of extract	Absorption maximum (nm)	Absorbance
S. nuxvomica	450	216	3.701
S. wallichiana	42	197	3.654

6.7.1 The effect of ethanolic root extracts of *S. nux-vomica, S. wallichiana* on viability and proliferation of MM cell lines

Recent studies have shown use of medicinal plant extracts in the treatment and prevention of various cancers. Several plant root extracts have been reported to show growth inhibition in various cancer cell lines (Moongakarndi *et al.*, 2004).The present

study was undertaken in order to understand the effect of *S. nux-vomica, S. wallichiana* root extracts and their efficacy on the growth and multiplication of MM cell lines RPMI 8226 and U266 B1. The detailed description of the methodology for the evaluation of *S. nuxvomica* and *S. wallichiana* ethanolic root extracts on MM were demonstrated (Figure 36). Cells cultured in RPMI 1640 medium supplemented with 10% FBS were incubated with different concentrations of *S. nux-vomica* and *S. wallichiana* root extracts and the viability was examined by MTT assay (Mosmann, 1983). Cell lines were incubated with root extracts for different time intervals *ie.,* 24, 48 and 72hrs. RPMI 8226 and U266 B1 cells showed remarkable difference in survival after 48 and 72hrs respectively. However the treatments after 24hr did not show much impact in proliferation, (Data not shown) while 100% Inhibition of proliferation was noticed in 48 and 72hrs treatments in a dose and time dependent manner.

The cells (RPMI 8226 and U266 B1) were exposed to *S. wallichiana* ethanolic root extract with concentrations (0, 0.5, 1, 2, 5, 10 mg / ml) for 24, 48 and 72hrs showed a remarkable difference in cell proliferation. The figure 37 demonstrates the effect of *S. wallichiana* ethanolic root extract on proliferation of the MM cell lines RPMI 8226 and U266 B1 after 48 and 72hrs suggesting that cell proliferation decreased in a dose and time dependent manner. Similar results were also observed when RPMI 8226 cells were incubated with the concentrations (0.5.5, 11, 22, 54,108 mg/ ml) of *S. nux-vomica* ethanolic root extract, for 24, 48 and 72hrs respectively. The figure 38 demonstrates effect of the *S. nux-vomica* ethanolic root extract on proliferation of the MM cell line RPMI 8226

after 48 and 72hrs suggesting that, there is substantial inhibition in the proliferation rate in a dose and time dpendent manner. The percentage of viable cells was calculated in comparison to untreated cells. The number of viable cells in control was taken as 100%. Values were expressed as mean ± SD of three independent experiments. There was no significant effect in 24 hrs (Data not shown) while noteworthy effect noticed after 48hrs in a dose and time dependent with both plant root extracts.

6.7.2 Determination of IC_{50} values of *S. nux-vomica, S. wallichiana* root extracts.

The IC_{50} values of *S. nux-vomica* and *S. wallichiana* root extracts was calculated with 50% decrease in cell proliferation of RPMI 8226 and U266B1 cells. The IC_{50} concentration of *S. nux-vomica* was found to be 10mg / ml (approximately) for RPMI 8226 cells, while the IC_{50} concentration of *S. wallichiana* was found to be 2mg / ml (approximately) in case of RPMI 8226 cells, and 1mg / ml (approximately) for U266B1 cells. Further experiments were carried out with IC_{50} concentration.

6.7.3 Morphological assessment of cells

Light and Phase contrast microscopy**:** Morphological alterations of RPMI 8226 cells were assessed following incubation with *S. nux-vomica* root extract for 48 and 72hrs. Images were captured by Light and Phase contrast inverted microscope**.** Cells grown in complete medium in the absence of *S. nux-vomica* root extract found to be round to polygonal, single or clustered cells in suspension and some are loosely adherent with characteristic features of multiple myeloma cells. (Figure 39 1a Light microscope and 1b Phase contrast microscope).The treated cells exhibited altered cellular morphology with cytoplasmic

shrinkage, membrane blebbing and convolution of cellular surfaces after 48 and 72hrs. Many cells displayed protuberances of the plasma membrane that would eventually separate into membrane-bound apoptotic bodies (Figure 39 2a and b; 3a and b).

Ultra structural and morphological features – analysis by SEM and TEM: Further studies were undertaken for exhaustive analysis of morphological and ultra structural alterations of RPMI 8226 on SEM (Scanning electron microscope) and TEM (Transmission electron microscope). To determine the antiproliferative effects associated with apoptosis, the ultra structural changes of RPMI 8226 cells exposed to *S. nux-vomica* root extract for 48hrs has been carried out. Apoptotic cell death was confirmed by scanning and transmission electron microscopy, which revealed characteristic ultra structural features of apoptosis. SEM studies of untreated cells found to be normal (Figure 40 a and b) while *S. nux-vomica* root extract treated cells revealed the presence of membrane blebbing, which might be due to a deep cytoskeleton rearrangement, causing progressive changes in cell shape, organelle distribution, and cell shrinkage. (Figure 40 c and d). Bidimensional image of sectioned samples of TEM revealed the detailed ultra structural changes in cells. Control cells exhibited distinguishable diffused interchromatin with prominent nucleus (Figure 41 a); however treated cells showed altered cellular morphology with marginalization of chromatids (Figure 41 b), cytoplasmic shrinkage while the plasma membrane remained well defined. The cells showed typical nuclear fragmentation and condensed chromatin with the formation of apoptotic bodies (Figure 41 c) while necrosis form of cell death is very low (Figure 41 d).

Nuclear staining assay and analysis using confocal microscope: A distinctive feature of apoptosis is the condensation and fragmentation of nuclear chromatin, which can be monitored by confocal microscope using specific DNA binding fluorescent dyes like DAPI [4,6,-Di amidino-2-2-phenylindole**]**. Cells were incubated with IC_{50} concentration of *S. nux-vomica* root extract for 48hrs, and then assessed for morphological signs of apoptosis by staining with DAPI. Nuclear condensation and fragmentation, hallmarks of apoptosis, were clearly observed in cells treated with *S. nux-vomica* root extract for 48hrs. Chromatin of apoptotic cells was segregated into compact and sharply delineated masses, very close to the nuclear envelope, where as control cells have shown intact nuclei. Control cells observed under lower magnification were very normal either in phase contrast emission (Figure 42 1a) or in fluorescence emission (Figure 42 1b) while at higher magnification showed prominent nucleus (Figure 42 2a and 2b), where as, treated cells exhibited altered cellular morphology with cytoplasmic shrinkage, membrane blebbing and formation of apoptotic bodies after 48hrs as observed at lower magnification (Figure 42 3a and 3b) and at higher magnification nuclear condensation and fragmentation of chromatin were clearly spotted. (Figure 42 4a and 4b).

6.7.4 Mitochondrial membrane potential assay by using Rhodhamine123 derivatives

Changes in the mitochondrial membrane potentials were measured by the uptake of cation Rhodamine123 in to mitochondria. The decrease in membrane potentials ($\Delta \psi$ m) is associated with mitochondrial dysfunction (Seuduto *et al.*, 1999). RPMI 8226 cells were treated with *S. nux-vomica* ethanolic root extract at various (0. 5.5, 11, 22, 44 mg/ ml)

concentrations for 24hr, harvested, washed with PBS and incubated with 100μM Rhodamine123 for 30min and subjected to FACS analysis. The changes in mitochondrial potentials were estimated by the uptake of lipophilic cation Rhodamine123 into mitochondria (Kroemer *et al.,* 2000). Untreated cells were used to determine the normal uptake of this cation, and the percentage of treated cells with decreased membrane potentials was calculated.The shift in fluorescent intensity of treated cells was plotted against the increasing concentrations of *S. nux-vomica* ethanolic root extract used (0. 5.5, 11, 22, 44 mg/ ml). The fluorescent intensity percentages calculated for treated cells were found to be 75.9, 53.9, 8.4 and 16.2% **(Figure 43)** respectively where fluorescent intensity of the control was taken as 100%. The results indicate mitochondrial membrane potentials decreased with increase in the concentration of *S. nux-vomica* ethanolic root extract.

6.7.5 Preparation of whole cell extracts and immunoblot analysis

One of the most important apoptotic pathways is activated by the release of apoptogenic protein, cytochrome *c* from mitochondria into the cytosol. The release of cytochrome *c*, one of the most important respiratory-chain proteins, from the mitochondria into the cytosol is the hallmark of cells undergoing apoptosis (Liu *et al.,* 1996; Desagher and Martinou *et al.,* 2000). To identify the molecular basis of apoptosis, the release of cytochrome *c* into the cytosol was analyzed in RPMI 8226 cell lines treated with IC_{50} concentration of *S. nux-vomica* ethanolic root extract by the Western blot analysis employing mouse monoclonal cytochrome *c* antibodies. Figure 44 a demonstrates that the levels of cytochrome *c* in the cytosol were elevated after treatment and the levels were

further increased at later time points (6, 12, 24, and 48hrs) in comparison to the control. Figure 44 b represents the elevated levels of cytochrome *c* in the cytosol which was further confirmed by densitometry while ß- actin was used as control for equal loading of the protein. (Figure 44 c)

6.7.6 Cellular DNA content analysis (FACS) by Flow cytometer.

The loss of DNA content in control and *S. nux-vomica* root extract treated cells was further verified and quantified by flow cytometric analysis. Loss of DNA is a typical feature of apoptotic cells. Propidium iodide (PI) staining of DNA, which is taken up in to nucleus of apoptotic and necrotic cells, was used to measure the relative number of dead cells (pullen *et al.,* 1981). Furthermore, since apoptosis, but not necrosis, involves degradation of DNA, the staining outline obtained with PI was used to establish whether cell death was due to apoptosis or necrosis. In the present study, control and RPMI 8226 cells treated with *S. nux-vomica* root extract were taken for FACS analysis. Flow cytometric analysis were performed at various (0, 22, 44mg/ ml) concentrations for 24 and 48hrs. Typical hypo-diploid peaks (sub G0/G1 peak) were observed in cells treated with (22, 44mg/ ml) for 24 h and 48hrs. The FACS analysis of control cells, showed prominent number of hypo diploid (sub-G0/ G1 phase) followed by S and G2/M phases. Only around 7.8% (Figure 45 a) of these cells showed hypo diploid DNA (sub G0/G1 peak). Hypo diploid DNA in treated cells increased to 9.8% (22 mg/ml) and 17.8% (44 mg/ml) in 24hrs (Figure 45 b and c) and to 15.18% (22 mg/ml) and 23.77% (44 mg/ml) (Figure 45 d and e) respectively after 48hrs. These studies thus reveal increase of hypo diploid cells(sub

G0/G1 peak) in response to *S. nux-vomica* root extract treatment in a concentration-dependent manner and the decrease of the cells in other phases of cell cycle. The number of hypo diploid cells(sub G0/G1 peak) which is expressed as a percentage of the total number of cells.

6.7.7 Flow cytometric analysis of CD 138 or Syndecan-1 expression in response to *S. wallichiana and S. nux-vomica* root extracts

The present study was undertaken to check the CD138 expression levels against the plant extracts in U226 cell line using anti-CD138 FITC labeled antibody for the detection. A flow cytometer analysis was performed in the control and treated cells, harvested after 48hrs and the CD138 expression was analyzed using a gating protocol for measuring mean FITC florescence intensity of labeled cells. The treatment of cells with extracts at the following concentrations of *S. wallichiana* root extract (0.5 and 2mg / ml), and *S. nux-vomica* root extract(5.5 and 22mg / ml) revealed marked difference in expression levels, there by decreasing the CD138 or Sydencan-1 expression levels in a dose dependent manner with increasing concentration of extracts. The two controls were kept in which positive control stained with anti-CD138 FITC labeled antibody for the detection of Sydencan-1 expression. 80% of cells positive to CD138 or Sydencan-1 expression while in negative control where cells are unstained showed negative to CD138 or Sydencan-1 expression (Figure 46 a and b). Cells treated with *S.wallichiana* ethanolic root extract (0.5 and 2 mg / ml) was found to show decreased levels of Sydencan-1 expression. At lowest concentration 0.5mg / ml, only 50% cells were positive while at

highest concentration 2mg / ml only 14.6% cells were positive to Sydencan-1 expression (Figure 46 c and d.). Similar results were observed incase of *S. nux-vomica* root extract (5.5 mg / ml 22 mg / ml). At lowest concentration 5.5mg / ml only 61.4% cells were positive while at highest concentration 2mg / ml only 3.2% cells were positive to Sydencan-1 expression (Figure 46 e and f). Over all, results suggest that a loss in the expression of Sydencan-1 was noticed in dose dependent manner with increase in concentration of *S. wallichiana & S. nux-vomica* root extracts.

6.7.8 Identification of active principles of root extracts

Strychnine and brucine are the major alkaloids, isolated and well characterized has been previously reported in other *Strychnos* species i.e., *S. nux-vomica, S .lucida, S. ignatti*. in Asia, *S. icaja* in Africa and *S. panamensis* in Central America.(De,1992). Exhaustive analysis of the root extracts of *S. nux-vomica,* and *S. wallichiana* has been carried out in order to identify the active principles that caused significant effect on the growth and multiplication of MM cell lines RPMI 8226 and U266 B1. The TLC, HPLC, IR, ^{1}H-NMR, ^{13}C-NMR and L.C. Mass analysis confirmed the presence of strychnine and brucine, which was common in *S. nux-vomica* and *S. wallichiana* root extracts. The L.C. Mass spectral analysis of crude ethanolic root extracts of *S. wallichiana* (Figure 47 a) and *S. nux-vomica* (Figure 47 b) showing molecular ion peak of strychnine at 335 MHz and brucine peak at 395 MHz respectively . The HPLC purified strychnine and brucine also showed similar results. The molecular ion peak of strychnine was noticed at 335 MHz (Figure 47 c*)* while brucine peak at 395 MHz (Figure 47 d*)* respectively.

6.7.9 The effect of HPLC purified brucine and strychnine from root extracts of *S. nux-vomica, S. wallichiana* on viability and cell proliferation

The present study was undertaken in order to identify the effective role of HPLC purified strychnine and brucine from root extracts of *S. nux-vomica, S. wallichiana* on viability, growth and multiplication of MM cell lines RPMI 8226 and U266 B1.The flow chart describes the methodology for the evaluation of effects of strychnine and brucine on MM cell lines (Figure 48). RPMI 8226 and U266 B1Cells cultured in RPMI 1640 medium supplemented with 10% FBS and were incubated with different concentrations of HPLC purified brucine and strychnine and the viability was examined by MTT assay. RPMI 8226 and U266 B1 cells cultured with or without brucine and strychnine showed remarkable difference in survival Cells were treated with strychnine (0.373, 747 µm, 1.12, 1.86, 3.73, 5.59, 7.47mM) and brucine (0, 31.6, 63.3, 95, 158.4, 316.8, 474.5, 633.7µm) at various concentrations and incubated for 24, 48 and 72hrs respectively. Cell proliferation was evaluated by MTT assay. The percentage of viable cells was calculated in comparison with control. The number of cells in control was taken as 100%. Inhibition of proliferation was observed in 24, 48 and 72 hrs treatments in a dose and time dependent with strychnine (Figure 49) and brucine (Figure 50) respectively. There was no significant effect within 24 hrs while noteworthy effect was noticed after 48 and 72hrs in a dose dependent manner with both compounds.

6.7.10 Determination of IC_{50} values for strychnine and brucine.

The IC_{50} value of strychnine and brucine was calculated with 50% decrease in cell proliferation. In the present investigation strychnine and brucine induced dose and time dependent inhibition in the growth of RPMI 8226 and U266B1 cells. The IC_{50} concentration of strychnine was found to be 4 mM (approximately) in RPMI 8226 and U266B1 cells, while the IC_{50} concentration of brucine was found to be 316μM (approximately) in RPMI 8226 and U266B1 cells after 48hrs. Further experiments were carried out with IC_{50} concentrations.

6.8. Morphological assessment of cells

Light microscopy: Morphological alterations of RPMI 8226 cells following treatment with strychnine and brucine were assessed after 24 and 48hrs. Images were captured by the Light microscope. Cells grown in complete medium in the absence of compounds found to be round to polygonal, single or clustered cells in suspension and some are loosely adherent with characteristic features of multiple myeloma cells after 24 and 48hrs (Figure 511a and 1b). The cells in response to treatment with HPLC purified compounds with various concentrations of strychnine (3.7mM and 7.4mM) and brucine (316μM and 633μM) after 24 and 48hrs respectively, exhibited altered cellular morphology with cytoplasmic shrinkage, membrane blebbing and convolution of cellular surfaces. Many cells displayed protuberances of the plasma membrane that would eventually separate into membrane-bound apoptotic bodies (Figure 51).

Nuclear staining assay and assessment by confocal microscope.

DAPI staining: A distinctive feature of apoptosis is the condensation and fragmentation of nuclear chromatin, which can be monitored by confocal microscope by staining with specific DNA binding fluorescent dyes like DAPI [4,6,-Di amidino-2-2-phenylindole**]**. Multliple myeloma cell lines RPMI 8226 and U266 B1 were exposed to strychnine and brucine (IC_{50} concentration for 48hr), and then assessed for morphological signs of apoptosis by staining with DAPI. Nuclear condensation and fragmentation, the hallmarks of apoptosis were clearly observed in RPMI 8226 and U266 B1 cells treated with strychnine and brucine for 48hrs. Chromatin of apoptotic cells was segregated into compact and sharply delineated masses, very close to the nuclear envelope, where as control cells have shown intact nuclei. The control and the treated cells observed under lower magnification with normal phase contrast emission (Figure 52 a and 53 a) and as well as fluorescence emission. (Figure 52 b and 53 b). Similarly the control and the treated cells were also monitored under higher magnification. Control cells stained with DAPI exhibiting prominent nucleus as observed under higher magnification (phase contrast emission Figure 52 c and 53 c; fluorescence emission Figure 52 d and 53 d). Treated cells with strychnine (phase contrast emission Figure 52 e and 53 e; fluorescence emission Figure 52 f and 53 f) and brucine (phase contrast emission Figure 52 i and 53 i ; fluorescence emission Figure 52 j and 53 j) exhibited altered cellular morphology with cytoplasmic shrinkage, membrane blebbing and formation of apoptotic bodies during a culture period of 48hrs at lower magnification, while the nuclear condensation and

fragmentation of DNA were clearly noticed under higher magnification after treatment with strychnine (phase contrast emission Figure 52 g and 53 g ; fluorescence emission Figure 52 h and 53 h) and brucine (phase contrast emission Figure 52 k and 53 k ; fluorescence emission Figure 52 l and 53 l).

Hoechst 33258 staining: Hoechst 33258 is a DNA binding fluorescent probe used for detecting apoptosis or necrosis. As shown in above data The DAPI florescence probe is used to confirm apoptosis. But here Hoechst 33258 staining has been tried, in order to reconfirm whether cell death was *via.* apoptosis or necrosis. U266 B1 cells were incubated with strychnine and brucine (IC_{50} concentration for 48hr), and then assessed for morphological signs of apoptosis by staining with Hoechst 33258. Nuclear condensation and fragmentation, hallmarks of apoptosis, were clearly observed in U266 B1cells treated with strychnine and brucine for 48hrs however, the control cells didn't display any of the given characteristics. Chromatin of apoptotic cells was segregated into compact and sharply delineated masses, very close to the nuclear envelope, where as control cells have shown intact nuclei. The overlaying of normal phase contrast image with fluorescence image revealed marked difference between control and treated cells.

The control and the treated cells observed under lower magnification with normal phase contrast emission (Figure 54 a), under fluorescence emission. (Figure 54 b) and finally overlaying the images of phase contrast emission with fluorescence emission (Figure 54 c). Similarly the control and the treated cells also monitored under higher magnification (Figure 54). Control cell stained with Hoechst 33258 florescence probe

exhibited a prominent nucleus, while overlaying images of normal phase contrast image with fluorescence image also reconfirms the marked difference in which distinct nucleus is highlighted which was observed under higher magnification.(Figure 54: 1d, 1e, and 1f). Treated cells with strychnine (Figure 54: 2a, 2b and 2c) and brucine (Figure 54: 3a, 3b and 3c) exhibited altered cellular morphology with cytoplasmic shrinkage, membrane blebbing and formation of apoptotic bodies during a culture period of 48hrs at lower magnification, the nuclear condensation and fragmentation of DNA were clearly noticed under higher magnification upon treatment with strychnine (Figure 54: 2d, 2e and 2f) and brucine (Figure 54: 3d, 3e and 3f) respectively.

6.9 Mitochondrial membrane potential assay by Rhodhamine123 derivatives

Changes in the mitochondrial membrane potentials ($\Delta \psi$ m) were measured by the uptake of cation Rhodamine123 into mitochondria. The decrease in membrane potentials ($\Delta \psi$ m) is associated with mitochondrial dysfunction (Seuduto *et al.*,1999). RPMI 8226 cells were treated with strychnine and brucine with IC_{50} concentrations for 24hr, harvested, washed with PBS and incubated with fluorescent dye Rhodamine123 for 30min at a concentration of 100μM and subjected to FACS analysis. We examined the transformations in the membrane potentials ($\Delta \psi$ m) of RPMI 8226 cells exposed to IC_{50} concentrations of strychnine and brucine. The cells were harvested and changes in mitochondrial potentials ($\Delta \psi$ m) were estimated by the uptake of lipophilic cat ion Rhodamine123 into mitochondria (Kroemer *et al.,* 2000) Untreated cells were used to determine the normal uptake of this cat ion, and the percentage of treated cells with altered

membrane potentials (Δ ψ m) was calculated. Decrease in the fluorescence intensity of treated cells was observed with strychnine and brucine treatment at specific IC_{50} concentrations for 24hrs upon comparision with control. Fluorescent intensity of control was taken as 100%. while there is gradual decrease in shift of fluorescent intensity with strychnine treated cells up to 48.4% (Figure 55 a) and incase of brucine treated cells up to 54.6 % (Figure 55 b) respectively.

6.10 Cellular DNA content analysis (FACS) in response to compounds by Flow cytometer

Loss of DNA content is a typical feature of apoptotic cells. The loss of DNA content in control and treated cells was further verified and quantified by the flow cytometric analysis. Propidium iodide (PI) staining of DNA, which is taken up in to nucleus of apoptotic and necrotic cells, was used to measure the relative number of dead cells (pullen *et al.,* 1981; Nicoletti 1991). Furthermore, since apoptosis, but not necrosis, involves degradation of DNA, the staining outline obtained with PI was used to establish whether cell death was due to apoptosis or necrosis. In the present study, control and RPMI 8226 cells treated with various concentrations of strychnine and brucine for 48hrs, were taken for FACS analysis. Flow cytometric analysis of the control and treated RPMI 8226 cells with strychnine (0, 1.8, 3.7, 5.6, 7.4 mM) and brucine at various (0, 158, 316, 474, 633µM) concentrations after 48hrs induced an increase in the amount of hypo diploid (sub-G0/ G1 phase) DNA, indicating internucleosomal DNA breakdown, as indicated for apoptotic cells. Moreover, histograms of the DNA fluorescence indicated that strychnine

and brucine caused cell cycle arrest at G2/M phase. The FACS analysis of control cells, showed prominent number of hypo diploid (sub-G0/ G1 phase) followed by S and G2/M phases, while the data in response to strychnine at various concentrations (0, 1.8, 3.7, 5.6, 7.4 mM.) after 48hrs induced typical hypo diploid peaks. Only around 9% (Figure 56 a) of control cells showed hypo diploid DNA (sub G0/G1 peak). Hypo diploid DNA in treated cells increased to (Figure 56 b*)* 42.7% (1.8mM), (Figure 56 c) 43.6% (3.7mM), (Figure 56 d) 47.7% (5.6mM), and (Figure 56 e) 56.4% (7.4mM), respectively. While brucine (0, 158, 316, 474, 633 µM) also reveals similar results. Only around 9% (Figure 57 a) of control cells showed hypo diploid DNA (sub G0/G1 peak). Hypo diploid DNA in treated cells increased to (Figure 57 b) 39% (158 µM), (Figure 57 c) 40.6% (316µM), (Figure 57 e) 48.7% (474 µM), and (Figure 57 e) 52.5 % (633 µM), respectively. These studies thus reveal increase of hypo diploid cells in sub-G0/ G1 phase in a concentration-dependent manner and the decrease of the cells in other S and G2/M phases of cell cycle in response to strychnine and brucine treatment. The number of hypo diploid cells which was expressed as a percentage of the total number of cells.

6.11 Flow cytometric analysis of CD 138 or Syndecan-1 expression analysis in response to HPLC purified strychnine and brucine

The present study is undertaken to check the CD138 expression levels against the brucine and strychnine in U226 cell line using anti-CD138 FITC labeled antibody for the detection. A flow cytometer analysis was performed in the control and treated cells, harvested after 48hrs and the CD138 expression was analyzed using a gating protocol for

measuring mean FITC florescence intensity of labeled cells. The treatment of cells with compounds at the following concentrations, brucine (63µM and 316 µM) and strychnine (747µM and 3.7mM) which were incubated for 48hrs revealed marked difference in expression levels, there by decreasing the CD 138 or Sydencan-1 expression levels in a dose dependent manner with increasing concentration of extracts. The two controls were kept in which one is used as positive control stained with anti-CD138 FITC labeled antibody for the detection of Sydencan-1 expression. 80% of cells were positive to CD 138 or Sydencan-1 expression while the other one is used as negative control where cells were unstained showed negative to CD138 or Sydencan-1 expression (Figure 58 a and b). Treated cells with strychnine (747µM and 3.7mM) expressed gradual decrease in Sydencan-1 expression levels, at lowest concentration 747µM only 62.3% cells were positive, while at highest concentration 3.7mM only 46.5% cells were positive to Sydencan-1 expression (Figure 58 c and d). Similar results obtained incase of brucine (63µM and 316 µM) at lowest concentration 63µM only 59.7% cells were positive, while at highest concentration 316 µM only 64.1% cells were positive to Sydencan-1 expression (Figure 58 e and f). Over all results indicate a loss in the expression of Sydencan-1 on MM cells with the increase in concentrations of strychnine and brucine in a dose dependent manner.

6.12 Discussion

Ethno botanical bioprospection takes advantage of traditional medicinal knowledge for identifying potential phytochemical drugs (Figure 1). The commercialization of medicinal plant resources has become rapid due to the identification, purification and characterization of active bio-molecules for various therapeutic purposes which have been vital for the present scenario. Because, the synthetic drugs exhibit severe side effects due to their mode of action. Hence, "bioprospection" plays an important role in the identification of the novel bio-molecules from the natural resources, especially from plants, having less side effects and they are cost effective. Hence, an active research is required for the identification of the plants which produces active compounds with therapeutic value. In modern times focus on plant research has increased all over the world, increased number of evidences put forward that more than 13000 plants have been studied in various indigenous traditional system of medicine. The potential role of various plants in cancer therapy as direct anti cancer agent, chemo preventive agent, and radiosentizer or immunity enhancer has been reported (Dhanhukar *et al.,* 2000). In current years the use of plant extracts is gaining more significance in curing various diseases and recent reports suggest that they are very effective in anticancer remedial programmes, due to the supplementary or synergistic effect of particular compounds of the extract. It is significant to avoid unwanted effects of cytotoxic drugs in cancer chemotherapy without compromising antitumor activity. The current research aimed at evaluating potential role of *Strychnos genus* for

bioprospection and targeted towards identifying lead molecules for the Multiple myeloma (MM) therapy.

The majority of plant extracts exhibit antiproliferation activity on cancer cells which is often much better than the effect of their particular bioactive compounds. In cancer research, use of natural drugs instead of synthetic ones for chemoprevention of cancer is rapidly evolving. Medicinal plants being the source of bioactive principles, there has recently been a surge of interest in natural bioresearch. Several preparations of medicinal plants such as polysaccharides, steroids, terpenoids, flavonoids, alkaloids, and pigments were shown to effect the multiplication of tumor cells. Aqueous extracts of medicinal plants possess bioactivity against tumors or lymphoma or carcinoma. Alkaloids from *Strychnos* species was shown to reduce viability of HepG2 cancer cell line (Deng *et al.,* 2006; 2006a).

Multiple myeloma is an incurable malignancy primarily localized in the bone marrow, characterized by the expansion of monoclonal Ig secreting cells with low proliferative activity affecting the immune system and causing progressive bone lesions. Epidemiologically, MM is the second most frequent hematological malignancy. It has almost a world-wide occurrence. Although the MM cells are sensitive to chemotherapy, in some patients development of drug resistance occurs. New therapeutic modalities are needed for this disease. Due to failure of many synthetic drugs several researchers are actively involved in search of new alternatives. Medicinal plant extracts and active principles look promising for the treatment of various cancers. However till today no one

has reported the importance of *Strychnos* extracts and its active principles for the treatment of MM.

Therefore, the present study has been undertaken to examine the mechanism of cytotoxicity of *S. wallichiana* and *S. nux-vomica* root extracts on myeloma cell lines. Root extracts were prepared by soxhlet extraction, freeze dried, filter sterilized, and dry weight was calculated and dissolved in 5% DMSO. The yield of *S. nux-vomica* root extract was found to be 450mg/ml while *S. wallichiana* root extract showed 42mg/ml (Figure 34, Table-16).Cytotoxicity screening models afford important data facilitating the selected plant extracts with potential antineoplastic properties for future work (Cardellina *et al.,* 1999) *S. wallichiana* and *S. nux-vomica* root extracts inhibited proliferation of RPMI 8226 and U266 B1 cell lines in a dose and time dependent manner. However 24hr treatment did not show much impact while inhibition of proliferation up to 100% was noticed in 48 and 72hr treatments in a dose and time dependent manner which was demonstrated (Figure 37 and 38). The data suggests that the *S. wallichiana* ethanolic root extract significantly inhibited the proliferation of MM cell lines (RPMI 8226 and U266 B1) in a dose dependent manner after 48 and 72hrs compared to 24hrs. Similarly results were noticed with *S. nux-vomica* ethanolic root extract on proliferation of MM cell lines (RPMI 8226) in a dose dependent manner after 48 and 72hrs compared to 24hrs. The IC_{50} values for the root extracts of *S. nux-vomica* and *S. wallichiana* was calculated with 50% decrease in cell proliferation. The IC_{50} concentration of *S. nux-vomica* was found to be 10mg / ml (approximately) in RPMI 8226 cells, while the IC_{50} concentration of *S.*

wallichiana was found to be 2mg / ml (approximately) in RPMI 8226 cells, and 1mg / ml (approximately) in U266B1 cells. Further experimentation was carried out with IC_{50} values.

The reduction in the viability of MM cells in the presence of root extracts of both the plants could be either due to apoptosis or necrosis. In order to test the factors responsible reduced viability of MM cells further studies were undertaken on the characteristic markers of apoptosis. Although the usage of these root extracts by the local tribes is well established in the ethno medicine however the mechanism of chemo preventive action of these extracts is largely unknown. Further experimentation was carried out in an effort to gain insight into effects of *S. nux-vomica* root extract on MM cell lines and to understand the mechanism involved in the antiproliferation activity.

Mode of cell death is a complex process. Apoptosis is a well-controlled, tightly-regulated physiological process, in which the cells play a part in self-destruction .A large body of evidence suggests that apoptosis is a central mechanism in embryogenesis and morphogenesis, immune system regulation, hematopoesis and control of normal tissue turnover (Vaux and Korsmeyer, 1999), but it has also been implicated in a variety of diseases (Arends and Wyllie, 1991).Failure of cells to undergo normal apoptotic cell death, or increased cell loss by apoptosis, may be involved in the pathogenesis of cancer, autoimmune disorders, neurodegenerative disorders, AIDS and myelodisplastic syndromes. While necrosis is defined as accidental cell death with membrane rupture, subsequent release of potentially inflammatory cell constituents into the surrounding

tissue. Regarding apoptosis, two major signaling pathways have been described: the 'extrinsic' pathway that is initiated by ligand-mediated activation of membrane death receptors, and the 'intrinsic' pathway that is controlled by members of the Bcl-2 family and mitochondria-derived proteins.

Here the experiments were carried out in order to identify the mode of cell death in response to *S. nux-vomica* root extracts on MM cell lines. Morphological alterations of RPMI 8226 cells subsequent to exposure of *S. nux-vomica* root extract for 48 and 72hrs were monitored by Light and Phase contrast microscopy**.** The treated cells exhibited altered cellular morphology with cytoplasmic shrinkage, membrane blebbing and convolution of cellular surfaces during a culture period of 48 hrs and 72hrs. Many cells displayed protuberances of the plasma membrane that would eventually separate into membrane-bound apoptotic bodies (Figure 39). Further studies were undertaken for extensive analysis of morphological and ultra structural alterations of RPMI 8226 cell line following exposure to *S. nux-vomica* root extract for 48hrs by using SEM and TEM to determine the antiproliferative effects associated with apoptosis or necrosis. Apoptotic cell death was confirmed by scanning and transmission electron microscopy, which revealed characteristic ultra structural features of apoptosis. SEM studies of untreated cells found to be normal while treated cells with *S. nux-vomica* root extract revealed the presence of membrane blebbing, which might be due to a deep cytoskeleton rearrangement, causing progressive changes in cell shape, organelle distribution, and cell shrinkage (Figure 40). TEM analysis revealed the ultra structural changes in cells. Control cells exhibited

distinguishable diffused inter chromatin with prominent nucleus; however treated cells showed altered cellular morphology with marginalization of chromatids, cytoplasmic shrinkage, well defined plasma membrane, typical nuclear fragmentation and condensed chromatin with the formation of apoptotic bodies while necrosis form of cell death is very low (Figure 41). Similar results were reported in HL-60 with active principles of *Strychnos icja* (Lansiaux *et al.,* 2002). The present study also evaluated and compared several of the available methods for detecting apoptosis. (DejanBaskic *et al*., 2006) These methods exploit the most important morphological and biochemical changes occurring in apoptotic cells. Unfortunately, no single assay can be used alone with perfect specificity and sensitivity. The morphological criteria for apoptosis, condensation of chromatin, cell shrinkage, as well as blebbing, although the oldest, remains probably the most specific of all assays. Therefore, most researchers agree that electron microscopy (TEM as well as SEM) is the only definitive way to identify apoptotic cells. Since these methods require expensive equipment and are not practicable in all laboratories one need alternative and simple methods for detection of apoptosis. The late stage of apoptotic cells undergoes secondary necrosis with degenerative changes, biasing the results towards exaggeration of necrotic cell death. Moreover, light microscopy identifies features that occur after major biochemical events, so the time at which apoptosis is analyzed must be optimized. Trypan blue staining though widely used for viability testing is valueless for detecting apoptosis.

Despite of many characteristics of apoptotic cells analyzed by current methods, chromatin condensation and nuclear fragmentation remains the hall mark of apoptosis

(Walker *et al.,* 1988; Cohen, 1993; Cohen 1997). It has been suggested that as a rule, classification of cell death in a given model should always include morphological examination coupled with at least one other assay (Renvoize *et al.,* 1998). Staining of apoptotic cells with fluorescent dyes such as DAPI and Hoechst 33258 stain is considered one of the accurate methods for evaluating the change in nuclear morphology. Fluorescence light microscopy with differential uptake of fluorescent DNA binding dyes like DAPI [4, 6,-Di amidino-2-2-phenylindole] staining is a method of choice for its simplicity, rapidity, and accuracy. In such an assay, apoptotic index and cell membrane integrity can be assessed simultaneously. 4'-6-Diamidino-2-phenylindole (DAPI) is known to form fluorescent complexes with natural double-stranded DNA, showing fluorescence specificity for AT, AU and IC clusters. Because of this property DAPI is a useful tool in various cytochemical investigations. When DAPI binds to DNA, its fluorescence is strongly enhanced, what has been interpreted in terms of a highly energetic and intercalative type of interaction, but there is also evidence that DAPI binds to the minor groove, stabilized by hydrogen bonds between DAPI and acceptor groups of AT, AU and IC base pairs. DNA strand breaks induced by *S. nux-vomica* root extract were characterized by nuclear staining assay, using confocal laser microscope once stained with specific DNA binding fluorescent dyes like DAPI (Coligan *et al.,* 1995). RPMI 8226 cells were exposed to IC_{50} concentration of *S. nux-vomica* root extract for 48hrs revealed morphological signs of apoptosis by staining with DAPI. Control cells containing intact genomic DNA with prominent nucleus while treated cells exhibited chromatin

condensation and nuclear fragmentation. (Figure 42) Similar results reported by staining with DAPI in C-Phycocyanin induced apoptosis in human chronic myeloid leukemia cell line –K562 (Subashini *et al.,* 2004) As we have shown, DNA fragmentation in treated RPMI 8226 cells by confocal laser microscopy correlate in all circumstances with apoptosis measured by all the independent methods of microscopy used in this study, namely, light, Phase contrast, SEM, and TEM. Although the morphological characteristics of apoptosis have been described in detail, little is known about the cellular machinery underlying the process. In addition, although chromatin condensation and internucleosomal DNA breaks are the two salient features of apoptotic cell death whether they are triggered by the same pathway or represent two independent events remains controversial.

The flow cytometer has lately turned out to be the instrument of option for analysis of cell kinetics and offers a mean for the quick and accurate analysis of a large population of individual cells. The flow cytometric analysis of *S. nux-vomica* root extract treated RPMI 8226 cells showed an increase in a dose-dependent manner in the hypo diploid apoptotic DNA content with a decrease in the number of cells at the S and G2 M phases of the cell cycle after 24 and 48 hrs respectively. These results suggest that the loss in DNA content was noticed with increase in concentration of the extract, which indicated induction of apoptosis at S and G2 M phase of the cell cycle (Figure 45). Similar results were obtained in *Rhodiola rose* rhizome extracts induced apoptosis in HL-60 cells (Majewska.*et al.,* 2006) Hanif *et al.,* (1996) reported comparable results were found by in the study of colon cancer cells treated with NSAIDs.

In mitochondria cytochrome *c* is required as an electron carrier in oxidative phosphorylation, a process which generates the greater part of intra cellular ATP. Cytochrome *c* resides in the space between outer and inner membranes of mitochondria where it smuggles up to the cytochrome *c* oxidase complex located in the inner membrane. Several apoptosis inducing agents are known to trigger mitochondrial uncoupling leading to the rupturing of the outer membrane. This in turn causes the release of pro apoptotic factors such as apoptosis inducing factor (AIF), cytochrome *c* in to the cytosol (Chen *et al.,* 2000). In cytoplasm, cytochrome *c* is known to get associated with caspase 9, Apaf-1 and dATP to form the apoptosome complex (Li *et al.,* 1997; Liux *et al.,* 1996) which in turn activates caspase 9, 7 and 3. Caspase activation leads to the cleavage of cellular substrates and apoptosis. The molecular mechanism responsible for the translocation of cytochrome *c* from mitochondria to cytosol is at present unknown. A simple hypothesis for how cytochrome *c* exits from mitochondria during apoptosis is that the permeability transition pore in, the mitochondrial membrane opens, causing mitochondrial swelling and rupture of mitochondrial outer membrane (Szabo and Joratti, 1991; 1992; Von Ashen *et al.,* 2000). The anti-apoptotic protein Bcl-2 acts on mitochondria to stabilize membrane integrity and prevent the opening of mega channel (Yang *et al.,* 1997; Susan *et al.,* 1998; Tsuji moto and Shimi zu, 2000). In the present study whether cytochrome *c* is released or not into the cytosol in response to *S. nux- vomica* root extracts was analyzed by using the western blot nalysis. These studies have shown the release of cytochrome *c* after treatment of RPMI 8226 cells with *S. nux- vomica* root extract. Cytochrome *c* release was observed as early as

6 hours after treatment with *S. nux vomica* root extract, with later increase up to 48hrs (Figure 44). In this present work that the release of cytochrome *c* from mitochondria to cytosol is an early event in the apoptotic process preceding morphological signs of apoptosis. In one recent study that RPMI 8226 cells in response to bruceantinine treatment showed that cytochrome *c* release from mitochondria is a consequence of the proteolytic processing of BID (a pro apoptotic member of the Bcl-2 family), secondary to the activation of caspase-8. Proteolytic generation of the cleaved product of BID results in translocation of BID to the mitochondria and insertion into the mitochondrial membrane where it inhibits the anti apoptotic action of Bcl-2 and results in the release of cytochrome *c* (Cuendet *et al.,* 2004).Similar reports were shown in many cell lines in response to various apoptotic inducing agents(Alimonti *et al.,* 2001) Several similar results were obtained with different naturally derived plant products in other cancer cell lines by Cytochrom *c* mediated pathway(Lansiaux *et al.,* 2002). Pterocarpans from *Platymiscium floribundum* in HL-60 human leukemia cells (Gardenia *et al.,* 2006). Release of cytochrome *c* from the mitochondria into the cytoplasm induced apoptosis in imexon-treated RPMI 8226 cells (Dvorakova *et al.,* 2001).

The leakage of cytochrome *c* is also a common feature of apoptosis triggered by different stimuli. This leakage is closely associated with mitochondrial depolarization and decrease in ATP synthesis (Pedersen, 1999).There is an increasing evidence that altered mitochondrial function is linked to apoptosis and decrease in mitochondrial transmembrane potential ($\Delta \psi$ m) is associated with mitochondrial dysfunction. Thus, the

effect of *S. nux-vomica* root extract on the mitochondrial transmembrane potentials (Δ ψ m) has been evaluated. The fluorescent probe Rhoda mine 123 was used and monitored using flow cytometry. The transformations in the membrane potentials (Δ ψ m) of RPMI 8226 cells exposed to different concentrations of *S. nux-vomica* ethanolic root extract was observed. Decrease in mitochondrial transmembrane potentials (Δ ψ m) in a dose dependent manner in treated cells was noticed with increasing concentration of *S. nux-vomica* ethanolic root extract (Figure 43). These results suggest that the treatment with *S. nux-vomica* root extract induces change in mitochondrial transmembrane potentials (Δ ψ m) which leads to leakage of mitochondrial proteins into cytosol indicates apoptosis induction. This study provides solid evidence that the decreased mitochondrial membrane potential (Δ ψ m) with simultaneous appearance of cytochrome *c* in the cytosolic fractions of cells exposed to *S. nux-vomica* root extract induces apoptosis. Cuendet *et al.,*(2004) reported similar results in RPMI 8226 cells in response to bruceantinine treatment and they found that mitochondrial dysfunction, in particular the induction of the mitochondrial membrane permeability transition, has been implicated in the cascade of events involved in the induction of apoptosis, Inhibition of the mitochondrial electron transport chain reduces the mitochondrial trans membrane potential (Δ ψ m), which may induce the formation of the mitochondrial permeability transition pore and thus activating the mitochondrial pathway of apoptosis. Similar identical findings were reported that, mitochondrial depolarization accompanies cytochrome *c* release in pheochromocytoma-6 cells undergoing apoptosis (Heiskanen *et al*., 1999). Several scientific evidences suggest the

pivotal role of mitochondria in the execution of apoptosis of the cells exposed to various stimuli (Desagher and Martinou, 2000; Green and Reed, 1998). Similar reports were found in the brucine induced apoptosis in HepG2 cells with typical characteristic features of programmed cell death. i.e., cell shrinkage, the formation of apoptotic bodies, DNA fragmentation, cell cycle arrest, mitochondrial depolarization as well as leakage of cytochrome *c* in to the cytoplasm. These results shown by Dengu *et al.,* (2006 a) will support the present work because the brucine was the one of the active ingredient in *S. nux-vomica.* Further morphological studies also supports that treatment of *S. nux-vomica* root extract induces apoptosis in RPMI 8226 cell lines, however, apoptosis is the primary mode of cell death in the cells studied, as indicated by nuclear shrinkage, chromatin condensation, and formation of apoptotic bodies assessed by the nuclear stain DAPI (Figure 42)

Heparan sulfate proteoglycans (HSPGs) expressed on the cell surface of MM cells or in the extracellular matrix of the bone marrow may modulate their function. Recently, genetic studies have provided convincing proof for an in vivo role of cell surface HSPGs in regulating growth and morphogenesis in *Drosophila*, mice, and humans. (Selleck, 2000).In recent times, biochemical, cell biological, and genetic studies have converged to disclose that integral membrane HSPGs are critical regulators of growth and differentiation of epithelial and connective tissues. Syndecan-1 is a transmembrane proteoglycan expressed on the surface of tumor cells of various origins and functional studies in myeloma reveal

that syndecan-1 may act as a multifunctional regulator of cell behavior in the tumor microenvironment.

In the present study the expression and function of Syndecan-1 on MM cell lines U266B1 was investigated. CD138 or Syndecan-1 expression in U266 B1 cell line was analyzed with flow cytometer by measuring the mean FITC florescence intensity of labeled cells using FITC conjugated anti-human CD138 antibody. The present investigation demonstrate that the expression of the syndecan-1 on MM cells in response to treatment with *S. wallichiana & S. nux-vomica* root extracts after 48hrs reduced the expression in a dose dependent manner and there by inhibiting the growth of multiple myeloma cells (Figure 46). This interaction promotes signaling, and regulates the activity of signaling pathways that control cell proliferation and survival. Cell surface–expressed syndecan-1 reduced gradually in a dose dependent manner with an increase in the effective concentration of *S. wallichiana* and *S. nux-vomica* root extracts suggest that these findings correlated with the existing hypothesis of Derksen *et al.,* (2002), it suggested that syndecan-1 regulates growth factor signaling in MM cells. For this purpose, they studied the interaction between syndecan-1 and he-patocyte growth factor (HGF), a putative paracrine and autocrine regulator of MM growth and demonstrated that syndecan-1 is capable of binding HGF and that this growth factor is indeed a potent stimulator of MM survival and proliferation. Dhodapkar and Sanderson (1999) showed that syndecan-1 (CD138), a heparin sulfate proteoglycan, expressed on and actively shed from the surface of most myeloma cells, induces apoptosis and inhibits the growth of myeloma tumor cells.

The soluble form of the syndecan-1 heparan sulfate proteoglycan acts as a tumor suppressor molecule that inhibits growth and induces apoptosis of some cancer cell lines *in vitro* (Pumphrey *et al.,* 2002). Jordan *et al*., (1998) group reported that syndecan-1 was rapidly lost by myeloma cells undergoing apoptosis. Dexamethasone induced a strong apoptosis of myeloma cells associated with the loss of syndecan-1 and they showed that syndecan-1 is a marker for viable myeloma cells which is rapidly lost by apoptotic cells. The sydecan-1 has a rapid turnover on the cell membrane and the ectodomain of the antigen is shed constitutively by cultured cells. (Wijidenes *et al.,*. 1997) The shedding of syndecan-1 show to be increased by tyrosine phosporylyation of its cytoplasmic domain (Reiland *et al.,* 1997). Such a rapid turnover of syndecan-1 on MM cells might explain its rapid loss by apoptotic cells due to decrease in protein synthesis and or in the increase in the protease activity. The loss of certain membrane antigens by apoptotic cells due to proteolytic cleavage has already been reported for granulocytic cells (Homburg *et al.,* 1995).Finally we may suggest that loss in cell surface expression of syndecan-1 was in a dose dependent by increasing the effective concentration of *S. wallichiana* and *S. nux-vomica* root extracts probably may lead to inhibition in proliferation and survival of MM cells.

Therefore it is hypothesized that *S. nux-vomica* root extract has an anti proliferative effect on myeloma cells by inhibiting DNA synthesis, causing cell cycle arrest followed by apoptosis. The *S. nux-vomica* root extract after gaining entry into the cell, induced the mitochondrial disruption leading to decreased membrane potentials and leakage of

mitochondrial cytochrome *c* into the cytosol which triggers apoptosis. More over the loss of syndecan-1 in MM cells upon treatment with root extracts may suggest a similar cell death programme operating in these cells upon treatment. The findings of the present work correlated well with the earlier studies explaning concomitant decrease in expression of syndecan-1 cell surface antigen in the MM cells undergoing apoptosis.

S. wallichiana root extract too has an anti proliferative effect on MM cells (U266B1) which was demonstated by the expression of syndecan-1 in MM cells. This suggests a similar cell death programme operating in these cells upon treatment.

In terms of cancer treatment, multi-drug resistance and side effects are two serious problems in chemotherapy (Setzer *et al.,* 2003). In recent years, considerable attention has been focused on identifying naturally occurring chemo preventive substances capable of inhibiting, retarding, or reversing the process of multistage carcinogenesis. The mechanisms accountable for executing the anti-proliferative effects comprise:

i) stimulation of alterations in the cell differentiation pattern, which plays a crucial role in the invasiveness and metastasis progression of the tumors, and ii) line of defense of pre neoplastic cell expansion or induction of apoptosis.

The present investigation was carried out in order to understand the role of active principles in *S. nux-vomica, S. wallichiana* root extracts that showed significant effect on the growth and multiplication of multiple myeloma cell lines RPMI 8226 and U266 B1. According to the previous reports alkaloids are the major bio active ingredients in which strychnine and brucine make up to 80% in *S .nux-vomica* along with other their derivatives

(Deng *et al.*, 2006). In one study the alkaloid fractions of *S. nux-vomica* was found to exhibit potential cytotoxicity towards cell proliferation of HeLa and K562 cell lines (Cai *et al.,* 1998).The assessment of ethanolic root extracts of *S. nux-vomica* and *S. wallichiana* showed positive results for the presence of alkaloids by dragendroffs reagent. Further spectral analysis was carried out for identifying the active principles. The TLC, HPLC and L.C Mass (Figure 47) analysis confirmed the presence of strychnine and brucine in *S. nux-vomica, S. wallichiana* root extracts. *Strychnos* species consists of several alkaloids among which strychnine and brucine were separated, characterized, and reported (Bisset, 1971, 1972). These compounds are gaining a lot of importance these days because of their various biological and pharmacological properties. However, most of its pharmacological importance is unknown except few. Strychnine and brucine are the major alkaloids, isolated and well characterized and have been previously reported in other *Strychnos* species i.e., *S. nux-vomica, S .lucida*, *S. ignatti* in Asia, *S. icaja* in Africa and *S. panamensis* in Central America (De, 1992).

Epidemiological studies showed that use of plant derived natural products was associated with reduced risk of developing several malignant diseases including colorectoral cancer (Giardiello *et al.,* 1993; Givannucci, 1994, 1995; Hanif *et al.,* 1996; Barnese and Lee 1998; Kawamori *et al.,* 1998; Reddy *et al.,* 2003). Several plant derived alkaloids, drugs derived from plants have largely benefited chemotherapy of cancer. The South African *S. icaja* is also used in the treatment of Cancer and the active principles from the root i.e. Sungicine, isosungicine induced apoptosis in Human cancer cells (HL-

60, HeLa cells, HCT-116 Colon cancer cells) (Lansuix *et al.*, 2002; Frederich *et al*., 2003). Resveratrol, a natural polyphenolic compound found in grapes, has been reported to have preventive effects on diverse molecular events associated with multi-stage carcinogenesis (Clement *et al.,* 1998). Studies have shown that in several model systems resveratrol has potential breast cancer chemo preventive activity. For example, resveratrol inhibited the development of preneoplastic lesions in carcinogen-treated mouse mammary glands in culture and suppressed the growth of human breast cancer cells in culture (Dubuisson *et al.,* 2002). Curcumin is isolated from *Curcuma* species gaining world wide attention for its potential role in treatment of various cancers. Phytoceuticals that have already been employed in cancer treatment include vincristine, vinorelbine, vindesine, vinblastine, etoposide, teniposide, paclitaxel and docitaxel. The improved safety profile of alkaloids makes it realistic to consider their long term use in individuals at low to moderate risk of cancer. Earlier studies from the Chinese laboratories (Dengu *et al.,* 2006) revealed that strychnine and brucine were inhibitors for Cox2. They also reported the anti-tumor effects of the four alkaloids: brucine, strychnine, brucine N-oxide and isostrychnine from the seed of *S. nux-vomica*, on the human hepatoma cell line (HepG2). When compared to their toxicities associated with the currently available synthetic anti-inflammatory drugs strychnine and brucine would likely provide safer therapeutic alternative since it is efficacious as currently used, if not more. But most importantly these alkaloids are from natural source and share common indole ring with other alkaloids, vincristin and vinblastin.

Hence these two compounds (strychnine and brucine) common in these plants (*S. wallichiana* and *S. nux-vomica*) and further experiments were carried out with these compounds. The detailed description of the methodology for the evaluation of strychnine and brucine on MM was demonstrated (Figure 48). The present study has been carried out in an effort to gain insight into effects of strychnine and brucine on MM cancer cell lines and to understand the mechanism involved in the mode of cell death. In the present investigation effects of strychnine and brucine were evaluated on viability, growth and multiplication of MM cell lines RPMI 8226 and U266 B1 after 24, 48, and 72 hours. However, 24 hr treatment did not show much impact while maximum percentage of Inhibition of proliferation was observed in 48 and 72 hr treatments in a dose and time dependent manner. Figure 49 and 50 demonstrates effect of the HPLC purified strychnine and brucine on proliferation of myeloma cell lines RPMI 8226 & U266 B1. The reduction in the viability of MM cells in the presence of both compounds may be due to apoptosis or necrosis. In order to identify the mechanism behind the reduced growth of myeloma cells and the mode of cell death further studies were undertaken.

Light microscope images of RPMI 8226 cells following exposure to strychnine and brucine at 24 and 48hrs, revealed morphological alterations of treated cells exhibiting altered cellular morphology with cytoplasmic shrinkage, membrane blebbing and apoptotic bodies while untreated cells remain unaffected (Figure 51). As mentioned earlier staining of apoptotic cells with fluorescent dyes such as DAPI and Hoechst 33258 is considered one of the approved methods for the evaluation of the change in nuclear morphology. DNA

strand breaks induced by strychnine and brucine were characterized by nuclear staining assay, using confocal laser microscope once stained with specific DNA binding fluorescent dyes like DAPI (Coligan JE, 1995). RPMI 8226 cells and U266B1 were exposed to IC_{50} concentration of strychnine and brucine for 48hr revealed morphological signs of apoptosis by staining with DAPI. Control cells containing intact genomic DNA with prominent nucleus while treated cells exhibited chromatin condensation and nuclear fragmentation (Figure 52 and 53). Similar results were obtained by staining with DAPI in C-Phycocyanin induced apoptosis in human chronic myeloid leukemia cell line –K562 (Subashini *et al.*, 2004).

Like DAPI, Hoechst 33258 is also a fluorescent probe used for staining DNA breaks in apoptotic cells for evaluating the change in nuclear morphology. Hoechst 33258 staining was applied to investigate and reconfirm whether myeloma cells underwent cell death via apoptosis or necrosis. U266B1 cells were exposed to IC_{50} concentration of strychnine and brucine for 48hrs stained with Hoechst 33258 revealed morphological signs of apoptosis (Figure 54). Control cells contained intact genomic DNA with prominent nucleus while treated cells exhibited marked evidence with nuclear condensation, membrane blebbing, nuclear fragmentation and apoptotic bodies, all of which are the characteristics of apoptotic programmed cell death.

In the present study as mentioned earlier in the above, several of the available methods were compared for detecting apoptosis. These methods exploit the most important morphological and biochemical changes occurring in apoptotic cells. Apoptotic cells

exhibit some morphological modifications that are readily detected by flow cytometry according to their light scatter properties (FSC/SCC) (Lecoeur *et al.,* 1997; Petit *et al.,* 1995). Cell shrinkage and increased granularity can be analyzed following the FSC and SCC criteria, respectively. Thus, the morphological alterations induced by the alkaloids tested are compatible with the presence of an increasing number of apoptotic cells. In the present study, the treatment of myeloma cells with strychnine and brucine induced an increase in the amount of sub-diploid DNA, indicating inter nucleosomal DNA breakdown, as expected for apoptotic cells. Sungicine isolated from *Strychnos icaja* also exhibited similar results (Lansiaux *et al.,* 2002).Some recent reports suggest that brucine induces apoptosis on Hep G2 cell lines in a similar manner (Deng *et al.,* 2006). Moreover, histograms of the DNA fluorescence indicated that strychnine and brucine caused cell cycle arrest at G0/G1 phase, thus preventing cells from entering S or G2/M phase (Figure 56 and 57) and finally inducing apoptosis. Therefore, whether the hindrance of myeloma cells cycle progression caused by strychnine and brucine is related to apoptosis need further clarification. Mitochondria have been shown to play a major role in programmed cell death. The leakage of cytochrome c from mitochondria is also a common feature of apoptosis triggered by different stimuli. This leakage is closely associated with mitochondrial depolarization and decrease in ATP synthesis (Pedersen, 1999). Mitochondrial dysfunction, in particular the induction of the mitochondrial membrane permeability transition ($\Delta \psi$ m), has been implicated in the cascade of events involved in the induction of apoptosis. Inhibition of the mitochondrial electron-transport chain reduces

the mitochondrial transmembrane potential (Δ ψ m), which may induce the formation of the mitochondrial permeability transition pore and the subsequent membrane permeability transition (Kroemer, 1995). Mitochondrial depolarization was assessed by flow cytometry using the Rhodamine123 dye, showing that this membrane potential (Δ ψ m) was altered by the treatment with strychnine and brucine, which also indicated apoptosis induction (Figure 55). These findings from flowcytometry measurements were corroborated by the morphological assessment data of myeloma cells staining with DAPI and Hoechst 33258 stain in response to treatment with strychnine and brucine.

Syndecan-1 is a transmembrane proteoglycan expressed on the surface of tumor cells of various origins and functional studies in myeloma reveal that syndecan-1 may act as a multifunctional regulator of cell behavior in the tumor microenvironment; it mediates cell-cell adhesion which was mentioned earlier. In the present study the expression and function of Syndecan-1 on MM cell lines U266B1 was investigated. CD138 or Syndecan-1 expression in U266 B1 cell line was analyzed with flow cytometer by measuring mean FITC florescence intensity of labeled cells using FITC conjugated anti-human CD138 antibody. The present investigation demonstrated that the expression of the syndecan-1 on MM cells in response to treatment with Strychnine and brucine after 48hrs reduced the expression in a dose dependent manner and there by inhibiting the growth of MM cells (Figure 58). This interaction promotes signaling, and regulates the activity of signaling pathways that control cell proliferation and survival. Cell surface expressed syndecan-1 reduced gradually in a dose dependent manner by increase in the effective concentration of

strychnine and brucine was supported with the existing hypothesis of Derksen *et al.,* (2002), it suggested that syndecan-1 regulates growth factor signaling in MM. Dhodapkar and Sanderson (1999) showed that syndecan-1 (CD138), a heparin sulfate proteoglycan, expressed on and actively shed from the surface of most myeloma cells, induces apoptosis and inhibits the growth of myeloma tumor cells. The soluble form of the syndecan-1 heparan sulfate proteoglycan acts as a tumor suppressor molecule that inhibits growth and induces apoptosis of some cancer cell lines *in vitro* (Pumphrey, 2002). Jourdan *et al.,* (1998) group reported that syndecan-1 was rapidly lost by myeloma cells undergoing apoptosis. Dexamethasone induced a strong apoptosis of myeloma cells associated with the loss of syndecan-1 and they showed that syndecan-1 is a marker for viable myeloma cells which is rapidly lost by apoptotic cells. The sydecan-1 has a rapid turnover on the cell membrane and the ectodomain of the antigen is shed constitutively by cultured cells. (Wijidenes *et al*., 1997) The shedding of syndecan-1 has been shown to be increase by tyrosine phosporylyation of its cytoplasmic domain (Reiland *et al.,* 1997). Such a rapid turnover of syndecan-1 on MM cells might explain its rapid loss by apoptotic cells due to decrease in protein synthesis and or in the increase in the protease activity. The loss of certain membrane antigens by apoptotic cells due to proteolytic cleavage has already been reported for granulocytic cells (Homburg *et al.,* 1995). Finally we may suggest that loss of cell surface expression of syndecan-1 gradually in a dose dependent by increasing the effective concentration of strychnine and brucine probably may lead to inhibition in proliferation and survival of MM cells.

We hypothesize strychnine and brucine as an emerging potential class of anticancer chemicals, exhibiting an anti proliferative effect on myeloma cells by inhibiting DNA synthesis, causing cell cycle arrest followed by apoptosis. These potential compounds after gaining entry into the cell, induced the mitochondrial disruption leading to decreased membrane potentials and leakage of mitochondrial proteins into the cytosol which triggers apoptosis. Morphological studies support that treatment with strychnine and brucine induces apoptosis in RPMI8266 cells. Nuclear staining assay by using fluorescence probes like DAPI and Hoechst 33258 revealed DNA breaks in apoptotic cells were clearly noticed with changing pattern of nuclear morphology. This result correlates with loss of syndecan-1 in U266B1 cells in a dose dependent manner treated with strychnine and brucine suggesting that cells are undergoing apoptosis. Evidences suggest that syndecan-1 is a marker for viable myeloma cells which is rapidly lost by apoptotic cells.

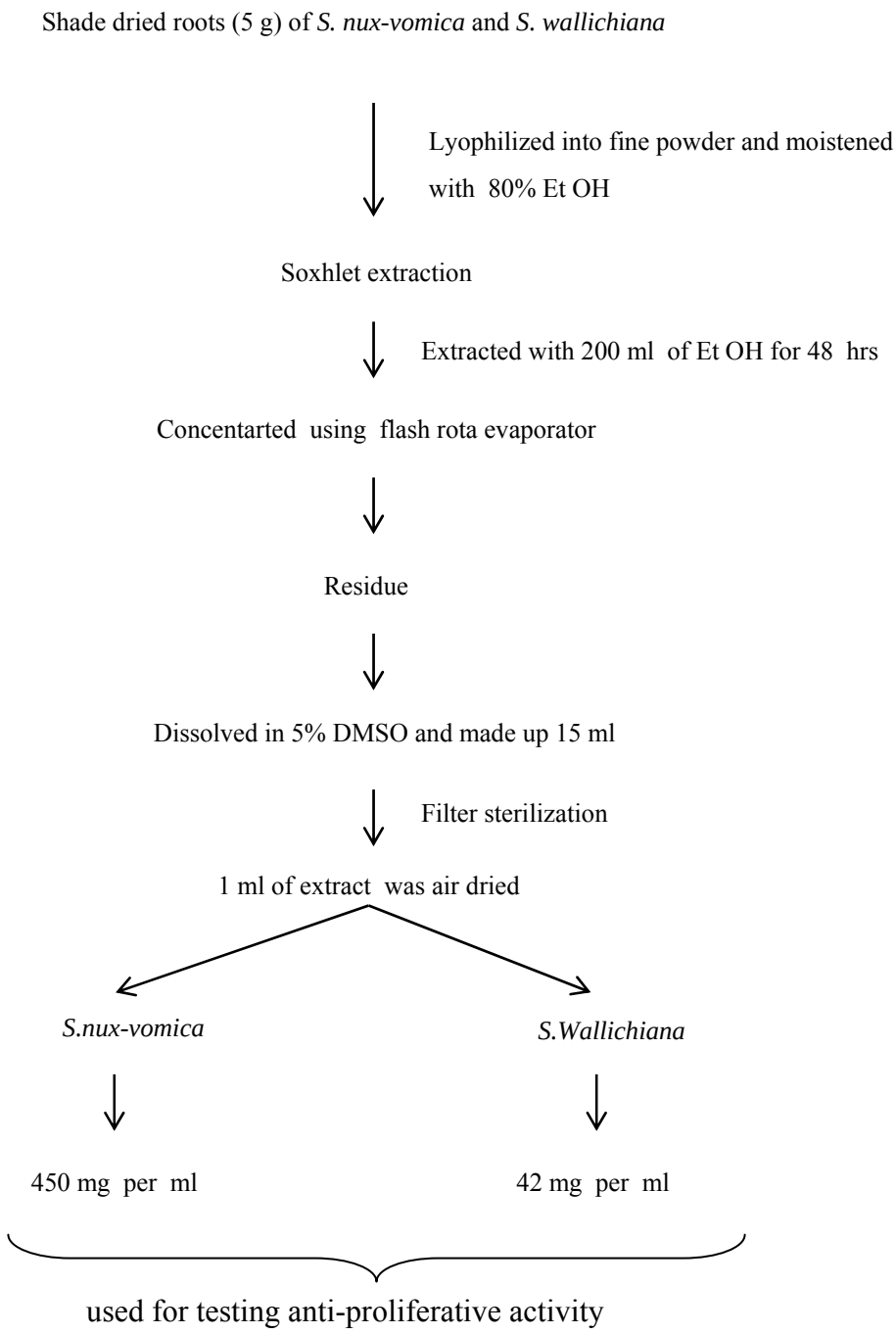


Figure 34: Procedure for preparation of ethanolic extracts from shade dried roots of *S. nux-vomica* and *S. wallichiana*

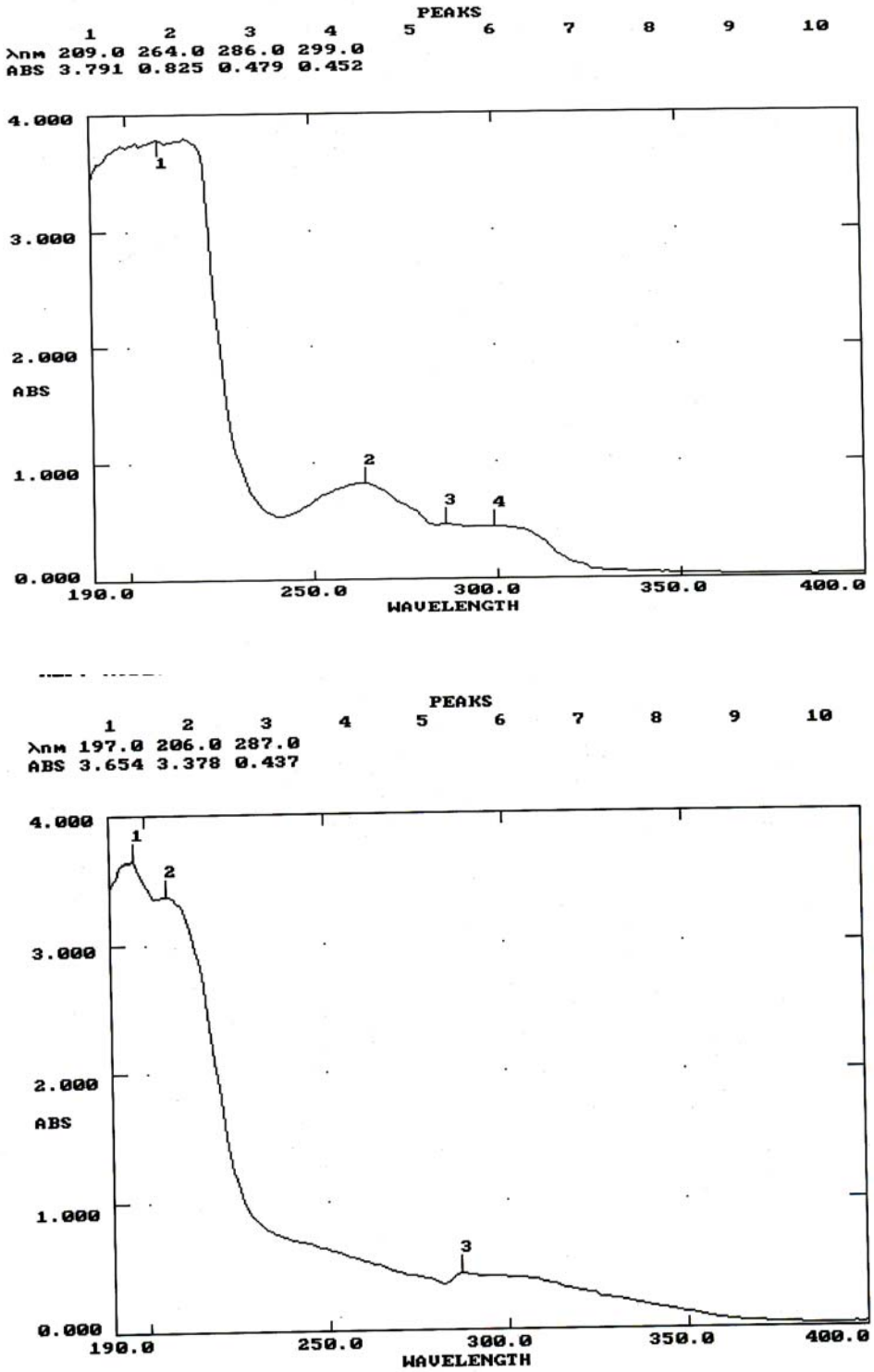


Figure 35. Absorption spectra of *S. nux-vomica* and S. *wallichiana* ethnolic root extract

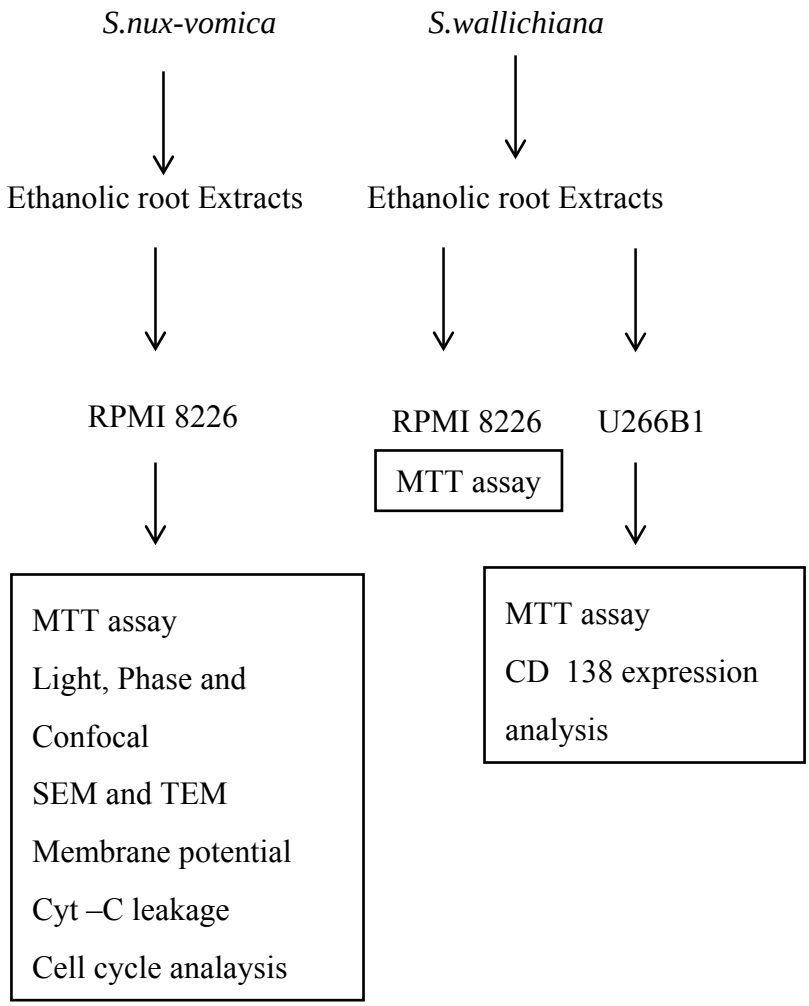


Figure 36: Flow chart representing the methodology for evaluation of *S.nuxvomica* and *S. wallichiana* ethanolic root extracts and its compounds on multiple myeloma.

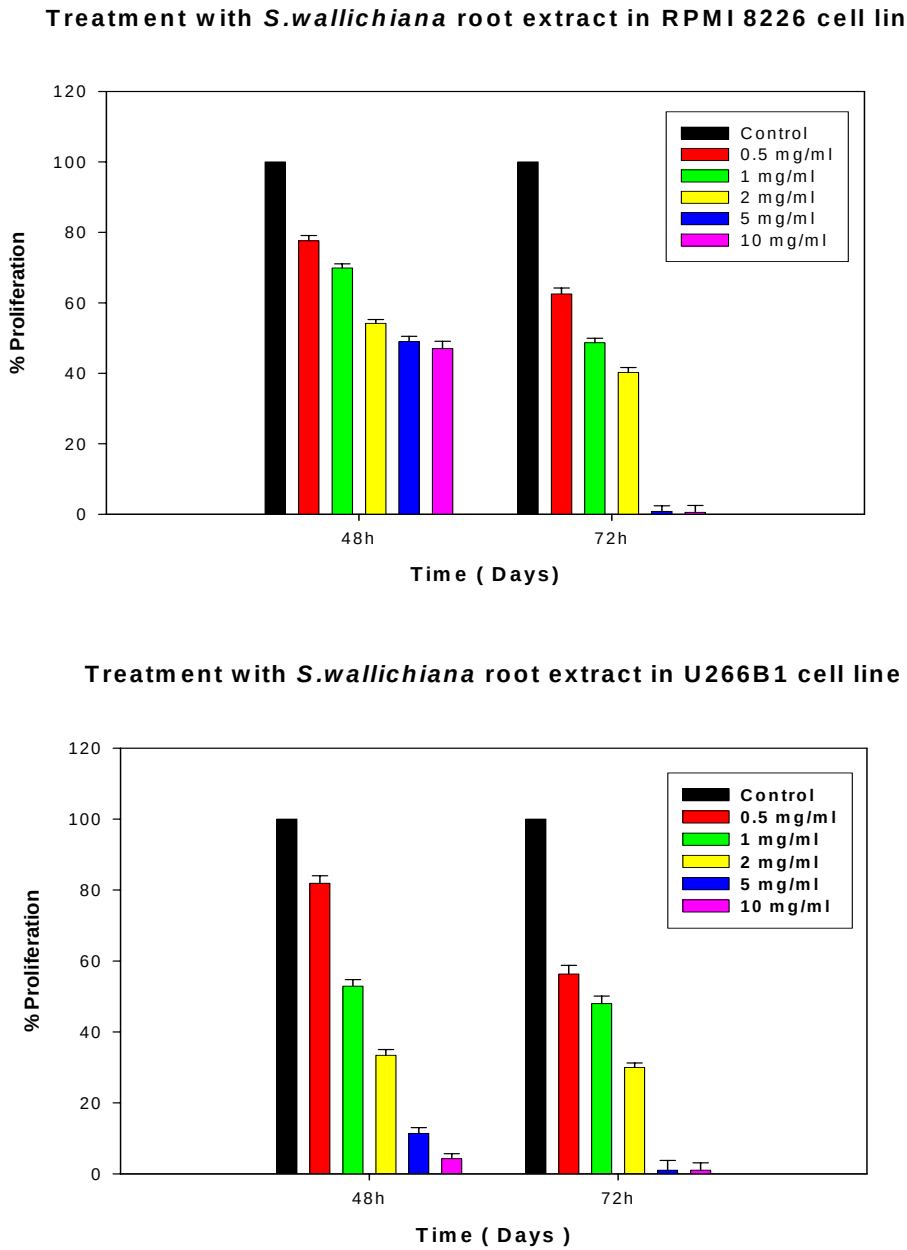


Figure 37: Effect of *S. wallichiana* ethanolic root extract on proliferation of myeloma cell lines RPMI 8226 & U266 by MTT assay. The cells were cultured in 10% FBS medium and treated with *S. wallichiana* ethanolic root extract (0.5,1,2,5,10 mg/ ml) for 48 and 72hr. The percentage viable cells were caluculated in comparision to un treated cells. The number of cells in control was taken as 100% . Values were expressed as mean ± SD of three independent experiment.

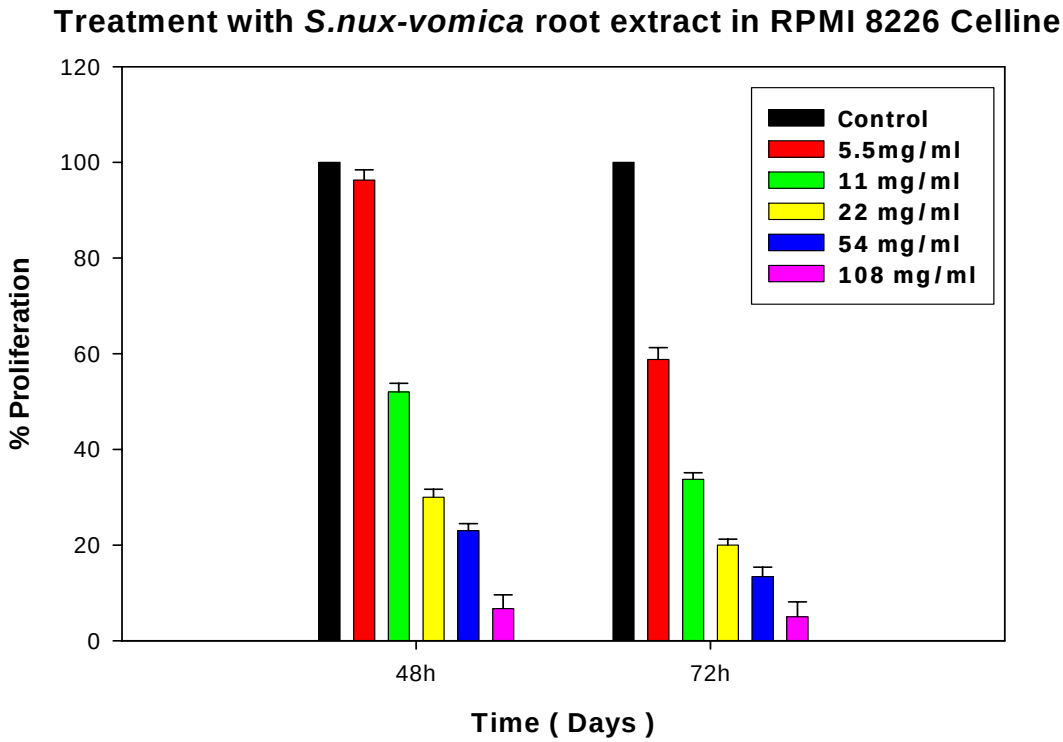


Figure 38: Effect of *S. nux-vomica* ethanolic root extract on proliferation of myeloma cell line RPMI 8226 by MTT assay. The cells were cultured in 10% FBS medium and treated with *S. nux-vomica* ethanolic root extract (0.5.5,11,22,54,108 mg/ ml) for 48 and 72hr. The percentage viable cells were caluculated in comparision to un treated cells. The number of cells in control was taken as 100% . Values were expressed as mean ± SD of three independent experiment.

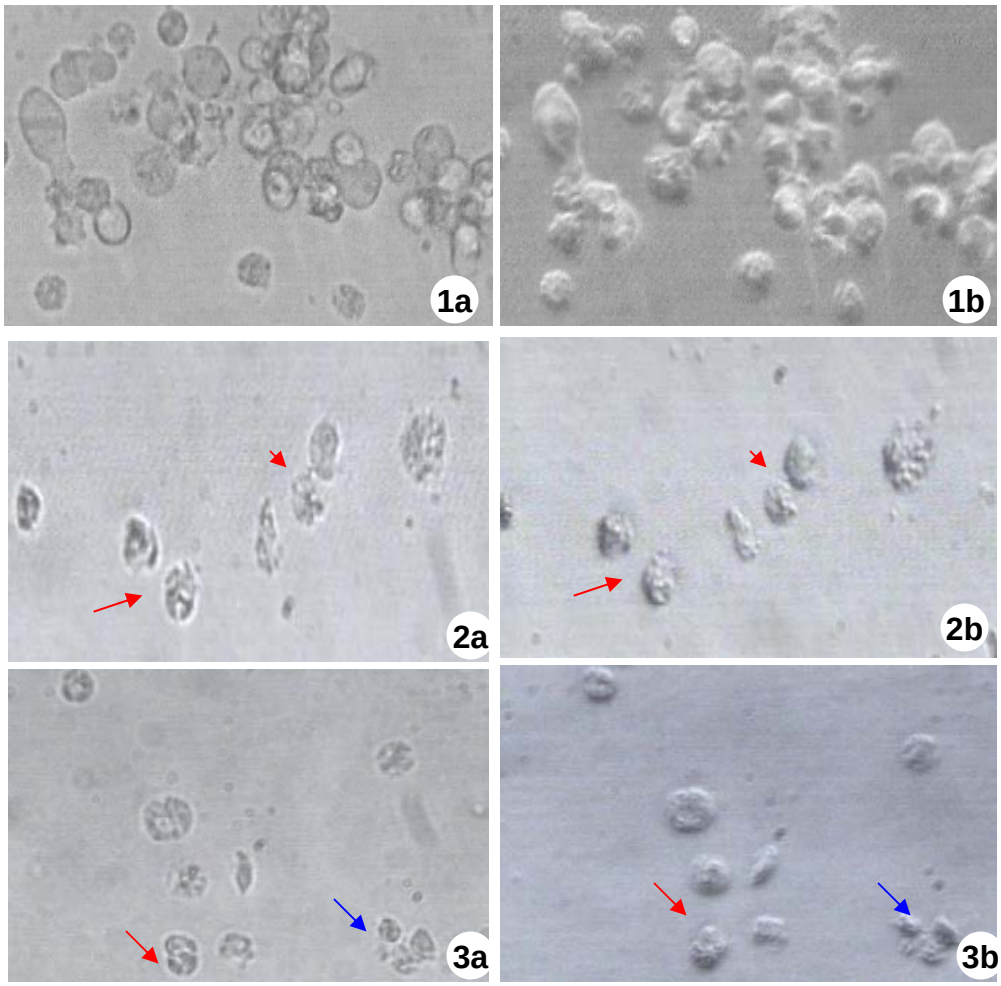


Figure 39 : Morphological alterations of RPMI 8226 cells following exposure to *S. nux-vomica* root extract for 48 and 72 hrs, images were captured by Light and Phase contrast inverted microscope

1a (Light microscope) and ,1b.(Phase contrast microscope) Control cells

2a and b; 3a and b. Treated cells were altered cellular morphology with cytoplasmic shrinkage and membrane blebbing during a culture period of 48 hrs and 72hrs. Red arrows representing nuclear shrinking, membrane blebbing while blue arrows represent apoptotic cell. Each experiment was performed in triplicate (magnification 40×).

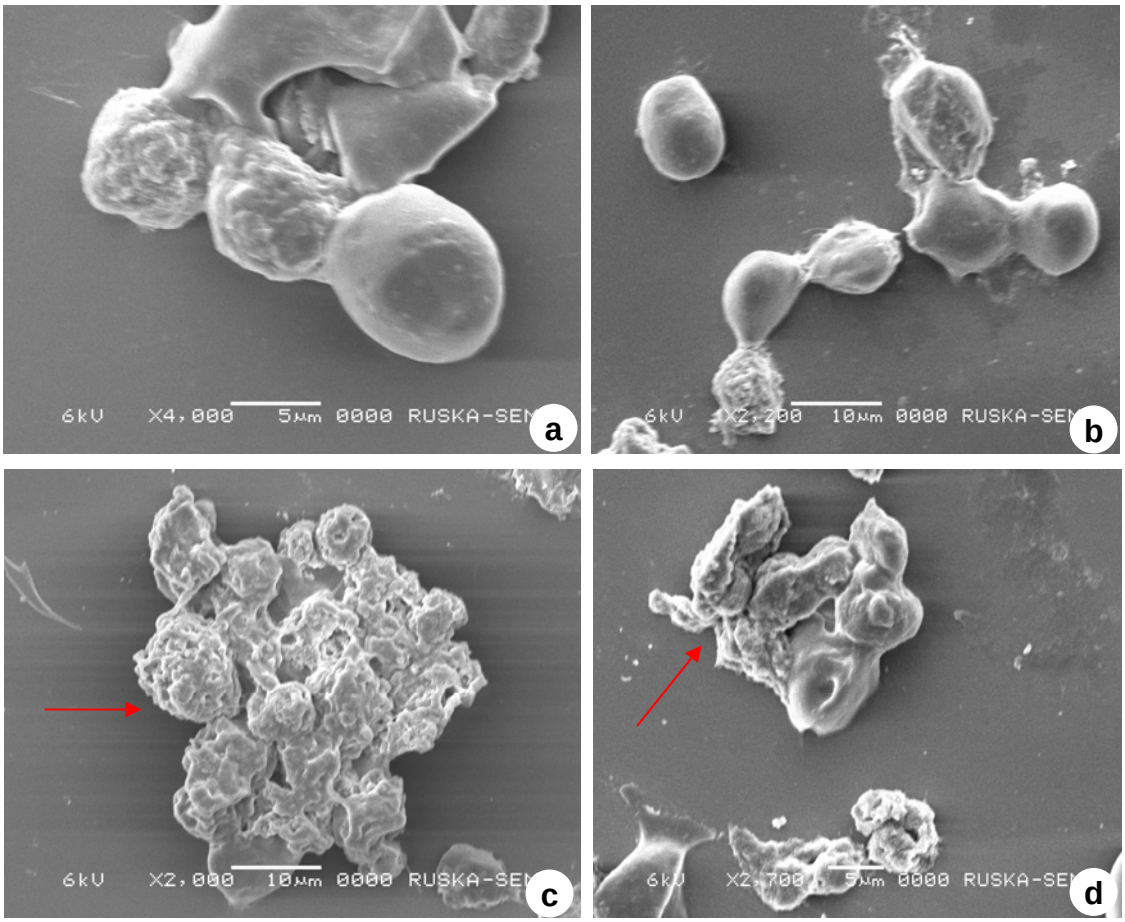


Figure 40: Morphological alterations of RPMI 8226 cells following exposure to *S. nux-vomica* root extract for 48 hrs, images were captured by Scanning electron microscope a and b Control cells

c and d. Treated cells were altered cellular morphology with cytoplasmic shrinkage and membrane blebbing during a culture period of 48hrs. Red arrows representing nuclear shrinking, membrane blebbing of the apoptotic cells.

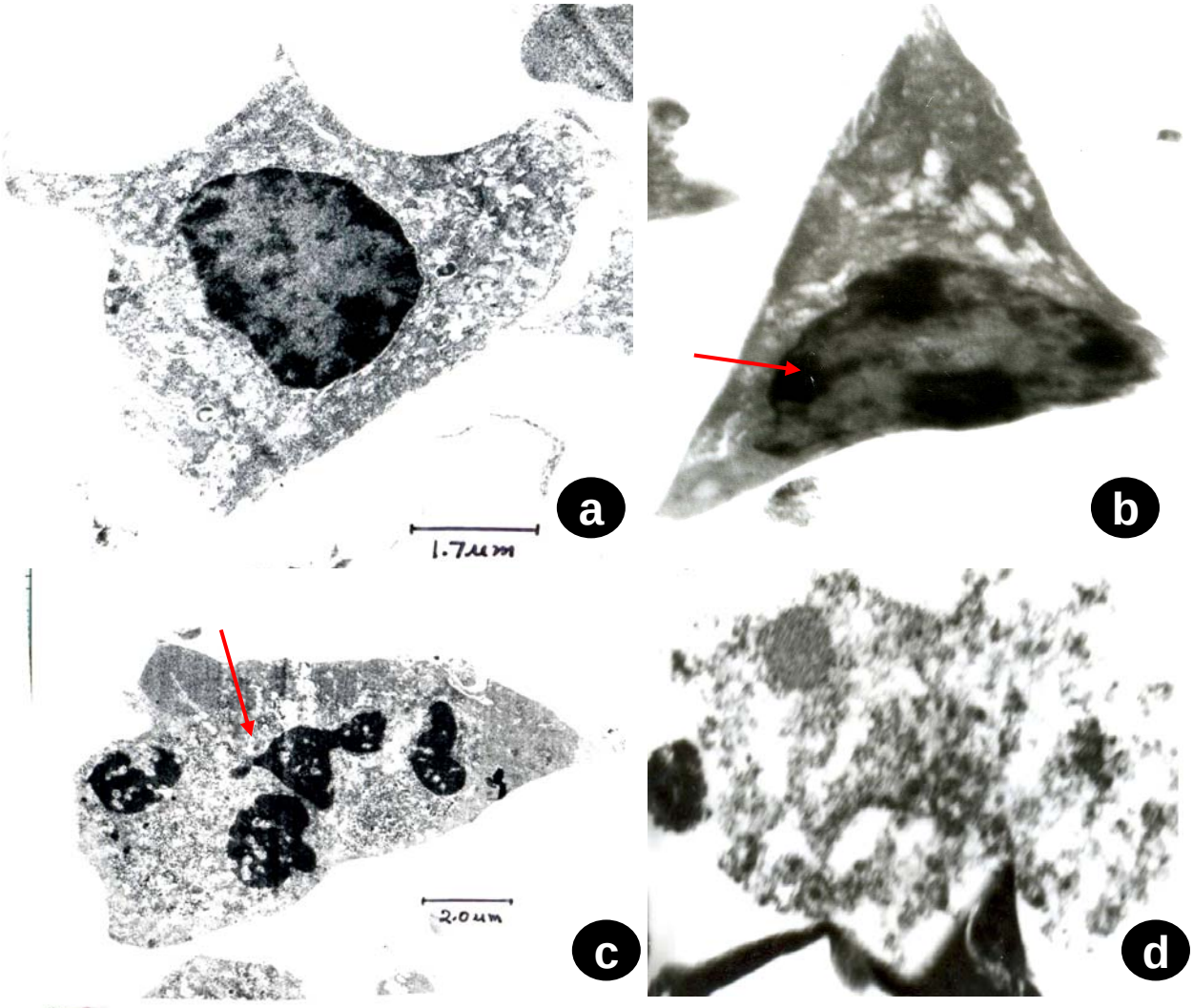


Figure 41: Ultra structural and morphological alterations of RPMI 8226 cells following exposure to *S. nux-vomica* root extract for 48 hrs, Cells were harvested , fixed in 2.5 % glutaraldehyde and images were analyzed by Transmission electron microscope.

a Control cells exhibited distinguishable diffused interchromatin with prominent nucleus

b. Treated cell exhibiting altered cellular morphology with marginalization of chromatids (represented by red arrow) and cytoplasmic shrinkage during a culture period of 48hrs.

c. Treated cell exhibiting nuclear fragmentation (represented by red arrow) during a culture period of 48hrs.

d. Treated cell exhibiting necrosis form of cell death.

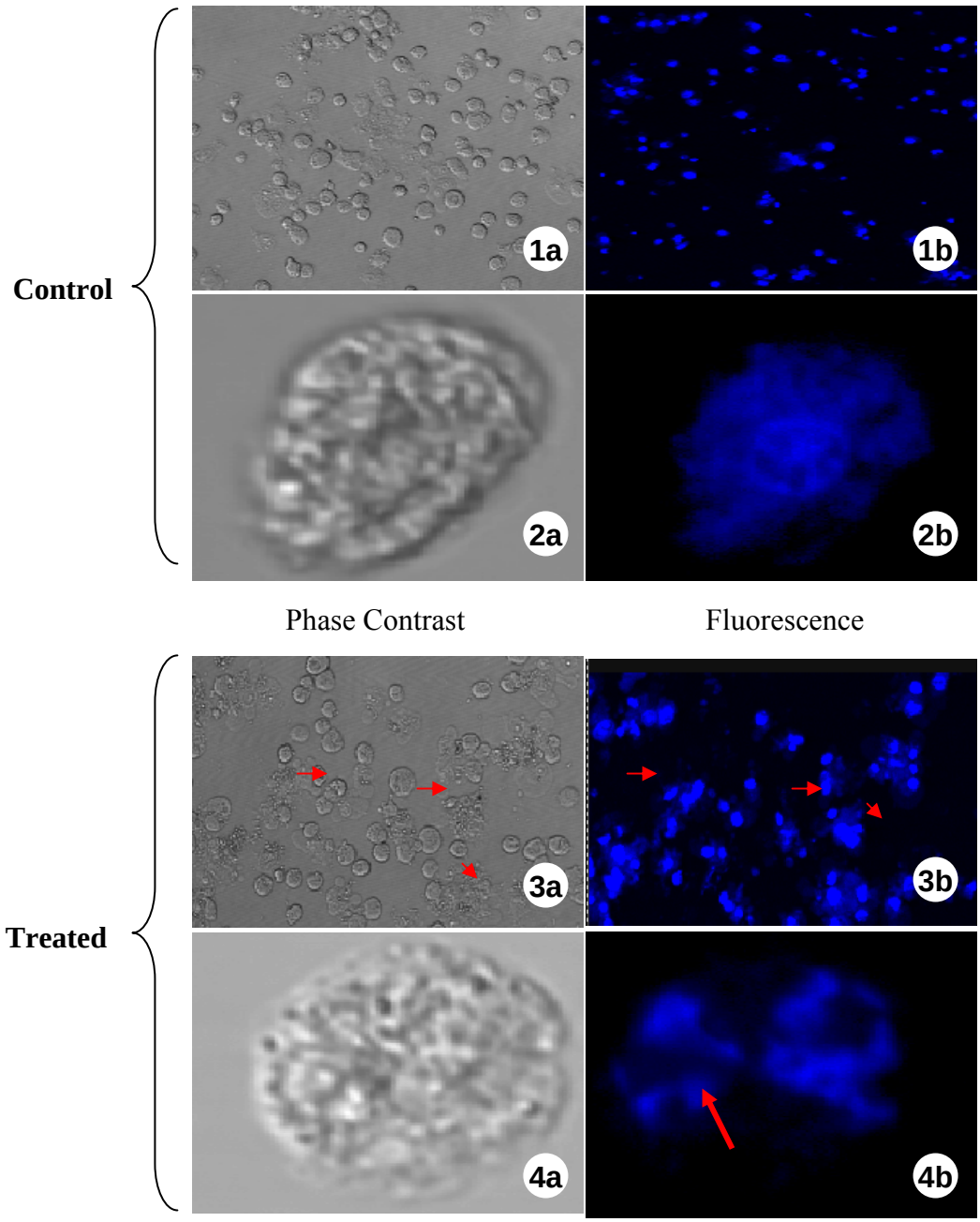


Figure 42: *S. nux-vomica* root treated RPMI 8226 cells after 48 hrs showing morphological alterations. The control cells and treated cells stained with DAPI [4,6,-Di amidino-2-2-phenylindole] and observed under the confocal microscope. Treated cells exhibiting altered cellular morphology with cytoplasmic shrinkage and membrane blebbing during a culture period of 48hrsin lower magnification. Red arrows representing nuclear shrinking, membrane blebbing of the apoptotic cells. (represented by red arrow). Each experiment was performed in triplicate (magnification 100×).

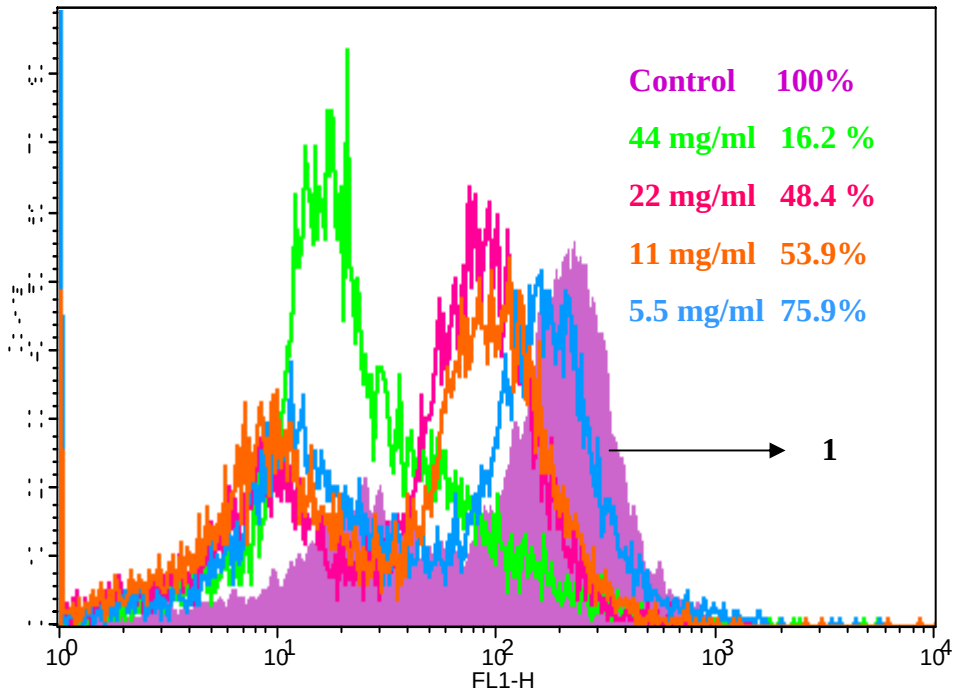


Figure 43: Flow cytometric analysis showing the the disruption of membrane potentials: Induction of mitochondrial dysfunction and relative mitochondrial membrane potential (ΔΨm) was measured by fluorescent emission from Rhodhamine 123 uptake by mitochondria. RPMI 8226 cells were treated with *S. nuxvomica* ethanolic root extract with (0. 5.5, 11, 22, 44 mg/ ml)concentrations for 24hr and stained with Rhodamine 123 and subjected to FACS analysis. Histogram 1 represents the florescence intensity from control cells. Remaining histograms represents fluorescence intensity from RPMI 8226 treated with extracts.

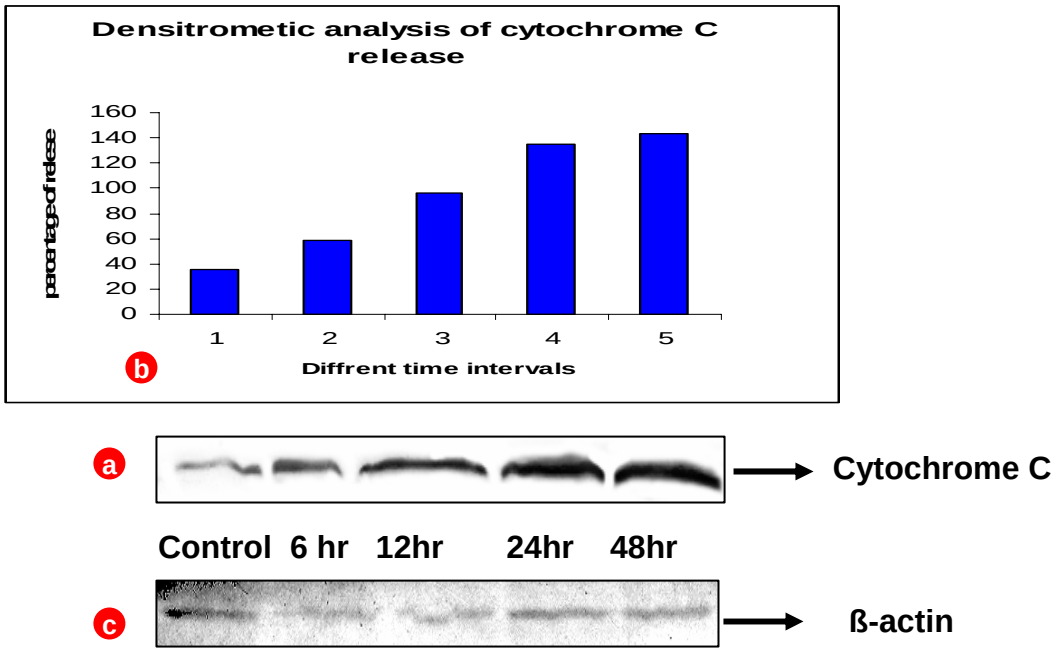


Figure 44: Westron blot analysis showing the effect of *S. nuxvomica* ethanolic root extract on cytochrome c release. ***a)*** Equal concentration of protein (50 µg) from the treated RPMI 8226 cells with IC 50 concentration for indicated times(0, 6, 12, 24, 48 hr) were analyzed by 15% SDS-PAGE and after electrophoresis, proeins on the gels were transferred to nitro cellulose membrane and probed with monoclonal cytochrome c antibodies. Lane 1: 0h, lane 2: 6h, lane 3: 12h, lane 4: 24h, lane 5: 48h. Elevated levels of cytochrome c was observed at different time intervals which also ***b)*** further confirmed by Densitometry ***c)*** ß- actin was used as control for equal loading of the protein.

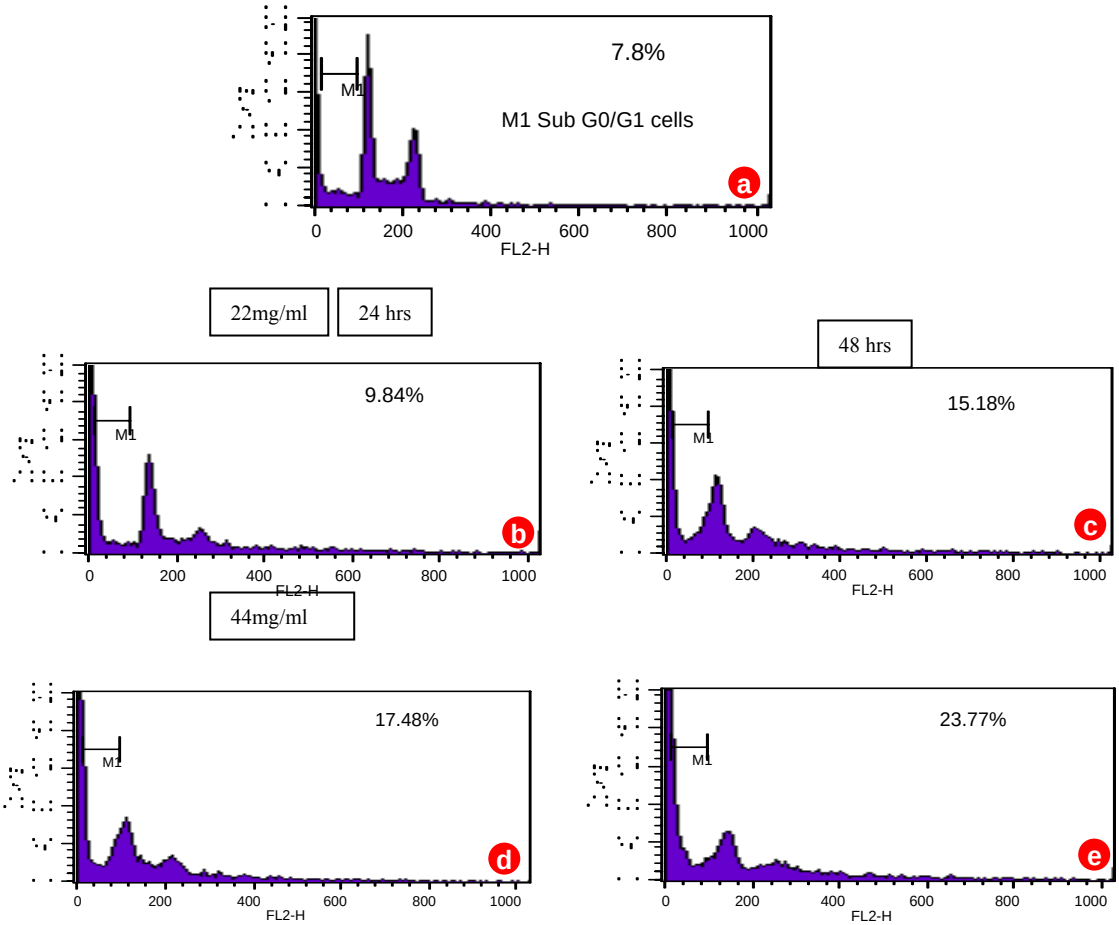


Figure 45: Flow cytometric analysis of the control and treated RPMI 8226 cells with *S. nuxvomica* ethanolic root extract at various (0, 22, 44 mg/ ml)concentrations for 24 and 48hrs. Cells were fixed in 70% ice cold ethnol and stained with propidium iodide and DNA content was quantified flow cytometer . The FACS analysis of control cells, showed prominent number of hypo dipliod (sub-G0/ G1 phase) followed by S and G2/M phases. Treatment with *S. nux-vomica* root extract cells showed increase in sub G0/G1 peak in a dose depend manner. The number of hypo diploid cells which is expressed as a percentage of the total number of cells.

a. Control RPMI 8226 cells *b.* Treated cells with 22 mg/ml extract after 24hrs. *c.* Treated cells with 44 mg/ml extract after 24hrs. *d.* Treated cells with 22 mg/ml extract after 48hrs. *e.*Treated cells with 44 mg/ml extract after 48hrs.

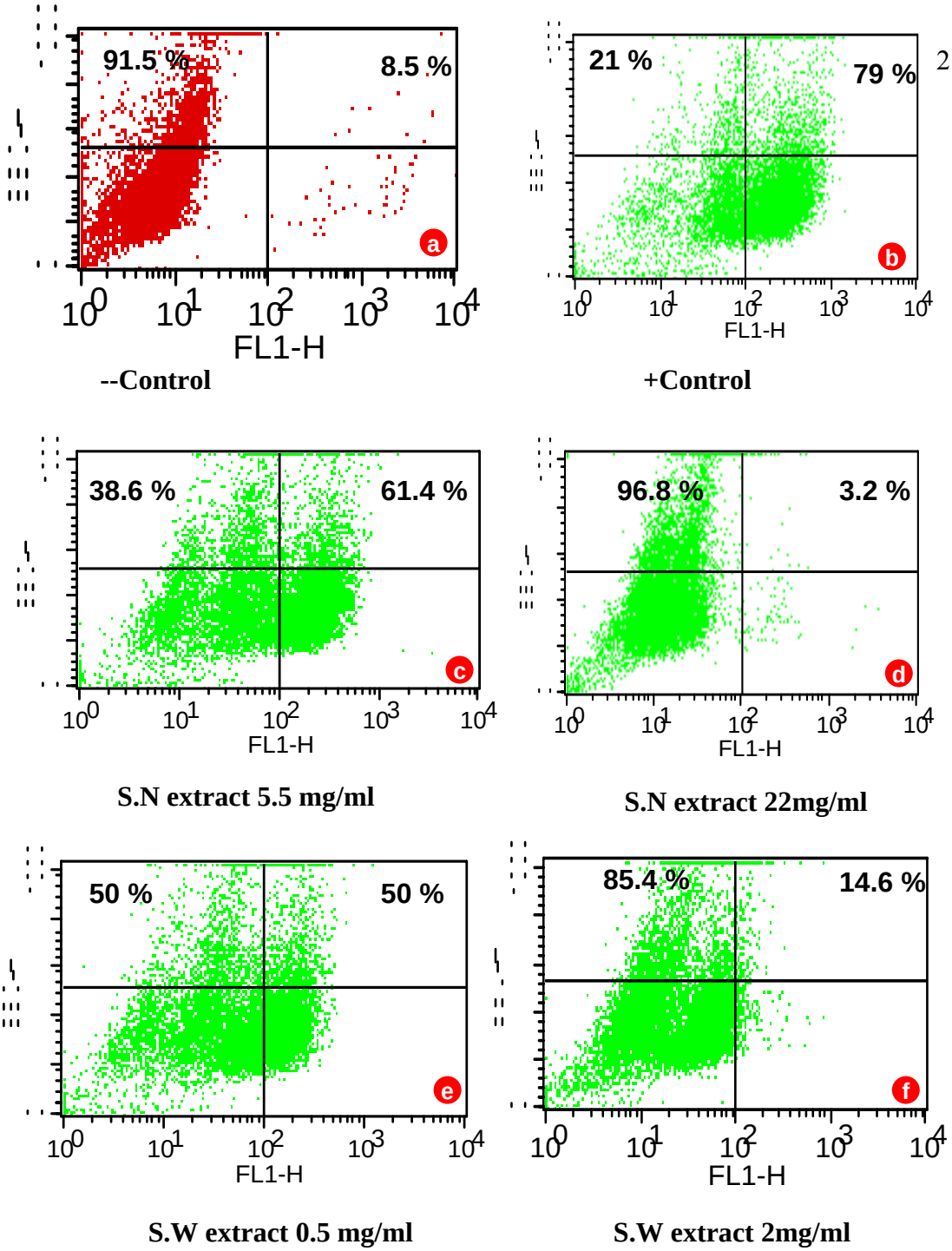


Figure 46: CD 138 expression analysis in U266 B1 cell line by flow cytometer using FITC conjugated anti-human CD138 antibody. The control and treated cells were harvested after 48hrs and the CD138 expression was analyzed using a gating protocol for measuring mean FITC florescence intensity of labeled cells.

a. Negative control of U266B1 cells *b.* Positive control of U266B1 cells. *c.* Treated cells
b. with 5.5 mg / ml of *S. nux-vomica* root extract. *d.* Treated cells with 22 mg / ml of *S. nux-vomica* root extract *e*. Treated cells with 0.5 mg / ml of *S. wallichiana* root extract. *f.* Treated cells with 2 mg / ml of *S. wallichiana* root extract.

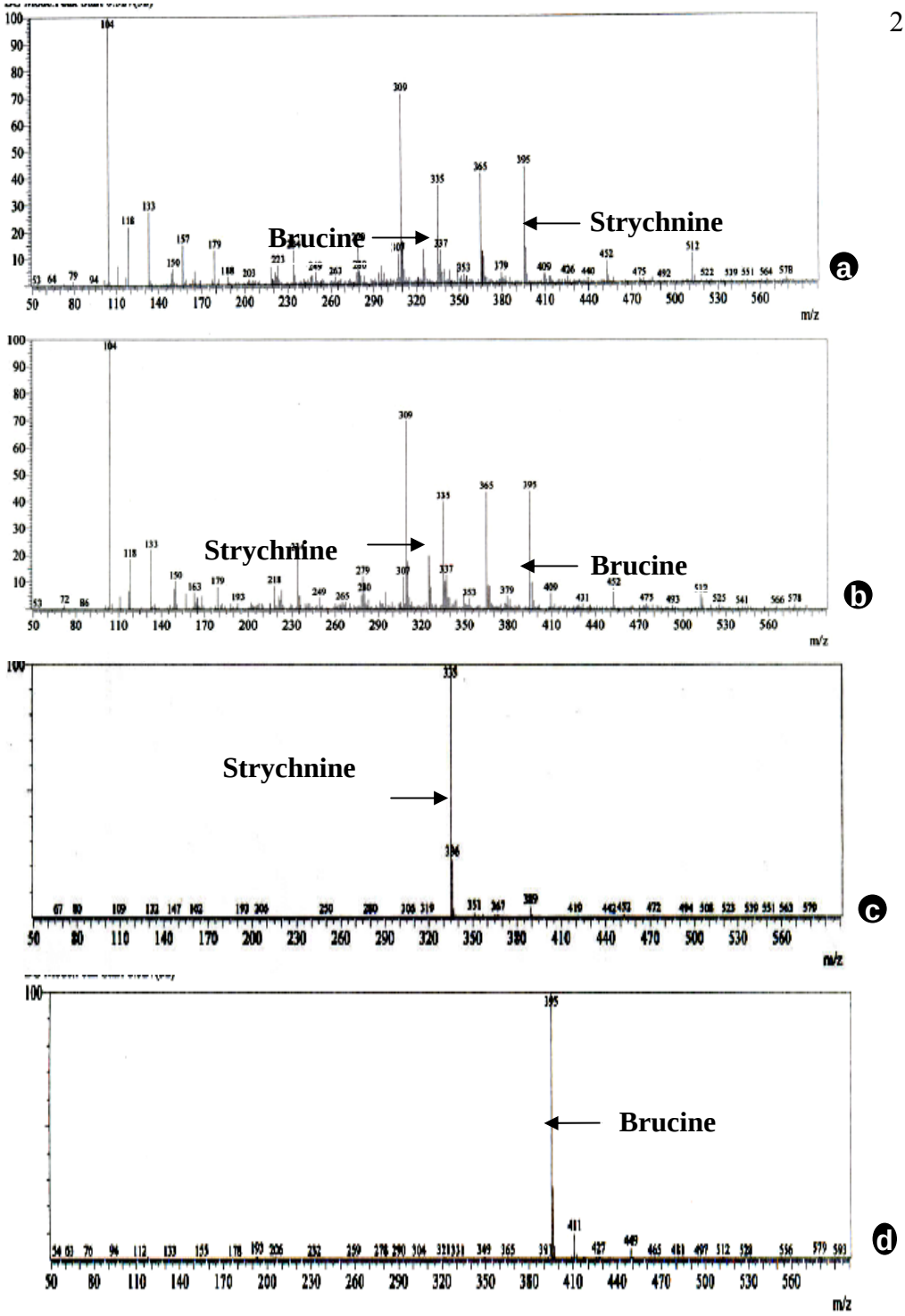


Figure 47: L.C. Mass spectral analysis of ethanolic root extracts and HPLC purified compounds

a) and b) L.C. Mass analysis of crude ethanolic root extracts of *S. wallichiana* and *S. nux-vomica* showing showing molecular ion peak of strychnine at 335 MHz and brucine peak at 395 MHz

c) and d). L.C. Mass analysis of HPLC purified strychnine and brucine showing molecular ion peaks strychnine 335 MHz and as well as brucine peak at 395 MHz.

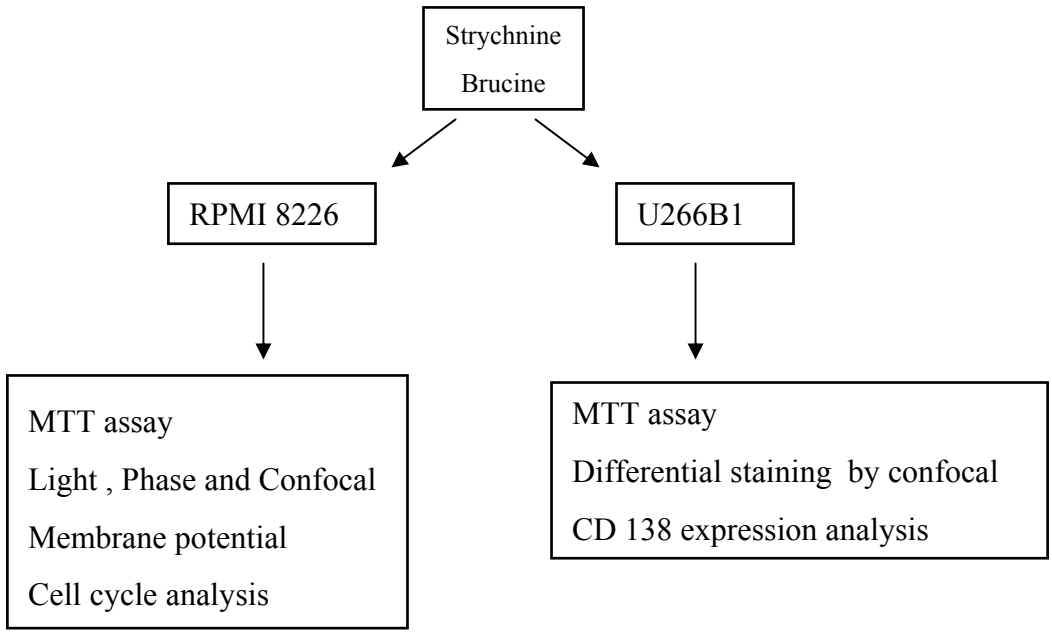


Figure 48: *S.nuxvomica* and *S. wallichiana e*thanolic root extracts subjected to HPLC purification and LC-Mass analysis. Sketch of analysis for evaluation of strychnine and brucine on multiple myeloma celllines.

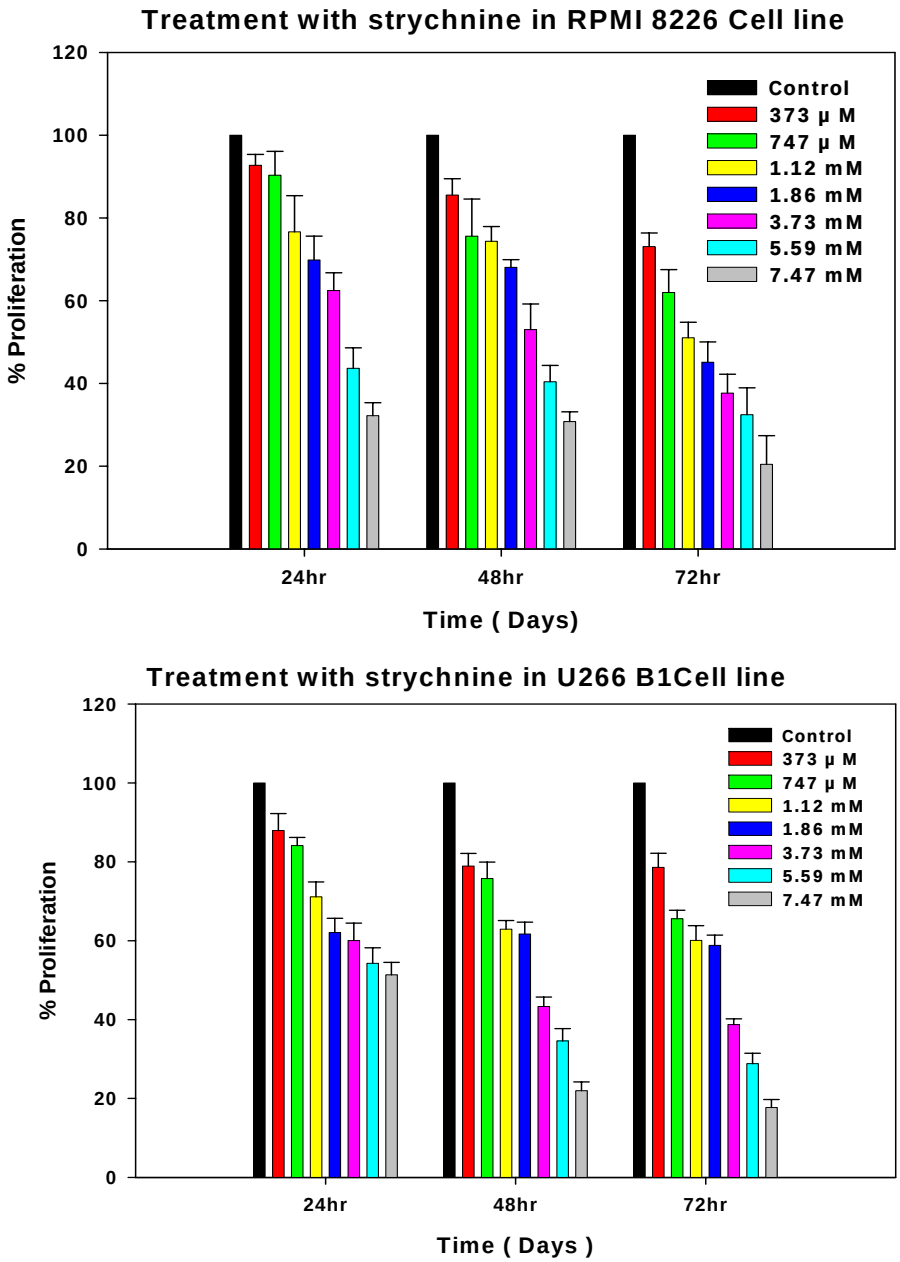


Figure 49: Effect of strychnine on proliferation of myeloma cell lines RPMI 8226 & U266 by MTT assay. The cells were cultured in 10% FBS medium and treated with strychnine (0.373, 747 µm, 1.12, 1.86, 3.73, 5.59, 7.47 mM) for 24, 48 and 72hr. The percentage viable cells were calculated in comparison with untreated cells. The number of cells in control was taken as 100% . Values were expressed as mean ± SD of three independent experiment.

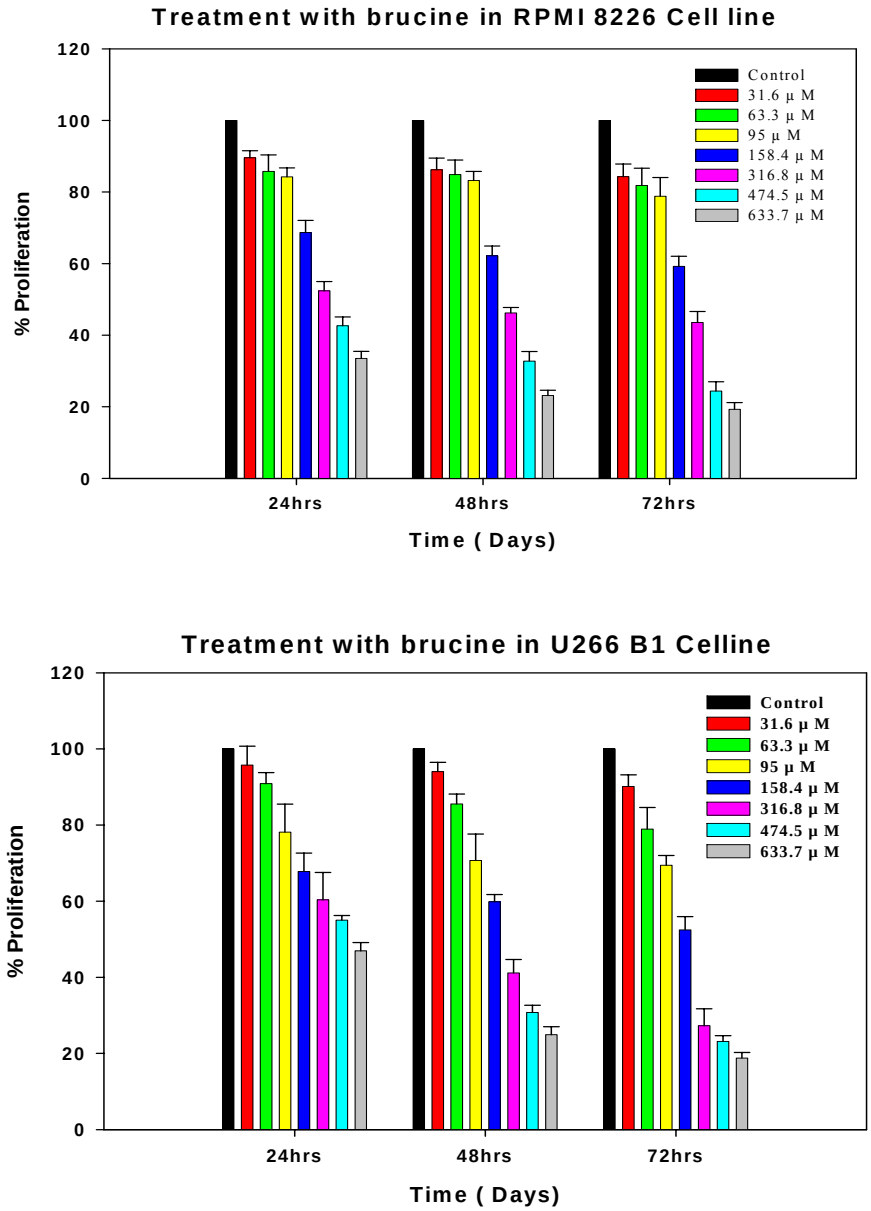


Figure 50: Effect of brucine on proliferation of myeloma cell lines RPMI 8226 & U266 by MTT assay. The cells were cultured in 10% FBS medium and treated with strychnine (0, 31.6, 63.3, 95, 158.4, 316.8, 474.5, 633.7 µm,) for 24, 48 and 72hr. The percentage viable cells were calculated in comparison with untreated cells. The number of cells in control was taken as 100% . Values were expressed as mean ± SD of three independent experiment.

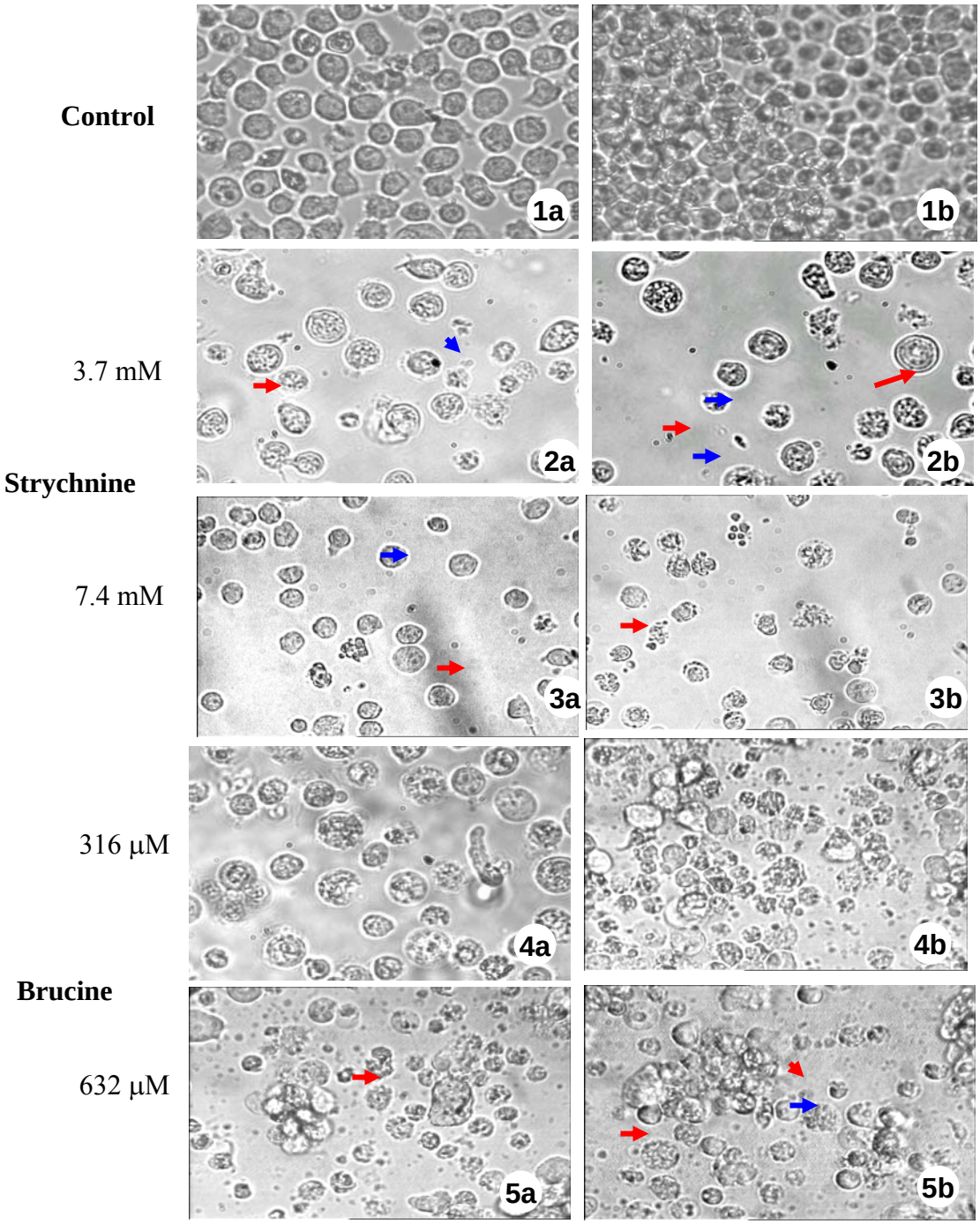


Figure 51: Morphological alterations of RPMI 8226 cells following exposure to strychnine and brucine for 24 and 48 h. Images were captured by Light microscope..

1 a and b Control cells 24 h and 48 h respectively.

2 a and 2 b 3.7 mM; 3a and 3 b 7.4 mM strychnine treated cells after 24 h and 48 h respectively

4a and 4 b 316 µM; 5a and 5 b 632 µM brucine treated cells after after 24 h and 48 h respectively. Red arrows representing nuclear shrinking, membrane blebbing while blue arrows represent apoptotic cell. Each experiment was performed in triplicate (magnification 40×).

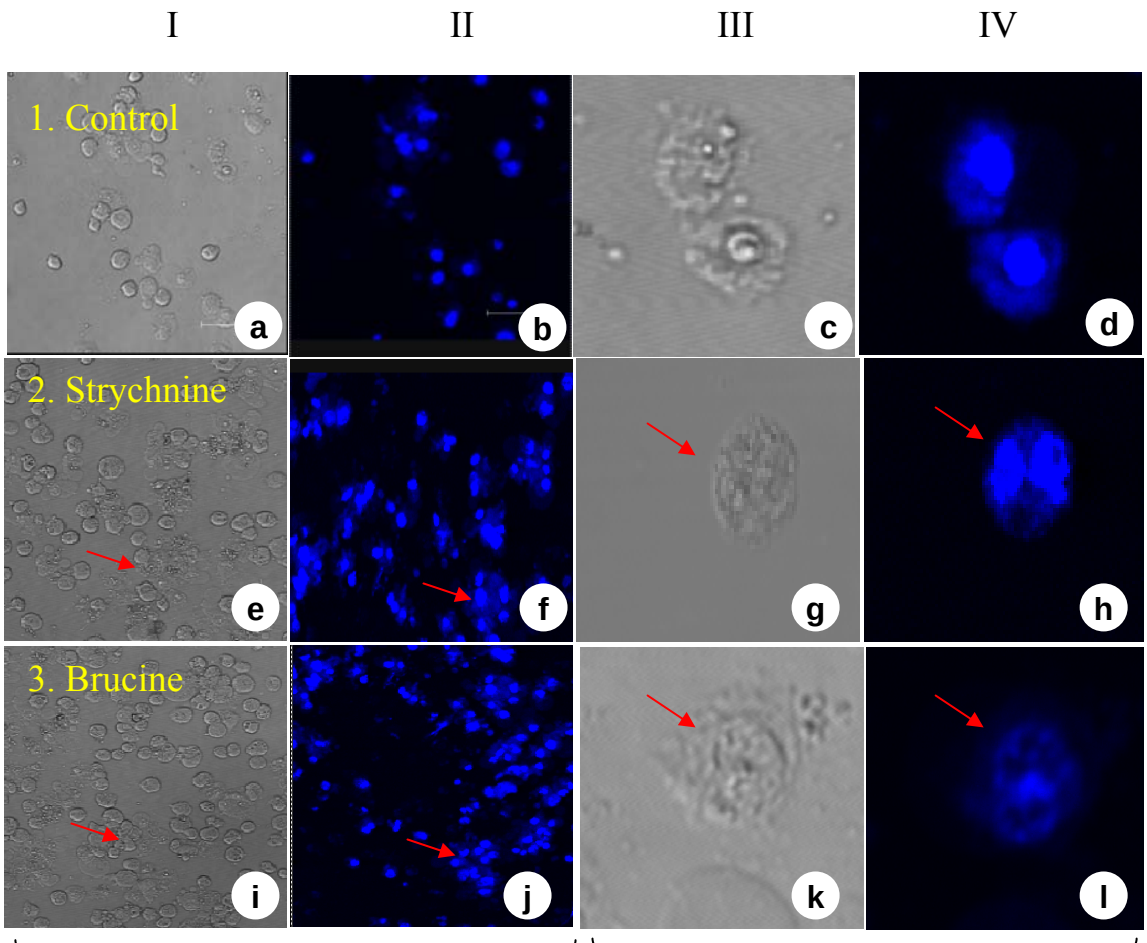


Figure 52: Morphological evidence of RPMI 8226 cells following exposure to strychnine and brucine with IC 50 concentration for 48 hrs. The control and treated cells were stained with DAPI [4,6,-Di amidino-2-2-phenylindole] and observed under the confocal microscope. Cells exhibiting varied extent of altered cellular morphology with cytoplasmic shrinkage and membrane blebbing be noted . Each experiment was performed in triplicate

Red arrows representing nuclear shrinking, membrane blebbing of the apoptotic cells.

Strychnine and brucine treated cells exhibit altered cellular morphology with typical nuclear fragmentation (indicated by red arrow).

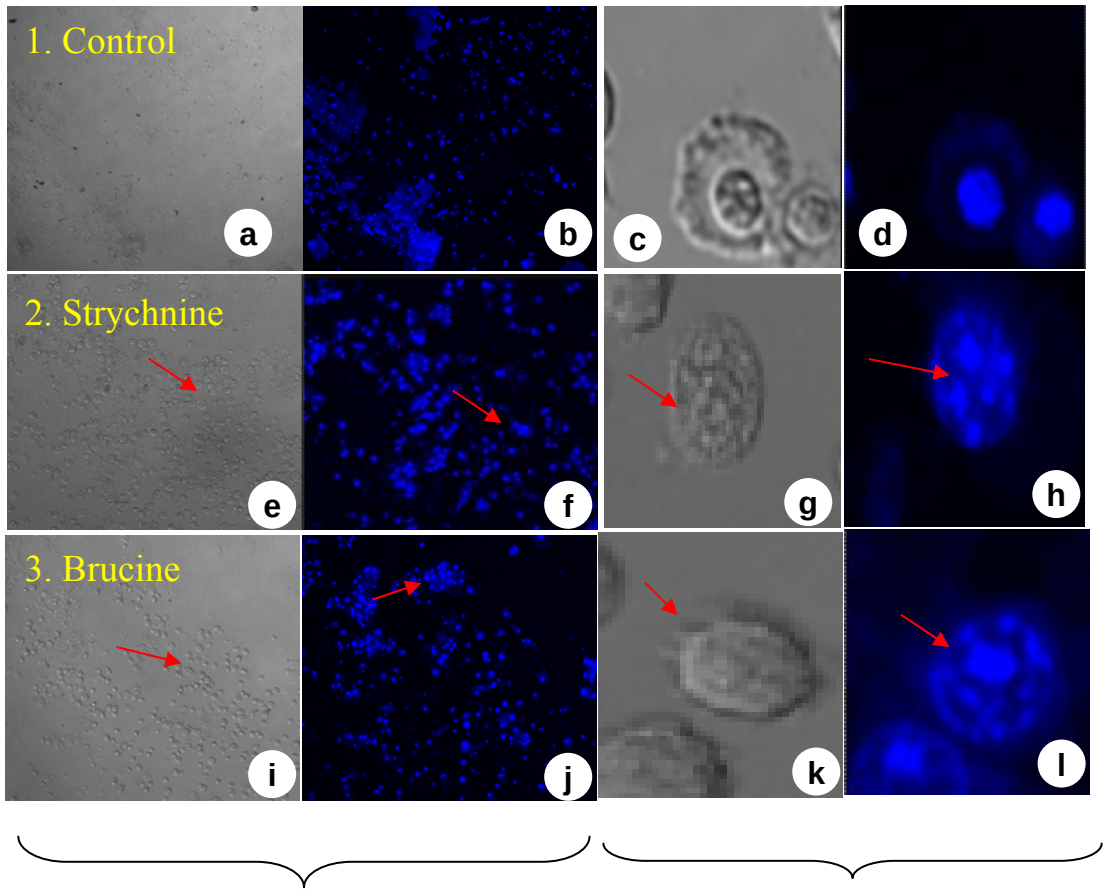
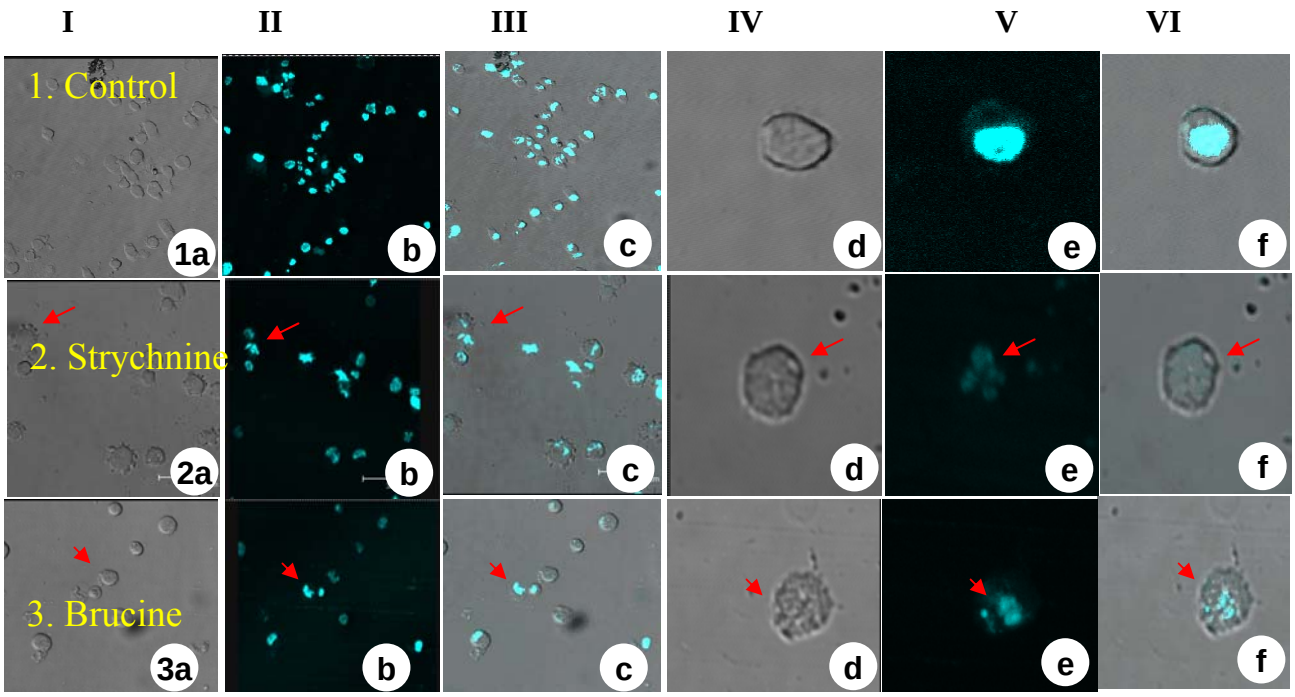


Figure 53: Morphological evidence of U266 B1 cells following exposure to strychnine and brucine with IC 50 concentration for 48 hrs. The control and treated cells were stained with DAPI [4,6,-Di amidino-2-2-phenylindole] and observed under the confocal microscope. Cells exhibiting varied extent of altered cellular morphology with cytoplasmic shrinkage and membrane blebbing be noted . Each experiment was performed in triplicate Red arrows representing nuclear shrinking, membrane blebbing of the apoptotic cells.

Strychnine and brucine treated cells exhibit altered cellular morphology with typical nuclear fragmentation (indicated by red arrow).



I	Phase contarst	IV	Phase contarst
II	Fluorescence emission	V	Fluorescence emission
III	Overlying of I and II	VI	Overlying of IV and VI

Figure 54: Morphological evidence of U266 B1 cells following exposure to strychnine and brucine with IC 50 concentration for 48 hrs. The control cells and treated cells stained with with Hoechst 33258 fluorescent probe and observed under the confocal microscope.

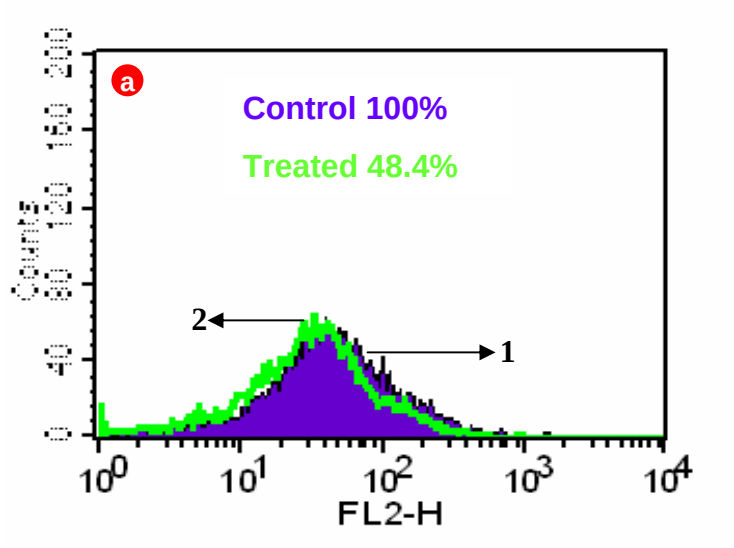
1 a-f: Control cell stained with Hoechst 33258 showing nucleus

2 a-c Strychnine treated cells exhibiting altered cellular morphology with cytoplasmic shrinkage and membrane blebbing during a culture period of 48hrs in lower magnification. Red arrows representing nuclear shrinking, membrane blebbing of the apoptotic cells.

2d-f. Treated cell with strychnine exhibiting altered cellular morphology with nuclear fragmentation(represented by arrow) in higher magnification.

3a-c. Treated cells with brucine exhibiting altered cellular morphology with cytoplasmic shrinkage and membrane blebbing during a culture period of 48hrs in lower magnification. Red arrows representing nuclear shrinking, membrane blebbing of the apoptotic cells.

3d-f. Treated cell with brucine exhibiting altered cellular morphology with nuclear fragmentation(represented by arrow) in higher magnification.



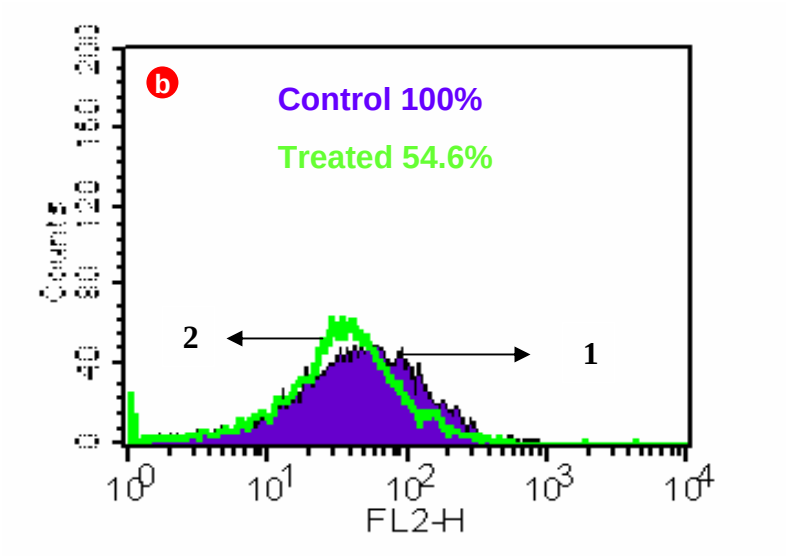


Figure 55: Flow cytometric analysis showing the disruption of membrane potentials: Induction of mitochondrial dysfunction and relative mitochondrial membrane potential (ΔΨm) was measured by fluorescent emission from Rhodhamine 123 uptake by mitochondria. RPMI 8226 cells were treated with strychnine and brucine with IC 50 concentrations for 24hr and stained with Rhodamine 123 and subjected to FACS analysis.

a. Histogram 1 represents the florescence intensity from control cells. Histogram-2 represents fluorescence intensity from RPMI 8226 treated with strychnine.

b. Histogram 1 represents the florescence intensity from control cells. Histogram-2 represents fluorescence intensity from RPMI 8226 treated with brucine.

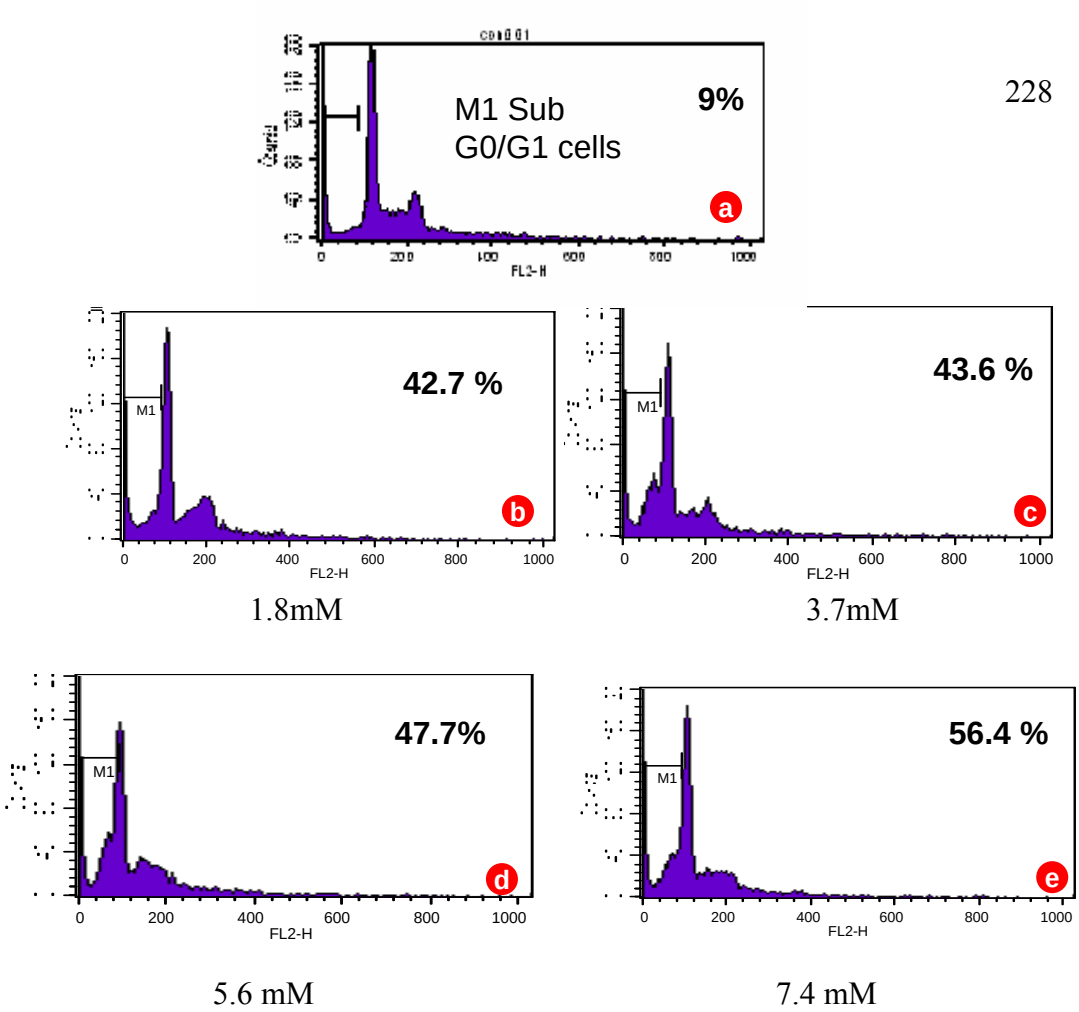


Figure 56: Flow cytometric analysis of the control and treated RPMI 8226 cells with strychnine at various (0, 1.8, 3.7, 5.6, 7.4 mM.)concentrations for 48hrs. Cells were fixed in 70% ice cold ethanol and stained with propidium iodide and DNA content was quantified flow cytometer . The FACS analysis of control cells, showed prominent number of hypo diploid (sub-G0/ G1 phase) followed by S and G2/M phases. Treatment with strychnine cells showed increase in sub G0/G1 peak in a dose depend manner. The number of hypo diploid cells which is expressed as a percentage of the total number of cells.

a. Control RPMI 8226 cells *b.* Treated cells with 1.8 mM strychnine . *c.* Treated cells with 3.7 mM strychnine . *d.* Treated cells with 5.6 mM strychnine . *e.* Treated cells with 7.4 mM strychnine..

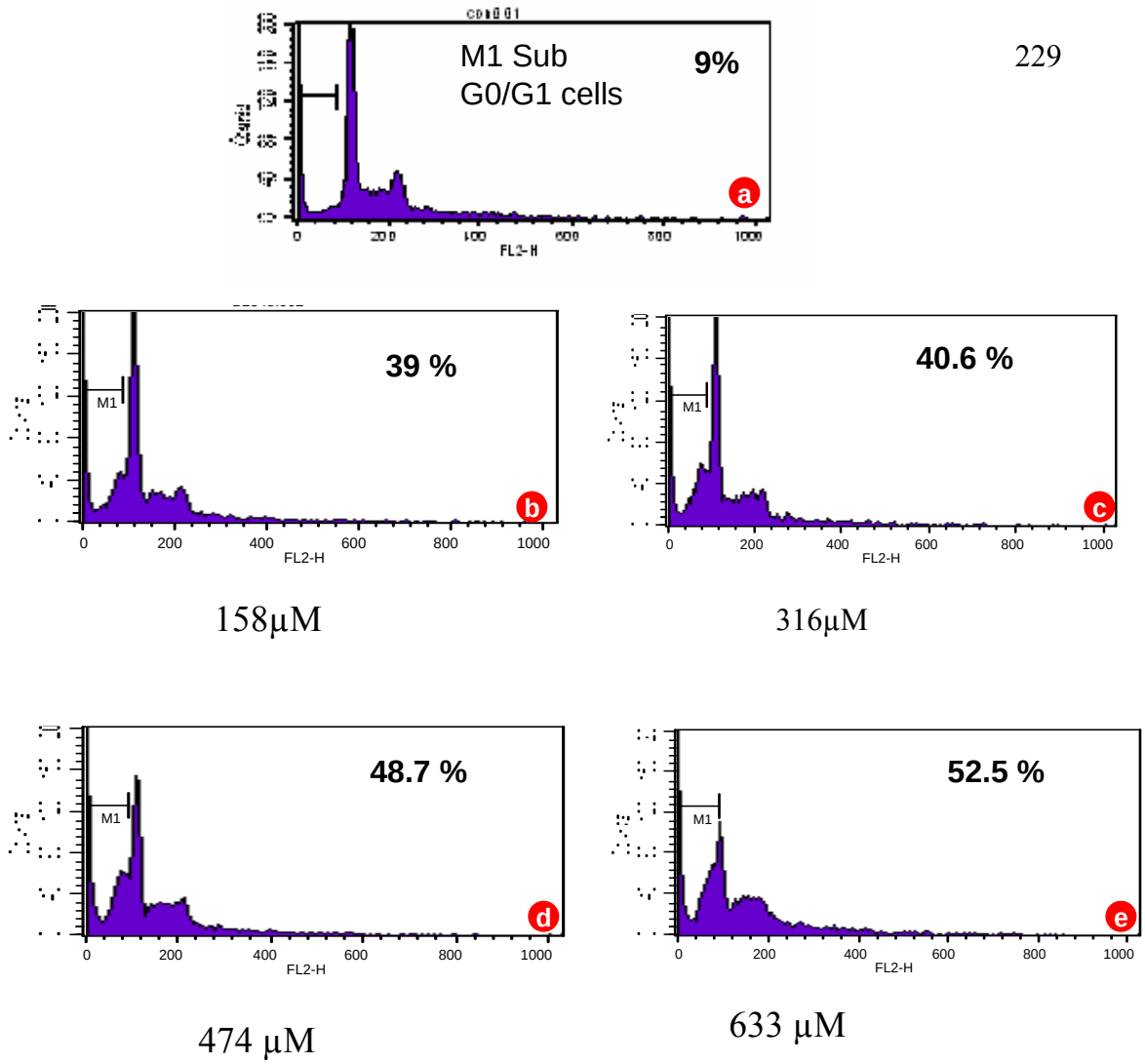


Figure 57: Flow cytometric analysis of the control and treated RPMI 8226 cells with brucine at various (0, 158, 316, 474, 633 µM) concentrations for 48hrs. Cells were fixed in 70% ice cold ethanol and stained with propidium iodide and DNA content was quantified flow cytometer . The FACS analysis of control cells, showed prominent number of hypo diploid (sub-G0/ G1 phase) followed by S and G2/M phases. Treatment with brucine cells showed increase in sub G0/G1 peak in a dose depend manner. The number of hypo diploid cells which is expressed as a percentage of the total number of cells.Control RPMI 8226 cells *b.* Treated cells with 158µM brucine . *c.* Treated cells with 316µM brucine.. *d.* Treated cells with 474µM . *e.* Treated cells with 633µM brucine.

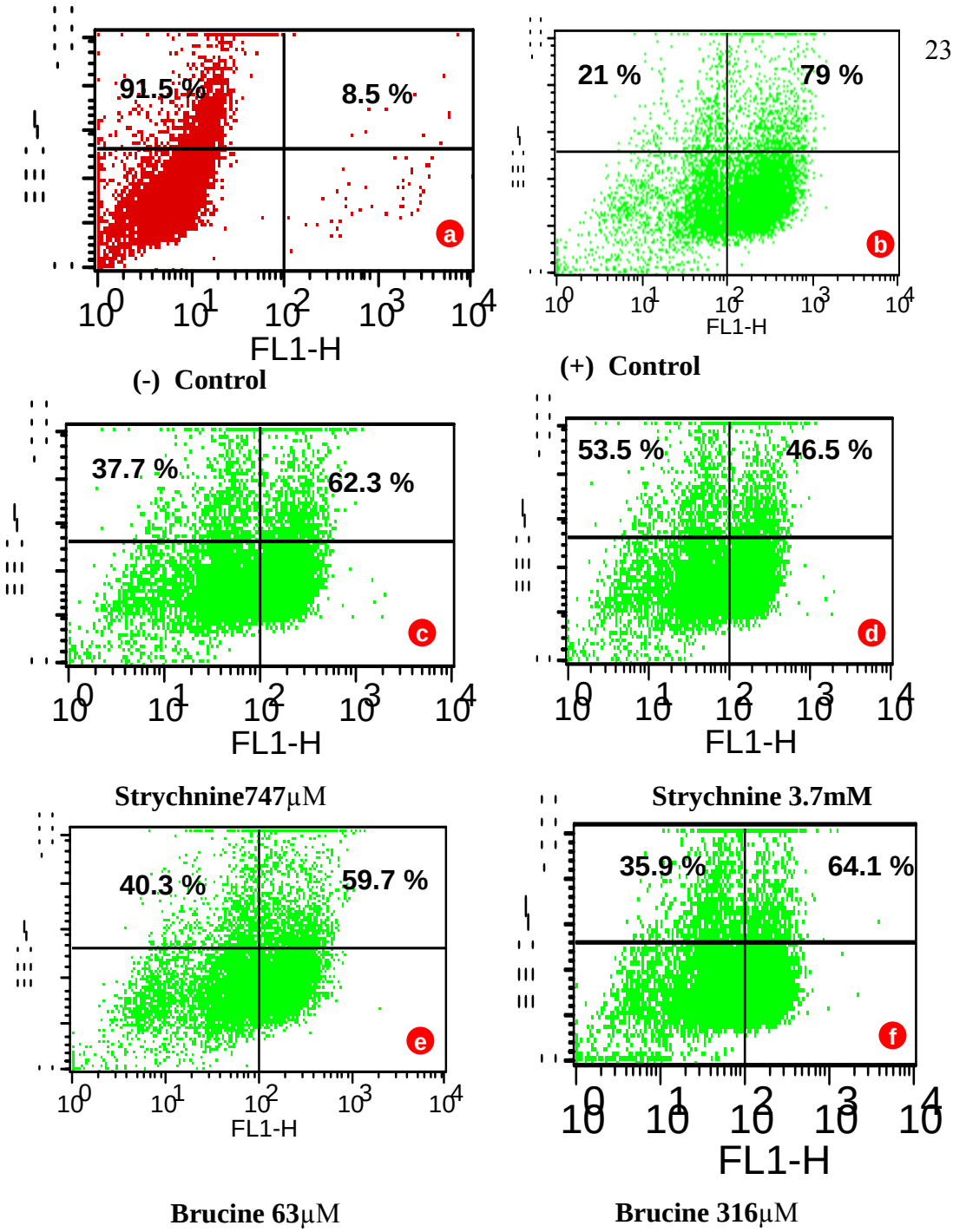


Figure 58: CD 138 expression analysis in U266 B1 cell line by flow cytometer using FITC conjugated anti-human CD138 antibody. The control and treated cells were harvested after 48hrs and the CD138 expression was analyzed using a gating protocol for measuring mean FITC florescence intensity of labeled cells.

a. Negative control of U266B1 cells *b.* Positive control of U266B1 cells. *c.* Treated cells a.with 747µM of strychnine . *d.* Treated cells with 3.7 mM of strychnine *e*. Treated b.cells with 63µM of brucine. *f.* Treated cells with 316 µM of brucine.

6.13 Conclusion

The present study evaluated and compared several of the available methods for detecting antiproliferation activity of *Strychnos* genus. Therefore, the present investigation has been carried out to identify the mechanism of cytotoxicity and as well as mode of cell death of on myeloma cell lines with *S. wallichiana* and *S. nux-vomica* root extracts. The *S. wallichiana & S. nux-vomica* root extracts inhibited proliferation in RPMI 8226 and U266 B1 in a dose dependent manner at different time intervals. 24 hr treatment did not show much impact while 100% Inhibition of proliferation was observed in 48 and 72hrs treatments in a dose dependent manner. The present study demonstrates that *S. nux-vomica* root extract, enters myeloma cells and alters mitochondrial membrane integrity, leading to cytochrome *c* leakage and nuclear disintegration. Mitochondrial depolarization was assessed by flowcytometry using the Rhodamine 123 dye, showing that this parameter was also altered by treatment with *S. nux-vomica* root extract, which also indicated apoptosis induction. Decreased mitochondrial membrane potentials with simultaneous appearance of cytochrome *c* in the cytosolic fractions of cells exposed to *S. nux-vomica* root extract. The translocation of mitochondrial cytochrome *c* into the cytoplasm observed in the present study supports such a possibility of RPMI 8226 cells undergoing apoptosis. Morphological assessment of cells by Light, phase contrast, SEM and TEM and nuclear staining assays with DAPI and, Hoechst 33258 stain further confirmed cells undergoing apoptosis, with typical apoptotic characteristics such as membrane blebbing, and chromatin condensation in the cells treated with *S. nux-vomica* root extract. The flow cytometric analysis of *S. nux-vomica* root extract treated 8226 cells showed an increase in a dose-dependent manner in the hypo diploid apoptotic DNA

content with a decrease in the number of cells at the S and G2 M phases of the cell cycle after 24 and 48 hrs respectively. These results suggest that loss in DNA content occurs with increase in concentration of extract and induction of apoptosis occurs at S and G2 M phase of the cell cycle. These biochemical alterations were reflected in morphological and ultra structural changes, typical of cells undergoing apoptosis. Data presented here demonstrate that in RPMI 8226 MM cells *S. nux-vomica* root extract causes mitochondrial alterations may be associated with the apoptotic cell death pathway. Apoptosis is a process of cell death that is critically regulated based on the expression of cell's intrinsic suicide machinery which further leads to the characteristic pattern of morphological, biochemical, and molecular changes. Syndecan-1 or CD138 expression on MM cells (U266B1) in response to treatment with *S. wallichiana* and *S. nux-vomica* root extracts after 48hrs reduced the expression in a dose dependent manner and there by inhibiting the growth of MM cells. Loss of syndecan-1 on MM cells in response to treatment with *S. wallichiana* and *S. nux-vomica* root extracts provides a solid proof for apoptosis induction In MM cell lines.

Analysis of root extracts by TLC, HPLC and L.C. Mass analysis confirmed the presence of strychnine and brucine from *S. nux-vomica* and *S. wallichiana* root extracts. The present data emphasize the importance of strychnine and brucine as an emerging potential class of anticancer chemicals, exhibiting an anti proliferative effect on myeloma cells by inhibiting DNA synthesis and triggering apoptosis. It is worthwhile to mention that the strychnine and brucine with this chemical group cause cell death by causing cell cycle arrest followed by apoptosis. In the present study, the treatment of myeloma cells with strychnine and brucine induced an increase in the amount of sub-diploid DNA,

indicating inter nucleosomal DNA breakdown, as indicated for apoptotic cells. Moreover, histograms of the DNA fluorescence indicated that strychnine and brucine caused cell cycle arrest at G0/G1 phase, thus preventing cells from entering S or G2/M phase and finally apoptosis occurred. Morphological analysis of cells by light microscope revealed cells were undergoing apoptosis .Nuclear staining assays with DAPI and, Hoechst 33258 stain further confirmed the apoptosis induction. Mitochondrial depolarization was assessed by flowcytometry using the Rhodamine123 dye, showing that this parameter was also altered by treatment with strychnine and brucine, which also indicated apoptosis induction. In addition syndecan-1 expression (U266B1) on MM cells in response to treatment with strychnine and brucine after 48hrs reduced the expression in a dose dependent manner and there by inhibiting the growth of MM cells. Nuclear staining assay by using fluorescence probes like DAPI and Hoechst 33258 revealed DNA breaks in apoptotic cells and this result correlates with loss of syndecan-1 expression in U266B1 cells in a dose dependent manner suggesting that cells undergoing apoptosis. Evidences suggest that syndecan-1 is a marker for viable myeloma cells which is rapidly lost by apoptotic cells.

Finally, we have to admit that the concentration strychnine and brucine used in this study was a little bit higher than its physiological concentration, which may not be honestly translated into clinical practices. However, the effect of strychnine and brucine, at smallest amount in this study has been proven to have strong anti proliferative activity on multiple myeloma cell lines. Similar results were demonstrated in the anti-tumor effects of alkaloids from the seeds of *Strychnos nux-vomica* on HepG2 cells and they have also successfully established an *in vitro* model to evaluate the possible mechanism

of alkaloids in *nux- vomica* in the therapy of liver cancer (Deng *et al.*,2006). The above experiments confirm *Strychnos* extracts and its alkaloids have antiproliferative properties while mitochondrial dysfunction may support execution of intrinsic pathway, however further experimentation is required to confirm detailed execution of pathway.

It is interesting to note that root extracts and purified compounds have significant effects on MM cells there by reducing the viability and growth. The ultimate goal we are would like to achieve is to find a more effective anti-cancer drug by properly modifying the structure of strychnine and brucine. In view of the fact that as many alkaloids such as harringtonine, vincaleukoblastine and vincristine which have strong anti-tumor effects share the indole ring in their chemical structures with strychnine and brucine. These provide new information for the design of cancer chemo preventive agents and the study of these functional groups in the future. Thus, the experimental model established in this above work will surely accelerate the understanding of the structure–activity relationships in strychnine and brucine and pave the way for the discovery of new drugs.

7 Summary

Ethno botanical bioprospection takes advantage of traditional medicinal knowledge for identifying potential phytochemical drugs (Figure 1). The commercialization of medicinal plant resources has become rapid due to the identification, purification and characterization of active bio-molecules for various therapeutic purposes which have been vital for the present scenario. Because, the synthetic drugs exhibit severe side effects along with their mode of action. Hence, “bioprospection” plays an important role in the identification of the novel bio-molecules from the natural resources, especially from plants, having less side effects and they are cost effective. Hence, an active research is required for the identification of the plants which produces active compounds with therapeutic value. The current research aimed at evaluating potential role of *Strychnos genus* in bioprospection and targeted towards identifying lead molecules for the Multiple myeloma (MM) therapy.

Genus *Strychnos* is commonly seed propagated, as vegetative propagation through conventional methods is inefficient, reduced span of seed viability and low germination rate while commercial exploitation for production led to dwindling in natural population. Consequently micropropagation techniques are the ideal for rapid production of these plants. Attempts were made to propagate the recalcitrant *S. nux-vomica and S. potatorum* by *in vitro* and *in vivo* techniques. In natural conditions, because of its hard seed coat, the percentage of germination Is very poor and lengthy (70-120 days to

germinate). This problem can be solved by *in vitro* culture of embryos, there by reducing the germination time and increasing the germination rate. *In vitro* shoot multiplication from *in vitro* raised explants was successful. Sporadic rooting was obtained in *S. potatorum* where as *S. nuxvomica* did not respond. Acclimatization to natural habitat was poor and further experimentation is required.

S. wallichiana is one of the rich sources of important bioactive indole alkaloids. In the present study, strychnine and brucine were isolated from the roots and seeds of *S. wallichiana.* Strychnine and brucine were separated by RP-HPLC. The purified strychnine and brucine were characterized by subjecting to the spectral analysis to reconfirm identity and elucidate the structure. The spectral data (IR, ^{1}H-NMR, ^{13}C-NMR and LC –MS) correlates with the existing literature and reconfirms the presence of strychnine and brucine in south Indian *S. wallichiana's* roots and seeds. The described HPLC procedure could be useful for the qualitative and quantitative analysis of alkaloids of the Loganiaceae family. Evaluation of the pharmacological importance of ethanolic root extracts (*S. nux-vomica, S. wallichiana)* on the MM cell lines (RPMI 8226, and U226B1) showed dose and time dependent growth inhibition. Qualitative and quantitative apoptotic studies were carried out thereby, the elucidation of the underlying molecular mechanisms. Morphological assessment of the cells (Light, Phase, SEM, and TEM) revealed cells were undergoing apoptosis. Nuclear staining assays (by using fluorescent probes like DAPI and Hoechst 33258) were performed by the confocal laser microscope. The flow cytometer analysis reveled loss in DNA content indicating that cell

cycle arrest at G0/G1 phase and preventing cells from entering S or G2/M phase and finally apoptosis occurred. Mitochondrial depolarization was assessed by the flowcytometry using Rhodamine123 derivatives and found that loss in membrane potentials,eventual leakage of mitochondrial proteins, cytochrome *c*, into cytosol and was detected by the Western blot analysis. Syndecan-1 (CD138) expressed on the surface of MM cells, acts as a multifunctional regulator of the cell behavior in the tumor microenvironment and was used as a standard marker for identification of the tumor cells in the MM patients. It is also used as standard marker for differentiating the viable and apoptotic cells. The present study was undertaken to check the CD138 expression levels in the U226 B1 cell line treated with the plant extracts. The flowcytometer analysis of CD138 expression level using FITC labeled CD 138 antibody showed dose dependent decrease in expression levels with increase in concentration of plant extracts indicating cells under apoptosis.

In order to understand the role of active principles in *S. nux-vomica* and *S. wallichiana* root extracts,attempts were made to analyze the extracts by the various analytical Methods (TLC, HPLC, IR spectra, ^{1}H-NMR, ^{13}C-NMR and LC-MS). The two potential bio-molecules (Strychnine and brucine) were identified, which are common in these plants. Strychnine and brucine showed dose and time dependent growth inhibition on the MM cell lines (RPMI 8226, and U226B1). Qualitative and quantitative apoptotic studies were carried out similarly as said above. The above results confirmed *Strychnos* extracts and its bio-molecules have anti proliferative properties. The eventual

accomplishment of this study is to find a more effective anti-cancer drug by properly modifying the structure of these active bio-molecules. This will provide new information for the design of new cancer chemo preventive agents. Thus, the experimental model was established in this work and will surely accelerate the understanding of the structure – activity relationships in these bio-molecules and pave the way for drug discovery. The executive summary of work carried out on "Bioprospecting Indian ***Strychnos"*** was demonstrated in the figure (59).

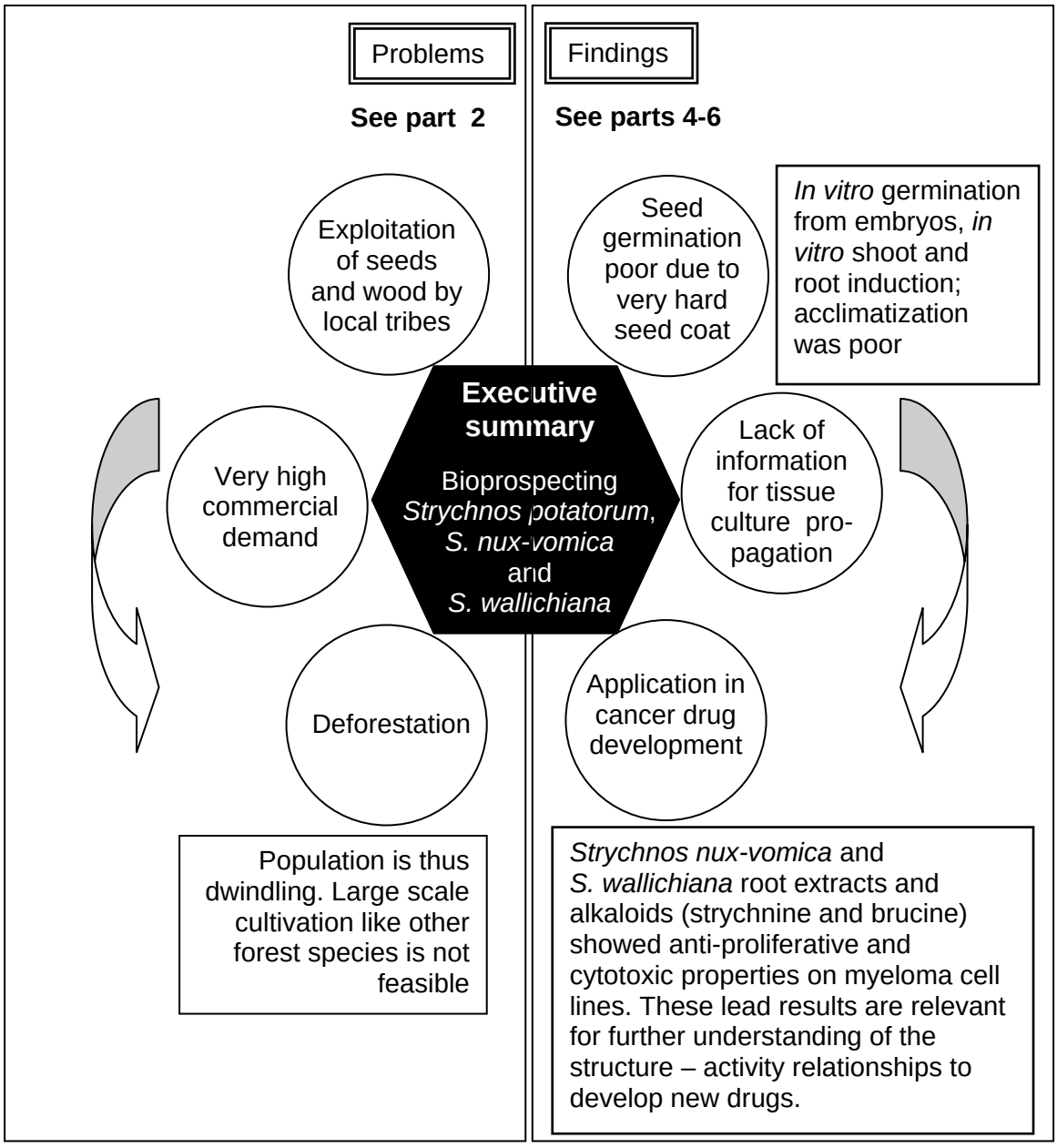


Figure 59. Executive summary of work carried out on "Bioprospecting Indian ***Strychnos*"**

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