IN VITRO CALLUS INDUCTION IN GURMAR (*Gymnema sylvestre*, R.Br.) FOR SECONDARY METABOLITE SYNTHESIS

By

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THESIS

Submitted in partial fulfilment of the requirement for the degree

Master of Science in Horticulture

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Kerala Agricultural University

Department of Plantation Crops and Spices

COLLEGE OF HORTICULTURE

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DECLARATION

I hereby declare that the thesis entitled "***In vitro* callus induction in Gurmar (*Gymnema sylvestre* R.Br.) for secondary metabolite synthesis"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

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Certified that this thesis entitled "***In vitro* callus induction in Gurmar (*Gymnema sylvestre* R.Br.) for secondary metabolite synthesis**" is a record of research work done independently by Mr. **Gholba Niranjan Dilip,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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GHOLBA NIRANJAN DILIP

DEDICATED

to

DR. DILIP GOVIND GHOLBA

my father,

who now mothers me

and whose constant inspiration

and encouragement have

seen me through this degree programme

In sweet memories of

Mrs. Meena Dilip Gholba

Kum. Abhir Shekhar Rege

Mankinds knowledge of medicine proved insufficient to save their lives !

CONTENTS

LIST OF TABLES

List of Tables (continued)

LIST OF FIGURES

LIST OF PLATES

ABBREVIATIONS

%	-	per cent
ABA	-	abscissic acid
AR	-	analytical reagent
BA	-	benzyl adenine
°C	-	degree celcius
cm	-	centimeter
2,4-D	-	2,4-dichlorophenoxy acetic acid
HCl	-	hydrochloric acid
HPLC	-	high performance liquid chromatography
GA_3	-	gibberellic acid
IAA	-	indole-3-acetic acid
IBA	-	indole-3-butyric acid
KN	-	kinetin
µl	-	micro litre
LS	-	Linsmaer and Skoog's medium
µg	-	micro gram
µg l^{-1}	-	micro gram per litre
µg ml^{-1}	-	micro gram per milli litre
g	-	gram
µM	-	micro molar
m	-	meter
M	-	molar
mg	-	milli gram
mg l^{-1}	-	Milli gram per litre
mg ml^{-1}	-	Milli gram per milli litre
min	-	minutes
mm	-	milli litre
½ MS	-	Murashige and Skoog's medium with half the salt concentration
MS	-	Murashige and Skoog's (1962) medium
NAA	-	naphthalene acetic acid
NaOH	-	sodium hydroxide
nm	-	nano meter
ODS	-	optical detector system
PCV	-	packed cell volume
PEG	-	polyethylene glycol
pH	-	hydrogen ion concentration
psi	-	per square inch
rpm	-	revolutions per minute
TLC	-	thin layer chromatography
UV	-	ultra violet
v/w	-	volume in volume
v/v	-	weight in volume

INTRODUCTION

INTRODUCTION

Diabetes mellitus is a metabolic disorder in which sugar levels in blood rise beyond normal (Bell and Hockday, 1996). More than 100 million people round the globe are affected by diabetes. Of this, about 20 per cent diabetic patients are Indians (Mohan, 1999).

Combating such a widely spread disorder demands effective and cheap therapy. Herbal medicines have a significant role in treating diabetes apart from insulin and related drugs. Most systems of traditional medicine use plant parts and their extracts to treat diabetes. Studies on hypoglycemic activity from plants revealed that about 100 plants have antidiabetic activities (Ivorra *et al.*, 1989 and Anon, 1996). The most commonly used plants are *Trigonella foenum-graecum, Gymnema sylvestre, Momordica charantia, Pterocarpus marsupium* etc. Some over-the-counter herbal drugs available against diabetes are 'cogent-db', 'Diabetea' and herbal teas (Rao, 1998).

Gymnema sylvestre R.Br. has a remarkable antidiabetic activity which has been proved scientifically (Shanmugasundaram *et al.*, 1981). The leaves of this vine belonging to the family Asclepiadaceae have been used in Ayurvedic formulations since many centuries. It has a unique ability to rejuvenate the impaired β-cells in the islets of Langerhans. Thus, these cells produce and secrete more insulin, which takes care of diabetes. *Gymnema* also reduces the secondary complications in diabetes and hence, increases the survival time of the patients. Unlike other drugs, *Gymnema* does not produce any toxicity or side effects, since it does not interfere with the normal metabolic pathways (Shanmugasundaram *et al.*, 1983). Owing to all these, *Gymnema sylvestre* is widely popular as a cheap and effective antidiabetic drug.

The existing formulations are prepared by harvesting leaves from the wild habitats. But, constant depletion of forest cover has reduced its supply and *Gymnema* has been designated "vulnerable" to extinction in the IUCN Red List (FRLHT, 1997). Commercial cultivation of *Gymnema* is problematic due to increasing land pressure and its inherent difficulty to propagate. But, obtaining the antidiabetic chemicals from it is equally necessary. Another alternative is to produce these chemicals from *in vitro* cultures of *Gymnema.*

In vitro techniques have been extensively studied to produce different secondary metabolites from various medicinal plants. With the dramatic commercial success of production of shikonin from *in vitro* cultures of *Lithospermum erythrorhizon*, the horizons of this field have widened greatly. It would be a great boon to the diabetic community, if antidiabetic compounds could be produced on large scale from *in vitro* cultures of *Gymnema sylvestre*.

Hence, this study was undertaken to explore the potentials of *in vitro* cultures of *Gymnema* to produce antidiabetic compounds. The objectives of this study were

1. To standardise the media combinations for initiation and proliferation of calli and cell suspension cultures.
2. To evaluate the effect of stress-inducing chemicals on the yields of secondary metabolites.
3. To standardise the techniques for qualitative and quantitative estimation of saponins from *in vitro* cultures of *Gymnema sylvestre*.

REVIEW OF LITERATURE

Gymnema sylvestre R.Br., belonging to the family Asclepiadaceae, has been described in the Indian *Materia Medica* as an antidiabetic plant and in Ayurveda, it has been recommended for the control of 'maturity onset diabetes'. The ability of its leaves to inactivate the taste and sensation of the tongue to sugar and bitter materials has been realised long back. It is also used as a stomachic, stimulant, laxative and diuretic. Literature pertaining to the topic is reviewed under different heads.

2.1 Antidiabetic herbal medicines

Many plants are used against diabetes in different traditional medicinal systems. Bever and Zahnd (1979) published a detailed review of all literature on plants with antidiabetic activity. Similar work was reported by Ivorra *et al.* (1989) in which they reviewed literature on antidiabetic plants, published in between 1978 to 1988. They have given a detailed report on 67 antidiabetic plant species. The prominent ones among them are *Gymnema sylvestre* R.Br., *Panax ginseng* Mey., *Pterocarpus marsupium* Roxb., *Aloe vera* L., *Momordica charantia* L., *Tecoma stans* Juss., *Trigonella foenum-graecum*, *Ganoderma lucidum* Karst., *Centaurea seridis* L., *Bumelia sartorum* Mart., etc.

Survey reports on indigenous hypoglycemic plants from different localities are available. In Bangladesh, Nahar (1993) screened the local flora for antidiabetic activity and reported that the most efficient ones were *Trigonella foenum-graecum*, *Gymnema sylvestre*, *Momordica charantia*, *Pterocarpus marsupium* and *Panax ginseng*. A survey of Canary Islands reported presence of 46 plants with hypoglycemic activity (Anon., 1997a). Recently, *Simlax glabra*, *Opuntia lindheimeri* and *Ruscus* spp. have been reported to possess antidiabetic activity (Anon, 1997b and 1997c).

2.2 *Gymnema sylvestre* R.Br.

Use of *Gymnema sylvestre* in traditional medicine systems is reported from all parts of India and in each language it has a specific name, indicating the widespread recognition of its medicinal properties. It is called as Mesasringi (Ram's horn) or Madhunasini (sweet-destroyer) in Sanskrit; Gur-mar or merasingi in Hindi; Chakkarakolli or Madhunashini in Malayalam and Periploca of the woods in English (Warrier *et al.*, 1995 and Shanmugasundaram *et al.*, 1981).

2.2.1 Botany of *Gymnema*

Gymnema sylvestre R.Br. exudes white latex from stem and leaves and is classified in the Asclepiadaceae family. It's corolla tube has 5 longitudinal ridges which sometimes produce fleshy lobes with lines or stiff hairs along each side (http://).

The genus *Gymnema* has about 25 species which are distributed in tropical and subtropical areas of Asia, South Africa and Oceania. FRLHT (1997) has reported the ecological status of *Gymnema sylvestre* to be 'vulnerable' in India, while, *G. khandalense* and *G. montanum* have become globally endangered.

Gymnema spp. are found from the Himalayan foot hills to tip of the Indian peninsula. *G. sylvestre* R.Br. is found mostly in the deciduous forests of Central and Southern India and in the Western Ghats, till an elevation of 600 m from mean sea level (Warrier *et al.*, 1995).

Thumburaj *et al.* (1996) collected and studied 12 ecotypes of *G. sylvestre* from different locations in Kerala and Tamil Nadu. They characterised the morphology of all accessions and found one from Yercaud to have maximum usable biomass (dry weight of leaf). Different ecotypes of *Gymnema* spp. collected from various areas are maintained at College of Horticulture, Vellanikkara (KAU, 1998).

2.2.2 Biochemicals in *Gymnema sylvestre* R.Br.

The antisweet and hypoglycemic ability of *G. sylvestre* has been intriguing the scientific community for quite a long time. For more than one century biochemical studies were conducted, which reported different compounds from *G. sylvestre*. These were eventually tested for antidiabetic and antisweet abilities. A summarised list of biochemicals reported from *G. sylvestre* is given in Table 1.

Table 1 Biochemicals from *G. sylvestre* leaves

CHEMICAL COMPOUNDS	REFERENCES
SAPONINS	
Gymnemic acids I - X VIII	Yoshikawa *et al.*, 1989a; 1989b; 1997a; 1997b
Gymnemasaponins I – V	Yoshikawa *et al.*, 1991
Gymnemasin A, B, C and D	Sahu *et al.*, 1996
Gymnemoside a, b, c, d, e and f	Yoshikawa *et al.*, 1997a; 1997b
Gymnemaside I to VII	Yoshikawa *et al.*, 1992a
Gypenoside II, V, XXVIII, XXXVII, XLIII, XLV, XLVII, LX, LXII, LXIII, LXXIV	Takemoto *et al.*, 1984a; 1984b, 1984c and Yoshikawa *et al.*, 1992a
Gynosaponin TN-2	Yoshikawa *et al.*, 1992a
POLYPEPTIDES	
Gurmarin	Imoto *etal.* (1991); Kamei *et al.* (1992) and Ota and Ariyoshi (1995)
SUGAR ALCOHOLS	
Conduritol-A	Miyatake *et al.* (1993)
OTHER CHEMICALS	
Hentriacontane	Power and Tutin, 1904

Tritriacontane	Power and Tutin, 1904
Pentatriacontane	Power and Tutin, 1904
Nonacosane	Power and Tutin, 1904
Quercitol	Power and Tutin, 1904
Anthraquinone	Mhaskar and Caius, 1930
Inositol	Mhaskar and Caius, 1930
Tartaric acid	Hooper, 1887
Phytol	Mhaskar and Caius, 1930
Calcium oxalate	Hooper, 1887

The details of each group of biochemicals are given below:

2.2.2.1 Saponins

Saponins from *G. sylvestre* are glycosides of triterpenoids. Triterpenoids in *G. sylvestre* are of two types, viz., oleanane-type and dammarane-type. To these structures are attached oxygen molecules, which bind with sugars like glucose, arabinose, rhamnose, xylose, etc. to form glycosides. Apart from sugar molecules, some other groups like tiglic acid, methyl butyrate, acetyl group, benzoyl group are also attached to the triterpenoid structures in the saponins by oxygen mediated linkages. Different combinations of the attached groups create a large number of saponins.

In 1887, Hooper reported the first systematic examination of *G. sylvestre* leaves. He isolated an acid, which is a glycoside and named it as gymnemic acid. In 1930, Mhaskar and Caius prepared salts of gymnemic acids to be used for clinical trials (Ivorra *et al.*, 1989).

Characterisation and structure elucidation studies were started by Sinsheimer and co-workers in 1968. They published a series of papers on isolation, purification and separation of gymnemic acids. Rao and Sinsheimer (1968) proposed a pentacyclic structure of gymnemagenin, which is the triterpenoid skeleton of gymnemic acids. Sinsheimer and

Rao (1970) isolated genins from mixture of gymnemic acids (saponins). They fractionated the genins to get gymnestrogenin, gymnemagenin and genins G, J, K and N. Using chromatographic techniques like thin layer chromatography (TLC), partition chromatography and column chromatography, they further obtained gymnemic acids A, B, C, D, V, W, X, Y and Z (Sinsheimer *et al.*, 1970). Rao and Sinsheimer (1971) reported a detailed study of chemical property of genins obtained from *G. sylvestre*.

Later on, Dateo and Long (1973) reported different chromatographic techniques for separation of genins and confirmed the obtained genins with previous reports. Chakravarti and Debnath (1981) refined the isolation and extraction methods for getting total saponins and total genins, using different solvents.

With the advent of sophisticated separation techniques like high performance liquid chromatography (HPLC), a lot many studies were reported wherein individual chemicals were separated and tested for antidiabetic and antisweet abilities.

Yoshikawa *et al.* (1989a) isolated gymnemic acids I-IV from water extract of *G. sylvestre* leaves and determined their structures. Yoshikawa *et al.* (1989b) further isolated gymnemic acids V to VII. They also found other saponins, namely gypenoside II, V, XLXII, XLV, XLVII LXXIV and gynosaponin TN-2 which were first reported from *Gynostemma pentaphyllum* Makino., by Takemoto *et al.* (1984a, 1984b, 1984c). Maeda *et al.* (1989) isolated homologues of gymnemic acids I and II and evaluated their antisweet ability.

Yoshikawa *et al.* (1991) identified gymnemasaponins I – V, established their structures and found that the triterpenoid skeleton in them was 23-hydroxylongispinogenin. Liu *et al.* (1992) finalised the structure of gymnemagenin and also found gymnemic acids VIII and IX. Yoshikawa *et al.* (1992a) found dammarane type triterpenoid saponins and named them gymnemasides I - VII. Also they found the presence of gypenosides XXVIII,

XXXVII, LV, LXII, LXIII, which also were first reported by Takemoto *et al.* (1984a, 1984b, 1984c) from *Gynostemma pentaphyllum* Makino. Yoshikawa *et al.* (1992b) isolated and identified five gymnemic acids which they named as gymnemic acids VIII to XII. But since Liu *et al.* (1992) had reported gymnemic acids VIII and IX previously, Yoshikawa *et al.* (1992b) renamed their gymnemic acids VIII and IX as XIII and XIV, respectively. Yoshikawa *et al.* (1993) further isolated and identified gymnemic acids XV - XVIII. Sahu *et al.* (1996) reported new saponins, namely gymnemasins A, B, C and D containing gymnemanol as the sapogenin. Yoshikawa *et al.* (1997a) reported new saponins and named them as gymnemosides - a and b and studied their hypoglycemic activity. Further, Yoshikawa *et al.* (1997b) found gymnemosides - c, d, e and f and studied their effect on intestinal glucose uptake in rats. The details of reported saponins from *G. sylvestre* are shown in Table 2.

The crude extract of *G. sylvestre* which exhibits antidiabetic and antisweet properties simultaneously, is a mixture of many different saponins. When the saponins are separated and tested individually, some show these activities, while some do not exhibit them. Suttisri *et al.* (1995) isolated different saponins from *G. sylvestre* extract and individually quantified their antisweet abilities. They considered the antisweet activity of gymnemic acid-I as a standard measure and denoting it an unit value, the antisweet abilities of all other saponins were quantified on a relative scale, which are shown in Table 3.

Table 3. Relative antisweet potentials of purified saponins from *G. sylvestre*

SAPONINS	ANTISWEET ABILITY (x gymnemic acid-I)
Gymnemic acid - I	1
Gymnemic acid - II	1
Gymnemic acids - III, IV, V, VI, X,	0.5

Table 2 Saponins reported from leaves of *G. sylvestre* R.Br.

Sl. No.	Name of saponin	Triterpenoid skeleton	Sugar moeity	Other groups attached	Economic activity	References
1	Gymnemic acid - I	Gymnemagenin	Glucuronic acid	Tigloyl, Acetyl	Highly antisweet	Yoshikawa *et al.*, 1989a, 1989b, 1992a and 1997b
2	Gymnemic acid - II	Gymnemagenin	Glucuronic acid	(2S)-Methyl butyroyl, Acetyl	Moderately antisweet	Yoshikawa *et al.*, 1989a, 1989b and 1997b
3	Gymnemic acid - III	Gymnemagenin	Glucuronic acid	(2S)-Methyl butyroyl	Slightly antidiabetic	Yoshikawa *et al.*, 1989a, 1989b, 1997a and 1997b
4	Gymnemic acid - IV	Gymnemagenin	Glucuronic acid	Tigloyl	Slightly antidiabetic	Yoshikawa *et al.*, 1989a, 1989b, 1997a and 1997b
5	Gymnemic acid - V	Gymnemagenin	Glucuronic acid	Tigloyl, Tigloyl	Moderately antisweet Slightly antidiabetic	Yoshikawa *et al.*, 1989b and 1997b
6	Gymnemic acid - VI	Gymnemagenin	Glucose (1→3) Glucuronic acid	Tigloyl	Moderately antisweet	Yoshikawa *et al.*, 1989b
7	Gymnemic acid - VII	Gymnestrogenin	Glucuronic acid	-	Slightly antidiabetic	Yoshikawa *et al.*, 1989b and 1997b

Table 2 (continued)

Sl. No.	Name of Saponin	Triterpenoid skeleton	Sugar moeity	Other groups attached	Economic activity	Reference
8	Gymnemic acid - VIII	Gymnemagenin	Glucuronic acid, Arabinose-2-Hexulose	(2S)-Methylbutyroyl	NR*	Liu *et al.*, 1992 Yoshikawa *et al.*, 1993
9	Gymnemic acid - IX	Gymnemagenin	Glucuronic acid, Arabinose-2-Hexulose	Tigloyl	NR	Liu *et al.*, 1992 Yoshikawa *et al.*, 1993
10	Gymnemic acid - X	Gymnemagenin	Glucuronic acid	Acetyl	Mildly antisweet	Yoshikawa *et al.*, 1992b and 1993
11	Gymnemic acid - XI	Gymnemagenin	Glucuronic acid	Tigloyl, Tigloyl	Moderately antisweet	Yoshikawa *et al.*, 1992b and 1993
12	Gymnemic acid - XII	Gymnemagenin	Glucose (1→3) Glucuronic acid	Tigloyl, Acetyl	Moderately antisweet	Yoshikawa *et al.*, 1992b and 1993
13	Gymnemic acid - XIII	Gymnemagenin	Glucuronic acid	(2S) - Methyl butyroyl	Mildly antisweet	Yoshikawa *et al.*, 1992b and 1993
14	Gymnemic acid - XIV	Gymnemagenin	Glucuronic acid	Tigloyl	Mildly antisweet	Yoshikawa *et al.*, 1992b and1993
15	Gymnemic acid - XV	Gymnemagenin	Glucuronic acid	Methyl crotonoyl Methyl butyroyl	Moderately antisweet	Yoshikawa *et al.*, 1993
16	Gymnemic acid - XVI	Gymnemagenin	Glucuronic acid	Methyl crotonoyl Methyl crotonoyl	Moderately antisweet	Yoshikawa *et al.*, 1993
17	Gymnemic acid - XVII	Gymnemagenin	Glucuronic acid	Benzoyl	Moderately antisweet	Yoshikawa *et al.*, 1993
18	Gymnemic acid - XVIII	Gymnemagenin	Glucuronic acid	Benzoyl	Moderately antisweet	Yoshikawa *et al.*, 1993

Table 2 (continued)

Sl. No.	Name of saponin	Triterpenoid skeleton	Sugar moeity	Other groups attached	Economic activity	References
19	Gymnemasaponin - I	23-hydroxy longispinogenin	Glucose	-	Not antisweet	Yoshikawa *et al.*, 1991
20	Gymnemasaponin - II	23-hydroxy longispinogenin	Glucose Glucose	-	Antidiabetic, Not antisweet	Yoshikawa *et al.*, 1991
21	Gymnemasaponin - III	23-hydroxy longispinogenin	Glucose Gentibiose	-	Mildly antisweet	Yoshikawa *et al.*, 1991
22	Gymnemasaponin - IV	23-hydroxy longispinogenin	Gentibiose Glucose	-	Antidiabetic, Mildly antisweet	Yoshikawa *et al.*, 1991
23	Gymnemasaponin - V	23-hydroxy longispinogenin	Gentibiose Gentibiose	-	Antidiabetic, Mildly antisweet	Yoshikawa *et al.*, 1991
24	Gymnemasin - A	Gymnemanol	Glucose (1→3) Glucuronic acid	Tigloyl	NR	Sahu *et al.*, 1996
25	Gymnemasin - B	Gymnemanol	Glucose (1→3) Glucuronic acid	-	NR	Sahu *et al.*, 1996
26	Gymnemasin - C	Gymnemanol	Glucuronic acid	Tigloyl	NR	Sahu *et al.*, 1996
27	Gymnemasin - D	Gymnemanol	Glucuronic acid	Tigloyl	NR	Sahu *et al.*, 1996

Table 2 (continued)

Sl. No.	Name of saponin	Triterpenoid skeleton	Sugar moeity	Other attached groups	Economic activity	References
28	Gymnemoside - a	Gymnemagenin	Glucuronic acid	Tigloyl, Acetyl	Moderately antidiabetic	Yoshikawa *et al.*, 1997a Yoshikawa *et al.*, 1997b
29	Gymnemoside - b	Gymnemagenin	Glucuronic acid	Acetyl, Tigloyl	Antidiabetic	Yoshikawa *et al.*, 1997a and 1997b
30	Gymnemoside - c	Gymnemagenin	Glucuronic acid	Benzoyl, Acetyl	Not antidiabetic	Yoshikawa *et al.*, 1997a and 1997b
31	Gymnemoside - d	Gymnemagenin	Xylose (1→6) Glucose (1→6) Glucose	-	Not antidiabetic	Yoshikawa *et al.*, 1997a and 1997b
32	Gymnemoside - e	23-hydroxy longispinogenin	Xylose (1→6) Glucose (1→6) Glucose and Glucose (1→6) Glucose	-	Not antidiabetic	Yoshikawa *et al.*, 1997a and 1997b
33	Gymnemoside - f	3β, 16β, 23, 28-tetra hydroxyolean-18-ene	Xylose (1→6) Glucose (1→6) Glucose and Glucose (1→6) Glucose	-	Mildly antidiabetic (inhibits intestinal glucose uptake)	Yoshikawa *et al.*, 1997a and 1997b

Table 2 (continued)

Sl. No.	Name of saponin	Triterpenoid skeleton	Sugar moeity	Other attached groups	Economic adivity	References
34	Gymnemaside - I	19-oxo,3β-20S-dihydroxydammar-24-ene	Glucose, Glucose	-	NR	Yoshikawa *et al.*, 1992a
35	Gymnemaside - II	19-oxo,3β-20S-dihydroxydammar-24-ene	Sophrose, Glucose	-	NR	Yoshikawa *et al.*, 1992a
36	Gymnemaside - III	19-oxo,3β-20S-dihydroxydammar-24-ene	Glucose (1→2) Arabinose Glucose	-	NR	Yoshikawa *et al.*, 1992a
37	Gymnemaside - IV	19-oxo,3β-20S-dihydroxydammar-24-ene	Glucose, Primeverose	-	NR	Yoshikawa *et al.*, 1992a
38	Gymnemaside - V	19-oxo,3β-20S-dihydroxydammar-24-ene	Sophrose, Primeverose	-	NR	Yoshikawa *et al.*, 1992a
39	Gymnemaside - VI	2α, 3β, 12β, 20S, 25-pentahydroxy dammar-23-ene	Rutinose	-	NR	Yoshikawa *et al.*, 1992a
40	Gymnemaside - VII	25-hydroperoxy, 2α,3β,12β,20S-tetrahydroxy dammar-23-ene	Primeverose	-	NR	Yoshikawa *et al.*, 1992a

Table 2 (continued)

Sl. No.	Name of saponin	Triterpenoid skeleton	Sugar moeity	Attached groups	Economic activity	References
41	Gypenoside - XXVIII	19-oxo,3β-20(S) dihydroxy dammar-24-ene	Sophrose	-	NR	Takemoto *et al.*, 1984b Yoshikawa *et al.*, 1992b
42	Gypenoside – XLVII	2α,3β,12β, 20(S), 26-pentahydroxy dammar-24-ene	Sophrose, Rutinose	-	NR	Takemoto *et al.*, 1984a Yoshikawa *et al.*, 1989a
43	Gypenoside – LV	3β,19,20(S)-trihydroxy dammar-24-ene	Glucose, Xylose (1→6) β, Glucose	-	NR	Takemoto *et al.*, 1984b Yoshikawa *et al.*, 1989a

Table 3 (continued)

XIII, XIV	
Gymnemic acids - XI, XII, XV, XVI, XVII, XVIII	1
Gymnemasaponins - III, IV, V	0.125

2.2.2.2 Polypeptides

Gurmarin is a polypeptide in *G. sylvestre* leaves. Imoto *et al.* (1991) found that it suppresses the effect of sweet taste stimulus on neural responses in rat and so has antisweet ability. Kamei *et al.* (1992) discovered that gurmarin has 35 amino acids. They also found the sequence of these amino acids in the polypeptide. Ota and Ariyoshi (1995) determined the disulphide linkages among the amino acids in the polypeptide.

2.2.2.3 Sugar alcohol

Conduritol-A, is a sugar alcohol in leaves of *G. sylvestre* which inhibits glucose absorption from intestine and hence exhibits antidiabetic ability. Miyatake *et al.* (1993) isolated conduritol-A and have standardised the procedure for it's purification.

2.2.3.4 Other chemicals in *G. sylvestre*

In 1887, Hooper found a resin with tingling activity from *G. sylvestre* leaves. He also found presence of tartaric acid and calcium oxalate. In 1904, Power and Tutin reported presence of hentriacontane and quercitol. Mhaskar and Caius in 1930 found phytol, inositol and anthraquinone in *G. sylvestre* leaves (Chopra *et al.*, 1958).

2.3 *In vitro* techniques

Plants are living chemical factories. They produce innumerable number of chemicals. Economic plants are getting scarce due to indiscriminate harvests from natural habitats. Plants can be raised in controlled environment by *in vitro* cultivation. The plant organs cultivated *in vitro* too produce chemicals of economic importance. Hence, *in vitro*

methods are useful as an alternative source to produce secondary metabolites.

2.3.1 Literature on *in vitro* production of secondary metabolites

2.3.1.1 Review on *in vitro* secondary metabolism

Bhalsing and Maheshwari (1998) have described the present status of secondary metabolic production by *in vitro* techniques. They have listed the plants which give strikingly high yields of secondary metabolites in *in vitro* cultures, as given in Table 4.

Table 4 *In vitro* yields of ssecondary metabolites

Secondary Metabolites	*Source plant species*	*Yield (% of dry wt.)*
Alkaloids		
Benzophenanthridine alkaloids	*Escholtzia californica*	1.7
Protoberberine alkaloids	*Berberis stolonifera*	10
Berberine	*Coptis japonica*	8.2
	Thalictrum minmus	12.1
Steroids and Terpenoids		
Diosgenin	*Dioscorea deltoidea*	7.8
Sterols	*Delphinium ajacis*	8-10
Ferruginol	*Solvia miltiorrhiza*	1.3
Solasodine	*Solanum khasianum*	2.07
Quinones		
Shikonin	*Lithospermum erythrorhizon*	12.4
Naphthaquinone	*Echium lycopsis*	12.3
Anthraquinones	*Galium* spp.	27

(Bhalsing and Maheshwari, 1998)

Production of shikonin from *Lithospermum erythrorhizon* cultures was the first successful case of *in vitro* production of any secondary metabolite on commercial scale. This was a remarkable breakthrough, as conventionally the plant roots took 3-4 years to grow and then yielded 1-2% shikonin, but the cultures yielded 15-20% shikonin in just 23

days (Fujita, 1983).

Jeon *et al.* (1995) reported production of ginkgolide B in cell cultures of *Ginkgo biloba* L., derived from leaves. Sankar (1998) reported significantly higher yield of ephedrine from *in vitro* cultures (0.02 %) than from plants (0.008 %). Within a short time span of one month, cultures produced ephedrine, while plants needed 7-8 months for the same. Sindhu (1999) reported very high yields of berberine from *in vitro* cultures (10.079 $\mu g\ g^{-1}$ callus) than from plant parts (0.013 $\mu g\ g^{-1}$ callus) of *Coscinium fenestratum*.

Chemicals produced from *in vitro* plant cell cultures are reported by Fowler (1983) as listed in Table 5.

Table 5 Chemicals from *in vitro* plant cultures

Alkaloids	Flavonoids & flavones	Growth regulators
Anthraquinones	Furanocoumarins	Steroids & derivatives
Aromatic chemicals	Latex	Tannins
Benzoquinones	Napthoquinones	Terpenes & terpenoids
Cardiac glycosides	Oils	Vitamins
Chalcones	Perfumes	
Dianthrones	Phenols	

(Fowler,1983)

2.3.1.2 Triterpene and saponin production by *in vitro* cultures

Triterpenoids and their derivatives are secondary metabolites of considerable importance in pharmaceutical industry. Many reports are seen on *in vitro* production of these chemicals.

Cheng and Liang (1981) reported production of saponins and sapogenins from *Panax notoginseng* cultures along with alkaloids from *Rauwolfia yunnanensis* and *Scopalia acutangula*. They found that *in vitro* yields were more than that from plants. Henry and Guignard (1982) studied the production of quillaic acid, a triterpene sapogenin

from *Saponaria officinalis* cell suspension cultures. The cells were cultured in air-lift bioreactors and yield was same as *in vivo*. Burnouf and Paupardin (1983) studied production of oleanane-type triterpenoids in calli cultures of *Chenopodium quinoa*. They found that the roots gave higher yields than calli. Kar and Sen (1985) reported production of sarasapogenin from calli cultures of *Asparagus racemosus*. Hayashi *et al.* (1988) examined production of triterpenoids in calli and cell suspensions of *Glycirrhiza glabra*. The cultures lacked glycirrhizin, a major oleanane-type triterpenoid, but produced betulinic acid and lupeol, which are lupane-type triterpenoids. Fujioka *et al.* (1989) reported production of chekisetsusaponins from callus tissue of *Panax japonicus*, which are the oleanane type triterpenoid saponins. In *Gypsophila* spp., along with calli, even multiple shoot cultures produced triterpenoid saponins (Pauthe-Dayde *et al.*, 1990). They found that root cultures produced maximum secondary metabolites (1.3 mg gm^{-1} of dry weight).

2.3.2 Methods of *in vitro* culturing to produce secondary metabolites

2.3.2.1 Callus culture

Explants are used for raising *in vitro* cultures. Different explants have been reported to give stable cultures. Leaf, root, bark are among the commonly used explants . For juvenile explants, tissues from seed, hypocotyl, cotyledons, buds are used (Narayanaswamy, 1977). Kar and Sen (1985) used shoot segments from *in vitro* derived cultures of *Asparagus racemosus* for raising calli. Sehgal and Khurana (1985) used endosperm from seeds of *Emblica officinalis* to get organogenesis. In *Panax japonicus*, tips of rhizomes were used as explant to evolve calli for producing oleanane-type triterpenoid saponins (Fujioka *et al.*, 1989). Seeds of *Gypsophila paniculata* were germinated *in vitro* and the cotyledons, hypocotyl and root explants from *in vitro* seedlings were used to raise calli by Pauthe-Dayde (1990). Hossain *et al.* (1994) used

nucellus as explant to develop multiple shoots from *Aegle marmelos*. They also used zygotic embryos to produce somatic embryos. Jeon *et al.* (1995) used leaf segments as explants to develop calli in *Ginkgo biloba*.

2.3.2.2 Cell suspension cultures

Gamborg and Shyluk (1981) have reported in detail the growth stages of cultured plant cells, viz; lag phase, log phase, retardation phase and stationary phase. Bhojwani and Razdan (1983) have enlisted the general protocol for initiating and maintaining cell cultures. Hayashi *et al.* (1988) used calli produced from roots of *Glycirrhiza glabra* to produce cell suspensions to synthesise triterpenoids. Sankar (1998) and Sindhu (1999) also used calli to initiate suspension cultures in *Sida* spp. and *Coscinium fenestratum* respectively.

2.3.2.3 Nutrient media for *in vitro* cultures

Culture medium is used as a source of nutrients for growing plant tissues. Different nutrient combinations are standardised and are in use under different names Murashige and Skoog (MS), White's medium, Gamborg B5 medium, Linsmaier and Skoog (LS) medium, Woody Plant medium, Nistch's medium etc. are some commonly used media in plant tissue culture (Narayanaswamy, 1977 and Gupta, 1995). Murashige and Skoog (MS) medium is most popular among them. Fujita (1983) found that calli of *Lithospermum erythrorhizon* showed different growth in different nutrient media. LS media gave good growth of calli but very less shikonin production, while White's medium produced shikonin, but calli growth was restricted. So, a two phase culture system was developed, wherein, growth was first achieved in LS medium and later on shikonin was produced in White's medium.

Kar and Sen (1985) cultured *Asparagus racemosus* on Murashige and Skoog medium to produce sarasapogenin. Hayashi *et al.* (1988) used Linsmaier and Skoog

medium to raise *Glycirrhiza glabra* cultures to produce triterpene glycosides. Pauthe-Dayde *et al.* (1990) used Gamborg's B5 and Murashige and Skoog medium to raise cultures of *Gypsophila* spp. to get triterpenoid saponins. Akashi *et al.* (1994) used Murashige and Skoog medium to culture *Taraxacum officinale* to produce triterpenoids.

2.3.2.3.1 Carbohydrate source

Mizukami et al. (1977) studied suitability of glucose, fructose and sucrose as carbohydrate source for calli cultures of *Lithospermum erythrorhizon*. They found that high sucrose concentrations increased the content of shikonin derivatives, but neither glucose nor fructose could achieve it. Dougall (1980) reported that with increase in concentration of sucrose from 1 to 5 %, the yield of shikonin increased in *L. erythrorhizon*. It remained fairly constant in between 7 and 10 % concentration, but the fresh weight of callus per flask decreased. Fowler and Stepan-Sarkissian (1985) studied in all 24 sugars including disaccharides and monosaccharides (including pentoses and hexoses). Of all these, they found that glucose and sucrose are the only ones which give good growth, metabolite synthesis and biomass yields. Also, sucrose was found to have more pronounced effect on metabolite synthesis than glucose at an equivalent initial concentration.

2.3.2.3.2 Growth regulators

Reports of considerable variation are seen regarding use of growth regulators *in vitro*. Murashige (1974) has given a detailed treatise on influence of phytohormones on growth of *in vitro* cultured tissues. He identified 2,4-D, NAA and IAA as popularly used auxins. He concluded that IAA has lesser potential than other auxins regarding callus initiation and proliferation. Goldsmith (1977) reported 2,4-D to be very potent to promote growth in cultures as it has little polar transport. Hence, it accumulates at one point and promotes growth at that point. In contrast, IAA is polar and disperses, thus reducing its

effect. Cheng and Liang (1981) studied effect of 2,4-D, NAA and Kinetin on saponin production in *Scopalia acutangula, Panax notoginseng* and *Rauwolfia serpentina.* They found that low concentrations of 2,4-D stimulated synthesis of saponins, but retarded growth of cultures. NAA was found to stimulate both growth and synthesis, while kinetin inhibited both. However, *Scopalia* cultures when raised on kinetin free medium and then transferred to kinetin containing medium, they showed presence of metabolites. Kar and Sen (1984) studied effect of 2,4-D and NAA on synthesis of sapogenin in *Asparagus racemosus.* When the cultures were fed with precursor cholesterol, it was found that 2,4-D induced more synthesis of sapogenins than NAA. The rate of synthesis of sapogenin was related to 2,4-D concentrations. In *Plumbago rosea*, Satheeshkumar and Bhavananda (1988) reported callus induction by adding 2,4-D to the medium.

Nissen and Sutter (1990) have studied in detail the *in vitro* stabilities of IAA and IBA. They found that during autoclaving, IBA was more stable than IAA. IBA was stable in liquid MS medium than IAA, but both were equally stable in solid MS medium. Both were degraded in cool fluorescent light in liquid and agar-solidified media. Activated charcoal at 5 % concentration adsorbed more than 97 % of both IAA and IBA in liquid MS medium. Both of them degrade during sterilization and culturing and so their availability is reduced to *in vitro* explants and hence are less effective.

2.3.2.3.3 Stress inducing compounds

The nutrient media has sources of all essential elements needed by the plant tissue for *in vitro* culture. Apart from this, for manipulating growth and metabolite production, many chemicals are added to the media, which are collectively grouped as media additives.

Change in osmotic pressure modifies the properties of cell membrane and hence influences the uptake and exchange of chemicals by the cell wall. Osmotic pressure is

increased by adding mannitol to the culture medium. Rudge and Morris (1986) studied the effect of mannitol at various concentrations on alkaloid accumulation in cultures of *Catharanthus roseus*. They noticed that lower concentrations of mannitol (0.05 to 0.2 M) induced fast growth of cells resulting in early loss of viability. Shorter growth span of cells led to lesser accumulation of alkaloids (0.12 % of dry wt.). Moderate concentration of mannitol (0.3 to 0.6 M) slowered the growth rate of cells, but increased their viability as well as the alkaloid content (0.2 %). At higher concentrations of mannitol (1.0 to 1.5 M), both growth and alkaloid production was decreased. Thus, they concluded that mannitol could increase osmotic pressure and induce stress to enhance metabolite production. *Panax notoginseng* cultures were supplied with mannitol to increase initial osmotic pressure by Zhang *et al.* (1995). They found that, mannitol increased saponin production and intracellular carbohydrate storage while reduced the plant cell volume, cell growth rate and consumption rates of nutrients.

Phloroglucinol was found to increase growth rates of cultures of *Cinchona ledgeriana* (Hunter, 1979). The cultures, without phloroglucinol showed oxidative browning and necrosis of cut surfaces and eventually died. Phloroglucinol increased the growth rate of cultures, which overtook the rate of browning and hence the cultures survived for longer time spans.

Activated charcoal in finely powdered form has high surface area. It being a very good adsorbent, much of the chemicals in nutrient media adhere to it strongly and so the availability of the nutrients and hormones to plant tissue is reduced. This reduces growth and creates stress conditions. Both these trigger synthesis of secondary metabolites (Fridborg and Eriksson, 1975). Activated charcoal in medium was found to inhibit growth of cultures of *Glycine max* and *Haplopappus gracilis*. Those cultures of *Allium cepa* which normally did not produce roots, showed rooting on adding charcoal to

medium (Fridborg and Eriksson, 1975).

Cejka (1985) has reported the composition of malt extract which is used to trigger growth. Malt extract has 70.1 % of reducing sugars and 1.44 % organic acids. Funk *et al.* (1987) used yeast extract to enhance production of secondary metabolites. It gave a steep rise in glyceollin production followed by a fall, in *Glycine max* cultures. While in case of *Thalictrum rugosum*, they found a continuous rise in berberine production.

2.3.3 *In vitro* studies in *Gymnema sylvestre*

Nazeem *et al.* (1991) were the first to report successful *in vitro* callusing in *Gymnema sylvestre*. They reported callus induction from leaf explants in MS medium. It was found that older leaves responded better than younger ones. Callus was produced on MS medium with 1 mg l^{-1} kinetin and 1 or 2 mg l^{-1} NAA.

Anu (1993) and Anu *et al.* (1994) have reported a detailed study on *in vitro* responses of *Gymnema sylvestre*. Anu (1993) found that, the period between January to April was best for raising cultures with minimum fungal contamination. In other months, prevalent rains increased relative humidity of air and also the fungal spore population in air. She also found that dipping explants in 0.1 per cent mercuric chloride solution for ten minutes is sufficient to clear adhering fungal contaminants. MS basal medium with half the concentration of salts was found to be best suitable for organogenesis.

Anu *et al.* (1994) found that, bud break was possible in ½ MS medium with 0.4 ppm kinetin and 2 to 5 mg l^{-1} IAA. Supplementing ½ MS medium with 0.4 mg l^{-1} BAP and 0.8 mg l^{-1} NAA also gave similar results. But the prior combination alone produced healthy shoots with normal leaves. Addition of 30 % coconut water and 2 mg l^{-1} adenine sulphate enhanced bud sprouting. MS medium with full strength all salts was best suited as the basal medium. Anu *et al.* (1994) concluded that the *in vitro* response was poor in case of *G. sylvestre*.

Sandhya and Vilasini (1996) tried explants like shoot tips, axillary buds, leaf discs, internodal segments and mature and immature seeds from *Gymnema sylvestre* to raise *in vitro* cultures. MS medium was found suitable for *in vitro* growth. Callusing was reported with 2 mg l^{-1} BA and 2.5 mg l^{-1} GA_3. Root initiation and shoot elongation was observed in MS medium with 1 mg l^{-1} BA and 2 mg l^{-1} IBA. Nodal bud sprouting was obtained with MS medium supplemented with 2 mg l^{-1} BAP and 0.1 mg l^{-1} NAA. Callus could be grown from leaf and internodal tissue when media had BA and 2,4-D. Immature seeds produced white callus, while mature seeds produced shoots and roots.

Komalavalli and Rao (1997) studied *G. elegans in vitro* and reported that seedling explants responded better than mature explants to produce multiple shoots and roots thereafter. Maximum multiple shoots were produced when MS medium was supplemented with 13 µM BA, 2 µM kinetin, 3 µM NAA, 100 mg l^{-1} ascorbic acid, 10% coconut milk and 1µM GA_3. When kinetin concentration was increased, callus production overtook shoot production. They also found that 15µM IBA gave rooting. But NAA and IAA promoted callusing instead of root development.

Reddy *et al.* (1998) used single node stem explants of *Gymnema* for culturing on MS medium to get shoots. Maximum of 7 shoots per explant were obtained on MS medium supplemented with 5 mg l^{-1} BAP and 0.2 mg l^{-1} NAA. Rooting was achieved on half strength MS medium without any growth regulators.

Reports on *in vitro* metabolite production are lacking in *Gymnema*.

2.4 Biochemical estimation of saponins

2.4.1 Extraction of crude saponins

Sinsheimer *et al.* (1970) extracted saponins from air dried leaf powder of *G. sylvestre* with 95% ethanol by soaking it for 18 hours. The extract was filtered and pH

was adjusted to 2, by 10 % HCl which precipitated all saponins in a crude blackish mass.

Chakravarti and Debnath (1981) used air-dried leaf powder of *G. sylvestre* for extracting saponins. They adopted successive extraction with petroleum ether, chloroform, ethyl acetate and alcohol.

From dried leaves of *Trigonella foenum-graecum*, Varshney *et al.* (1984) extracted saponins. The dried leaves were defatted with petroleum ether and extracted with ethanol. The solvent was removed and resulting syrupy mass was extracted successively with petroleum ether, carbon tetrachloride, chloroform, ethyl acetate and acetone. The residue obtained was dissolved in methanol and saponins were precipitated in large volumes of ether or acetone.

2.4.2 Separation and purification of saponins

Usually in plants, saponins are found in mixtures. The crude saponin mass thus extracted, is a diverse mixture and needs to be separated and purified into saponins and non-saponin-impurities.

Maeda *et al.* (1989) subjected crude saponin from *G. sylvestre* to column chromatography using silica gel 60 and eluted it with chloroform: methanol: water (65 : 35 : 10, lower phase) to get three fractions.

Liu *et al.* (1992) purified the crude saponins from *G. sylvestre* by HPLC (ODS-18) and eluted with methanol (50 %, 70 %, 100 %) subsequently to get eleven fractions. Fractions 5 and 6 were further subjected to HPLC (ODS, recycled liquid chromatography) to get 4 fractions each, by elution with 0.8 % methanol: ammonium carbonate (65 : 35). The third fraction from both, yielded gymnemic acid VIII. The second fraction from fifth fraction was subjected to recycle liquid chromatography with methanol: 0.25 % potassium dihydrogen phospate (2 : 1) to obtain gymnemic acids IV and IX. The tenth fraction was purified by HPLC (ODS) by eluting with methanol: 1 %

ammonium carbonate (3 : 2) to obtain gymnemic acid V.

Yoshikawa *et al.* (1993) subjected crude saponins to column chromatography on Servachrome XAD-2 and eluted with 40-70 % methanol, to get four fractions. The second fraction was repeatedly chromatographed on silica gel with chloroform: methanol: water (65 : 35 : 10, lower layer) and then purified by HPLC (Develosil ODS) with 23-28% methyl cyanide to get gymnemic acids XVII and XVIII. Fraction three was similarly separated and purified to get gymnemic acids XV and XVI.

Yoshikawa *et al.* (1997b) extracted crude saponins from dry leaves from *G. sylvestre* by reversed-phase silica gel column chromatography followed by normal phase chromatography with methanol, to get 8 fractions. Fraction two was separated by HPLC (ODS) to obtain gymnemosides-a, b, c and gymnemic acids I, II and V. Fraction 3 was subjected to HPLC (ODS) and eluted with methanol : 1% aqueous acetic acid (75 : 25) to get gymnemic acids III and IV. Similarly fraction 5 yielded gymnemoside-d, fraction 6 yielded gymnemic acid VII and gymnemasaponin V while fraction 8 yielded gymnemosides-e and f.

2.4.3 Characterisation and quantification of saponins

Saponins are quantified by many other methods. Burnouf and Paupardin (1983) quantified triterpenoid saponins from *Chenopodium quinoa* using a human erythrocyte hemodialysis assay, utilising the property of saponins to cleave blood.

Stepanova (1985) purified the triterpene saponins from *Glycirrhiza glabra* and were quantified by dissolving in ethanol and measuring its optical density at 258 nm wavelength by spectrophotometry.

The total triterpene saponins from *Astragalus dasyanthus* were dissolved in ethanol and 0.7 % vanillin solution in 65 % sulphuric acid was added to the mixture and it was maintained at 60 °C for 1 hour. The mixture was cooled and its optical density was measured at 560 nm wavelength in a spectrophotometer. Reference solution was made

from a standard of dasyanthoside-A (Shemeryankina, 1986).

The purified saponins from *Gymnema* were tested by spectoscopy (NMR, FAB-MS, UV and IR) to identify or confirm their structures and molecular properties (Maeda, 1989; Yoshikawa *et al.*, 1989a; Yoshikawa *et al.*, 1989b and Liu, 1992).

Ionkova and Kartnig (1997) quantified triterpene saponins from transformed hairy roots of *Astragalus mongholicus* by densitometric methods.

HPLC analysis directly gave computed plots with peaks for each saponin, which were used for quantification (Yoshikawa *et al.*, 1997a). Alternatively, the purified and identified chemicals were quantified by weighing and expressing it as percentage of initial dry weight of sample.

2.3.5. TLC for saponin estimation

Thin layer chromatography has been used quite popularly for qualitative and quantitative determination of saponins. Kesselmeier and Ruppel (1979) studied saponins from *Avena sativa* L. They separated saponins on TLC plates with silica gel coat by using running solvent systems like chloroform :methanol : acetic acid: water (170 : 30 : 20 : 6); chloroform :methanol : water (70 : 30 : 4) and ethyl acetate :methanol : water (100 : 16.5 : 13.5).

Furuya *et al.* (1983) quantified saponins from *Panax ginseng* by eluting on silica gel 60 TLC plates with upper layer of n-butanol : ethyl acetate : water (4 : 1 : 5). Spots were developed by spraying 10 % sulphuric acid followed by heating at 105 °C for 10 min. Quantification was done by scanning and densitometric analysis at 530 and 700 nm wavelengths.

Vanhaelen and Vanhaelen-Fastre (1984) studied saponins from *Eleutherococcus senticosus* Maxim., *Panax ginseng* Meyer and *Picorrhiza kurroa* Royle. All were spotted on silica-gel 60 TLC plates. The mobile phases used for *P. ginseng* and *E. senticosus*

was 1,2-dichloroethane: ethanol: methanol: water (65 : 22 : 2 : 7), while that used for *P. kurroa* was dichloromethane: methanol: water (40 : 10 : 1). The spots were developed with a 1:1 mixture of 1 % vanillin in ethanol and 5 % sulphuric acid in ethanol followed by heating at 120 °C for 2.5 min. The plates were scanned at 530 nm wavelength and the peaks were generated. Quantification was done with respect to peaks generated by standard samples of reference saponins. For *P. kurroa*, scanning was done at 268 and 285 nm wavelengths.

Dioscorea deltoidea saponins were eluted on TLC plates with lower phase of chloroform: methanol: water (65 : 35 : 10). The furostanol saponins were developed by spraying Ehlrich's reagent while others were detected with methanol: H_2SO_4 (1 : 1) (Drapeau *et al.*, 1986).

Mathur *et al.* (1994) estimated saponins from *Panax quinquefolium* on TLC plates by elution with chloroform: methanol: water (13: 7: 2, lower phase). The spots were visualised by spraying with 10 % sulphuric acid (v/v) followed by heating at 100 °C for 10 minutes. Quantification was done using densitometry by scanning at 530 and 700 nm wavelength.

Mukherjee *et al.* (1996) investigated the crude extract of *G. sylvestre* leaves to identify the components by TLC techniques. The results are shown in Table 6.

Table 6 TLC investigations on tinctures of *G. sylvestre*

Running solvent system	*R_f values*	*Spray reagent*	*Chemicals identified*
Chloroform : acetone (95 : 5)	0.083, 0.45, 0.77	Vanillin- phosphoric acid	Saponins/steroids
Ethyl acetate : petroleum ether (3 : 1)	0.06, 0.21, 0.78	Antimony trichloride (10%)	Flavonoids
Chloroform : Methanol (6 : 4)	0.7,0.77, 0.85, 0.96, 0.48	Modified anisaldehyde sulphuric acid	Sugars

MATERIALS AND METHODS

MATERIALS AND METHODS

The study entitled '*In vitro* callus induction in Gurmar (*Gymnema sylvestre* R.Br.) for secondary metabolite synthesis' was carried out in the Plant Tissue Culture Laboratory of the Centre for Plant Biotechnology and Molecular Biology, and Biochemistry Laboratory of the All-India Co-ordinated Research Project on Medicinal and Aromatic Plants, College of Horticulture, Vellanikkara between May, 1998 to November, 1999. The materials used and the methodology adopted in this study are described below.

3.1 Culture media

The medium suggested by Murashige and Skoog, 1962 (MS medium with full strength of all salts) was reported to be the best basal medium for callus induction in *Gymnema sylvestre* by Anu (1993) and hence, was used as the basal medium in the present study. The composition is given in Appendix I.

3.1.1 Preparation of MS medium

All the chemicals of AR grade, used as ingredients and additives in the MS medium were procured from M/s Merck India Ltd., Sisco Research Laboratories, Pvt. Ltd., British Drug House and Sigma Ltd. Borosilicate glasswares of Corning, Vensil and Borosil brands were used. The glassware was cleaned by soaking in solution of potassium dichromate in sulphuric acid for half an hour. Later, the glass containers were washed with jets of tap-water to remove all traces of potassium dichromate solution. Then, they were further cleaned with 0.1% Teepol detergent solution and were washed thoroughly with water and rinsed twice with double distilled water. These were then dried in hot air oven at more than 100 °C for 24 hours and later stored in cupboards free of dust till further use.

Medium was prepared by following the standard procedure adopted by Gamborg and Shyluk (1981). Stock solutions of major and minor elements were prepared and stored in precleaned amber coloured bottles in refrigerated conditions.

A cleaned steel vessel, rinsed with distilled water was used to prepare the medium. Aliquots from all stock solutions were pipetted in proportionate volumes in the vessel. Little amount of distilled water was added to it and later on required quantities of sucrose (carbohydrate source) and inositol were added and dissolved in it. The desired volume was made up by adding distilled water. The pH of medium was adjusted in between 5.5 to 5.8 using NaOH or HCl.

For solid medium, agar from ^{M}/s Sisco Research Laboratories Pvt. Ltd. was added at 0.75 % ($^{w}/_{v}$) concentration, after adjustment of pH. The medium was stirred and heated to melt the agar and was poured when hot, into culture vessels which were plugged with cotton. For solid media, test tubes (15 cm x 2.5 cm) were used, while for liquid media, conical flasks (100 ml volume) were used as culture vessels. About 15 ml medium was poured into each test tube and 30 ml medium in each conical flask. Vessels containing media were sterilized in an autoclave by applying a pressure of 15 pounds psi for 20 minutes. After this, the culture vessels were kept in culture rooms till further use.

3.1.2 Growth regulators

Different plant growth regulators were added to the basal MS medium. The stock solutions of growth regulators at 1000 mg l^{-1} were prepared and stored under refrigeration and aliquots were taken from them for use after dilution. These aliquots were added to the medium, before the pH was adjusted.

The growth regulators used were auxins (2,4-D, NAA, IAA and IBA) and cytokinins (BA and Kinetin). The different concentrations in which the phytohormones were used, are given in Table 7.

Table 7 Growth regulators used in media

Plant Growth Regulators	*Respective concentrations in mg l^{-1}*
2,4-D	0.25, 0.5, 1, 2 and 5
NAA	0.25, 0.5, 1, 2 and 5
IAA	0.25, 0.5, 1, 2 and 5
IBA	0.25, 0.5, 1, 2 and 5
2,4-D + BA	0.25 + 0.1, 0.25, 0.5, 1 and 2
2,4-D + BA	2 + 0.1, 0.25, 0.5, 1 and 2
2,4-D + KN	0.25 + 0.1, 0.25, 0.5, 1 and 2
2,4-D + KN	2 + 0.1, 0.25, 0.5, 1 and 2
NAA + BA	2 + 0.1, 0.25, 0.5, 1 and 2
NAA + BA	5 + 0.1, 0.25, 0.5, 1 and 2
NAA + KN	2 + 0.1, 0.25, 0.5, 1 and 2
NAA + KN	5 + 0.1, 0.25, 0.5, 1 and 2

3.1.3. Media additives

Details of other chemicals added to the media for manipulating *in vitro* production of secondary metabolites are listed in Table 8.

Apart from ABA, all other additives were added to the medium before sterilization. ABA was filter sterilized and added after the medium was autoclaved. Stock solutions were prepared for ABA, phloroglucinol and silver nitrate. All others were weighed and added directly to the medium before the final volume was made.

Table 8 Media additives used to manipulate *in vitro* synthesis of secondary metabolites

Media additives	Concentration	
	Solid medium	Liquid medium
ABA	0.1, 0.5 and 1 mg l^{-1}	0.5 mg l^{-1}
Peptone	1 and 2 %	1 %
Yeast extract	1 and 2 %	1 %
Malt extract	1 and 2 %	1 %
Activated charcoal	0.1, 0.25 and 0.5 %	0.25%
Phloroglucinol	25, 50 and 100 mg l^{-1}	50 mg l^{-1}
Silver nitrate	5, 10 and 15 mg l^{-1}	10 mg l^{-1}
Polyethylene glycol	2 and 10 %	10 %
Mannitol + Sucrose	1 % + 2 % and	
	2 % + 1 %	
Mannitol	3 %	3 %
Sucrose	1 and 2 %	1 %
Agar	0.75 and 0.9 %	

The composition of basal medium had 3% sucrose as the carbohydrate source. It was not added to the media when mannitol and/or sucrose were added as mentioned in Table 8.

3.2. Collection and preparation of explants

3.2.1 Source of explants

Explants for *in vitro* culture were collected from potted plants of *Gymnema sylvestre* R.Br. maintained in the glasshouse of College of Horticulture, Vellanikkara. These plants were maintained free from diseases by regular spraying of fungicides, namely Emisan 0.3 % and Bavistin 0.1 % at weekly intervals.

From the potted plants and staked vines of *Gymnema sylvestre* in the glasshouse, branches of 2.5 mm diameter were selected, which were green to greenish brown in colour. One to 1.5 feet long terminal portion of these branches were cut with a sharp razor blade and were enclosed in a sterile glass cintainer.

For obtaining root explants, young plants maintained in polybags were used. The ploybags were emptied and the roots were carefully separated from all adhering soil. The arial parts of these plants were cut off by a razor blade and discarded. The roots were then washed thoroughly in water and only 1 to 1.5 mm thick roots were selected these were cut and separated. They were enclosed in another sterile glass container.

The explants in closed sterile glass containers were taken to the Plant Tissue Culture Laboratory.

3.2.2 Preparation of explants

In the laboratory, the stem was cut into pieces of about 10 cm length. These and the roots were separately washed in a frothing solution of detergent (Teepol, 0.01 %). Then to remove all traces of the detergent, they were thoroughly washed 3 to 4 times under running tap water.

From the 10 cm long stem segments, the leaves were severed at the node, such that the leaves retained the entire petiole. These stem segments were cut into nodal and internodal segments of not more than 1.5 cm length. Care was taken to maintain the axillary bud at the centre of the nodal segments.

These and the roots were separately wiped with clean swabs of cotton soaked in 70 % ethanol.

3.2.3 Surface sterilization

The explants were surface sterilized under aseptic conditions maintained inside a laminar air-flow cabinet. The nodes, internodes, leaves and roots were separately dipped in 0.1% mercuric chloride solution added with 3 to 4 drops of Teepol detergent (0.01%). These containers were shaken intermittently. The nodal segments were retained such for 12 minutes while roots, leaves and internodes were dipped only for 6 minutes. Later on, they were thoroughly washed 5 times with sterile distilled water and were spread on sterile filter papers to drain inside the laminar air-flow chamber.

Explants free from adhering water were trimmed at edges to remove all drying-off tissues. The explants tested for callus induction were:

1. Leaf explants:
    a. Leaf lamina (one cm^2 area around midrib)
    b. Leaf petiole (stalk of leaf with adjacent part of lamina)
2. Stem explants:
    a. Nodal segment (1.5 cm long with one node and axillary bud)
    b. Internodal segment (1.5 cm long)
3. Root explant:
    a. Root segment (1.5 cm long)

3.3. Inoculation procedure

Inoculation was carried out under strict aseptic conditions inside laminar air-flow chambers from M/s Klenzaids or Kirloskar Pvt. Ltd. makes. The floor of the work-bench, inside the chamber was wiped thoroughly with 100 % ethanol to remove any traces of dust or adhering dirt. Sterilized forceps, petridishes, surgical blades, knives and blotting papers

were kept inside the chamber and the whole chamber was sterilized with UV light for 30 minutes. Surface sterilised explants were inoculated in the culture tubes. One explant per test tube was placed horizontally on the surface of medium.

In case of treatments having media additives, the explants were inoculated on medium consisting of MS + 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}). After one month, the callus was subcultured, at the rate of approximately one gram per tube in media having stress inducing additives and the above basal medium.

For inoculating the liquid media, calli were used as explants. Calli grown in test tubes were removed under aseptic environments inside the laminar air-flow chambers and were made free of any adhering pieces of media. The calli were cut into pieces of approximately one gram and one such piece was transferred to each conical flask

3.4. Culture conditions

The cultures were incubated in a closed room providing 3000 lux fluorescent light for a period of 16 hours followed by 8 hours of dark period daily. The temperature was maintained at 26 ± 2 °C and humidity between 60 and 80 %.

The liquid cultures were kept on rotary shakers at 105 rpm and cultured at 28 ± 2 °C temperature in diffused light.

To find the effect of low temperature on secondary metabolite production, one treatment was maintained under refrigerated condition at 6 to 7 °C temperature.

3.5. Subculture intervals

Subculturing was done in aseptic conditions similar to those employed during inoculation of explants. Subculturing of calli was done every 4 weeks in fresh medium, having respective composition. Approximately one gram of callus was subcultured in each

tube. The liquid cultures were subcultured after every 7 days into respective fresh media. The conical flasks were allowed to stand for 5 minutes for settling the cell aggregates and then 5 ml of the supernatent was poured into another sterilised conical flask with 25 ml of fresh medium.

3.6 Layout of experiments

Following experiments were conducted.

(A) Influence of auxins and explants

The cultures were raised on MS media supplemented with auxins namely 2,4-D, NAA, IAA and IBA, each at concentrations of 0.25, 0.5, 1, 2 and 5 mg l^{-1}. The control was full MS basal medium.

For each of the levels of auxins and for control, explants like node, internode, petiole, leaf lamina and root segments were used.

The effect of auxins and explants on callusing was recorded.

(B) Influence of combinations of auxins with cytokinins

From the previous experiment, specific levels of auxins were selected and each of them was combined with cytokinins. 2,4-D (0.25 and 2 mg l^{-1}) and NAA (2 and 5 mg l^{-1}) were each combined with BA and kinetin at 0.1, 0.25, 0.5, 1 and 2 mg l^{-1} concentrations each. The media containing only auxins in concentrations mentioned above, but lacking any cytokinins, were used as control. The experiment was conducted to select the best combination of auxins with cytokinins.

Internode, petiole and leaf lamina were the explants used for culturing. But, the effect of explants was not evaluated.

From these 12 high callus yielding treatments were selected and their saponin yields at 2 months age were computed. The treatment combination producing maximum saponins per day per tube was selected as the best.

(c) Influence of stress producing chemicals

From the second experiment, a best combination was selected. It was used as basal for the experiment and also as control. The leaf lamina, petiole and internodal explants were cultured on this media (MS + 2,4-D 2 mg l^{-1} + BA 1 mg l^{-1}) to develop callus. When the callus was of one month age, it was subcultured into the media containing stress inducing chemicals (as mentioned in table 8) along with the basal medium. One gram of callus was subcultured in each tube during subculturing. The age of callus was recorded after subculturing. Saponins were estimated at one and two months age after subculturing. The medium producing maximum saponins per day per tube was selected as the best.

3.7 Observations of growth of cultures

3.7.1. Observations on growth of callus

The observations taken for calli cultures were:

(a) Percentage of cultures initiating calli

Of all the inoculated tubes, those, which showed signs of callusing, were counted and were expressed as percentage of total number of inoculated tubes.

(b) Number of days for callus initiation

The time interval between the day of inoculation to the day when first visible signs of growth were seen, was counted as number of days for callus initiation.

(c) Percentage of tubes producing full-tube callus

Of all the inoculated tubes, those which produced full-tube or more callus, were counted and this was expressed as percentage of total number of inoculated tubes.

(d) Number of days to produce full tube callus

The time taken for callus to grow and cover the complete surface of medium in test tube was recorded.

(e) Callus index

This was computed to get an overall view of the initaition and proliferation of callus as influenced by any medium. At the end of the culture period, based on the growth of the callus in each tube, the tubes in each treatment were classified into different categories as shown in Figure 1. The categories were decided by judging the extent of surface of media covered by the calli. These categories of callus growth are given in Table 9. Each growth category was attributed a value, correlated to its growth and was denoted as 'growth score' (G_i). This value increased with the extent of growth. The values ranged from a minimum of 0 to a maximum of 9.

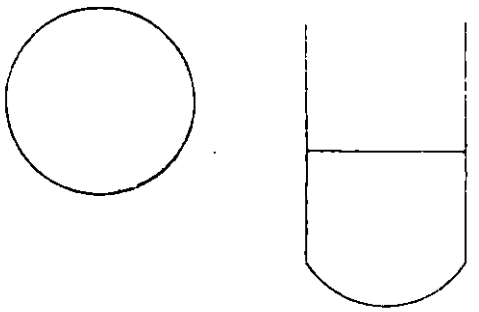
The number of tubes in each category was expressed as the percentage of the total number of tubes inoculated in that treatment and was denoted as 'P_i'. The product of P_i and G_i for each growth category was computed. The sum of such products for all categories in a treatment was termed as the 'callus index' (C.I.).

Callus index was computed by the formula:

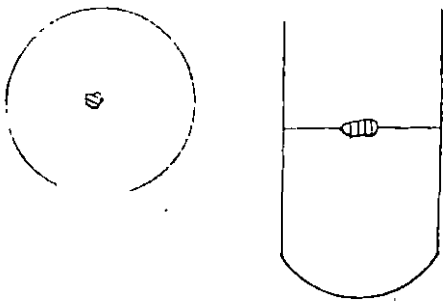
$$\text{C.I.} = \sum_{i=1 \text{ to } n}^{n} (P_i \times G_i)$$

Figure.1 DIFFERENTIAL GROWTH OF CALLUS OBSERVED FROM IN VITRO CULTURES OF GYMNEMA SYLVESTRE R.Br.

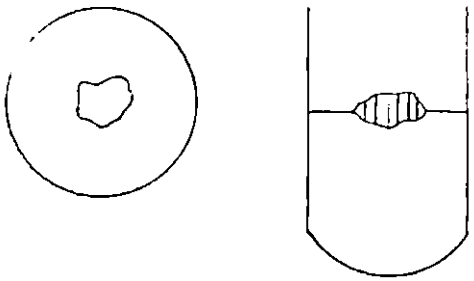
in top view and cross sectional view of test tube.



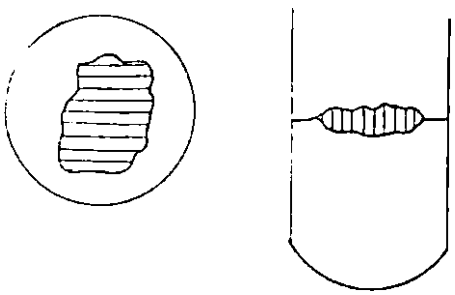
No callus initiation



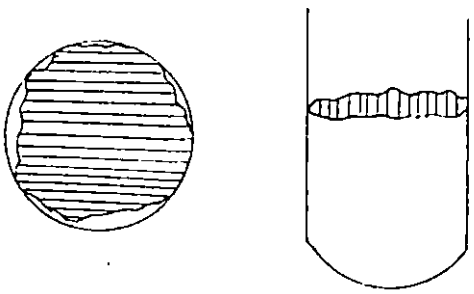
Specks of callus



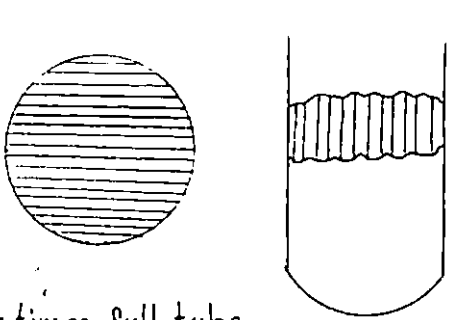
¼ tube callus



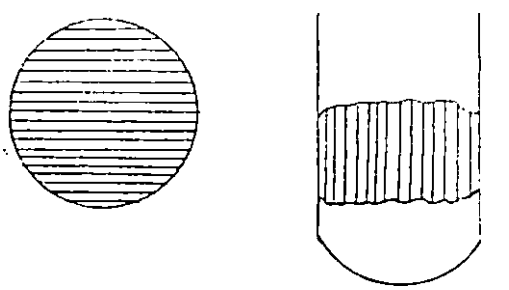
½ tube callus



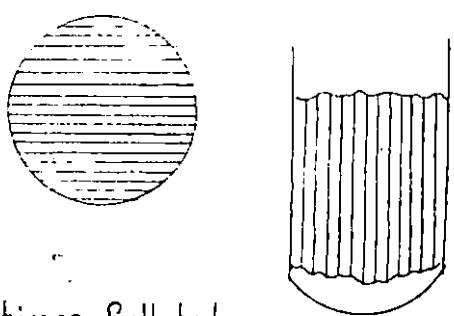
Full tube callus



1.5 times full tube



2 times full tube



3 times full tube

where, P_i = percentage of tubes showing calli in a particular growth category,

G_i = growth score of a particular growth category

i = number of a particular growth category

n = total number of growth categories in a treatment

Table 9 Differentiation of callus growth into various categories and respective average weight of callus per tube in each category

Extent of callus growth at end of culturing period	Growth score (G_i)
No callus initiated	0
Very little callus	1
Callus covering about 1/4th surface of medium	2
Callus covering about 1/2 surface of medium	3
Callus covering full-tube	4
Callus covering 1.5 times full-tube	5
Callus covering 2 times full-tube	6
Callus covering 2.5 times full-tube	7
Callus covering 3 more times full-tube	8
Callus covering 3.5 times full-tube	9

P_i being the percentage of tubes in each growth category, the sum of percentages for any treatment is always 100. Hence, callus index gave the estimate of callus produced by 100 tubes for a particular treatment.

Since, the growth score ranged from 0 to 9, the values of callus index ranged between 0 to 900. So the maximum obtainable value of callus index was 900. So the treatments having callus indices near 900 were considered as the best, while those with values near 0 were considered to have poor callusing ability.

(f) Callus growth rate

The callus growth rate was computed on per day and per tube basis. To have an exact estimate of the growth, the weight of callus produced per tube was found. For this, some representative calli from each growth category were removed from the tubes and weighed. The average weight of callus per tube was computed for each category (denoted as W_i). For incorporating the time factor, the number of days needed to produce callus in each growth category was computed (denoted as D_i) and used.

The growth rate was computed by the following formula:

$$\text{G.R.} = \sum_{i=1 \text{ to } n}^{n} \frac{P_i \times W_i}{100 \times D_i}$$

where, P_i = percentage of tubes producing calli in a particular growth category,

W_i = average representative weight of callus per tube in a particular growth category

D_i = number of days needed to achieve the specified growth in any category

i = number of a particular growth category

n = total number of growth categories in a treatment

Callus growth rate was expressed as g $(\text{tube})^{-1}$ $(\text{day})^{-1}$ of callus.

Since the summation of percentage of tubes in each category (Σ P_i) is 100, division was done by 100 to obtain callus growth rate on per tube basis. The growth rate is superior to the callus index, because it measures the growth in absolute terms by considering the actual weight of callus produced per tube. Also, it considers the time factor.

Hence, it highlights the treatment producing maximum callus within minimum time.

(g) Callus morphology

The colour and texture of the calli were noted. Friable calli being made up by the adhesion of granules, the influence on the size of these granules was recorded.

3.7.2. Observations on growth of cell suspension cultures

The observations recorded on the growth of cell suspension cultures were as below.

(a) Cell number per ml of medium

Cell suspension cultures were homogenized by agitation and about 1 ml of the suspension was poured on a watch glass under sterile conditions of a laminar air-flow chamber. From this, about 0.2 ml was pipetted and about 0.1 ml was placed on the grid on each side of a hemocytometer. A cover slip was placed on it and the number of cells were counted in all 9 squares on the grid of the hemocytometer (in a volume of 1 µl). Readings from both grids were noted and their average was computed to per ml basis.

(b) Cell viability

The drop of suspension culture taken previously on the slide was mixed thoroughly with 10 µl Evan's blue solution (0.01 %) and observed under a magnification of 40X through a light microscope. The blue stained cells were either dead or ruptured or broken. All the fields on the slide were screened and the number of total cells and number of stained cells were counted. From this, the number of living (unstained) cells was computed by subtracting the number of stained cells from the number of total cells. It was then expressed as the percentage of total cells counted. This was denoted as cell viability.

No. of living cells = (Total no. of cells) – (No. of stained cells)

Cell viability = $\frac{\text{(No. of living cells) x 100}}{\text{Total no. of cells}}$

(c) Packed cell volume

The conical flasks containing the cell suspensions were allowed to stand for 5 minutes and then 5 ml volume of the supernatant culture was removed and centrifuged at 2000 rpm for 5 minutes in a graduated centrifuge tube. The cell mass accumulated at base of the tube. Its volume was noted and expressed on percentage basis.

3.8 Biochemical estimation of saponins

Total saponins were estimated from calli and cell suspension cultures as a measure of antidiabetic chemicals synthesized *in vitro*. A thin layer chromatography-densitometry method was developed for qualitative as well as quantitative estimation of the saponins. This method is described below. The experiments conducted to standardize this method are described thereafter.

3.8.1. Extraction of crude saponins from *in vitro* cultures

3.8.1.1 Extraction from calli

The calli were collected from test tubes and all adhering pieces of nutrient media were separated and discarded. The cleaned calli were weighed and 10 gm callus was taken per sample. It was ground in mortar and pestle with 30 ml of 60 % ethanol solution. The ground calli in solvents were retained for 6 hours for extraction, filtered through Whatman No.4 filter paper and were heated to evaporate only alcohol. The original volume was maintained by addition of distilled water. It was allowed to cool and was mixed with an

equal volume of a mixture of chloroform : methanol (1:1) and agitated in a separating funnel, which separated it into two layers. The lower layer of chloroform was separated and the solvent was evaporated to get a fraction, which contained saponins with minimum impurities. The non-saponins in the upper aqueous layer, were discarded. The extract from the chloroform fraction was taken for estimation of saponins.

3.8.1.2 Extraction from liquid cultures

To determine the amount of total saponins produced by cell cultures, five ml of liquid culture was extracted by grinding in 60% ethanol followed by extraction and fractionation with chloroform : methanol (1 : 1) similar to the method described above. The chloroform extract thus obtained, was used for estimation of saponins.

3.6.2. Preparation of TLC plates

TLC plates were prepared by coating 300 µm thick layer of silica gel-G on glass plates of 20 cm x 20 cm size. Forty grams of silica gel G of SRL brand was weighed and added to 85 ml of distilled water. Immediately, it was shaken vigorously for 30 seconds to get a homogenous slurry which was poured in the trough of the TLC plate gel applicator of CAMAG brand. The plates were passed one by one beneath the trough and each was coated with a 300 µm thick coat of silica gel G. About eight plates were coated with a slurry made from 40 grams of silica gel G. The plates were coated quickly, within 2 minutes, to avoid setting of the silica within the applicator itself. The plates were allowed to dry for about ten minutes, after which they were placed in aluminium racks and kept in chromatographic ovens for heating at 110 °C for 30 min. This desiccated the plates and activated them for further use.

3.8.3 Preparation of sample

For calli and cell suspension cultures the chloroform fraction was taken in test tubes and was added with 1 ml of 100 % ethanol. The tubes were shaken well till a clear golden yellow solution was obtained. This solution was used for spotting on TLC plates.

Apart from estimating the extract of cell suspension cultures, another estimation was done to find whether saponins were secreted by the plant cells into the culture medium. Five ml of the liquid culture was centrifuged at 7000 rpm for 5 minutes. This settled the cells at the bottom. Cell free liquid medium constituted the supernatant. Five µl of the supernatant was taken in capillary tubes and spotted on TLC plates for this estimation.

3.6.4. Spotting on TLC plates

Spotting on TLC plates was done with the help of capillary tubes. The capillary tubes were calibrated to 5, 10, 15 and 20 µl marks. Using micropipettes, 5, 10, 15 and 20 µl volumes of distilled water were successively pipetted. These volumes were sucked by the capillary tubes and the respective levels of water in them were marked to get calibrated capillary tubes. Samples were spotted on activated TLC plates with calibrated capillary tubes. Usually, 20 µl volume was spotted per sample. Some samples had less quantity of saponins, which could not be visualised by spotting 20 µl sample. For them, 40 µl of the sample was spotted.

Spotting was done 2 cm above the lower edge of the plate in a straight line parallel to the lower edge. A distance of 1.5 cm was maintained between two consecutive spots. Spotting was done either in circular spots or in horizontal streaks. Circular spots were not allowed to exceed 6 mm in diameter. Streaks were restricted to 1 cm in length and 2 mm in

breadth. Sample was not allowed to spread and exceed the specified limits. This was done by intermittent application of small doses of the sample. After application of each small dose, the solvent was evaporated from the point of application by spraying hot air from a hair dryer. When it dried, repeated dose of sample was applied on the same spot. This was done till all the 20 µl volume of the sample was completely applied. On each plate, two spots of the reference standard sample of 26 % gymnemic acid, procured from M/s Laila Impex, Hyderabad, were spotted in concentrations of 5.2 and 10.4 mg ml^{-1} respectively, to aid in quantification. These concentrations were specifically used because they produced spots containing 26 and 52 µg saponins respectively when 5 µl volume of each was applied.

3.8.5 Running solvent systems

Running solvent systems were prepared by mixing different solvents in desired ratios. For purpose of quantifying the saponins, the elution was done in a solvent mixture of chloroform : acetone : methanol in the ratio of 5 : 1 : 1.5. About 70 ml of this solvent system volume was used to elute four plates at a time in a CAMAG TLC developing glass tank. The solvent system was poured in the tank and lid was placed tightly. Adjacent to the walls of the tank were placed filter paper sheets. The tank was shaken once vigorously. It was retained as such for 30 min to saturate with the vapours of the volatile components of the solvent system. Then the spotted plate was placed in the tank such that the edge of the plate below the spots was immersed in the solvent system, but spots were above the surface of solvent system. The lid was closed tightly to avoid the loss of volatile solvents. All chromatograms were eluted between 29 to 30 °C temperature and at 72 to 75 % relative humidity, to maintain uniformity in the elution pattern. In approximately 30 minutes, the solvent eluted the spots vertically upto 2/3 rd length of the plate. Then the plate was removed for developing.

3.8.6 Preparation of the spray reagent

To detect the saponins on the TLC plates, vanillin sulphuric acid spray reagent was used. It was prepared in 2 solutions and sprayed separately one after other, each, approximately 5 ml. First to be sprayed was 5 per cent (v/v) sulphuric acid in ethanol, while the second to be sprayed was 3 per cent (w/v) vanillin solution in ethanol.

3.8.7 Developing the chromatogram

The eluted plates were placed under an exhaust flow of air to evaporate the solvents from the silica gel coat on the plate. When the reverse side (uncoated) of the plate became free of all moisture droplets, the plate was considered to be free of running solvent. Using a Vensil reagent sprayer, each plate was sprayed with a minimum of 10 ml of vanillin sulphuric acid spray reagent. The spraying was done in an exhaust chamber. The eluted chromatogram was uniformly sprayed all over, with fine droplets of spray reagent. Care was taken to avoid use of excess force in spraying, as it would scrape out the silica gel coat, which would result in loss of a part of the chromatogram.

The sprayed plate was kept at 110 °C for 3 to 5 minutes in a chromatographic oven to develop coloured spots. The plate was removed and observations were documented.

3.8.7 Quantification of saponins

Quantification was done by using densitometric techniques. The developed plate, was scanned when it was hot. Scanning was done immediately before the colour of the saponin spots faded due to cooling. Scanning was done using the Herolab RH scanner attached to AlphaImager 1200 computer software, which analyses scanned data. Adequate setting of aperture and focus was achieved. The balance of black, white and gamma radiation intensities was achieved to get maximum contrast of spots against the white

background. Adjustments were done such that the scanned image resembled the view as perceived by human eyes. The image was stored and analysis was done in the 'SPOT DENSO' mode of the AlphaImager software. The spots of saponins and standard reference sample were selected by delineating their boundaries. The background values were deducted and the known values for standard reference spots were fed. Using cubic-spline fit, the data were generated from the saponin spots.

The standard spots were having 26 and 52 µg of gymnemic acid (as computed from HPTLC data provided by M/s. Laila Impex, Hyderabad). Using these, other saponin spots were quantified and saponin content of each was expressed in µg units. This result was then computed on per gram basis for calli and per ml basis for cell suspension cultures.

3.8.9 Experiments conducted to standardise some steps in the estimation of saponin using TLC

3.8.9.1 Standardisation of solvents to extract saponins

Ethanol at strengths of 100, 60, 50 and 30 % was evaluated to find out its ability to extract saponins. For this experiment, callus from the medium MS + 2 mg l^{-1} 2,4-D + 1 mg l^{-1} BA was used. Ten grams of the callus was ground in 30 ml of each of the ethanol solutions. It was kept as such for 6 hours for extraction and was then filtered through Whatman No. 4 filter paper. The filtrate was evaporated under vacuum for obtaining solvent-free sample.

The weights of the extracted samples were noted for each solvent. They were expressed as percentage of initial weights of the callus. Also, the samples were eluted and their chromatograms were evaluated for the clear separation of saponins. Using densitometric techniques, the saponin spots from each sample were quantified with respect to standard gymnemic acid. The best one was used further for isolating saponins from other chemicals present in the extracts.

3.8.9.2 Selection of appropriate method to separate saponins from other compounds present in the extracts

The alcohol extracts have many organic substances other than saponins, which interfere with the saponin spots of the thin layer chromatograms. They were to be eliminated from the saponins for getting better and clearer chromatograms. To eliminate other compounds from saponins in the alcohol extracts, a method suitable for *in vitro* samples, as suggested by Dr. M.R. Heble, Head, Plant Cell Technology Division, BARC, Mumbai, was evaluated (hereafter referred to as 'fractionation method'). Herein, the calli were ground with a solution of 60% ethanol in distilled water and kept for 6 hours. It was filtered through Whatman No. 4 filter paper and only alcohol was evaporated. While heating, the loss in volume due to evaporation of alcohol was made up by addition of distilled water and the original volume was maintained. When all the alcohol was completely evaporated, it was allowed to cool. Then, it was mixed with an equal volume of a mixture of chloroform : methanol (1:1) and agitated in a separating funnel, which separated it into two layers. The lower layer of chloroform was collected and the chloroform solvent was evaporated to get saponins with minimum impurities. The non-saponin impurities, which collected in the upper aqueous layer, were discarded.

The samples obtained from both these fractions were compared with those obtained from ethanol extracts for their chromatographic expressions of saponins.

3.8.9.3 Evaluation of the different methods of applying samples on TLC plates

Effect of application of sample in circular spots and horizontal streaks was examined under various concentrations of saponins. This was done to examine reduction of interference of non-saponin materials with saponins to improve the clarity and visibility of the chromatogram.Samples in different concentrations were spotted by these methods.

3.8.9.4 Standardisation of running solvent systems

The composition of running solvent system being a very crucial factor in resolution of the saponin spots (Hamilton and Hamilton, 1987), many different running solvent systems were tried with the following objectives:

(a) to get a single condensed spot having all saponins to aid quantification and

(b) to fractionate the saponins in maximum number of spots to get a profile for characterization.

The solvent systems tried are listed in Table 10. For evaluating the performance of these running solvent systems, samples were eluted in each of them and the chromatograms were compared. The samples were extracted from callus grown on MS + 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}) medium. Ten µl volume of the sample solution of 1 mg ml^{-1} concentration, was spotted on the plates. The plates were eluted and developed and the chromatograms were evaluated on following lines:

(a) *Capacity to elute the applied sample*

The capacity of a running solvent to elute more compounds from a sample was judged by the quantity of the sample eluted upwards. To get an idea about the quantity of sample eluted upwards, the quantity of sample remaining uneluted was noted. The uneluted sample, which remained at the point of application, was graded based on the intensity of colour produced by it.

When the entire sample was eluted, no colour was seen at the point of application and the running solvent was graded as 'very good'. When small quantity of the sample remained uneluted, yellow colour was seen, and 'good' grade was allotted. When more sample remained uneluted, shades of brown colour were noticed. Within these, grading was done as 'satisfactory', 'poor' and 'very poor', with increasing shades of brown.

(b) Elution pattern

Whether the solvent system condensed the saponins in one spot or produced a profile of many spots was observed. A running solvent system producing a profile of more than 12 distinct saponin spots was selected as suitable for characterization studies, whereas, one giving a single condensed spot of saponins was judged as the best for quantification.

(c) Clarity of the chromatogram

Whether the saponin spots were distinct or were mixed with other organic compounds, was noted. Whether these organic compounds masked or interfered with the visibility of saponin spots was recorded. Screening was done with the objective of getting high fidelity in the chromatograms.

(d) Accumulation at solvent front

The extent of accumulation of compounds at the solvent front was noted. This was graded as nil, less, moderate and high. It was considered as a measure of compounds rendered useless for quantification. So lesser the accumulation, better was the running solvent.

Table 10. Running solvent systems tried for suitability to elute the saponins from callus of *Gymnema sylvestre* on thin layer chromatograms.

Solvent system number	*Running solvent systems*	*Ratio of solvents*
1.	Chloroform (100 %)	
2.	Ethyl acetate (100 %)	
3.	Methanol (100 %)	
4.	Water (100 %)	
5.	Methanol (100 %) followed by elution in Chloroform (100 %)	

6.	Hexane : Ethyl acetate	3 : 1
7.	Chloroform : Ethyl acetate : Acetone : Methanol	5 : 0.5 : 1 : 1
8.	Chloroform : Ethyl acetate : Acetone : Methanol : Water	25 : 3 : 5 : 16 : 1
9.	Chloroform : Ethyl acetate : Acetone : Water	30 : 19 : 3 : 2
10.	Chloroform : Ethyl acetate : Methanol	97 : 2 : 1
11.	Chloroform : Acetone : Methanol	10 : 1 : 1
12.	Chloroform : Acetone : Methanol	7 : 1 : 1
13.	Chloroform : Acetone : Methanol	5 : 1 : 1
14.	Chloroform : Acetone : Methanol	5 : 1 : 1.5
15.	Chloroform : Acetone : Methanol	5 : 1 :2
16.	Chloroform : Acetone : Methanol	5 : 1 :3
17.	Chloroform : Acetone : Methanol : Water	64 : 10 : 16 : 1
18.	Chloroform : Methanol	98 : 2
19.	Chloroform : Methanol	95 : 5
20.	Chloroform : Methanol	90 :10
21.	Chloroform : Methanol	80 : 20
22.	Chloroform : Methanol	50 : 50
23.	Chloroform : Methanol	25 : 75
24.	Chloroform : Methanol	80 : 20, followed by repeated elution with chloroform : methanol (98:2)
25.	Chloroform : Methanol	80 : 20, followed by repeated elution with chloroform : methanol (95:5)
26.	Chloroform : Methanol : Water	60 : 40 : 10
27.	Chloroform : Methanol : Water	65 : 50 : 10
28.	Ethyl acetate : Methanol	70 : 30
29.	Ethyl acetate : Methanol : Water	200 : 10 : 10
30.	Ethyl acetate : Methanol : Water	100 : 10 : 10
31.	Ethyl acetate : Methanol : Water	100 : 13.5 : 10

32.	Ethyl acetate : Methanol : Water	100 : 20 : 10
33.	Ethyl acetate : Methanol : Water	80 : 20 : 10
34.	Ethyl acetate : Methanol : Water	80 : 30 : 10
35.	Ethyl acetate : Methanol : Water	80 : 40 : 10
36.	Ethyl acetate : Methanol : Water	70 : 33 :5
37.	Ethyl acetate : Methanol : Water	70 : 40 : 10
38.	Ethyl acetate : Methanol : Water	40 : 40 : 10
39.	Ethyl acetate : Methanol : Water	40 : 40 : 10 followed by repeated elution with Ethyl acetate : Methanol : Water (70 : 33 : 5)
40.	Three times successive elution in Chloroform : : Methanol	90 : 10

3.8.9.5 Standardisation of spray reagent

The spray reagents evaluated for detecting saponins were:

(1) Vanillin sulphuric acid reagent

It was prepared in 2 solutions and sprayed separately one after other, each, approximately 5 ml. First was 5 per cent (v/v) sulphuric acid in ethanol and second was 1 per cent (w/v) vanillin solution in ethanol.

(2) p-anisaldehyde sulphuric acid

In 50 ml glacial acetic acid, 5 ml sulphuric acid and 0.5 g *p*-anisaldehyde were added and were used fresh every time. On every plate, 10 ml of the reagent was sprayed.

Coloured spots were used for visual certification of the presence of saponins. The colours and R_f values of the coloured spots were recorded for different spray reagents. These were compared for selecting the suitable one for detecting saponins. For better visualization of the saponins at lower concentrations, some modifications were made in the compositions of the spray reagents. They are listed as below:

(1) Vanillin sulphuric acid

(a) 5% sulphuric acid in ethanol and 1% vanillin in ethanol

(b) 5% sulphuric acid in ethanol and 2% vanillin in ethanol

(c) 5% sulphuric acid in ethanol and 3% vanillin in ethanol

(d) 5% sulphuric acid in ethanol and 4% vanillin in ethanol

(e) 5% sulphuric acid in ethanol and 5% vanillin in ethanol

(f) 10% sulphuric acid in ethanol and 1% vanillin in ethanol

(g) 25% sulphuric acid in ethanol and 1% vanillin in ethanol

(2) p-anisaldehyde sulphuric acid reagent

(a) 1% *p*-anisaldehyde in acetic acid

(b) 2% *p*-anisaldehyde in acetic acid

Their ability to develop bright spots of saponins even when saponins were in low concentration was evaluated.

3.8.10 Observations on the saponin production from the *in vitro* cultures

The following observations were recorded for extracts from callus cultures as well as cell suspension cultures.

(a) R_f values of saponin spots

The R_f values of the coloured saponin spots were recorded for each sample.

(b) Colour of saponin spots

The colours of the saponin spots were recorded. The saponins were identified by purple, blue violet and red colours.

(c) Saponin yields

From the TLC plates, saponins were quantified and expressed as μg of saponins per gm of callus. In case of cell suspension cultures, the saponin yield was expressed as μg of saponins per ml of culture and μg of saponins per ml of medium.

(d) Rate of production of saponins

This measure accounts for the saponins produced per tube per day in each treatment. Thus, it helps to compare the treatments and select the more economic ones. It was calculated as below,

Saponin Yield Index = (Growth rate) x (Saponin yields)

Division was done with the age at which saponins were estimated, to get the per day yield of saponins. Multiplication of saponin yields with the growth rate results in highlighting the treatment which produces maximum callus as well as maximum saponins per tube per day.

3.9 Statistical analysis

All the experiments had two replications. Using the completely randomised design, the analysis of variance was done. The means were compares by Duncan's multiple range test at 5 per cent level of significance.

RESULTS

RESULTS

The results of the, study '*In vitro* callus induction in Gurmar (*Gymnema sylvestre* R.Br.) for secondary metabolite synthesis' are presented in this chapter.

The results are presented under five major heads, viz.,

(a) Influence of explants and auxins on callus induction.

(b) Influence of combinations of auxins and cytokinins on callus growth and secondary metabolite production.

(c) Influence of other media additives on secondary metabolite production.

(d) Cell suspension cultures.

(e) Standardisation of biochemical techniques.

4.1 Influence of auxins and explants on callusing

4.1.1 Effect of auxins on callus growth

The effect of auxins on callus induction and proliferation is presented in Table 11.

4.1.1.1 Effect on percentage of tubes initiating calli

Among the auxins, 2,4-D had the highest mean value of callus initiation percentage (65.024). Next were NAA and IAA, which showed statistically par performances. The least percent callus initiation was from IBA (17.16 %). All the auxins, at all concentrations were superior to control. With increasing concentrations of 2,4-D and NAA, increase in percentage of callus initiation was noted. In both the auxins, increase was prominent above 1 mg l^{-1} concentration. No such trend was observed with respect to increasing concentrations of IAA and IBA.

The highest callus initiation was found from 5 mg l^{-1} 2,4-D (70.59 %) followed by 5 mg l^{-1} NAA (68.97 %).

Table 11 Effect of auxins on callus growth in *Gymnema sylvestre* R.Br.

Auxins	Percent tubes initiating callus	Days for callus initiation	Maximum growth of callus	Days for producing maximum callus	Callus index	Callus growth rate
Control (MS basal)	0^{d}	0	Nil	-	0	0
2,4-D						
0.25 mg l^{-1}	56.25	25^{c}	Full-tube	60^{f}	39.7	6.4
0.5 mg l^{-1}	58.07	23^{bc}	Full-tube	60^{f}	35.8	6.0
1 mg l^{-1}	54.8	23^{bc}	Full-tube	45^{d}	47.9	10.6
2 mg l^{-1}	61.4	20^{ab}	Full-tube	45^{d}	44.6	10.1
5 mg l^{-1}	70.59	19^{a}	Full-tube	43^{d}	41.1	10.6
Mean	*65.02*^{a}	*22*^{A}			*41.85*	*8.7*
NAA						
0.25 mg l^{-1}	28.58	35^{e}	¼ tube	50^{e}	7.7	1.5
0.5 mg l^{-1}	44.69	35^{e}	¼ tube	40^{e}	17.0	2.3
1 mg l^{-1}	24.4	33^{de}	¼ tube	38^{bc}	7.3	1.9
2 mg l^{-1}	50	30^{d}	¼ tube	33^{a}	23.8	7.3
5 mg l^{-1}	68.97	30^{d}	¾ tube	50^{e}	50.3	9.6
Mean	*47.22*^{b}	*32.6*^{B}			*21.2*	*4.5*
IAA						
0.25 mg l^{-1}	42.31	35^{e}	¼ tube	38^{bc}	9.1	2.3
0.5 mg l^{-1}	35.56	35^{e}	Specks	38^{bc}	8.6	2.2
1 mg l^{-1}	29.42	34^{e}	Specks	36^{abc}	9.2	2.5
2 mg l^{-1}	41.88	33^{de}	Specks	36^{abc}	8.0	2.2
5 mg l^{-1}	36.95	33^{de}	Specks	36^{abc}	7.5	2.1
Mean	*41.54*^{b}	*34*^{B}			*8.5*	*2.3*
IBA						
0.25 mg l^{-1}	14.29	45^{f}	¼ tube	50^{e}	4.4	0.7
0.5 mg l^{-1}	22.58	45^{f}	Specks	48^{de}	4.6	0.9
1 mg l^{-1}	12.5	44^{f}	Specks	48^{de}	2.4	0.2
2 mg l^{-1}	26.93	42^{f}	Specks	48^{de}	1.2	0.1
5 mg l^{-1}	11.76	42^{f}	¼ tube	46^{de}	1.8	0.3
Mean	*17.16*^{c}	*43.6*^{C}			*2.9*	*0.7*
Grand Mean	40.7	34.33			14.9	4.1

4.1.1.2 Effect on the number of days required for callus initiation

The auxin treatments needed significantly lesser days to initiate callus than control (Table 11). The average performances of all the all levels of each auxin showed that 2,4-D needed least time (22 days) to initiate callus. NAA needed 32.6 days while IAA needed 34 days, but their performances were statistically on par. Performance of IBA was very poor as it took maximum number of days (43.6) among all auxins, though this was significantly lesser than control (60 days).

With the increase in concentrations of auxins, a uniform decrease was observed in the number of days needed for callus initiation. Higher concentrations of 2,4-D and NAA (2 and 5 c) were better than respective lower concentrations.

Fastest callus initiation was noticed in just 19 days from 5 mg l^{-1} 2,4-D treatment.

4.1.1.3 Effect on proliferation of callus

The maximum callus produced in a period of 60 days was recorded as a measure of callus proliferation. Maximum proliferation observed for 2,4-D was the production of full-tube callus growth (Table 11). NAA could proliferate callus, which covered only ¼ th surface area of the culture tube. NAA at 5 mg l^{-1} concentration only, could produce callus, that filled ¾ th surface of the culture tube. IAA and IBA produced very little callus. Hence, maximum callus proliferation in *G. sylvestre*, was exhibited by media supplemented with 2,4-D at all concentrations. Since, control failed to prodece any callus, all auxins performed superior to control.

4.1.1.4 Effect on number of days required for callus proliferation

Since the maximum growth produced by all auxins was different, the number of days needed to produce it too was different and hence could not be compared. In general, increase in auxin concentrations, was found to reduce the days needed for maximum proliferation of callus (Table 11). Thus, faster proliferation was obtained with 2 and 5 mg l^{-1} concentration of all

auxins, than at any other concentration. The fastest full-tube callus was produced in 43 days by 5 mg l^{-1} 2,4-D treatment.

4.1.1.5 Effect on callus index

The values for calli indices (Table 11) showed that, all the treatments performed significantly superior than the control. The mean values of callus index for all concentrations of auxins showed that 2,4-D had a maximum callus index of 41.85, followed by NAA (21.25), IAA (8.51) and IBA (2.9). The different concentrations of 2,4-D showed different values of callus index, but no uniform increase or decrease was noted in callus index with increase in concentration of 2,4-D. With increasing concentrations of auxins, only NAA showed an increasing trend in values of calli indices. Callus index showed a drastic increase at 2 and 5 mg l^{-1} concentrations of NAA. The variation in values of callus indices from different levels of IAA and IBA showed no significant variation. All were nearly similar to each other.

Highest callus index of 50.3 was obtained from 5 mg l^{-1} NAA treatment followed by 44.63 mg l^{-1} 2,4-D and 41.15 from 5 mg l^{-1} 2,4-D.

With the increasing concentrations of 2,4-D, the calli indices did not show any continuous rise or fall. Alternate rise and fall was noted in the calli indices. So, 0.25 and 2 mg l^{-1} concentrations of 2,4-D were considered for further experimentation. IAA and IBA showed not significant decrease in calli indices with increase in concentrations.

4.1.1.6 Effect on callus growth rate

The growth of callus expressed as the quantity of callus produced per tube per day. These values are presented in table 11. The control treatment failed to produce any callus and so its growth rate was zero. All auxins performed significantly better than control. Mean value of growth rate for 2,4-D at all concentrations was the highest of 8.77 mg $(day)^{-1}$ $(tube)^{-1}$, followed by 4.55 mg $(day)^{-1}$ $(tube)^{-1}$ from NAA. IAA showed a slower growth rate of 2.3 mg $(day)^{-1}$ $(tube)^{-1}$ while IBA was slowest with a growth rate of 0.72 mg $(day)^{-1}$ $(tube)^{-1}$.

The highest growth rate of 10.64 mg $(day)^{-1}$ $(tube)^{-1}$ was noticed from 2,4-D (1 mg l^{-1}), followed by 10.62 from 2,4-D (5 mg l^{-1}) and then 10.13 mg $(day)^{-1}$ $(tube)^{-1}$ from 2,4-D (2 mg l^{-1}).

As in the previous cases, only NAA showed an increasing trend in growth rate with increase in its concentration. 2,4-D did not show any such trend as its values oscillated. IAA and IBA had nearly similar growth rates at all concentrations and so no trend was noticed.

4.1.2 Effect of explants on callusing

The effect of various concentrations of auxins in combination with different explants on callus initiation and proliferation is shown in Table 12.

None of the explants produced any callus in control medium (full MS) but produced callus when media had auxins. This indicated that the presence of auxins was necessary to induce callus from all the explants of *G. sylvestre*.

The mean callus index values for all level of auxins showed that, when the media had 2,4-D, maximum callus index was exhibited by internode (70.04), followed by petiole (49.5). With increasing concentrations of 2,4-D, none of the explants showed any general trend of increase or decrease in calli indices.

NAA containing media produced maximum callus index with petiole explant (25.8) followed by internode (22.22) and leaf lamina (21.14). In NAA containing media, all explants showed a distinct trend of rise in callus index with rise in NAA concentrations. From 0.25 to 1 mg l^{-1} levels, the rise in callus index was less, but beyond 2 mg l^{-1} concentration the rise was quite prominent. At 5 mg l^{-1} concentration, this rise in calli indices was further continued in leaf and root explants, but the stem explants showedreduction in callus index at 5 mg l^{-1} of NAA.

When media contained IAA, maximum callus index was produced by internode (14.7), followed by node (13.16). Similarly in case of IBA containing media, maximum callus was produced by node (9.52) followed by internode (3.52). With respect to increasing concentration

Table 12 Effect of explants and auxins on callus indices in *Gymnema sylvestre* R.Br.

Auxins	Explants				
	Leaf lamina	Leaf petiole	Node	Internode	Root
Control (MS basal)	0	0	0	0	0
2,4-D					
0.25 mg l^{-1}	50.0	36.6	40	65.5	0
0.5 mg l^{-1}	17.2	64.3	46.5	49	5.4
1 mg l^{-1}	27.9	43.7	76.5	89.2	2.1
2 mg l^{-1}	85.8	25.9	12.2	84	30.0
5 mg l^{-1}	24.9	77.0	60.9	62.5	3.3
Mean	*41.1*	*49.5*	*47.5*	*70.0*	*8.16*
NAA					
0.25 mg l^{-1}	3.3	5.7	17.5	7.3	4
0.5 mg l^{-1}	1.8	15.0	8.6	13.6	8
1 mg l^{-1}	2.8	8.3	21.3	4.4	0
2 mg l^{-1}	11.6	30.0	28	50	2
5 mg l^{-1}	86.2	70.0	13.3	35.8	36.3
Mean	*21.1*	*25.8*	*17.7*	*22.2*	*10.1*
IAA					
0.25 mg l^{-1}	1.8	10	16.6	17.4	0
0.5 mg l^{-1}	0	6.6	15	15	6.6
1 mg l^{-1}	0	7.5	6	14.5	18.3
2 mg l^{-1}	2	4.4	18.2	15.4	0
5 mg l^{-1}	2	6.6	10	11.1	8
Mean	*1.1*	*7.0*	*13.16*	*14.7*	*6.5*
IBA					
0.25 mg l^{-1}	0	0	15	2.8	1.8
0.5 mg l^{-1}	0	3.3	10	4.0	6
1 mg l^{-1}	2.2	2.5	5	2.5	0
2 mg l^{-1}	0	6.6	14.1	8.3	0
5 mg l^{-1}	0	5	4	0	0
Mean	*0.4*	*3.5*	*9.62*	*3.5*	*1.5*
Grand Mean	**15.98**	**21.15**	**21.99**	**27.6**	**6.5**

of IAA and IBA, no trend of increase or decrease in callus index was noticed for any explant. In the case of IAA and IBA containing media, stem explants performed better in callus production, followed by petiole explants.

The mean callus index value for each explant derived from all auxin treatments indicated that maximum callus index of 27.62 was produced by internode, followed by node (21.99), then petiole (21.45), leaf lamina (15.98) and lastly root (6.59).

For high callusing auxins like 2,4-D and NAA at all concentrations, explants like internode and petiole were best. For low callusing auxins like IAA and IBA, explants like internode and node were ideal to get some quantity of callus. Internode explants performed exclusively superior in all auxins. Following internode were petiole and node, which performed at par with one another. Leaf lamina and root explants ranked last in callus initiation and proliferation. The root explants performed consistently poor in all auxins throughout the range of 0.25 to 5 mg l^{-1} concentrations.

The stem and leaf explants showed preference towards 2,4-D, followed by NAA, but the root explant preferred for callusing.

4.1.4 Effect of combinations of auxins and explants on colour and texture of callus

The colour of callus of 60 days age, developed by auxin media by using different explants is shown in Table 13. The colour of callus after culturing for 60 days indicated that the leaf explants (lamina and petiole) showed wide variation in colour producing white, green, lemon yellow and translucent colours in nearly equal proportions. Whereas, the stem explants produced mostly green coloured callus. The root explants produced callus of white colour in response to all auxins.

The 2,4-D containing media produced more of white callus from all explants, whereas media containing NAA produced more of lemon yellow callus. IAA and IBA containing media produced uniformly green callus for all explants, as and when callus was produced.

Table 13 Effect of explants and auxins on callus colour at 60 days age in *Gymnema sylvestre*

Auxins	Leaf lamina	Leaf petiole	Node	Internode	Root	Mean effect of auxins
Control (MS basal)	-*	-	-	-	-	-
2,4-D						
0.25 mg l^{-1}	Green	White	Green	Green	-	Green
0.5 mg l^{-1}	White	White	White/Green	White	-	White
1 mg l^{-1}	Translucent	White	Green	White/Green	-	White
2 mg l^{-1}	White	White	White	White	White	White
5 mg l^{-1}	Translucent	Translucent	White/ Translucent	White	-	White
Predominant colour/s	Translucent White	White	White	White	White	White
NAA						
0.25 mg l^{-1}	-	-	Green	-	-	Green
0.5 mg l^{-1}	-	Green	Green	Green	-	Green
1 mg l^{-1}	-	Translucent	-	-	-	Translucent
2 mg l^{-1}	Yellow	Yellow	Green	Translucent	-	Yellow
5 mg l^{-1}	Yellow	Yellow	Green	Yellow	-	Yellow
Predominant colour/s	Yellow	Yellow	Green	Yellow	-	Green Yellow
IAA						
0.25 mg l^{-1}	-	-	-	Green	-	Green
0.5 mg l^{-1}	-	Green	Green	Green	Green	Green
1 mg l^{-1}	-	Green	Green	Green	-	Green
2 mg l^{-1}	-	Green	-	Green	-	Green
5 mg l^{-1}	-	-	Green	Green	-	Green
Predominant colour/s	-	*Green*	*Green*	*Green*	*Green*	*Green*
IBA						
0.25 mg l^{-1}	-	-	-	Green	-	Green
0.5 mg l^{-1}	-	-	-	-	-	-
1 mg l^{-1}	-	Green	-	-	-	Green
2 mg l^{-1}	-	-	-	-	-	-
5 mg l^{-1}	-	-	-	-	-	-
Predominant colour/s	-	Green	-	Green	-	Green
Colour score shown by explants	Green (1) White (1) Translucent(2) Yellow(2)	Green (5) White (4) Translucent (2) Yellow (2)	Green (11) White (4) Translucent (1	Green (10) White (4) Translucent (1) Yellow (3)	Green (1) White (1)	Green (28) White (14) Translucent (6) Yellow (7)

*No callus was produced in these treatments, so colour could not be reported

The callus colour at low concentrations of 2,4-D and NAA was mostly green for all explants. But with increase in auxin concentration, the colours change towards more white in 2,4-D and more lemon yellow in NAA media.

After 60 days, the calli showed browning, which may be due to phenolic interference. In some cases, translucent calli changed to green.

The texture of callus produced by each explant in each treatment is shown in Table 14. Friable and compact type of texture was noted in calli from *G. sylvestre*. Friable texture was noticed more commonly. Despite various explants and various concentrations of auxins, mostly all treatments produced friable callus.

It was also found that friable callus had green and lemon yellow as more predominant colours. The friable calli were composed of loosely attached calli particles. The size of these particles was bigger in green and lemon yellow calli than in white and transluscent calli. Some calli, in young stages showed white flaky regions, which later on grew into larger particles and merged into friable calli.

The smaller the size of particles forming friable callus, higher was the water content of callus and vice-versa. Ageing and browning was found to increase the water content.

4.2 Effect of combinations of auxins with cytokinins on callusing and saponin production

4.2.1 Influence on callusing

4.2.1.1 Influence of auxins with cytokinins on callus initation

(A) Influence on percentage callus initiation

The percentage of tubes initiating calli in each combination of auxins and cytokinins are shown in Table 15. The mean per cent callus initiation values for auxin and cytokinin combinations were superior to the mean value of all control treatments.

Table 15 Influence of combination of auxins and cytokinins on the percentage of tubes initiating callus

AUXINS / CYTOKININS	2,4-D			NAA			Mean percentage of tubes initiating callus
	0.25 mg l^{-1}	2 mg l^{-1}	Mean	2 mg l^{-1}	5 mg l^{-1}	Mean	
BA							
0 mg l^{-1} (control)	64.7	59.1	61.9	70	76.4	73.2	67.55^{d}
0.1 mg l^{-1}	88.8	91.66	90.23	80	81.25	80.62	85.42^{abcd}
0.25 mg l^{-1}	90	94.4	92.2	100	88.8	94.9	93.32^{ab}
0.5 mg l^{-1}	100	86.9	93.45	100	85.7	92.85	93.15^{ab}
1 mg l^{-1}	94.1	100	97.05	85.7	100	92.85	94.95^{a}
2 mg l^{-1}	91.3	73.9	82.6	100	83.3	91.65	87.12^{abcd}
Mean	*92.84*	*89.37*	*91.1*	*93.14*	*87.82*	*90.48*	*90.79*
Kinetin							
0 mg l^{-1} (control)	64.7	59.1	61.9	70	76.4	73.2	67.55^{d}
0.1 mg l^{-1}	82.6	100	91.3	85.7	80	82.85	87.07^{abc}
0.25 mg l^{-1}	88	85.7	86.85	87.8	80	82.85	86.2^{abcd}
0.5 mg l^{-1}	73.9	91.6	82.75	82.3	47.3	64.8	73.77^{bc}
1 mg l^{-1}	60	85.7	72.85	37.5	90	63.75	68.3^{cd}
2 mg l^{-1}	70.83	92.3	81.03	100	90	95	88.23^{ab}
Mean	*75.06*	*91.06*	*82.95*	*78.66*	*78.12*	*78.28*	*80.61*
Grand Mean	82.2^{A}	87.38^{A}	84.79	84.45^{A}	82.37^{A}	83.41	84.05

Among the combinations of cytokinin and auxins, apart from 1 mg l^{-1} KN + 0.25 mg l^{-1} 2,4-D, 1 mg l^{-1} KN + 2mg l^{-1} NAA and 0.5 mg l^{-1} KN + 5 mg l^{-1} NAA, all performed better than respective controls.

The highest callus initiation of 100 % was recorded from many combinations, viz; 2,4-D (0.25 mg l^{-1}) + BA (0.5 mg l^{-1}), 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}), 2,4-D (2 mg l^{-1}) + KN (0.1 mg l^{-1}), NAA (0.25 mg l^{-1}) + BA (0.25 and 0.5 mg l^{-1}), NAA (2 mg l^{-1}) + KN (2 mg l^{-1}), NAA (5 mg l^{-1}) + BA (1 mg l^{-1}).

The average percent callus initiation recorded was 84.05 when media had auxins combined with cytokinins. But, when only auxins were provided in the medium, the average percent callus initiation was only 40.7. This showed that combination of auxins with cytokinins gave better callus initiation than when only auxins were provided.

(B) Effect on number of days needed to initiate callus

The days needed for callus initiation are enlisted in Table 16. The number of days needed for callus initiation, by all treatments were significantly lower than or equal to that needed by control. This suggested that all the treatments initiated faster callus than control.

Among the combinations of auxins with cytokinins, 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}) took least time (12 days) for callus initiation. Among the various concentrations of cytokinins, earliest callus initiation was seen with BA and kinetin at 2 mg l^{-1} levels (17 and 16 days respectively) irrespective of the combination with auxins.

With increasing concentrations of 2,4-D and NAA in the combinations with cytokinins, the time needed to initiate callus was reduced significantly. Thus, higher auxin levels in the combinations (2 mg l^{-1} of 2,4-D and 5 mg l^{-1} of NAA), were better than lower ones for faster initiation of callus. With increasing concentrations of cytokinins in the combimations, a gross decrease was seen in days needed for initiation.

Table 16 Influence of combination of auxins and cytokinins on the number of days needed to initiate callus in *Gymnema sylvestre* R.Br.

AUXINS / CYTOKININS	2,4-D			NAA			Mean number of days for callus initiation
	0.25 mg l^{-1}	2 mg l^{-1}	Mean	2 mg l^{-1}	5 mg l^{-1}	Mean	
BA							
0 mg l^{-1} (control)	33	20	26.5	25	25	25	25.75^{d}
0.1 mg l^{-1}	25	18	21.5	25	20	22.5	22^{bcd}
0.25 mg l^{-1}	20	20	20	14	18	16	18^{abc}
0.5 mg l^{-1}	20	20	20	25	20	22.5	21.25^{bcd}
1 mg l^{-1}	23	12	17.5	23	15	19	18.25^{abc}
2 mg l^{-1}	20	15	17.5	20	13	16.5	17^{ab}
Mean	*21.6*	*17*	*19.3*	*21.4*	*17.2*	*19.3*	*19.3*
Kinetin							
0 mg l^{-1} (control)	33	20	26.5	25	25	25	25.75^{d}
0.1 mg l^{-1}	20	20	20	20	15	17.5	18.75^{abc}
0.25 mg l^{-1}	25	20	22.5	27	20	23.5	23^{cd}
0.5 mg l^{-1}	30	15	22.5	20	18	19	20.75^{abc}
1 mg l^{-1}	25	13	19	25	13	19	19^{abc}
2 mg l^{-1}	18	13	15.5	20	13	16.5	16^{a}
Mean	*23.6*	*16.2*	*19.9*	*22.4*	*15.8*	*19.1*	*19.5*
Grand Mean	23.54^{B}	16.9^{A}	20.22	22.18^{B}	17.27^{A}	19.72	19.97

Plate 1

1 A Inability of a leaf explant to initiate callus even after 30 days of culturing in MS medium containing 2 mg l^{-1} IBA

(Growth category – no callus initiated)

1 B Little initiation of callus from internodal explant when cultured on MS medium supplemented with 1 mg l^{-1} NAA.

(Growth category – specks of callus)

1 C Callus covering half the surface of medium when cultured on MS medium with 1 mg l^{-1} 2,4-D using leaf explant.

(Growth category - ½ tube callus)

1 D Callus covering full surface of the medium and penetrating into the medium when cultured on MS medium supplemented with 2,4-D (2 mg l^{-1}) + kinetin (1 mg l^{-1})

(Growth category – two times full-tube callus)

(explant used was leaf petiole)

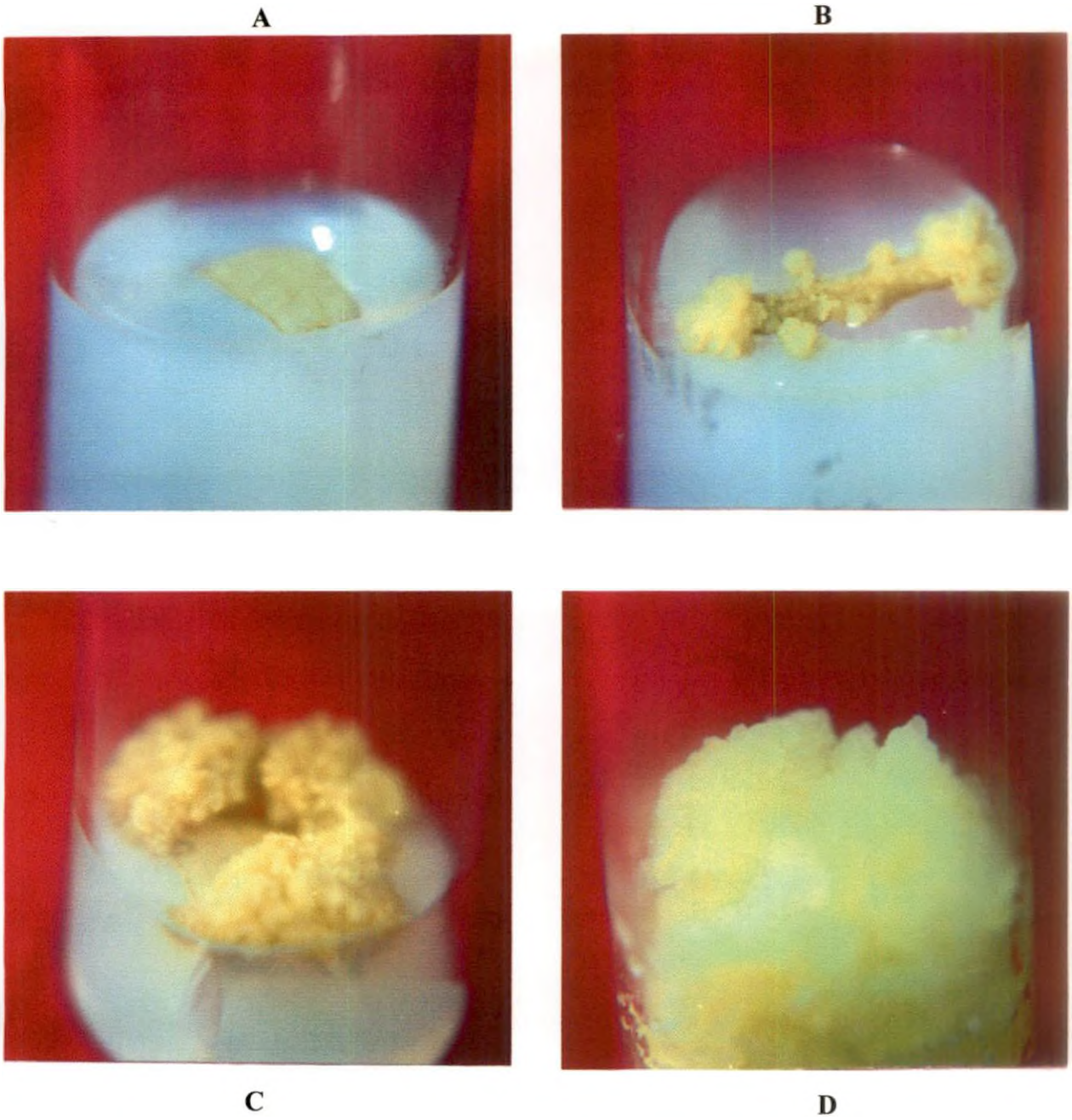


Plate 1. Initiation and proliferation of callus from explants of *Gymnema sylvestre* R.Br.

The average time for callus initiation needed when auxins and cytokinins were used in media, was 19.97 days, whereas only auxins required 34.33 days (Table 11). It reflected that, combination of auxins with cytokinins resulted in faster initiation of callus.

4.2.1.2 Influence on callus proliferation

4.2.3 Effect of cytokinins and auxins on callus proliferation

(A) Effect on callus proliferation (percentage of full-tube callus production)

Production of full-tube callus being considered as the criterion for callus proliferation, the percentages of tubes producing it are presented in Table 17. Not many treatment combinations showed superior callus production than respective controls. Some treatments containing NAA as the auxin, even failed to produce full-tube callus. Highest proliferation was found from 2 mg l^{-1} 2,4-D + 2 mg l^{-1} KN and 2 mg l^{-1} 2,4-D + 1 mg l^{-1} BA, who produced 80 per cent or more tubes having full-tube callus. When mean values were computed for the auxin and cytokinin combinations, superior callus proliferation was shown by 2,4-D + BA (53.08 %) and 2,4-D + KN (56.55 %) than control (20.94 %) as well as other combinations.

When in all combinations with cytokinins, 2,4-D at 2 mg l^{-1} concentration showed higher proliferation than 0.25 mg l^{-1} level except when combined with 0.25 V KN. No such trend was seen in combinations of NAA with cytokinins. With increasing levels of cytokinins in the combinations, no substantial rise was seen in their ability to proliferate callus. Amond the various levels of cytokinins combined with auxins, only 2 mg l^{-1} BA exhibited highest proliferation of 51.92 % tubes showing full-tube callus. Rest all performed poorly and were statistically on par with control (20.9 % full-tube callus).

From table 17, it was seen that the auxins with cytokinins resulted in average production of 38.1 % tull-tube callus. But, from table 11, it is seen that when media had only auxins, full-tube callus was produced rarely, and that too only by 2,4-D. Aapart from 2,4-D, no oter auxin

Table 17 Influence of combination of auxins and cytokinins on the percentage of tubes producing full-tube callus in *Gymnema sylvestre* R.Br.

AUXINS / CYTOKININS	2,4-D			NAA			Mean % full-tube callus
	0.25 mg l^{-1}	2 mg l^{-1}	Mean	2 mg l^{-1}	5 mg l^{-1}	Mean	
BA							
0 mg l^{-1} (control)	41.07	31.8	36.48	5	5.8	5.4	20.94^{b}
0.1 mg l^{-1}	61.5	75	68.25	36	0	18	43.12^{ab}
0.25 mg l^{-1}	33.3	44.4	38.85	50	16.6	33.3	36.07^{ab}
0.5 mg l^{-1}	52.9	56.52	54.71	0	0	0	27.35^{ab}
1 mg l^{-1}	41.17	80	60.58	0	71.4	35.7	48.14^{ab}
2 mg l^{-1}	39.13	47.8	43.46	66.66	54.16	60.38	51.92^{a}
Mean	*45.6*	*60.77*	*53.08*	*30.52*	*28.43*	*29.47*	*41.27*
Kinetin							
0 mg l^{-1} (control)	41.07	31.8	36.48	5	5.8	5.4	20.94^{b}
0.1 mg l^{-1}	47.8	64.2	56	0	13.3	6.65	31.32^{ab}
0.25 mg l^{-1}	68	42.8	55.4	71.4	0	25.7	29.45^{ab}
0.5 mg l^{-1}	43.47	66.6	55.03	11.76	0	5.85	30.45^{ab}
1 mg l^{-1}	28	78.5	53.25	6.25	50	28.12	40.68^{ab}
2 mg l^{-1}	41.6	84.6	63.1	0	50	25	44.05^{ab}
Mean	*45.77*	*37.34*	*56.55*	*17.75*	*22.6*	*20.2*	*38.37*
Grand Mean	45.27^{A}	61.12^{A}	53.15	22.39^{B}	23.72^{B}	23.05	38.1

managed to produce full-tube callus on their own. This indicated that, combination of auxins with cytokinins in media gave superior callus proliferation than media with only auxins.

(B) Effect on number of days required for callus proliferation

The days needed to generate full-tube callus being as a measure of time required for callus proliferation, are shown in Table 18. It was found that the control treatments took more time for producing full-tube callus than all those combinations of auxins and cytokinins which produced full-tube callus. The minimum time for producing full-tube callus was found to be 30 days from the day of inoculation, which was exhibited by the following treatments combinations, viz; 2 mg l^{-1} 2,4-D + 1 mg l^{-1} BA, 2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} KN, 5 mg l^{-1} NAA + 2 mg l^{-1} BA, 5 mg l^{-1} NAA + 1 mg l^{-1} KN, 5 mg l^{-1} NAA + 2 mg l^{-1} KN.

In combination with all levels of cytokinins, 2 mg l^{-1} 2,4-D proliferated faster (35.27 days) than 0.25 mg l^{-1} concentration (54.9 days). But, in case of NAA, since many treatments falied to produce full-tube callus, such comparison was not feasible. With increase in concentrations of BA and KN, no significant trend was seen in decrease of time for callus proliferation. Only 2 mg l^{-1} BA in combination with auxins proliferated faster in an average of 387 days. Aall other levels of cytokinins neede more days and were statistically on par with control which neded 65.75 days for the same.

4.2.1.3 Influence on callus index

The overall growth of callus produced by various combinations of auxins (2,4-D and NAA) and cytokinins (BA and kinetin) was expressed in terms of callus indices which are presented in Table 19.

All treatments, except 0.25 mg l^{-1} 2,4-D + 1 mg l^{-1} KN, 2 mg l^{-1} NAA + 1 mg l^{-1} KN, and 5 mg l^{-1} NAA + 0.1 mg l^{-1} BA and 5 mg l^{-1} NAA + 0.5 mg l^{-1} KN, showed significantly superior callus production over respective controls. The highest callus index of 170.1 was exhibited by 2,4-D (2 mg l^{-1}) + KN (1 mg l^{-1}), followed by 168.5 from 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}).

Table 18 Influence of combination of auxins and cytokinins on the number of days needed to produce full-tube callus in *Gymnema sylvestre* R.Br.

AUXINS / CYTOKININS	2,4-D			NAA			Mean number of days for full-tube callus
	0.25 mg l^{-1}	2 mg l^{-1}	Mean	2 mg l^{-1}	5 mg l^{-1}	Mean	
BA							
0 mg l^{-1} (control)	5.8	5.4	20.94^{b}	60	60	60	65.75^{b}
0.1 mg l^{-1}	53	36	44.5	45	-* (90)	67.5	56^{ab}
0.25 mg l^{-1}	50	33	44	25	50	42.5	42^{ab}
0.5 mg l^{-1}	48	32	40	-	-	-	65^{ab}
1 mg l^{-1}	50	29	39.5	-	31	60.5	50^{ab}
2 mg l^{-1}	55	30	42.5	37	30	33.5	38^{a}
Mean	*51.2*	*32*	*41.6*	*59.4*	*58.2*	*58.8*	*50.2*
Kinetin							
0 mg l^{-1} (control)	5.8	5.4	20.94^{b}	60	60	60	65.75^{b}
0.1 mg l^{-1}	50	36	43	-	45	67.5	55.25^{ab}
0.25 mg l^{-1}	60	35	47.5	55	-	72.5	60^{ab}
0.5 mg l^{-1}	58	30	44	40	-	65	54.5^{ab}
1 mg l^{-1}	50	32	41	50	30	40	40.5^{ab}
2 mg l^{-1}	50	32	41	-	30	60	50.5^{ab}
Mean	*53.6*	*33*	*43.3*	*65*	*57*	*61*	*52.15*
Grand Mean	54.9^{B}	35.27^{A}	45.08	62^{B}	57.8^{B}	59.9	52.47

* In these treatments, no full-tube callus was produced. So, these cells had no values. But to aid in statistical analyses, '-' was replaced by '90 days'. Ninety days was the maximum period for which these cultures were maintained. So, this value reflects that these particular treatments took the longest possible time to develop full-tube calli and hence performed poorer than control.

Table 19 Influence of combination of auxins with cytokinins on callus index and growth rate in *Gymnema sylvestre*

Phytohormones		Callus index	Callus growth rate	Phytohormones		Callus index	Callus growth rate
Auxins (mg l^{-1})	Cytokinins (mg l^{-1})			Auxins (mg l^{-1})	Cytokinins (mg l^{-1})		
2,4-D	-			NAA	-		
0.25	-	39.7	6.4	2	-	23.8	7.3
2,4-D	BA			NAA	BA		
0.25	0.1	121.6	22.8	2	0.1	76.4	16.9
0.25	0.25	92.4	18.4	2	0.25	100	28.5
0.25	0.5	108.6	22.6	2	0.5	60	17.1
0.25	1	89.79	17.9	2	1	51.4	9.3
0.25	2	89.1	16.2	2	2	106.5	28.7
2,4-D	KN			NAA	KN		
0.25	0.1	100.3	20.1	2	0.1	51.4	9.3
0.25	0.25	103.6	17.2	2	0.25	52.1	9.4
0.25	0.5	81.7	14.0	2	0.5	51.3	12.8
0.25	1	55.6	11.1	2	1	26.8	5.3
0.25	2	71.5	14.3	2	2	60	10.9
2,4-D	-			NAA			
2	-	44.6	10.1	5		50.3	9.6
2,4-D	BA			NAA	BA		
2	0.1	148.9	41.3	5	0.1	48.7	8.85
2	0.25	122.5	37.1	5	0.25	64.9	12.9
2	0.5	125.9	39.3	5	0.5	51.4	9.3
2	1	168.5	58.1	5	1	121.1	39.1
2	2	91.4	30.4	5	2	87.9	29.3
2,4-D	KN			NAA	KN		
2	0.1	116.2	32.2	5	0.1	57.2	12.7
2	0.25	92.5	36.4	5	0.25	50	9.1
2	0.5	143.1	47.7	5	0.5	28.4	5.1
2	1	170.1	53.1	5	1	97	19.4
2	2	131.2	41.0	5	2	89	29.6

Grand mean of callus index for all treatments is 88.9

Grand mean of callus growth rate for all treatments is 47.74

In combinations of 2,4-D with all cytokinins levels, it was seen that 2 mg l^{-1} 2,4-D showed higher callus index than 0.25 mg l^{-1} 2,4-D, eexcept when combined with 0.25 mg l^{-1} KN. No such trend was noticed when NAA combined with cytokinins.

The mean callus index when auxins combined with cytokinins was 88.9, whereas when only auxins were used, it was only 14.9 (Table 11). This indicated that the addition of cytokinins to auxins enhanced the callusing, than when only auxins were used.

4.2.1.4 Influence on callus growth rate

From Table 19, the values of growth score expressed as mg callus produced per day per tube, revealed that all the treatment combinations had a greater growth rate than respective controls except NAA (2 mg l^{-1}) + KN (1 mg l^{-1}) and NAA (5 mg l^{-1}) + KN (0.5 mg l^{-1}).

The fastest growth rate of 58.1 mg $(day)^{-1}$ $(tube)^{-1}$ was recorded from medium containing 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}), followed by 53.15 mg $(day)^{-1}$ $(tube)^{-1}$ from 2,4-D (2 mg l^{-1}) + KN (1 mg l^{-1}).

The mean growth rate when auxins were combined with cytokinins was 47.47 mg $(day)^{-1}$ $(tube)^{-1}$, whereas, when only auxins were used it was only 40.18 mg $(day)^{-1}$ $(tube)^{-1}$. Thus, faster production and proliferation of callus was noticed when media had contribution of auxins and cytokinins.

4.2.1.5 Influence of auxins and cytokinins on callus morphology

The colour and texture of callus produced with auxin and cytokinin combinations are listed in Table 20. The colour of callus was restricted mostly to green. Other colours like white and lemon yellow too were produced. In control treatments, use of 2,4-D alone produced white and translucent colours. When 2,4-D combined with BA, it produced green and white colours and when it combined with kinetin, it produced green and translucent colours in the calli. But the reverse outcome was never seen.

Table 20 Influence of combinations of auxins and cytokinins on callus morphology in *Gymnema*

Phytohormones		Callus colour	Callus texture	Phytohormones		Callus colour	Callus texture
Auxins (mg l^{-1})	Cytokinins (mg l^{-1})			Auxins (mg l^{-1})	Cytokinins (mg l^{-1})		
2,4-D	-			NAA	-		
0.25	-	White Translucent	Friable	2	-	Green	Friable
2,4-D	BA			NAA	BA		
0.25	0.1	Green	Friable	2	0.1	Green	Friable
0.25	0.25	Green	Friable	2	0.25	Green	Friable
0.25	0.5	White/Green	Friable	2	0.5	Green	Friable
0.25	1	Green	Friable	2	1	Green	Friable
0.25	2	Green	Friable	2	2	Green/Yellow	Friable
Mean		*Green*	*Friable*	*Mean*		*Green*	*Friable*
2,4-D	Kinetin			NAA	Kinetin		
0.25	0.1	Translucent Green	Friable	2	0.1	Green/Yellow	Friable
0.25	0.25	Translucent Green	Friable	2	0.25	Green	Friable
0.25	0.5	Green	Friable	2	0.5	Green	Friable
0.25	1	Green	Friable	2	1	Green	Friable
0.25	2	Translucent Green	Friable	2	2	Green	Friable
Mean		*Translucent Green*	Friable	*Mean*		*Green*	Friable
2,4-D	-			NAA	-		
2	-	White Translucent	Friable	5	-	Translucent, Green/Yellow	Friable
2,4-D	BA			NAA	BA		
2	0.1	Green/White Yellow	Friable	5	0.1	Translucent Green/Yellow	Friable
2	0.25	Green/White	Friable	5	0.25	Translucent Green/Yellow	Friable
2	0.5	Green	Friable	5	0.5	Translucent Green	Friable
2	1	Green/Yellow	Friable	5	1	Green Yellow	Friable
2	2	Green	Friable	5	2	Green/Yellow	Friable
Mean		*Green/White Yellow*	*Friable*	*Mean*		*Translucent Green, Yellow*	*Friable*
2,4-D	Kinetin			NAA	Kinetin		
2	0.1	Translucent Green	Friable	5	0.1	Green	Friable
2	0.25	Translucent Green	Friable	5	0.25	Translucent Green/Yellow	Friable
2	0.5	Translucent Green	Friable	5	0.5	Green	Friable
2	1	Green	Friable	5	1	Green/Yellow	Friable
2	2	Green	Compact	5	2	Green/Yellow	Friable
Mean		*Translucent Green*	*Friable*	*Mean*		*Green/Yellow*	*Friable*

In control treatments, NAA exhibited green colour along with yellow and translucent colours. When BA and KN combined with NAA, same colours were repeated. No addition or deletion of colours was noted in combinations as in the case of 2,4-D, where each of the cytokinins favoured one colour and suppressed the other.

All treatment combinations except one produced friable callus. Only NAA (5 mg l^{-1}) + KN (2 mg l^{-1}) produced compact callus. The friable callus was composed of granular particles of callus mass which were loosely attached to each other.

4.2.2 Influence of auxins and cytokinins on saponin production

4.2.2.1 Influence on saponin yields

Out of all the combinations of auxins and cytokinins, twelve best combinations were selected based on their growth rates. They were evaluated for production of saponins at 60 days age of callus and the results are presented in Table 21.

The highest amount of saponin produced was 270 μg g^{-1} from medium containing MS + 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}). Following this was the yield of 241 μg g^{-1} saponins from MS + 2,4-D (2 mg l^{-1}) + KN (0.5 mg l^{-1}). The combination of 2,4-D (2 mg l^{-1}) with KN (0.5 and 2 mg l^{-1}) also performed quite well in yield of saponins. Rest all others had saponin yields below 200 μg g^{-1}.

4.2.2.2 Influence on the rate of production of saponins

The highest rate of production of saponins at 15.68 μg $(day)^{-1}$ $(tube)^{-1}$ of saponins was reported ffrom MS + 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}). Next to it was MS + 2,4-D (2 mg l^{-1}) + KN (1 mg l^{-1}) which produced saponins at the rate of 12.59 μg $(day)^{-1}$ $(tube)^{-1}$ (Table 21).

Table 21 Saponin yields from some superior combinations of auxins and cytokinins

Phytohormones ($mg\ l^{-1}$)		Callus index	Callus growth rate [$mg\ (day)^{-1}$ (tube)-1]	Saponin yield ($\mu g\ g^{-1}$)	Rate of saponin production [$\mu g\ (day)^{-1}$ $(tube)^{-1}$]
Auxins	Cytokinins				
2,4-D	BA				
2	0.1	148.9	41.36	161	6.65
2	0.25	122.5	37.12	158	5.86
2	0.5	125.9	39.34	146	5.74
2	1	168.5	58.1	270	15.68
2	2	91.45	30.48	134	4.08
2,4-D	Kinetin				
2	0.1	116.2	32.27	187	6.03
2	0.5	143.1	47.77	241	11.51
2	1	170.1	53.15	237	12.59
2	2	131.2	41.00	209	8.56
NAA	BA				
5	1	121.5	39.08	142	5.7
5	2	87.9	29.3	134	3.92
NAA	Kinetin				
5	2	89	29.66	111	3.29

Apart from these, only 2,4-D (2 mg l^{-1}) + KN (0.5 mg l^{-1}) had a saponin production rate of 11.59 µg $(day)^{-1}$ $(tube)^{-1}$, while all rest produced saponins below the rate of 10 µg $(day)^{-1}$ $(tube)^{-1}$.

4.3 Influence of stress on callusing and saponin production

4.3.1 Influence of stress inducing chemicals

4.3.1.1 Influence on callusing

4.3.1.1.1 Influence on callus morphology

The colour of callus was usually greenish with a white tinge. Peptone rendered yellow/orange tinge to calli, while yeast extract induced orange callus and phloroglucinol offered reddish tinge. The intensity of callus colour increased with increasing concentrations of stress inducing compounds. In rest all treatments, callus colour was similar to that of control (yellowish green). All the treatments produced uniform friable callus.

4.3.1.1.2 Influence on callus index

The callus production in control was depicted by a callus index of 389 (Table 22). Many treatments exhibited superior callus production than control.

(a) Carbon sources

Peptone, yeast extract (YE) and malt extract (ME) were the carbohydrate sources evaluated (Table 22). All except peptone (2 %) produced a higher callus index than control. Among them, ME (1 %) produced the highest callus index of 705.7 followed by YE (1 %) producing a callus index of 651. It was noticed that all these three sources of carbon produced more callus at 1 % concentration than at 2 % concentration.

Table 22 Effect of stress inducing media additives on callusing and synthesis of saponins from *in vitro* cultures of *Gymnema sylvestre*

Stress inducing chemicals	Colour of callus	Texture of callus	Callus index	Callus growth rate mg (day)$^{-1}$ (tube)$^{-1}$	Saponin yield (µg gm^{-1})		Rate of saponin production µg (day)$^{-1}$ (tube)$^{-1}$
					One month	Two months	
Control (Basal)	Green	Friable	389.3	43.26	510.25	10.019	22.07
Carbon sources							
Peptone, 1%	Chrome Yellow	Friable	537.4	59.72	475.5	14.42	28.39
Peptone, 2%	Orange-yellow	Friable	184.3	20.48	247.28	44.97	5.06
Yeast extract, 1%	Pale brown	Friable	651.0	72.33	192.65	12.64	13.96
Yeast extract, 2%	Blackish brown	Friable	522.0	58.00	182.73	0	10.59
Malt extract, 1%	Cream yellow	Friable	705.7	78.41	417.78	0	32.75
Malt extract, 2%	Deep yellow	Friable	610.2	67.80	233.08	0	15.80
Growth retardant							
ABA, 0.1 mg g^{-1}	Whitish green	Friable	384.3	42.70	236	23.76	12.92
ABA, 0.5 mg g^{-1}	Green	Friable	476.3	52.92	236	23.76	12.48
ABA, 1 mg g^{-1}	Green, Yellow	Friable	417.0	46.33	121.25	25.97	5.61
Auxin synergist							
Phloroglucinol, 25 mg g^{-1}	Whitish, cream	Friable	422.8	46.97	67.36	14.3	3.16

Table 22 (continued)

Phloroglucinol, 50 mg g^{-1}	Cream, reddish	Friable	246.1	27.35	104.61	4.65	2.86
Phloroglucinol, 100 mg g^{-1}	Reddish white	Friable	228.3	25.36	338.21	16.39	8.57
Antioxidant							
$AgNO_3$, 5 mg g^{-1}	Cream white	Friable	307.7	34.18	220.89	10.01	7.55
$AgNO_3$, 10 mg g^{-1}	White	Friable	326.9	36.33	59.82	23.19	2.17
$AgNO_3$, 15 mg g^{-1}	White	Friable	417.5	46.38	120.52	44.2	5.59
Osmoregulants							
PEG, 2%	Cream white	Friable	541.7	60.18	0	0	0
PEG, 10%	Cream	Friable	586.9	65.21	0	0	0
Mannitol (1%) + Sucrose (2%)	White, Cream	Friable	363.7	40.41	684.89	11.34	27.68
Mannitol (2%) + Sucrose (1%	Pale cream	Friable	185.7	20.63	543.37	15.48	11.21
Mannitol (3%)	Cream, green	Friable	73.0	8.11	725.79	109.85	5.88
Nutrient depletion							
Sucrose, 1%	Whitish	Friable	169.5	18.83	582.41	0	10.95
Sucrose, 2%	Cream	Friable	281.1	31.23	67.23	0	2.09
Agar 0.9% (Basal)	Cream	Friable	400.2	44.47	333.75	0	14.84

Table 22 (continued)

Activated charcoal, 0.1%	Cream, green	Friable	149.0	16.55	725.58	0	12.02
Activated charcoal, 0.25%	Cream, yellow	Friable	259.7	28.86	222.78	84.73	6.42
Activated charcoal, 0.5%	Cream, green	Friable	191.7	21.31	160.24	81.29	3.41
Stress from low temperature culturing							
Refrigeration (Basal)	Pale brown	Friable	412.2	45.80	430.49	0	19.71

(b) Growth retardant

The growth retardant absaicic acid (ABA), produced more callus than control when at 0.5 and mg l^{-1} concentrations. But, at 0.1 mg l^{-1} concentration, it produced slightly lower callus (384) than control (389).

(c) Auxin synergists

Phloroglucinol, which is an auxin synergist, produced a higher callus index (422) than control only at 25 mg l^{-1} concentration. When used at 50 and 100 mg l^{-1} concentration, it produced lesser quantities of callus than control. A uniform decrease was noticed in callus index with increase in concentration of phloroglucinol.

(d) Antioxidants

Silver nitrate, an antioxidant, produced more callus than control only at 15 mg l^{-1} concentration and not at 5 and 10 mg l^{-1} concentrations. With increase in its concentration the callusing too increased.

(e) Osmoregulants

Polyethylene glycol (PEG) superior callus than control at 2 and 10 % concentrations, though it was more at 10 % concentration. Mannitol, another osmoregulant, however produced lesser callus than control at all levels of use (1 to 3 %). The callus index decreased uniformly from 363.75 to 73, when its concentration was increased from 1 to 3 %.

(f) Nutrient depletion

Availability of nutrients in the medium was reduced by either providing sucrose in deficient amounts (1 and 2 %), or in creasing agar to 0.9 % or addition of activated charcoal. Sucrose produced lesser callus index when provided in deficient amounts, but the callusing increased with increase in concentration of sucrose. Increase in agar did not significantly differ in callusing, as it produced a callus index of 400.25, which was close to 389.35 of control,

having agar at 0.75 %. Activated charcoal retarded callus growth and at concentrations of 0.1, 0.25 and 0.5 % produced lesser callus than control.

From all the above treatments, highest callus index of 705.7 was produced from malt extract (1 %), followed by an index of 651.05 from yeast extract (1 %).

4.3.1.1.3 Influence on callus growth rate

Table 22 presents the rate of callus growth expressed as mg of callus produced per day per tube. The control treatment produced 43.26 mg $(day)^{-1}$ $(tube)^{-1}$ of callus.

(a) Carbon sources

Peptone (1 %) showed a higher rate of callus production of 59.7 mg $(day)^{-1}$ $(tube)^{-1}$ which, lowered to 20.48 mg $(day)^{-1}$ $(tube)^{-1}$ at 2 % concentration. Similarly, yeast extract showed reduction in growth rate from 72.33 to 58 mg $(day)^{-1}$ $(tube)^{-1}$, while malt extract, exhibited from 78.4 to 67.8 mg $(day)^{-1}$ $(tube)^{-1}$.

(b) Growth retardant

At 0.1 mg l^{-1} concentration, ABA has a slower rate of callus production, but it increased to 52.9 mg $(day)^{-1}$ $(tube)^{-1}$ at an concentration of 0.5 mg l^{-1} and further decreased to 46.3 mg $(day)^{-1}$ $(tube)^{-1}$ at the concentration of 1 mg l^{-1}.

(c) Auxin synergist

Phloroglucinol only at 25 mg l^{-1} concentration managed to produce callus at the rate of 46.3 mg $(day)^{-1}$ $(tube)^{-1}$,while at other concentrations, produced callus at lower concentrations than control.

(d) Antioxidant

Only 15 mg l^{-1} concentration of silver nitrate produced callus at the rate of 46.3 mg $(day)^{-1}$ $(tube)^{-1}$, while other concentrations produced callus at slower rate than control.

(e) Osmoregulants

The rate of callus production was higher than control and amounted to 60.18 and 65.21 mg $(day)^{-1}$ $(tube)^{-1}$ from 2 and 10 % concentrations of PEG respectively. Mannitol, at all concentrations produced callus at a slower rate than that of control.

(f) Nutrient depletion

The rate of callus production was slower than control, when sucrose was deficient, but it increased rapidly with increase in concentration of sucrose. At 1 % concentration of sucrose, its growth rate was 18.83 mg $(day)^{-1}$ $(tube)^{-1}$, while it was 31.23 at 2 % concentration. Control, which had 3 % sucrose had a callus growth rate of 43.26 mg $(day)^{-1}$ $(tube)^{-1}$.

Agar at 0.9 % showed a faster growth rate of 44.4 mg $(day)^{-1}$ $(tube)^{-1}$. Activated charcoal at all concentrations showed retarded growth, which quite slower than control.

The fastest growth rate of 78.4 mg $(day)^{-1}$ $(tube)^{-1}$ was obtained from the medium containing malt extract (1 %), followed by 72.33 mg $(day)^{-1}$ $(tube)^{-1}$ from yeast extract (1 %) containing medium. The slowest growth rate of 8.1 mg $(day)^{-1}$ $(tube)^{-1}$ was obtained when medium was supplemented with mannitol at 3 % concentration.

4.3.1.2 Influence on saponin production

4.3.1.2.1 Influence on yield of saponins

4.3.1.2.1.1 Saponin yields from one month old callus

From one month old callus, the control treatment produced 510.25 $\mu g\ g^{-1}$ saponins (Table 22). Only 5 other treatments had yields higher than the yield from control. The highest yield of 725 $\mu g\ g^{-1}$ was produced by mannitol (3 %) as well as activated charcola (1 %). Saponin yield of 684.89 $\mu g\ g^{-1}$ was obtained from the combination of mannitol (1 %) + sucrose (2 %), followed by 582.41 $\mu g\ g^{-1}$ from sucrose (1 %) and 543.37 $\mu g\ g^{-1}$ from mannitol (2 %) + sucrose (1 %). All other stress yielding chemicals yielded lesser saponins than that produced by control.

Peptone, yeast and malt extracts exhibited reduction in saponin yields with increase in concentration from 1 to 2 %, thouhg the yields were lower than control.

ABA also registered a decline from 302 μg g^{-1} at 0.1 mg l^{-1} concentration to 121 μg g^{-1} at 1 mg l^{-1} concentration.

Phloroglucinol registered a reverse trend. It exhibited increase in saponin yields with increase in its concentration. At 25 mg l^{-1} concentration, it produced 67 μg g^{-1} saponins, but at 100 mg l^{-1} concentration, it yielded 338 μg g^{-1} saponins.

Silver nitrate yielded 220 μg g^{-1} saponins at 5 mg l^{-1} concentration, but the yield decreased to 59 μg g^{-1} at 10 mg l^{-1} concentration. Again a rise was seen upto 120 μg g^{-1} when it was added at a concentration of 15 mg l^{-1}.

From the media containing PEG, the extracts did not elute owing to higher viscosity and hence, the saponins could not be estimated. All treatments containing mannitol registered high saponin yields.

Sucrose, at 1 % concentration yielded 582.41 μg g^{-1} of saponins, but the yield was found to reduce drastically to 67.23 μg g^{-1} at 2 % concentration. Agar at 0.9 % reduced the saponin yields to 333.75 μg g^{-1}. Activated charcoal showed drastic yield in saponin from 725.58 μg g^{-1} at 0.1 % concentration to 160.24 μg g^{-1} at 0.5 % concentration.

4.3.1.2.1.2 Saponin yields from two month old callus

The yields of saponins at two months age was very lower than the respective yields at one month age of calli, in all the treatments (Table 22).Many treatments even failed to detect saponins at two months age.

The yield of the control treatment fell to 10.1 μg g^{-1} after the second month from 510.25 μg g^{-1} at the first month (Table 22). Peptone, at 1 % concentration, yielded 14.4 μg g^{-1} saponins. The yield increased to 44.97 μg g^{-1} at 2 % concentration. Yeast extract yielded 12.64 μg g^{-1}

saponins at 1 % concentration. Yeast extract at 2 % and malt extract at 1 and 2 % concentrations failed to detect any presence of saponins.

ABA at 0.1 mg l^{-1} concentration gave a fairly high yield of 62.4 μg g^{-1} saponins. When applied at 0.5 and 1 mg l^{-1} concentrations, ABA gave similar yields of 23.7 and 25.9 μg g^{-1} saponins respectively.

Phloroglucinol at 25 mg l^{-1} concentration yielded 14.3 μg g^{-1} saponins, but the yield decreased to 4.6 μg g^{-1} at 50 mg l^{-1} concentration and further increased to 16.39 μg g^{-1} at 100 concentration.

Silver nitrate recorded a uniform increase in the saponin yields from 10 μg g^{-1} at 5 mg l^{-1} concentration to 44.2 μg g^{-1} at 15 mg l^{-1} concentration.

PEG failed to detect presence of saponins. Mannitol showed increase in saponin yields with increase in its concentration. At 1 % concentration, it yielded only 11.3 μg g^{-1} saponins, but at 3 % concentration, the highest yield of 109.8 μg g^{-1} was obtained.

Sucrose (1 and 2 %) and agar (0.9 %) failed to detect the presence of saponins. Except at 0.1 % concentration, activated charcoal gave very high yields of 84.7 and 81.2 μg g^{-1} from 0.25 and 0.5 % concentration respectively.

The maximum saponin yield from two month old calli was 109.8 μg g^{-1} obtained from mannitol (3 %).

4.3.1.2.2 Influence on rate of production of saponins

The rate of production of saponins in callus expressed as μg of saponins per day per tube are presented in Table 22.

The control treatment produced callus at the rate of 22.07 μg $(day)^{-1}$ $(tube)^{-1}$. Only three other treatments had rates higher than the control. The fastest production of saponins at a rate of 32.75 μg $(day)^{-1}$ $(tube)^{-1}$ was from medium containing malt extract at 1 % concentration,

followed by 28.3 µg $(day)^{-1}$ $(tube)^{-1}$ from peptone (1 %) and 27.68 µg $(day)^{-1}$ $(tube)^{-1}$ from the combination mannitol (1 %) + sucrose (2 %). The lowest rate of saponin production was reported as 2.09 µg $(day)^{-1}$ $(tube)^{-1}$ from sucrose (2 %) containing medium.

Peptone, yeast and malt extract showed uniform decrease in rate of saponin production with increase in their concentrations. ABA also registered a similar trend.

At concentrations of 25 and 50 mg l^{-1}, phloroglucinol had lower saponin production rates which increased drastically at 100 mg l^{-1} concentration. Silver nitrate showed no specific trend of rise or fall in rate of saponin production with increase in its concentration.

With increase in concentrations of mannitol and sucrose, the rate of saponin production showed a decline. Agar (0.9 %) registered a slower rate of saponin production of 14.8 µg $(day)^{-1}$ $(tube)^{-1}$. Activated charcoal too showed a decline in rate of saponin production with increase in its concentration.

4.3.1.2.3 Influence on chromatographic properties of the saponins produced *in vitro*

The details of saponins detected by TLC and quantified by densitometry are listed in Table 23.

Saponins were identified by mostly purple and blue colour of the spots. But, in some cases, red and violet colours were also seen for saponins. Most treatments exhibited saponin spots corresponding to R_f 0.7, but some treatments exhibited spots at R_f values of 0.3 and 0.5.

From the samples of some treatments, no saponin spots were seen. They failed to detect the presence of saponins.

The standard gymnemic acid exhibited a purple spot at R_f 0.5. According to Yoshikawa *et al.* (1992), purple colour is shown by the dammarane-type saponins, while blue colour is shown by oleanane-type saponins. Violet colour was also shown by both these types of saponins.

Table 23 Influence of stress inducing chemicals on the chromatographic properties of the saponins produced from *in vitro* cultures of *Gymnema sylvestre* R.Br.

Media	One month				Two months			
	R_f value	Colour of spot	Saponin yield ($\mu g\ ml^{-1}$)	Total saponins	R_f value	Colour of spot	Saponin yield ($\mu g\ gl^{-1}$)	Total saponins
Control (MS + 2 mg l^{-1} 2,4-D + 1 mg l^{-1} BA)	0.7	Purple	510.25	510.25	0.5	Red	10.019	10.019
Carbon sources								
Peptone, 1%	0.5 0.7	Red Violet	104.09 371.4	475.5	0.7	Blue	14.42	14.42
Peptone, 2%	0.5 0.7	Red Violet	103.41 143.38	247.28	0.7	Blue	44.97	44.97
Yeast extract, 1%	0.55 0.7	Violet Violet	111.63 81.01	192.65	0.7	Blue	12.64	12.64
Yeast extract, 2%	0.55 0.7	Violet Violet	132.04 50.68	182.73	-	-	-	-
Malt extract, 1%	0.55 0.7	Violet Violet	283.45 134.32	417.78	-	-	-	-
Malt extract, 2%	0.55 0.7	Violet Violet	152.24 80.74	233.04	-	-	-	-
Growth retardant								
ABA, 0.1 mg l^{-1}	0.5 0.7	Red Violet	141.66 161.03	302.7	0.5 0.7	Red Blue	30.46 31.98	62.45
ABA, 0.5 mg l^{-1}	0.5 0.7	Red Violet	84.09 151.9	236	0.7	Blue	23.76	23.76
ABA, 1 mg l^{-1}	0.5 0.7	Red Violet	40.5 80.74	121.25	0.7	Blue	25.97	25.97
Auxin synergist								
Phloroglucinol, 25mg l^{-1}	0.7	Violet	67.36	67.36	0.7	Blue	14.3	14.3
Phloroglucinol, 50 mg l^{-1}	0.7	Purplish Violet	104.61	104.61	0.7	Blue	4.65	4.65
Phloroglucinol, 100 mg l^{-1}	0.4 0.7	Violet Purple	202.42 185.78	388.21	0.7	Blue	16.39	16.39
Antioxidant								
$AgNO_3$, 5 mg l^{-1}	0.7	Blue	220.89	220.89	0.7	Blue	10.01	10.01
$AgNO_3$, 10 mg l^{-1}	0.7	Blue	59.82	59.82	0.7	Blue	23.19	23.19
$AgNO_3$, 15 mg l^{-1}	0.7	Blue	120.52	120.52	0.7	Blue	44.2	44.2
Osmoregulants								
PEG, 2%	-	-	-	-	-	-	-	-
PEG, 10%	-	-	-	-	-	-	-	-
Mannitol, 1% + Sucrose, 2%	0.3 0.5 0.7	Purple Blue Blue	180.35 193.51 311.02	684.89	0.3	Red	11.34	11.34
Mannitol, 2% + Sucrose, 1%	0.3 0.5 0.5	Purple Blue Blue	313.32 80.74 149.3	543.37	0.3	Red	11.34	11.34

Table 23 (continued)

Mannitol, 3%	0.3 0.5 0.7	Purple Blue Blue	136.27 180.03 409.47	725.79	0.3	Red	109.85	109.85
Nutrient depletion								
Sucrose, 1%	0.3 0.5 0.7	Purple Blue Blue	63.41 150.72 368.2	582.41	-	-	-	-
Sucrose, 2%	0.3 0.5 0.7	Purple Blue Blue	13.01 13.39 40.8	67.23	-	-	-	-
Agar, 0.9%	0.5 0.7	Blue Blue	162.77 170.97	333.75	-	-	-	-
Activated charcoal, 0.1%	0.55 0.7	Violet Violet	545.76 179.8	725.58	-	-	-	-
Activated charcoal, 0.25%	0.4 0.7	Violet Violet	112.82 109.9	222.78	0.7	Blue	84.73	84.73
Activated charcoal, 0.5%	0.7	Violet	160.24	160.24	0.7	Blue	81.29	81.29
Stess from low temperature culturing								
Cool temperature	0.5 0.7	Blue Blue	151.33 269.15	420.49	-	-	-	-

Control medium exhibited only one saponin spot at R_f 0.7 was seen, but from two month old callus, a single red spot was observed at R_f 0.5.

Peptone at 1 and 2 % concentrations produced a red spot at 0.5 R_f and a violet spot at 0.7 R_f from one month old callus. But, the two month old callus, only one blue spot was seen at R_f 0.7. Both yeast and malt extract exhibited 2 violet spots at R_f values of 0.55 and 0.7 from one month old callus. After two months, yeast extract exhibited only one blue spot at R_f 0.7. Malt extract failed to exhibit spots from two month old callus.

ABA, from one month old callus, exhibited red spot at R_f 0.5 and a violet spot at R_f 0.7. At two month age, it showed only one blue spot at R_f 0.7. At the concentration of 0.1 mg l^{-1}, ABA showed an extra red spot at R_f 0.5.

Phloroglucinol showed variation in colour and R_f value of spots with change in concentration. At one month age, 25 mg l^{-1} concentration exhibited a violet spot at R_f 0.7, while 50 mg l^{-1} concentration exhibited a purplish violet spot at R_f 0.7. But 100 mg l^{-1} concentration produced 2 spots, a violet at R_f 0.4 and a purple at R_f 0.7. At two months age, all concentrations uniformly exhibited a single blue spot at R_f 0.7.

Silver nitrate uniformly exhibited a single blue spot at R_f 0.7 from one and two month old callus.

Presence of mannitol and sucrose, either singly, or in combination exhibited three spots from one month old callus. One purple spot at R_f 0.3 and two blue spots, one at R_f 0.5 and other at R_f 0.7 were reported. At two months age, mannitol treatments showed only one red spot at R_f 0.3, while sucrose treatments failed to detect saponins.

Agar (0.9 %) produced 2 blue spots at R_f values of 0.5 and 0.7 from one month old callus, but no spots were exhibited from two month old callus. Activated charcoal produced violet spots from one month old callus, but the R_f values differed with its concentration. At 0.1% concentration, 2 violet spots at R_f 0.55 and 0.7 were noticed, but at 0.25 % concentration, their

R_f values were 0.4 and 0.7. But, at 0.5 % concentration, it showed only one violet spot at R_f value of 0.7.

4.3.2 Influence of stress induction by manipulating culture conditions on callusing and saponin production

Stress induction by manipulating culture environment was carried out by culturing at 6 to 7 °C temperature. Basal medium combination, used as control, was the same here also.

It produced pale brown coloured friable callus, at a slightly higher callus index of 412.2 than when cultured at room temperature (389.35). The rate of callus production was little faster at 45.8 mg $(day)^{-1}$ $(tube)^{-1}$ than when cultured at room temperature, which proliferated at the rate of 43.26 mg $(day)^{-1}$ $(tube)^{-1}$.

After one month, the culture from low temperature yielded 430.49 μg g^{-1} saponins, which were less than control (510.25 μg g^{-1}). After two months, it failed to detect presence of saponins. The rate of production of saponins was 19.71 μg $(day)^{-1}$ $(tube)^{-1}$, which was lesser than 22.07 μg $(day)^{-1}$ $(tube)^{-1}$, the rate of saponin production of control.

From one month old calli obtained from the low temperature cultures, 2 blue spots of saponins were noticed on thin layer chromatograms, 0.5 and 0.7 R_f values.

4.4 Cell suspension cultures

4.4.1 Growth of cell suspension cultures

4.4.1.1 Cell viability of the cell suspension cultures

All the treatments showed a uniform decrease in the cell count (Table 24) with increase in age of cultures, except peptone (1 %) containing medium. This treatment showed slight increase at the end of 3rd and 4th week of culturing. Also, at this stage, it contained a very large number of small sized cells, which were in dividing and multiplying stage.

Table 24 Growth of cultures and synthesis of saponins in cell suspensions of *Gymnema sylvestre* R.Br.

Media additives	Age in weeks	Cell viability (% of live cells)	Cell count (No. of cells ml^{-1})	Packed cell volume (%)	Yield of saponins secreted in the medium ($\mu g\ ml^{-1}$)	Saponin yield in extract ($\mu g\ ml^{-1}$)
Control (MS + 2 mg l^{-1} 2,4-D + 1 mg l^{-1} BA)	I	18.5	78,888	10	432.5	Not detected
	II	6	22,777	2	120	
	III	17	13,888	<2	210	
	IV	25	3,333	<2	347.5	
	Mean				277.5	
Peptone, 1%	I	22	1,33,888	10	1274.5	Not detected
	II	30	19,444	3	420.5	
	III	23	11,666*	2	123	
	IV	50	13,333**	2	458	
	Mean				569	
Yeast extract, 1%	I	7	1,73,888	10	766	477.25
	II	14	36,666	4	207	0
	III	8	13,333	3	354	308.25
	IV	0	3,888	2	377.5	117.75
	Mean				426.3	225.8
Malt extract, 1%	I	25	46,111	10	282.5	0
	II	0	6,111	4	322	50
	III	0	5,000	2	381	0
	IV	0	4,111	<2	415.5	0
	Mean				350.2	14.75
ABA, 0.5 mg l^{-1}	I	45	14,222	8	141.5	329.25
	II	25	33,333	4	32	279
	III	5	11,666	4	0	0
	IV	0	7,777	2	9.5	314
	Mean				45.75	230.56
Phloroglucinol, 50 mg l^{-1}	I	40+	2,56,111	12	2313.5	Not detected
	II	17	22,777	6	1367	
	III	0	3,333	4	708	
	IV	0	1,111	2	615	
	Mean				1250.8	

Table 24 (continued)

$AgNO_3$, 10 mg l^{-1}	I	0	1,15,555	8	591	Not detected
	II	5	5,555	2.8	429	
	III	0	10,000	2	-	
	IV	15	1,666	2	-	
	Mean				255	
PEG, 10%	I	18	41,111	16	-	Not detected
	II	50	10,000	10	-	
	III	5	4,444	6	-	
	IV	0	2,777	2	162	
	Mean				40.5	
Mannitol, 3%	I	0	93,333	12	637.5	300.5
	II	0	23,333	4	400.5	340
	III	0	6,666	2	485.5	223.5
	IV	0	2,777	1	957	235.75
	Mean				620.12	279.4
Sucrose, 1%	I	9	76,666	6	328.5	86.75
	II	0	10,555	3	108	-
	III	5	3,333	2	79.5	-
	IV	10	2,222	2	66	-
	Mean				145.5	21.68
Activated charcoal, 0.25%	I	0	71,111	12	-	-
	II	5	5,555	6	-	-
	III	No cells seen	4,444	3	638.5	114.75
	IV		1,111	2	125.5	25
	Mean				191	34.93

*small sized cells
** very small sized cells
+dividing cells

Control treatment registered 95.7 % reduction in cell viability at the end of 4 weeks of culturing. Highest reduction in viability of 99.5 % was noticed from the cultures containing phloroglucinol (50 mg l^{-1}), while the lowest reduction in viability of 45.3 % was recorded from cultures grown in medium containing ABA (0.5 mg l^{-1}).

4.4.1.2 Cell count of the cell suspension cultures

It was found that, with the increase in age of cultures, from 1 week to 4 weeks, some cultures showed rise in cell viability (Table 24) while others showed decline. Control medium and treatments with peptone (1%), silver nitrate (10 mg l^{-1}) and sucrose (1%) showed a rise in cell viability, while others recorded a decline. Control recorded a rise of 35 % in viability at the end of 4 weeks of culturing, while peptone (1 %) recorded 127 % increase in viability. Hundred per cent loss in viability was recorded from media containing ABA (0.5 mg l^{-1}), yeast extract (1%), malt extract (1 %), phloroglucinol (50 mg l^{-1}) and PEG (10 %).

4.4.1.3 Percent packed cell volume of the cell suspension cultures

For all the treatments, the packed cell volume showed a uniform decrease with increase in age (Table 24). Highest initial packed cell volume (PCV) of 16 % was noted in PEG (10 %) treatment and a lowest of 6 % in sucrose (1 %) treatment at the end of first week of culture. All the treatments showed reduction in PCV to 2 % or less, at the end of 4 weeks of culturing.

4.4.2 Saponin yields from cell suspension cultures

4.4.2.1 Yield of saponins secreted into the liquid medium

The control treatment showed presence of saponins at all four weeks of culturing (Table 24). The yields of saponins decreased from 423.5 μg ml^{-1} at end of the first week to 120 μg ml^{-1} at end of the 2nd week, but later on, it increased steadily to 347.5 μg ml^{-1} after 4 weeks. The mean saponin yield per week secreted in the liquid medium was 277.5 μg ml^{-1}.

Peptone (1 %) cultures showed a very high initial yield of secreted saponins of 1274 μg ml^{-1} at one week, which gradually decreased to 123 μg ml^{-1} at the end of 3 weeks, but again increased to 458 μg ml^{-1} after 4 weeks. The mean yield of saponins per week was 569 μg ml^{-1}, which was more than that of control.

Yeast extract (1 %) too registered a decline from 766 μg ml^{-1} at first week to 207 μg ml^{-1} at 2nd week. But, later on, the yield increased to 371.5 μg ml^{-1} at the end of 4 weeks. The mean saponin yield per week was 426.3 μg ml^{-1}.

Malt extract (1 %) registered a uniform increase in saponin yields from 282.5 μg ml^{-1} at 1st week to 415.5 μg ml^{-1} after 4 weeks. The mean saponin yield per week was 350.2 μg ml^{-1}.

Phloroglucinol (50 mg l^{-1}) yielded the highest saponins at one week age, amounting to 2313.5 μg ml^{-1}. The yields steadily decreased to 315 μg ml^{-1} after 4 weeks. The mean saponin yield per week of 1250.8 μg ml^{-1}, was the highest in all the treatments.

Silver nitrate (10 mg l^{-1}) showed presence of saponins only in 1st and 2nd weeks, amounting to 591 and 429 μg ml^{-1}.

Mannitol (3 %) yielded 637.5 μg ml^{-1} saponins at the 1st week. The yield decreased to 400 μg ml^{-1} after the 2nd week, but further increased to 957 μg ml^{-1} after 4 weeks. The mean saponin yield per week was 620.1 μg ml^{-1}.

Sucrose (1 %) showed uniform decrease in the yield of saponins from 328.5 μg ml^{-1} after 1st week to 66 μg ml^{-1} after 4 weeks. The mean saponin yield per week was 145.5 μg ml^{-1}, which was lower than control.

Activated charcoal (0.25 %) showed presence of saponins only after 3rd and 4th week, amounting to 638 and 125 μg ml^{-1} saponins respectively.

Among all these treatments, only peptone (1 %), yeast extract (1 %), malt extract (1 %), phloroglucinol (50 mg l^{-1}) and mannitol (3 %) yielded higher saponins than the control.

4.4.2.2 *Yield of saponins from the cell cultures*

The cultures of control, peptone (1 %), phloroglucinol (50 mg l^{-1}), silver nitrate (10 mg l^{-1}) and PEG (10 %) failed to show presence of saponins n the chromatograms (Table 24).

ABA (0.5 mg l^{-1}) containing cultures yielded 329.2 µg ml^{-1} saponins after 1 week, which reduced to 279 µg ml^{-1} after 2 weeks, but further increased to 314 µg ml^{-1} after 4 weeks. The 3 week old culture failed to detect presence of saponins. Mean saponin yield was 230.5 µg ml^{-1}.

Yeast extract registered a decline in saponin yields from 477.2 µg ml^{-1} after 1st week to 117.7 µg ml^{-1} after 4 weeks. At the 2nd week, saponins were not detected. The mean saponin yield per week was 225.8 µg ml^{-1}.

Saponins from malt extract (1 %) could be detected only at 2nd week which amounted to 50 µg ml^{-1}.

Mannitol (3 %) yielded 300.5 µg ml^{-1} saponins after 1st week. The final yield after 4th week was 235.7 µg ml^{-1}, but the mean saponin yield of 279.4 µg ml^{-1} was the highest among all treatments.

Saponins could be detected from sucrose (1 %) only at 2nd week of culturing which amounted to 86.7 µg ml^{-1}.

4.4.3 Variation in chromatographic properties of the saponins produced in the cell suspension cultures

4.4.3.1 Saponins secreted in the liquid media

All treatments, except phloroglucinol (50 mg l^{-1}) and mannitol (3 %), exhibited a single blue spot for saponins at R_f between 0.85 and 0.95 (Table 25).

Phloroglucinol (50 mg l^{-1}) treatment produced a purple spot at R_f 0.7 from 1st and 2nd week samples. For all the 4 weeks, it showed a blue spot at R_f 0.9 or 0.95.

Table 25 Influence of stress inducing chemicals on the chromatographic properties of the saponins produced from the cell suspension cultures of *Gymnema sylvestre* R.Br.

Media additives	Age in weeks	Culture media			Culture extract		
		R_f	Colour of spot	Saponin ($\mu g\ ml^{-1}$)	R_f	Colour of spot	Saponin ($\mu g.ml^{-1}$)
Control (MS + 2 mg l^{-1} 2,4-D + 1 mg l^{-1} BA)	I	0.9	Blue	432.5			
	II	0.9	Blue	120			
	III	0.9	Blue	210			
	IV	0.9	Blue	347.5			
Mean				277.5^{BC}			
Peptone, 1%	I	0.8	Blue	1274.5			
	II	0.8	Blue	420.5			
	III	0.85	Blue	123			
	IV	0.85	Blue	458			
Mean				569^{B}			
Yeast extract, 1%	I	0.85	Blue	766	0.95	Blue	477.25
	II	0.85	Blue	207	-		0
	III	0.85	Blue	354	0.95	Blue	308.25
	IV	0.85	Blue	377.5	0.95	Blue	117.75
Mean				426.3^{BC}			225.8^{A}
Malt extract, 1%	I	0.95	Blue	282.5	-	-	-
	II	0.95	Blue	322	0.9	Blue	59
	III	0.9	Blue	381	-	-	-
	IV	0.9	Blue	415.5	-	-	-
Mean				350.2^{BC}			14.75^{B}
ABA, 0.5 mg l^{-1}	I	0.85	Blue	141.5	0.9	Blue	329.25
	II	0.85	Blue	32	0.9	Blue	2.79
	III	-	-	0	-	-	0
	IV	0.85	Blue	9.5	0.9	Blue	314
Mean				45.75^{C}			230.56^{A}
Phloroglucinol, 50 mg l^{-1}	I	0.7	Purple	882.5			
		0.9	Blue	1431			
	II	0.7	Purple	486			
		0.9	Blue	881			
	III	0.95	Blue	708			
	IV	0.95	Blue	615			
Mean				1250.8^{A}			
$AgNO_3$ 10 mg l^{-1}	I	0.95	Blue	591			
	II	0.95	Blue	429			
	III	-	-	-			
	IV	-	-	-			
Mean				255^{BC}			

Table 25 (continued)

PEG, 10 %	I	-	-	-			
	II	-	-	-			
	III	-	-	-			
	IV	0.95	Blue	162			
Mean				40.5^{C}			
Mannitol, 3 %	I	0.05	Red	302.5	0.23	Purple	300.5
		0.4	Purple	335			
	II	0.05	Red	243	0.23	Purple	340
		0.4	Purple	157.5			
	III	0.05	Red	361	0.23	Purple	223.5
		0.4	Purple	124.5			
	IV	0.05	Red	67.5	0.23	Purple	235.75
		0.4	Purple	889.5			
Mean		0.05		243.5			-
		0.4		376.6			
		Total mean		620.1^{B}			279.4^{A}
Sucrose, 1 %	I	0.9	Blue	161.5	0.7	Purple	86.75
		0.95	Blue	167			
	II	0.9	Blue	108	-	-	-
	III	0.95	Blue	79.5	-	-	-
	IV	0.95	Blue	66	-	-	-
Mean				145.5^{BC}			21.68^{B}
Activated charcoal, 0.25 %	I	-	-	-	-	-	-
	II	-	-	-	-	-	-
	III	0.95	Blue	638.5	0.95	Blue	114.75
	IV	0.95	Blue	125.5	0.95	Blue	25
Mean				191^{BC}			139.75^{B}

Mannitol (3 %) exhibited a red spot at R_f 0.05 and a purple spot at R_f 0.4.

4.4.3.2 Saponins from the cell cultures

ABA (0.5 mg l^{-1}), yeast extract (1 %), malt extract (1 %) and activated charcoal (0.25 %) containing culture media produced a blue spot of saponins at R_f 0.9 or 0.95 (Table 25).

Cultures with sucrose (1 %) exhibited a purple spot at R_f 0.7. Cultures with mannitol (3%) exhibited a purple spot at R_f value of 0.23.

4.2 Standardisation of biochemical techniques to quantify saponins

4.2.1 Selection of solvent to extract saponins from calli

The ability of ethanol at different strengths to extract all possible saponins with minimum quantity of other organic matter was judged by the parameters shown in Table 26.

Table 26 Evaluation of extraction efficiency of saponins by different solvents

Solvent number	Extracting solvent	Dry weight of extract (%)	Saponin Yield ($\mu g\ g^{-1}$)
	Ethanol extracts		
1	100 % Ethanol	1.3269	23.68
2	80 % Ethanol	1.5036	4.56
3	50 % Ethanol	1.3638	10.72
4	30 % Ethanol	1.4749	7.46
	Fractionation method		
5	Chloroform extract	0.5783	3.46
6	Aqueous extract	1.536	8.18

The total amounts of organics harvested by each of the solvents were expressed on dry weight basis. This data showed that 60 per cent ethanol was found to be the most efficient among them. It extracted maximum organics amounting to 1.5036 % of callus weight. Following it were 30 per cent ethanol, 50 per cent ethanol and lastly 100 per cent ethanol solvent

systems. The dry weight of these extracts did not show any specific trend of increase or decrease with decrease in the concentrations of ethanol.

The fractionation method provided the chloroform fraction which extracted the least amount of dry matter (0.5783 %), while the aqueous fraction extracted high quantity of dry matter (1.536 %).

The chromatograms developed from these extracts are shown in Plate II (a). The standard sample of gymnemic acid (no. 7 & 8) showed a red spot at R_f 0.5. None of the extracts, except the chloroform fraction showed presence of saponins at R_f 0.5, or at any other R_f value. Also, except the chlorohorm fraction, all others showed a uniform streak of blackish charred material throughout the entire chromatogram.

The chloroform fraction (sample no. 5) exhibited a purple saponin spot at R_f value of 0.5, which was quite distinct and free of interference from non-saponin chemicals (shown in square). Also non-saponin compounds accumulated at R_f 0.95 in the chloroform extract, which was quite far off from the saponin spot (denoted by NS). So, the saponin spot was neither mixed with other chemicals nor was it masked by them. Thus, the chloroform extract gave best view of the saponins. The aqueous extract (sample no. 6) failed to show any visible saponin spot (red/purple coloured) but showed presence of most of the organic compounds, which were charred black.

Densitometric quantification with reference to the standard gymnemic acid sample was done and the data of saponin yields is shown in Table 26. It revealed that, the extract from 100 per cent ethanol gave the highest saponin yield of 23.68 $\mu g\ g^{-1}$, while the chloroform fraction showed the least saponin yield of 3.46 $\mu g\ g^{-1}$. Others exhibited intermediate yields.

4.2.2 Application of samples

Samples were applied in either circular spots or horizontal streaks as shown in Plate II (b). The Plate 2B shows application of 5, 10 and 15 µl of same callus extract, each in spots and

Plate 2

2 A Effect of different methods of extraction on visibility and separation of saponins on thin layer chromatograms

1. 100 % ethanol
2. 60 % ethanol
3. 50 % ethanol
4. 30 % ethanol
5. Chloroform fraction
6. Aqueous fraction
7. and 8. Standard gymnemic acid
9. Leaf extract of *Gymnema sylvestre*

2 B Effect on visibility of saponins due to application of samples in circular spots or horizontal streaks

1. 5 µl volume in spot
2. 5 µl volume in streak
3. 10 µl volume in spot
4. 10 µl volume in streak
5. 15 µl volume on spot
6. 15 µl volume in streak

2 C Effect on visibility of saponins due to application of concentrated samples in circular spots or horizontal streaks

1. Application in spot
2. Application in streak

2 D Effect of different spray reagents on spotting of saponins

1. *p*-Anisaldehyde sulphuric acid reagent
2. Vanillin sulphuric acid reagent

2 E Effect of different concentrations of vanillin on visibility of weak saponin spots

1. 1 % vanillin
2. 2 % vanillin
3. 3 % vanillin
4. 4 % vanillin
5. 5 % vanillin

2 F Effect of increasing concentrations of sulphuric acid in vanillin sulphuric acid reagent on visualisation of saponins

1. 5 % sulphuric acid
2. 10 % sulphuric acid
3. 25 % sulphuric acid

All the samples were eluted in chloroform : acetone : methanol (5 :1 : 1.5)

Samples in Plate 2A, 2B and 2C were developed by spraying with vanillin sulphuric acid.

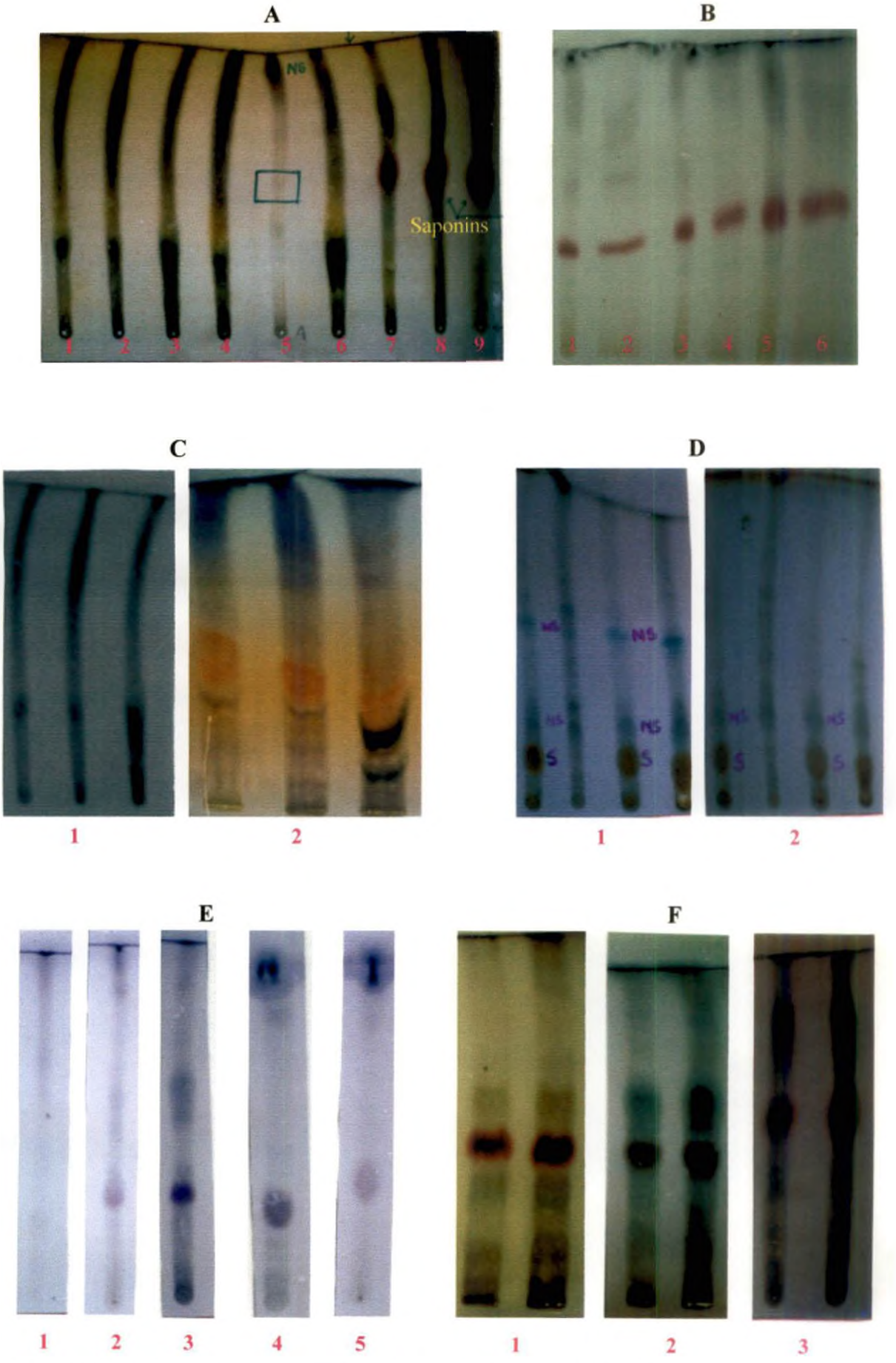


Plate 2. Thin layer chromatography of triterpenoid saponins from *in vitro* cultures of *Gymnema sylvestre* R.Br.

streaks, successively. The sample numbers 1 and 2 were application of 5 µl volume of callus extract in spot and streak respectively. The spot application produced a concise circular spot of saponins at R_f value about 0.5. The streak application showed a weak and thin band for saponins at R_f value of 0.5.

In contrast, 15 µl sample, when applied in a spot (sample no. 5), produced a big and bright spot of saponins which spread and mixed its boundaries with other compounds below and above R_f 0.5. But, when applied in a streak, (sample no. 6) it produced a clear and concise band of saponins with distinct boundaries. Since 5 µl volume had lesser saponins than 15 µl, it depicted that, in lower concentrations (like 5 µl), spot application gave better visibility, while in higher concentrations (like 15 µl or more), streak application gave better visibility.

Also Plate II (c) showed the difference in visibility of spots when highly concentrated samples were applied in circular spots and in streaks. When applied in circular spots, the eluted compounds assimilated at their respective R_f values. These highly concentrated compounds could not restrict themselves at their respective R_f values and mixed with adjacent spots to produce long smears. In such smears, different spots could not be distinguished. When the same sample was applied in streaks, the spots of the eluted compounds restricted themselves to their respective R_f values and could not mix with other compounds due to their affinity/covalent bonding properties. This avoided formation of smears. So, respective bands could be visualised clearly.

4.2.3 Selection of a best suitable running solvent system

The running solvent systems experimented for their suitability to elute saponins from *G. sylvestre* are listed in Table 27. The elution patterns of chromatograms obtained from each are depicted in Fig.2 .

Initially, individual solvents at 100 per cent strength were evaluated for eluting saponins. Chloroform eluted saponins to R_f of about 0.1, while ethyl acetate eluted them till R_f 0.2. In

Table 27 Performance of various running solvent systems on elution of saponins from *in vitro* cultures of *Gymnema sylvestre* R.Br.

Solvent system No.	Running solvent system	Elution of sample from point of application	Pattern of elution Clarity and fractionation/condensation of saponin spots	Accumulation of compounds at the solvent front
1	Chloroform (100%)	Poor	(a) All accumulated below R_f 0.2 (b) Poor separation of saponins	Less
2	Ethyl acetate (100%)	Moderate	(a) Saponins mostly eluted only till R_f 0.2 (b) Other material was mixed with saponins	Non-saponins acculmulated at the solvent front
3	Methanol (100%)	High	(a) Saponins seen only beyond R_f 0.9 (b) Saponins mixed with impurities	High
4	Water (100%)	Poor	(a) Saponins moved beyond R_f 0.9 (b) Some chemicals were not eluted	Very high
5	Elution in methanol (100%) followed by elution in chloroform (100%)	High	(a) Saponins were properly separated above R_f 0.6 (b) Saponins condensed at R_f 0.5 and mixed with impurities	Little
6	Hexane : Ethyl acetate (3 : 1)	Very poor	(a) Saponins were distributed in 7 spots near R_f 0.2, 0.5 and beyond 0.8 (b) Other meterial was either not eluted or was at the solvent front	High
7	Chloroform : Methanol (98 : 2)	Satisfactory	(a) Saponins were seen in 6 spots with more differentiation above R_f 0.5 (b) Below R_f 0.5, more condensation was seen	Less
8	Chloroform : Methanol (95 : 5)	Less	(a) Saponins showed 10 spots, but were merged with each other and were not distinct or fractionated (b) Differentiation was more at R_f 0.2 and not above that	High Dense and dark spot

Table 27 (continued)

9	Chloroform : Methanol (90 : 10)	Better, but not complete	(a) Saponins were in 12 spots (b) Some of the other chemicals were separated while rest were at the solvent front	High Less dense spot
10	Chloroform : Methanol (80 : 20)	Good, but not removed complete	(a) Good separation of saponins into 10 spots from R_f 0.05 to 0.9 (b) Other chemicals were mixed with saponins	Very high, appears as a dense spot
11	Chloroform : Methanol (50 : 50)	High	(a) Most of chemicals eluted along with solvent front (b) Saponins were seen at R_f 0.9	Very high, Dark spot
12	Chloroform : Methanol (25 : 75)	High	(a) All chemicals moved to solvent front (b) Saponins had mixed with others chemicals at R_f0.9	Very high, Dark spot
13	Chloroform : Methanol (80 : 20) followed by elution with Chloroform : Methanol (98 : 2)	Very good. Complete removal	(a) Distinct and clear, 15 saponin spots (b) Separation was more about R_f 0.5 (c) Saponins below R_f 0.2 were not much separated (d) Saponins were separated from impurities	Very less
14	Chloroform : Methanol (80 : 20) followed by elution with Chloroform : Methanol (95 : 5)	Very good, Complete removal	(a) Saponins were separated into 14 spots (b) Separation was more between R_f 0.3 to 0.7 (c) Saponins were separated from other chemicals	Dense and prominent
15	Chloroform : Methanol : Water (65 : 50 : 10)	Very good, complete removal	(a) Saponins were condensed in two spots, one at R_f 0.95 and other at R_f 0.6 (b) At R_f 0.95, saponins had mixed with other chemicals (c) Spot at R_f 0.95 developed colour first and then the one at R_f 0.6 developed colour	Dense clustering was seen
16	Chloroform : Methanol : Water (60 : 40 : 10)	Good, but not complete	(a) Saponins were fractionated between R_f 0.7 and 0.9 (b) Saponin spot at R_f 0.5 was mixed with other chemicals (c) Separation of saponins was poor	Nil
17	Chloroform : Ethyl acetale : Methanol (97 : 2 : 1)	Poor	(a) Saponins were seen near R_f 0.5 (b) Separation of saponins from other material was poor	Less

Table 27 (continued)

18	Chloroform : Acetone : Methanol (10:1:1)	Poor	(a) Saponins were predominant at R_f 0.2 (b) Separation was not so good. Saponins spots were surrounded by other chemicals (c) Saponins had condensed mostly into one bright spot at R_f 0.2 but some were seen as faint spots above R_f 0.5 (d) Above R_f 0.5, separation was unclear	Less
19	Chloroform : Acetone : Methanol (7:1:1)	Poor	(a) Saponins were in a major clustered spot at R_f 0.35 and into a few faint spots above R_f 0.5 (b) Separation was relatively poor (c) Non-saponin chemicals were scattered below the saponin spot	Less
20	Chloroform : Acetone : Methanol (5 : 1 : 1)	Very good	(a) Saponins were seen in one clear and condensed spot at R_f 0.4 (b) Non saponin was material separated away and condensed near R_f 0.9 (c) Saponins remained distinct and away from other materials	Less
21	Chloroform : Methanol : Water (5 : 1 : 1.5)	Very good	(a) Saponins condensed into one spot at R_f 0.5 (b) Non-saponins were distributed upto R_f 0.3 and beyond R_f 0.9 (c) Saponins remained distinct from non-saponins	Less
22	Chloroform : Methanol : Water (5 : 1 : 2)	Good	(a) Saponin spot was at R_f 0.7 (b) Non-saponin chemicals formed a smear below and above the saponin spot	High, Black spots
23	Chloroform : Methanol : Water (5 : 1 : 3)	Good	(a) Saponin spot was at R_f 0.75 (b) Non-saponin chemicals formed a smear and also concentrated at the solvent front	High, Dark smears
24	Chloroform : Acetone : Methanol : Water	Good	(a) Saponins were in one spot, condensed at R_f 0.45 (b) Non-saponins were in a smear below the saponin spot	High

Table 27 (continued)

	(5 : 1 : 1 : 0.5)		(c) Pigments and other material were at solvent front	
25	Chloroform : Ethyl acetate : Acetone : Water (30 : 3 : 1 9 : 2)	Moderate	(a) Saponins were condensed at R_f 0.4 (b) Non saponins were fractionated between R_f 0.1 and 0.4 (c) Pigments were fractionated beyond R_f 0.95	High
26	Chloroform : Acetone : Methanol : Water (64 : 10 : 16 : 1)	Poor	(a) Saponins were condensed in one spot at R_f 0.45 (b) Some saponins were mixed with other chemicals and formed a smear below R_f 0.4 (c) Clarity was poor when sample was concentrated	High, Dark clusters
27	Chloroform : Ethyl acetate : Acetone : Methanol : Water (25 : 3:5 : 16 : 1)	Poor	(a) Saponins were seen at R_f values of 0.4 and 0.2 (b) All saponins were mixed with other chemicals (c) Non-saponins were spread throughout the chromatogram	High, Small clusters
28	Ethylacetate : Methanol (70 : 30)	Very poor	(a) Saponins were seen at R_f 0.7 and 0.8 (b) Below R_f 0.7, saponins were mixed with other chemicals to form a long smear	Very high, Horizontal smear at solvent front
29	Ethyl acetate : Methanol : Water (200 : 10 : 10)	Good	(a) Most of the sample was eluted to solvent front (b) Saponins and others were mixed above R_f 0.9	Very high, Dense clusters
30	Ethyl acetate : Methanol : Water (100 : 10 : 10)	Good	(a) Saponins were seen in 2 spots at R_f 0.5 and 0.8 (b) Non-saponins smeared below R_f 0.2 and above 0.8 (c) Separation of saponins from other chemicals was poor	High
31	Ethyl acetate : Methanol : Water (100 : 13.5 : 10)	Good	(a) Saponins were in 4 to 6 spots (b) Some non-saponin chemicals condensed at R_f 0.2 while others formed smears	High, Prominent clusters
32	Ethyl acetate : Methanol : Water (100 : 20 : 10)	Good	(a) Saponins were found at R_f 0.55 and 0.88 (b) Saponins were mixed with other chemicals (c) Non-saponins were clustered below R_f 0.5	Less, Tiny clusters
33	Ethyl acetate : Methanol : Water (80 : 20 : 10)	Good	(a) Saponins were mostly in one distinct spot at R_f 0.65 but, some were mixed with pigments and formed a smear from R_f 0.8 to the solvent front (b) Non-saponins clustered below R_f 0.5	Less

Table 27 (continued)

34	Ethyl acetate : Methanol : Water (80 : 30 : 10)	Good	(a) Separation was poor; continuous smear was seen from R_f 0.0 to the solvent front (b) Saponins had mixed with other chemicals and smeared from R_f 0.7 to the solvent front	Less
35	Ethyl acetate : Methanol : Water (80 : 40 : 20)	Good	(a) Separation of saponins from other chemicals was poor (b) Saponins were mixed with other chemicals and smeared above R_f 0.8	More
36	Ethyl acetate : Methanol : Water (70 : 33 : 5)	Very good	(a) Saponins had condensed into one smear near R_f 0.5 (b) Non-saponins were found above R_f 0.75 (c) Some mixing of saponins with other chemicals was seen at R_f 0.7	Less
37	Ethyl acetate : Methanol : Water (70 : 40 : 10)	Poor	(a) One big saponin smear was noticed above R_f 0.7 (b) Saponins and other chemicals were mixed into a smeare above R_f 0.7	Less
38	Ethyl acetate : Methanol : Water (40 : 40 : 10)	Good	(a) Saponins were seen in 2 spots above R_f 0.9 (b) Between R_f 0.7 and 0.9, saponins were mixed with non-saponins (c) Separation is poor	Nil
39	Ethyl acetate : Methanol : Water (40 : 40 : 10) followed by elution in Ethyl acetate : Methanol : Water (70 : 33 : 5)	Good	(a) Saponins were seen in 2 spots at R_f 0.5 and 0.65 (b) Non-saponins had mixed with saponins and smeared beyond R_f 0.65 (c) Poor separation was noticed	Nil
40	Thrice successive elution in Chloroform : Methanol (90 : 10)	Good	With each elution, (a) More of the applied sample was removed from point of application (b) High separation of spots was seen at R_f 0.2 (c) Smears separated into spots, giving more clarity (d) Saponin spot fractionated into two	Less

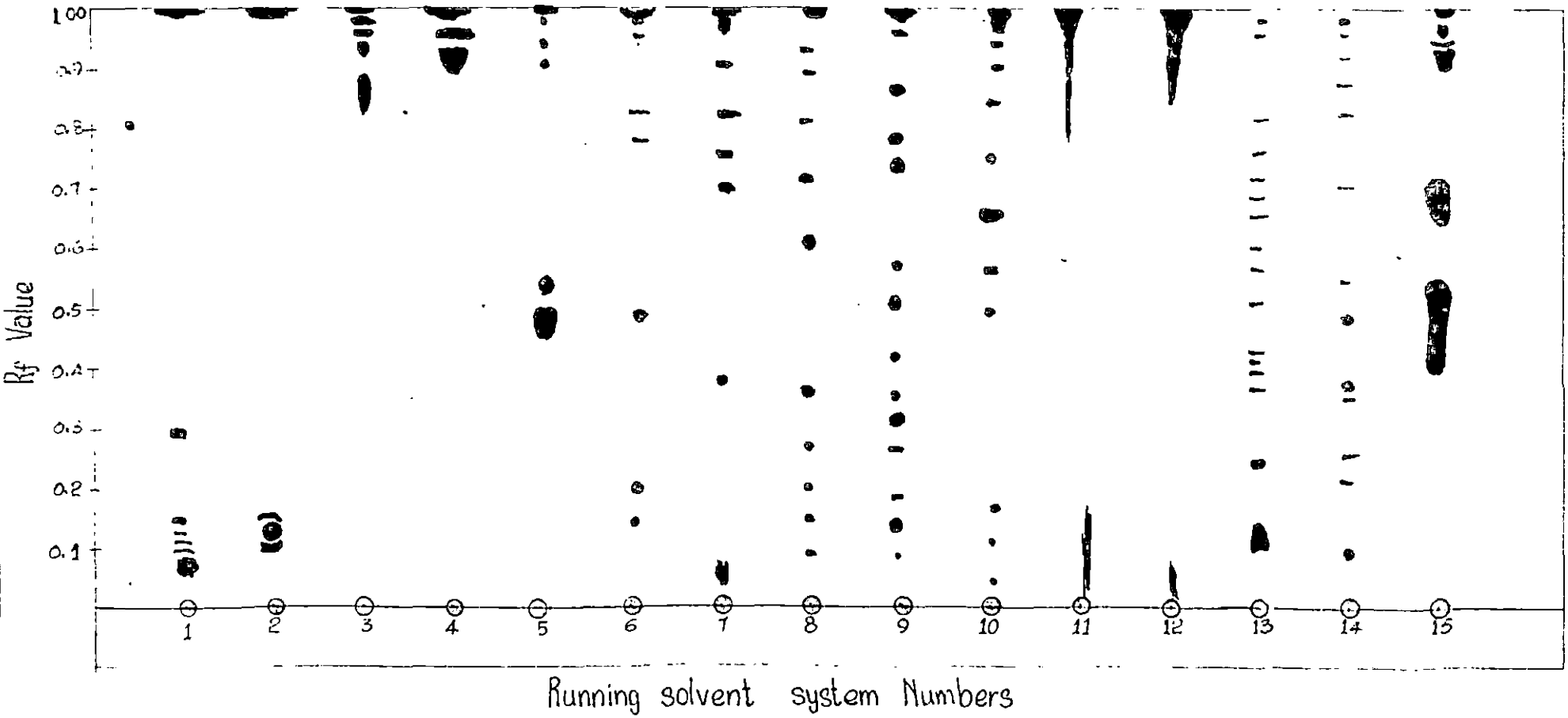


Figure. 2 Elution pattern of saponins using different running solvent systems

SAPONINS

NON SAPONIN ORGANIC MATTER

UNELUTED SAMPLE

SAPONINS

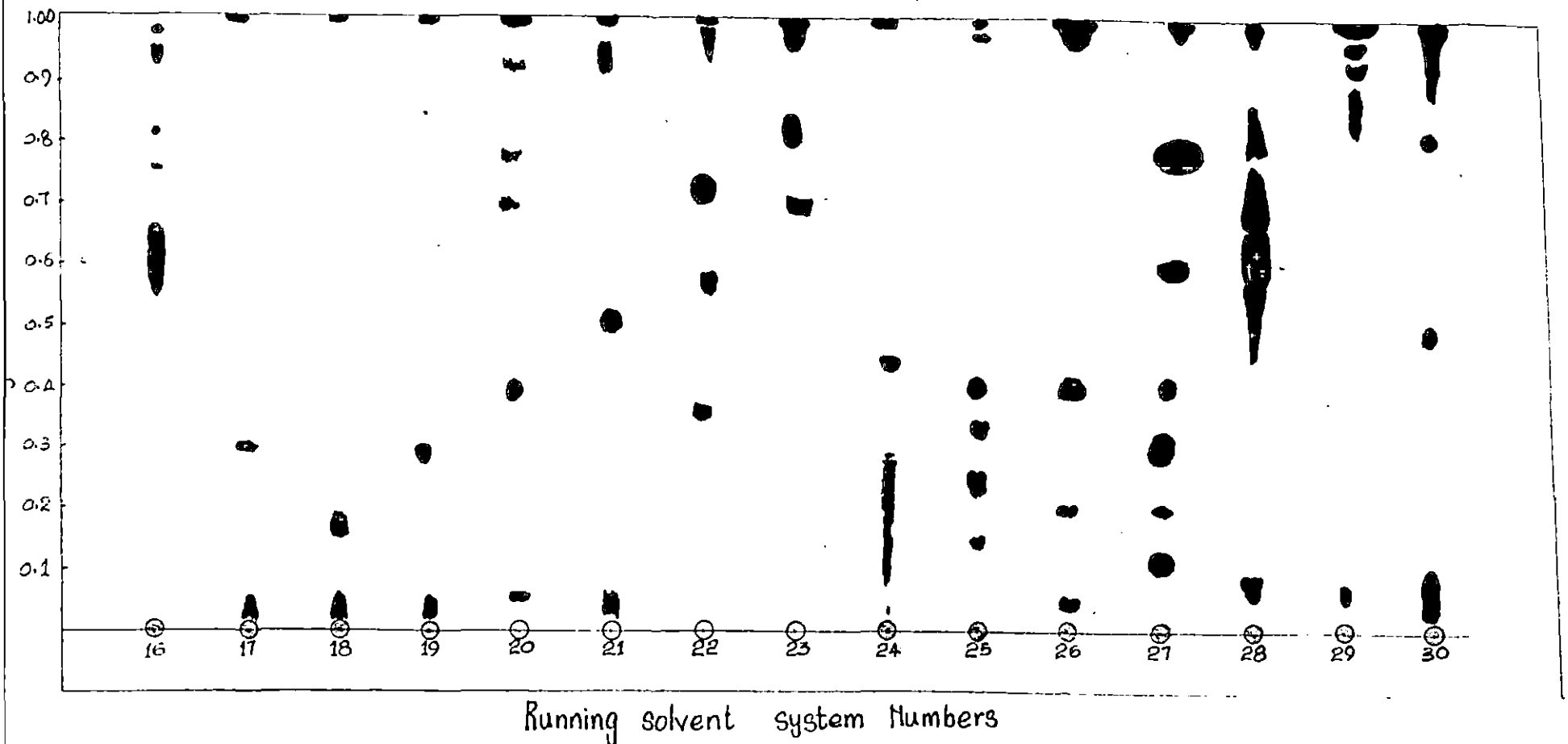


Figure 2 [Continued]

SAPONINS

OTHER ORGANIC MATTER

UNELUTED SAMPLE

SAPONINS

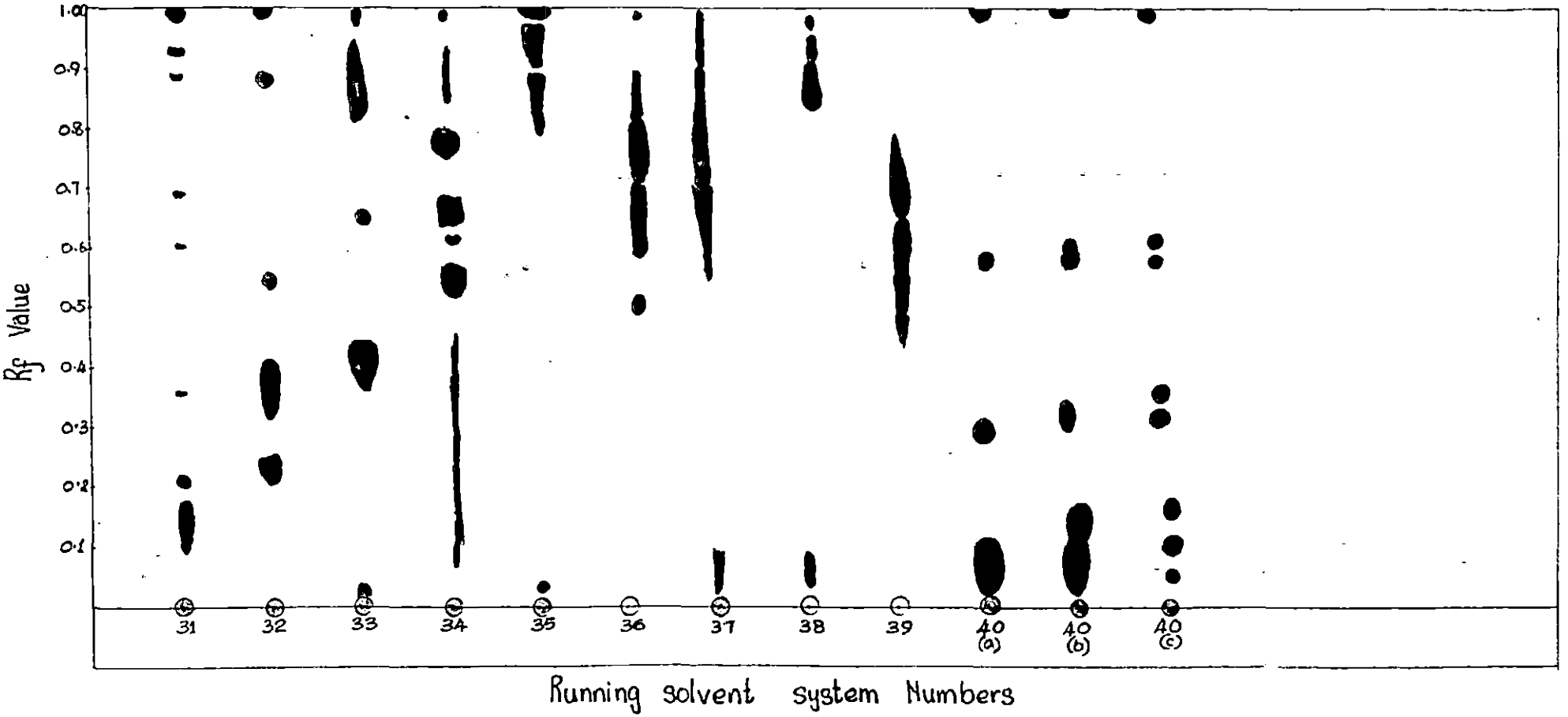


Figure 2 [Continued]

SAPONINS

OTHER ORGANIC MATTER

UNELUTED SAMPLE

SAPONINS

both the cases, the entire sample was not eluted from the point of application (yellow colour at R_f 0.00).

Methanol had a very good elution of the entire sample from point of application. It moved most of the compounds along with the solvent front and the saponins were seen above R_f 0.95. Water too showed saponins around R_f 0.95.

In all these cases the saponins were found to be closely associated or mixed with non-saponin materials. To improve the separation of saponins and to aid better elution of the applied sample, successive elution was tried with methanol followed by chloroform (no. 5). As a result separation of saponins into four spots, 1 at R_f 0.5 while others above R_f 0.9 was obtained.

To get an even better clarity, combinations of different solvents, in various proportions, were evaluated. The data regarding their performances is detailed in Table 27.

The mixture of hexane and ethyl acetate in the ratio of 3 : 1 separated the spots into 7 distinct spots. But much of the applied sample remained uneluted. A better fractionation of the saponins was obtained by using a mixture of chloroform and methanol. In a ratio of 80 : 20, they separated the saponins into 10 spots, but elution of sample was quite poor. When successive elution was done with 98 : 2 ratio of chloroform : methanol mixture, it resulted in separation into 15 spots of saponins. This combination (no. 13) was best as it gave a distinct profile of the saponin spots, which could be used for characterisation studies.

When mixture of chloroform, methanol and water was used for elution of sample from the point of application was very good. But, the saponins were seen into several spots and these spots were mixed with other compounds, rendering poorer clarity to the chromatogram. When in ratio of 65 : 50 : 10 (no. 15), many compounds accumulated at the solvent front into a dense spot. But, when methanol content was reduced and the ratio was modified to 60 : 40 : 10 (no. 16), ther was no accumulation at the solvent front.

The mixture of chloroform : ethyl acetate : methanol (97 : 2 : 1) showed poor elution and poorer separation of the saponins. The saponins remained mixed with other compounds.

One of the objectives of this experiment was to condense the saponins into a single spot, which would aid in quantification using densitometry. For this purpose, a mixture of chloroform, acetone and methanol in the ratio of 5 : 1 : 1.5 was found best. It exhibited a single saponin spot at R_f 0.5 with no interference whatsoever from other compounds. The visualisation of saponins was quite clear and also the R_f value being 0.5, any distortion in the elution pattern did not affect its visibility.

Addition of water to chloroform and methanol (no. 21 to 24) enhanced the elution of the sample from the point of application, but it accumulated more compounds at the solvent front.

The mixture of chloroform, ethyl acetate, acetone, methanol and water showed poor elution of the sample and the saponins remained mixed with the non-saponin compounds.

The combination of ethyl acetate : methanol (70 : 30) showed very poor elution. When water was added to it in various ratios (no. 28 to 39), the elution was found to be improved. The accumulation of compounds at the solvent front reduced to moderate levels. But, the saponins were neither completely fractionated nor completely condensed. They were also interspersed and mixed with nom-saponin compounds. The performances of these solvents (no. 28 to 39) were not impressive.

Another experiment was done to improve separation of spots, which had very close R_f values. A plate was eluted three times successively in the same solvent. Their pattern (40 a, b and c) showed that the closely condensed spots separated into distinct spots.

4.2.4 Standardisation of spray reagents

Spray reagents were evaluated for their efficiency of expressing saponins as specific coloured spots. The colours of saponin spots obtained from different spray reagents are shown in Plate II (d).

The *p*-anisaldehyde sulphuric acid reagent (ASR) exhibited purple, blue, green and violet spots. Vanillin sulphuric acid (VSR) exhibited purple and violet spots. Both showed brownish black patches of charred organic compounds.

Both the reagents commonly exhibited purple and violet spots, which also tallied with the R_f values of the standard gymnemic acid. Thus, it indicated that, purple and violet colours were specific for saponins and were shown by both the spray reagents.

ASR exhibited certain green and blue spots, corresponding to that R_f. VSR did not show any coloured spots. To confirm their chemical nature, samples lacking saponins were eluted and sprayed with both reagents. Even then, ASR spray produced blue and green spots as before. More over these spots were at R_f value other than that of the saponin spot obtained from the standard gymnemic acid. This inferred that ASR had identified some other group of compounds, besides saponins. But, since VSR identified saponins only and nothing else, it was selected as the appropriate spray reagent to detect saponins from *Gymnema sylvestre*.

When saponins were present in lower quantities, these reagents were unable to detect them. So the concentrations of the ingredients of these reagents were modified. As a result, better detection of saponins was obtained, as seen in Plate II (e), with increase in concentration of vanillin upto three per cent. The visibility of saponin spots was improved, but beyond this concentration, no significant improvement was noticed.

When concentration of sulphuric acid in VSR was increased, visibility was distorted, as it increased the charring and browning Plate II (f). So, the best combination of VSR was three per cent vanillin with five per cent sulphuric acid.

Similarly, in case of ASR, two per cent concentration of *p*-anisaldehyde showed better visibility of saponins than 1 per cent concentration

So, the best spray reagent to detect the saponins from *Gymnema sylvestre* was found to be vanillin sulphuric acid reagent with three per cent vanillin and five per cent sulphuric acid.

DISCUSSION

DISCUSSION

The results obtained in the study "*In vitro* callus induction in Gurmar (*Gymnema sylvestre* R.Br.) for secondary metabolite synthesis" are discussed in this chapter.

5.1 Influence of auxins and different explants of *Gymnema sylvestre* on callusing

5.1.1 Influence on callus initiation, proliferation and callus index

2,4-D, NAA, IAA and IBA were the auxins evaluated at (0.25, 0.5, 1, 2 and 5 mg l^{-1} concentrations each) for their potential to produce callus from explants of *Gymnema sylvestre* R.Br. (Fig.3).

It was found that, 2,4-D was the best auxin as it gave highest percentage of callus initiation, highest proliferation and highest callus index (Table 11). NAA was the next in performance. IAA and IBA performed very badly and their mean performance was nearly same as that of control (hormone-free MS medium).

Goldsmith (1977) had reported that the movement of 2,4-D in plant tissue is very less than compared to the movement of IAA. As a result, the applied IAA disperses to all the cells of the plant tissue, but 2,4-D remains accumulated where it was applied. Hence, concentration of 2,4-D is more at some points and it triggers growth in these areas. But since IAA has a tendency to disperse, its concentration at any certain place is very less for triggering growth. So as a result, callus growth was less in case of IAA, within the range of concentrations examined.

Nissen and Sutter (1990) reported that IAA and IBA when added to the nutrient medium for culturing plant tissues, they undergo considerable degradation during heat sterilization and also during culturing due to exposure to fluorescent light.

As a result the available quantity of intact IAA and IBA to the explant is greatly reduced. Thus their effect on growth of explant is very less.

Murashige (1974) had observed that 2,4-D and NAA had better callusing ability while IAA and IBA were poor callus inducers.

These theories help to explain the results of this study on *Gymenma sylvestre*. On the culture medium, all explants were placed horizontally to have maximum surface area in contact with the medium. This provided uniform availability of all auxins to each of the explants. Hence, all the explants had equal chance of absorbing same quantity of each of the auxins. According to Goldsmith (1977) and Nissen and Sutter (1990), after absorption, 2,4-D might have stayed at the peripheral cells, near the cambium, while IAA and other might have diffused inwards. The concentration of 2,4-D might have been higher at the cambial and peripheral cells than other auxins, it triggered more growth than other auxins.

Thus, 2,4-D was found to initiate callus in higher percentage of tubes than any other auxin. Similarly, availability of 2,4-D was more as it did not degrade like IAA and IBA, so continued growth and proliferation was more from 2,4-D than from IAA and IBA.

So more initiation and more proliferation gave higher callus index for 2,4-D than all other auxins. Hence, among the auxins evaluated, 2.4-D was the best. The ability of NAA was found to be next to 2,4-D, by Murashige (1974). Similar results were seen in this study.

Within the concentration range of 0.25 to 5 mg l^{-1}, IAA and IBA failed to show any callusing. Owing to the fact of its degradation (Nissen and Sutter, 1990), its ability of callusing needs to be evaluated at higher concentrations.

2,4-D has been found to be useful for callus induction in *Plumbago rosea* (Satheeshkumar and Bhavananda, 1988) and *Catharanthus roseus* (Mumtaz *et al.*, 1990). Sankar (1998) also found that 2,4-D produced highest amount of callus

initiation in *Sida* spp. as compared to NAA & IAA. The calli indices and growth scores of callusing in *Sida* spp. revealed that 1 mg l^{-1} 2,4-D was the best level of auxin to get good callusing. On the contrary, John (1996) found that 2,4-D gave a lower callus index in *Holostemma* and that the performance of NAA and 2,4-D was on par in case of callus initiation. Sindhu (1999) reported that the combination of 2,4-D (1 mg l^{-1}) and IAA (2.5 mg l^{-1}) gave high callus index in *Coscinium fenestratum*.

NAA was reported to induce calli from *Papaver bracteatum* (Ilahi and Ghauri, 1994) and in *Catharanthus roseus* (Akram and Yurekhi, 1995). Sankar (1998) also reported that NAA was next to 2,4-D in callus growth in *Sida* spp., while IAA performed even poorer than NAA.

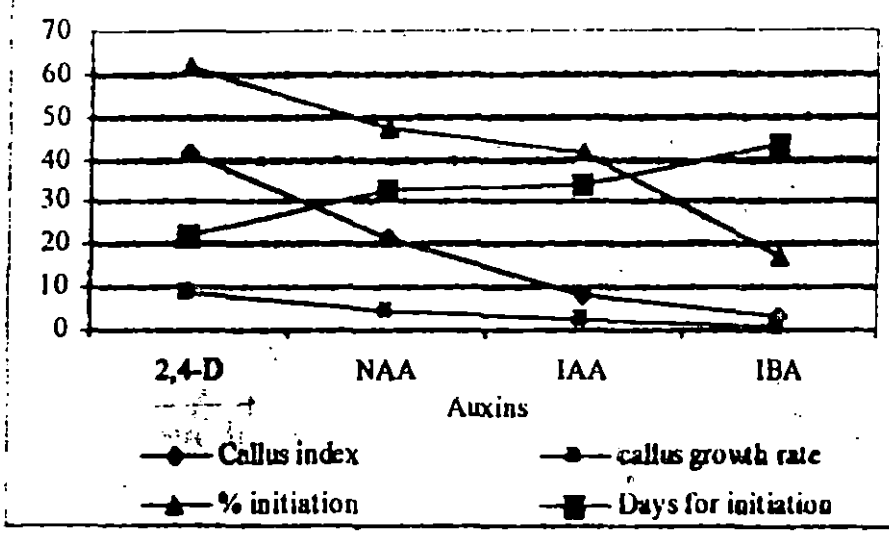
5.1.2 Influence on number of days required for initiation and proliferation of callus and growth rate

Among the evaluated auxins, the number of days required for callusing was least for 2,4-D followed by NAA and lastly by IAA and IBA. The fastest growth rate was recorded from 2,4-D, followed by NAA and lastly by IAA and IBA. So, the quickest proliferation of callus was obtained from 2,4-D.

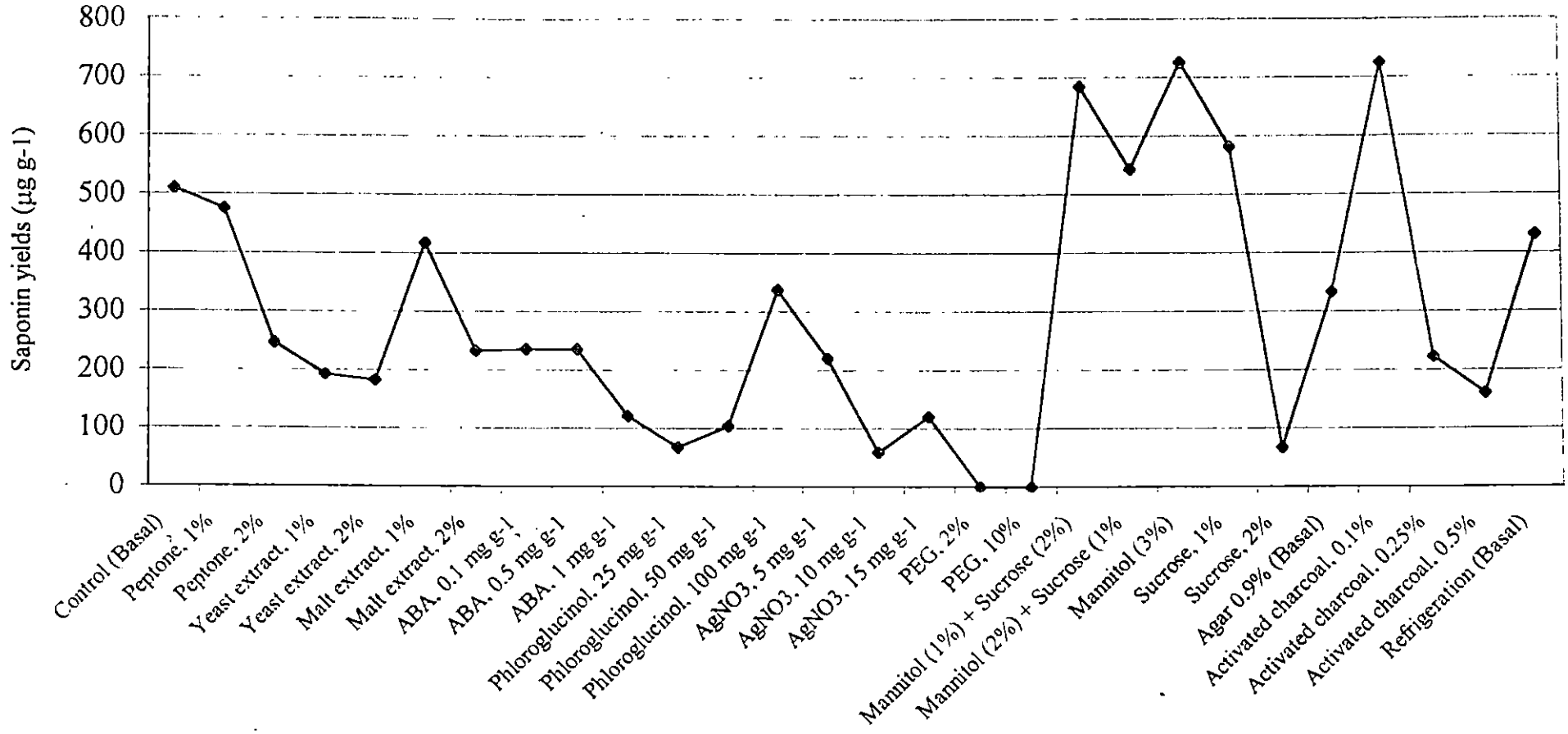
As per the report from Goldsmith (1977), 2,4-D tends to accumulate at certain points. More concentration of 2,4-D stimulates growth. Since continuous and sustained stimulus is provided to the explant from 2,4-D, the growth initiation is earlier than from other auxins. So, the time for callus initiation and proliferation is least for 2,4-D.

On the same lines, stimulus from IAA and IBA was less due to lesser concentration available to the explants. Concentrations of IAA and IBA reduced due to degradation. As a result, IAA and IBA needed more time for callus initiation and proliferation.

Fig 3 Effect of various auxins on callusing in Gymnema sylvestre R.Br.



Influence of stress on synthesis of saponins from in vitro cultures of Gymnema sylvestre R.Br.



Growth rate was computed as the ratio of amount of growth of the callus, to the time required to attain that much growth. Since 2,4-D had maximum callus index and needed least time for callusing, its growth rate was highest. Conversely, all other auxins had lower callus growth and needed more time for callusing and as a result, their growth rate was less. Hence, in *Gymnema sylvestre*, fastest growth of callus was observed when 2,4-D was the auxin.

2,4-D was found to produce early and fast callusing in *Sida* spp. by Sankar (1998).

5.1.3 Influence on colour and texture of callus

2,4-D containing media expressed white colour in calli, while NAA expressed yellow colour. IAA and IBA produced uniformly green callus from all the explants. Stem explants produced green callus, while the leaf explants produced white, lemon yellow and green callus.

Anu (1993) reported production of green callus from *G. sylvestre*. Sindhu (1999) found that all auxins and cytokinins produced uniformly brown callus in *Coscinium fenestratum*, except in a few cases where IBA containing media produced yellow callus with leaf explants.

In this study, it was noticed that, at lower connections of 2,4-D and NAA, callus colour was green, but when auxin concentrations were increased, the calli from 2,4-D media expressed more of white colour, while that from NAA containing media expressed yellow colour. This fact suggested that, at lower concentrations of auxins, the explants expressed their potential to develop colour in callus, indicating that they have potential to develop chlorophyll. But at higher concentrations, the auxins started to dominate the expression of colour, thereby inhibiting or inactivating chlorophyll production. Thus, the expression of callus colour seemed to be an effect of auxin concentrations.

In stem explants, irrespective of the concentration of auxins, green colour was predominant in callus. In leaf explants, a mixture of different colours was seen. This might be due to the inherent differences in the tissues of leaf and stem, which might have contributed to their specific responses to auxins.

After 60 days, some translucent calli converted into green colour. This could be due to development of chlorophyll. Development of chlorophyll in certain areas only, may indicate that the structural differentiation of cells could be occurring, which is a progress towards organogenesis.

The texture of callus developed by all the explants in all auxins at all concentrations was mostly friable. This suggested that auxins had no role to play in influencing texture of calli. Also, it suggested that all explants of *G. sylvestre* had an inherent and uniform ability to produce friable callus.

Friable callus was reported by Anu (1993) in *G. sylvestre* and by O'Dowd *et al.* (1993) in *Ephedra geradiana*.

5.1.4. Effect of explants on callus induction and proliferation

The stem explants (node and internode) had maximum percentage of callus initiation in all auxins, followed by leaf explants (lamina and petiole), while last was root explant.

Internode was found to produce maximum callus index in all auxins. Next to it were petiole and nodal segment, which performed nearly similar. The performances of leaf lamina and root explant in initiating and proliferating callus were very poor.

It was noticed that leaf explant withered away faster than the stem explants. Stem explants survived the shock of culturing, but the leaf explants did not. This might be because, the total volume of tissue in leaf lamina is quite less than in node or internode or petiole, owing to their larger girths. Thus the lamina had lesser nutrients

than other explants to tide over the shock of culturing. So, before it could recover from the shock and start the uptake of nutrients from medium, it withered. This resulted in very low callus initiation from leaf lamina.

For induction of combined growth from root tissue, combination of cytokinins and auxin is needed (Jacobs, 1979). Since, in this study only auxins were used, it could not induce callus from the roots.

Satheeshkumar and Bhavanandan (1989) reported use of leaf as explant to initiate callus in *Plumbago rosea*. Nazeem *et al.* (1990) reported the use of leaves from *G. sylvestre* to produce callus. They found that the older leaves produced more callus than the younger ones. In *Taxus baccata*, hypocotyl showed good callusing (Zhiri, *et al.*, 1995). Sandhya and Vilasini (1996) reported that in *G. sylvestre*, shoot tips, axillary buds, leaf discs and immature seeds produced callus. But, among the explants, relative superiority was not reported with respect to callusing.

In *Sida* spp., Sankar (1998) found that stem explants produced better callusing than leaf explants. Also the stem explants had a faster growth rate than the leaf explants. Root explants did not produce satisfactory callus in *Sida* spp. Sindhu (1999) found that, in *Coscinium fenestratum*, leaf and petiole gave better callusing than stem explants.

5.2 Influence of combination of auxins with cytokinins on callusing and saponin production

5.2.1 Influence on callus initiation, proliferation, callus index and growth rate

Combination of cytokinins with auxins showed more callus initiation, higher proliferation, higher callus index and faster growth rate than when only auxins were used.

The highest callus index was produced from the combination of 2,4-D (2 mg l^{-1}) + KN (1 mg l^{-1}) and 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}). Also these treatments showed higher percentages of callus initiation and proliferation.

The fastest growth rate was obtained from 2,4-D (2 mg l^{-1}) + BA (1 mg $^{-1}$).

Auxins are primarily known to induce cell elongation, while cytokinins induce cell division. Both these processes are essential for growth. So, a combination of cytokinins and auxins is more able and efficient to give higher callus growth than by use of auxins alone. Also, the combined stimulus from auxins as well as cytokinins may have enhanced the rate of growth of the callus.

2,4-D being found as the most potent auxin in this study, when it was supplemented with cytokinins (BA or KN), showed enhanced callusing.

Anu (1993) reported maximum callus index from *G. sylvestre* callus cultured on medium containing BA and 2,4-D combination, followed by BA and NAA combination. All other combinations of BA and kinetin with IAA, NAA and 2,4-D showed an inferior performance. Highest callus index was obtained by Anu (1993) from medium containing 1 mg l^{-1} 2,4-D + 1 mg l^{-1} BA. In *Sida* spp, Sankar (1998) reported that 1 mg l^{-1} 2,4-D + 0.3 or 0.5 mg l^{-1} kinetin gave highest callus index. For leaf explants the medium with 1 mg l^{-1} NAA+1 mg l^{-1} kinetin exhibited higher callus index. In case of stem explants, 1 mg l^{-1} 2,4-D + 1 mg l^{-1} kinetin gave highest callus index.

Mayashi *et al.* (1988) reported production of callus from *Glycirrhiza glabra* by incorporating 100 µM NAA and 1 µM BA in the medium. Sindhu (1999) reported that BA produced satisfactory callusing in *Coscinium fenestratum*. She also found that 1 mg l^{-1} BA + 2 mg l^{-1} IAA gave highest callus index.

The minimum number of days required for callus initiation, as found by Anu (1993) in *Gymnema sylvestre* were from media containing BA and 2,4-D. This is quite consistent

with the finding of this study, that 2 mg l^{-1} 2,4-D + 1 mg l^{-1} BA initiated and proliferated callus in least time, thus having the highest growth rate.

Song *et al.* (1994) reported that combination of BA and NAA, each at 1 mg l^{-1} concentration gave good callus proliferation in *Eucommia ulmoides* Oliv. Valk *et al.* (1995) found BA to have deleterious effect on callusing when combined with auxins. Also, Joseph (1997) reported suppression in callus proliferation when kinetin was applied in combination with 2,4-D in *Kaempferia galanga*. The results from the above reports are not consistent with this study on *Gymnema sylvestre*.

5.2.2 Influence on colour and texture of callus

All the combinations of auxins with cytokinins expressed predominantly green callus. When 2,4-D combined with BA and kinetin, apart from green colour, it produced white and translucent callus respectively, but the reverse combination of colours was never seen.

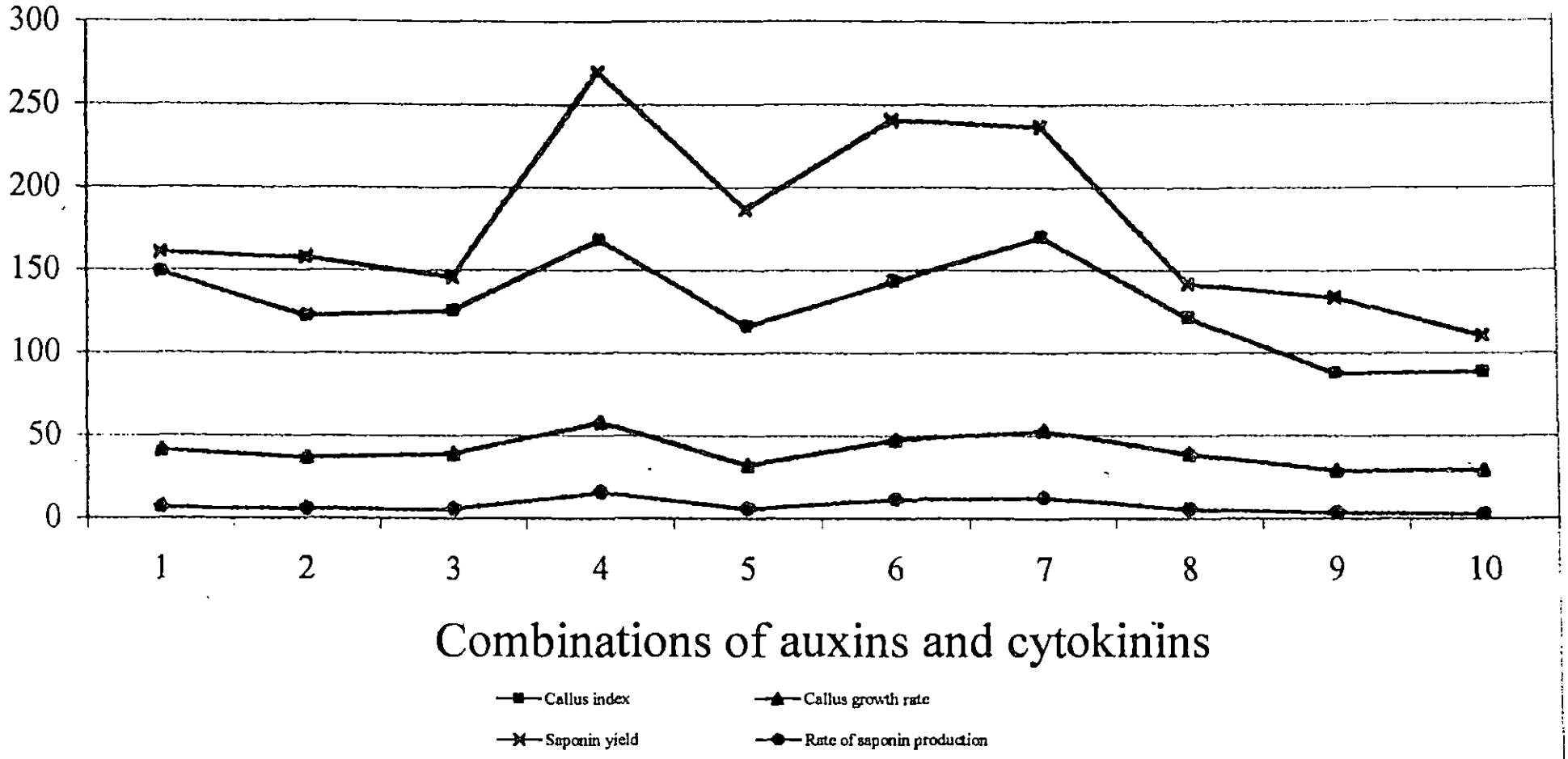
The predominance of green callus was reported in *Gymnema* by Nazeem *et al.* (1991) and Anu (1993). Sankar (1999) also reported green calli in *Sida* spp.

In this study all treatments produced friable calli except one containing NAA (5 mg l^{-1}) + kinetin (2 mg l^{-1}). This fact again suggests that the inherent tendency of *G. sylvestre* has an inclination towards producing friable callus.

5.2.3 Influence on saponin yields and rate of saponin production

Based on the growth scores, twelve superior media combinations were selected and their saponin yields were computed and compared. The rate of saponin production per day per tube showed that 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}) produced maximum saponins in least time followed by 2,4-D (2 mg l^{-1}) + kinetin (1 mg l^{-1}). The high yielding combinations contained 2,4-D as the auxin (Fig. 4)

Fig.4 Growth of callus and production of saponins from some selected combinations of auxins and cytokinins



Cheng and Liang (1981) studied *Scopolia acutangula* and *Rauwolfia yunnanensis* for alkaloids and *Panax notoginseng* for sapogenins. They found that at lower concentrations, 2,4-D stimulated synthesis of metabolites, but retarded growth of cultures. Also, NAA was found to stimulate growth and metabolite synthesis, while kinetin inhibited both. Burnouf *et al.* (1983) reported production of saponins from *Chenopodium quinoa* calli grown in MS + NAA (8 mg l^{-1}) + kinetin (1 mg l^{-1}). Kar and Sen (1984) cultured *Asparagus racemosus* Willd. for production of steroidal saponins. They found that, 2,4-D induced synthesis of saponins at a higher rate than NAA. Also the rate of saponin synthesis was correlated with 2,4-D concentrations. Both these findings are in exact unison with the findings of this study.

Hayashi *et al.* (1988) produced triterpeniod saponins from *Glycirrhiza glabra* calli from LS medium + 100 µM NAA + 1 µM BA. Akashi *et al.* (1994) produced triterpenoids from cultures of *Taraxacum officinale* by cultivating them on 2,4-D (1 mg l^{-1}) and kinetin (0.1 mg l^{-1}). Cultures of *Panax quinquefolium* when grown in MS + 2,4-D (1 mg l^{-1}) + kinetin (0.25 mg l^{-1}) produced saponins (Mathur *et al.*, 1994).

5.3 Influence of stress on callusing and saponin production

5.3.1 Influence of stress inducing chemicals

5.3.1.1 Influence on callus index and growth rate

The effects of stress inducing chemicals on callus index and growth are presented below.

(a) Carbon sources

Peptone, yeast extract and malt extract showed more callus production than control. Since peptone, yeast extract and malt extract provided additional carbon source to the callus, it helped in faster and increased callus growth.

(b) Growth retardant

ABA being a growth retardant had a retarding effect on calli growth, thus it produced lesser callus than control.

(c) Auxin synergist

Phloroglucinol, which is an auxin synergist, when provided at 25 mg l^{-1} concentration, exhibited more callusing than control, but at 50 and 100 mg l^{-1} concentrations showed less callusing than control. Hunter (1979) found phloroglucinol to give good *in vitro* growth in *Cinchona ledgeriana*. It was found to reduce the oxidative browning and enhanced the life span of cultures. Sankar (1998) reported promotion of callus in *Sida* spp. at 100 and 125 mg l^{-1} concentrations of phloroglucinol. Increase in agar to 0.9 % also influenced enhanced callus growth.

(d) Antioxidant

Silver nitrate was evaluated as an antioxidant. At all the concentrations evaluated, it produced lesser callus than the control.

(e) Osmoregulants

PEG gave higher callusing than control at 2 and 10 % concentrations. Being an osmoregulant, it has been reported to sustain growth of callus in *Coscinium fenestratum* (Sindhu, 1999).

Incorporation of mannitol in the medium reduced callusing greatly. Mannitol is known to increase osmotic potential of the medium. This adversely affects the cell growth. Cell division is restricted and cells remain small and stressed (Rudge and Morrisson, 1986 and Zhang *et al.*, 1995). This cause to lesser callusing in case of *Gymnema sylvestre*.

(f) Nutrient depletion

Sucrose at lower concentrations of 1 and 2 % reduced callus growth. Sucrose was provided as the lone source of carbohydrates in the medium. At these concentrations, the

quantity of carbohydrates available to the explant were reduced. This induced stress and retarded the callus growth (Mikuzami *et al.*, 1977).

Activated charcoal showed high reduction in callusing. Since it possesses the property to adsorb nutrients from the media, activated charcoal starved the callus of nutrients and hormones, thus showing reduction in growth. Fridborg and Eriksson (1975) stated this phenomenon and also found growth inhibition in calli of *Glycine max* and *Haplopappus gracilis*. They reported that auxins are also adsorbed by activated charcoal. This reduced the quantity of auxins available for callus growth, thus resulting in retarded growth. Similar retardation was observed by Sankar (1999) in calli of *Sida* spp.

5.3.1.2 Influence on saponin production

5.3.1.2.1 Influence on saponin yields from one and two month old calli

Saponins from calli were evaluated at one and two month's age. All the treatments showed higher saponin yields at one month age while reduced yields were observed in two month old calli. Yeoman *et al.* (1980) have suggested that the secondary metabolites may get degraded after it is produced in the cells. When rate of production of the metabolite is more than its rate of degradation, the metabolite accumulates in the cell. But, when the rates are reverse, more of the metabolite is degraded and lost. This may have happened in case of the *Gymnema* calli, wherein the rate of degradation of saponins might be higher than its rate of production at two month age, than the rate of production at one month, thereby causing reduction in yields.(Fig.5).

The biosynthetic pathway of saponins in *Gymnema* follows the mevalonic acid route. This pathway leads to formation of a vast range of compounds important to the plant cell, like fatty acids, flavonoids, gibberellins, steroids, alkaloids, carotenoids and saponins (Bhalsing and Maheshwari, 1998). Each of this group of compounds is synthesised by creating a diversion from the main pathway. The saponins are at the terminal position in this

pathway. It can so happen that after one month of age, the cell may divert the pathway towards production of any of these compounds by compromising the production of saponins by the way of feed-back inhibition. Thus, fresh synthesis of saponins will be reduced. This possibility coupled with the degradation of stored saponins may account for reduction in saponin yields in the second month. Assays have to be carried out for other products of the mevalonate pathway at both ages, to see if this hypothesis is true.

Saponin yield at one month age of callus was highest in 0.1 % activated charcoal containing media, followed by mannitol and sucrose combinations and then peptone (1 %). These yields were higher than control. At two month's age, 3 % mannitol yielded the highest amount of saponins, followed by 0.25 and 0.5 % activated charcoal.

Activated charcoal has been reported to block the availability of nutrients and hormones and mimic stress conditions for calli (Fridborg and Eriksson, 1975). In conditions of stress, it is common that the more secondary metabolites are synthesized. Since, activated charcoal produced stress it showed increase in saponins in *Gymnema* calli. Possibility of adsorption of certain inhibitors by the charcoal cannot be avoided in this context.

Combination of mannitol with sucrose was the next best treatment for obtaining higher yields of saponins. Rudge and Morris (1986) reported that mannitol increased the osmotic potential and reduced cell division. This favoured the accumulation of more carbohydrates per cell. Mannitol induced stress to these cells. Thus, it further increased the production of secondary metabolites. They found that alkaloid production was increased in *Catharanthus roseus* due to mannitol. In cultures of *Panax notoginseng*, saponins were increased by supplementing mannitol in the medium (Zhang *et al.*, 1995). Mannitol did not impressively increase ephedrine yields in *Sida* cultures (Sankar, 1998). In *Coscinium fenestratum*, mannitol did not give enhanced yield of berberine (Sindhu, 1999).

5.3.1.2.2 Influence of media additives on rate of production of saponin

The rate of saponin production, was expressed as μg gm^{-1} of callus per tube per day. It revealed that only malt extract (1 %), mannitol (1 %) + sucrose (2 %) and peptone (1 %) containing media were superior to control. These treatments showed economically significant results as they respectively produced 48.44, 25.43 and 23.62 % more saponins per tube respectively, than control. Rest of the treatments performed inferior to the control.

The higher yield of callus from malt extract rendered it a higher rate of saponin production. Though activated charcoal showed high saponin yields, the callus induction was poor and so its saponin index value was low. This index tried to find the treatment having maximum biomass production as well as maximum saponin production. It considered the economic usage of medium per test tube. When more biomass (callus) was produced per tube, media could be said to be utilized in a better way. The treatment giving maximum saponins from minimum number of tubes, being the most economic, was found by the rate of production of callus. Higher saponin yields could be due to either more callusing (as in 1 % malt extract) or due to more saponin production (as in 1 % mannitol + 2 % sucrose).

5.3.1.2.3 Influence on the types of saponins produced by *in vitro* cultures

Saponins were detected by thin layer chromatography. Purple, violet, red and blue coloured spots were exhibited for saponins. Yoshikawa *et al.* (1992a) reported that in case of *G. sylvestre*, dammarane type saponins showed pink and violet coloured spots, while oleanane type saponins showed blue and violet colour on TLC plates sprayed with 30 % sulphuric acid. *Gymnema sylvestre* has a large number of saponins with diverse chemical structures (Tables 1 and 2). These saponins have different polarities and hence exhibit different R_f values on chromatograms.

The reference spots of gymnemic acid were seen at R_f 0.5. Most calli samples showed saponins corresponding to R_f values of 0.5 and 0.7. Mannitol and sucrose media alone produced spots at R_f 0.3. This meant that diverse saponins were produced in calli, which were distributed at different R_f values in the chromatogram. Hamilton and Hamilton (1987) have reported that more polar compounds have lower R_f values. Thus, it can be said that when mannitol and sucrose were added to the media, they might have produced saponins which were more polar than the standard gymnemic acids, which was seen at R_f 0.5 supplied by Laila Impex Ltd, Hyderabad. Similarly, it can be said that all treatments showing saponins at R_f 0.7 produce saponins of lower polarity than the standard gymnemic acid, since they have a higher R_f value than that of the standard spot. The standard sample is derived from leaves of *G. sylvestre* plants. It means that all the saponins in the leaf are condensed in the standard and are seen at R_f 0.5. But the calli produced some newer and different saponins, which were located at R_f 0.3 and R_f 0.7. This fact suggested that, *de novo* synthesis could have occurred in the calli. These saponins need to be further purified and cross checked with those already produced in the leaves of *G. sylvestre*, so that the hypothesis about *de novo* synthesis can be accepted or rejected. The standard gymnemic acid exhibited purple coloured spot. Some treatments showed purple colour spots at R_f 0.3 and R_f 0.5, which could be inferred as being dammarane-type triterpene saponins (Yoshikawa *et al.*, 1992a). The blue and violet spots at R_f 0.7 could be of oleanane-type saponins.

At two month's age, nearly all treatments showed saponins only at R_f 0.7. This meant that, the saponins which were seen at R_f 0.5 in first month, might have been degraded or might have been chemically restructured into saponins of lower polarity.

Predominance of blue/violet spots was more than purple spots, which indicated that oleanane-type saponins were more than dammarane-type saponins. The reports on

saponins from *G. sylvestre* leaves also showed that only 7 saponins have been found to be of dammarane-type, while more than 30 are of oleanane type (Table 1 and 2).

5.3.2 Influence of stress induction by modifying culture conditions

Control medium containing MS + 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}) was cultured at room temperature and at 6 to 7 °C temperature. Low temperature significantly favoured callusing, but the rate of production of saponins was lower than at room temperature. Low temperature is known to retard the rate of reaction of enzymes. This tries to retard the normal growth of plant cells. So, to enhance the growth, metabolic pathways favour increased production of primary metabolites. This is achieved by compensating the production of secondary metabolites. This situation might have ocured in this study, which lead to reduced saponin yields.

5.4 Cell suspension cultures of *Gymnema sylvestre*

5.4.1 Growth of cell suspension cultures

All the cultures showed decrease in cell viability, cell count per ml and the percentage of packed cell volume (PCV) with increase in their age.

The data showed that, the percentage of packed cell volume and cell content per ml after one week were nearly similar for most of the treatments. The cell sizes, were quite large in all treatments, at all stages, except in peptone (1%) medium, wherein small and fast dividing cells were seen at 3rd and 4th week age. Based on the above facts, it can very well be presumed that the cells were in the lag phase for first seven days. The initial inoculum was the same for all treatments (1 gm callus approximately per flask). This accounted for near similar values of cell counts at one week age for most of the treatments. More over uniformly large cell size indicated the lag phase, wherein cells are known to increase in size and accumulate nutrients to undergo mitosis in the oncoming log phase. In peptone containing medium, cell growth was fastened by peptone and so cells started dividing much

early. As a result, enormous number of small sized cells were seen, indicating that the log phase had begun. This fact further confirmed that all other treatments had cells in lag phase. Since other media failed to fasten the cell division process, these cultures could not reach the log phase within one week. Moreover, observations at all the 4 weeks showed cells in lag phase because subculturing was done every 7 days interval. During each subculture, cells in lag phase were transferred to fresh medium. When subcultured in fresh medium, cells undergo lag phase (Gamborg and Shyluk, 1981). Hence each subculture created a lag phase. This delayed the onset of log phase instead of hastening it. It was also evident that the lag phase extended above 7 days. Though it could end shortly after 7 days, since it was seen to be advanced when medium was supplied with peptone (1 %).

Sankar (1998) also found that, in all species of *Sida*, lag phase began after 10 days and the cell density was maximum at 17 days after which, a decline was observed in growth. In case of *Gymnema*, it can be said that lag phase extended till a minimum of first 7 days. It is expected that, subculturing should be done when cell density is maximum and cells are in lag phase since it gives successive increase in cell count. But in this case, subculturing was done in the lag phase, so no increase in cell count and cell density was noticed with passage of time.

For growth observation of cell suspension cultures, the cultures were allowed to stand for 5 minutes and then 5 ml of the supernatant was taken. This supernatant showed reduction in cell viability with increase in age. The reasón is that at the first subculture the cells were in lag phase. The cell viability was good and living cells were in suspended form. Only the dead and broken cells were floating. Since only the top layer was taken, it happened to consist more of dead. This layer was subcultured as well as analysed. This inoculum had small quantity of living cells, and growth was again in lag phase due to subculturing. Dilution combined with poor growth rate (lag phase) resulted in low cell

count by end of the second week. This low-density culture again had floating dead cells which were transferred during 3rd subculture. This process was repeated till 4 subcultures. Hence it showed that viability, cell density and percentage of PCV decreased over time.

Cell count at one week age was relatively low in case of BA, malt extract and PEG incorporated media, than all other treatments. Of them, ABA being a growth retardant and PEG being an osmoregulant reduced growth of cells. Addition of malt extract to the medium had caused very good growth in case of callus cultures, but it did not show similar results for cell suspensions.

Peptone at 1% concentration was found to increase cell viability. Peptone seemed to have positive effect on growth and vigor of cells (Table No.2 4).

5.4.2 Saponin production from cell suspension cultures

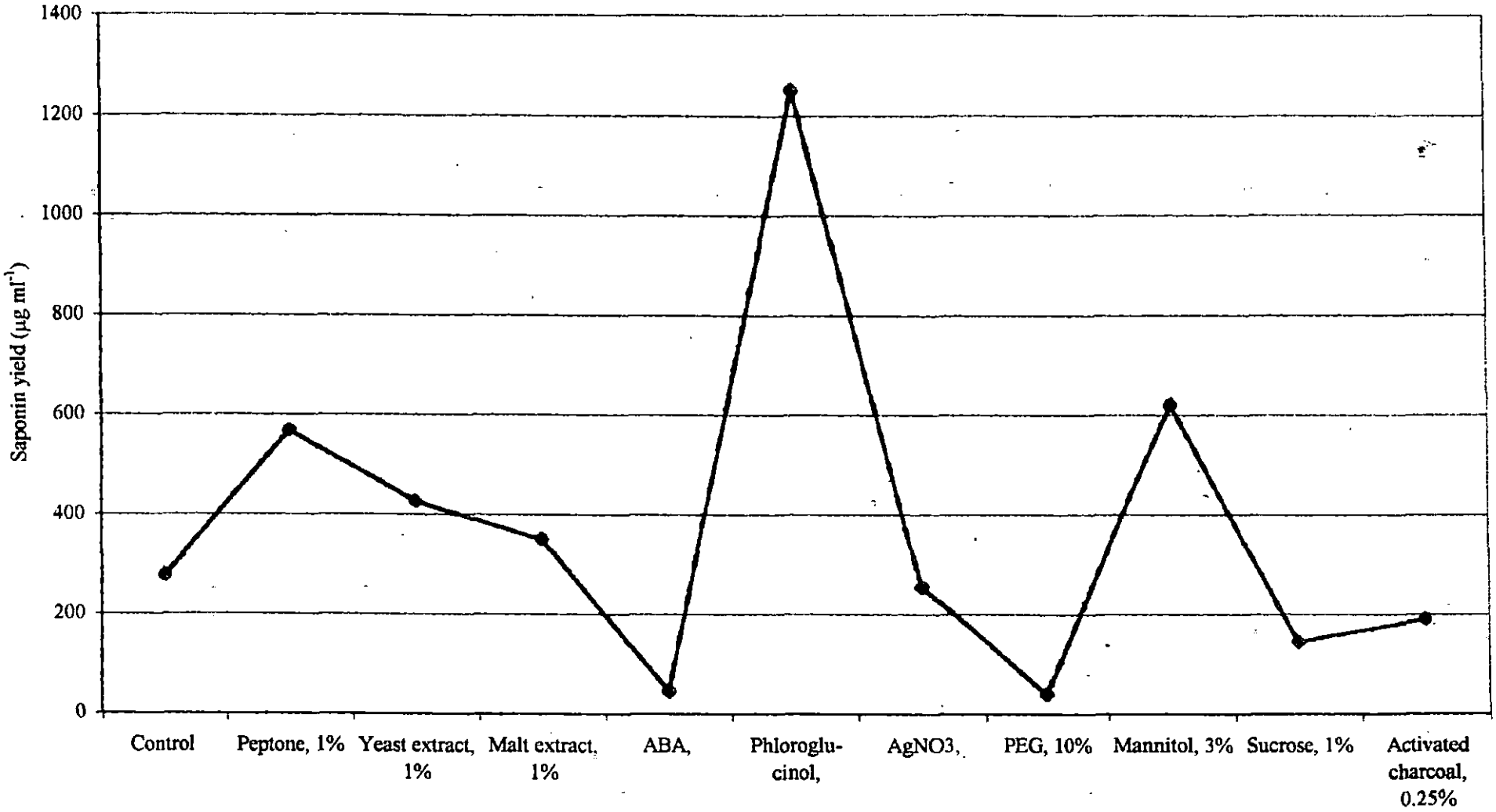
5.4.2.1 Yield of saponins secreted in the liquid medium

The saponin yields from media showed that control, ABA, peptone, yeast extract and mannitol containing media produced higher yields at one week age followed by a decline and again an increase in yield, till 4 weeks age. Malt extract (1%) showed continuous increase in saponin production with increase in age of cultures. Phloroglucinol (50 mg l^{-1}) and sucrose (1%) containing media showed continuous decline of saponins with passage of time (Fig.6).

The highest mean saponin yield per week was reported from phloroglucinol 50 mg l^{-1} treatment followed by mannitol 3% and peptone 1% containing media.

Rudge and Morris (1986) cultured cells of *Catharanthus roseus* for production of alkaloids by providing mannitol in the culture media. They found that the amount of serpentine alkaloid was initially 1 mg g^{-1} which decreased till 5 days age and then increased to 4 mg g^{-1} by 40 days age of culture. Such a trend of initial decrease followed by increase was also seen in this study.

Fig.6 Yield of saponins secreted in the media of cell suspension culturea of Gymnema sylvestre R.Br.



Malt extract has high content of reducing sugars (70.4%) with many acidic compounds (1.44 %) as reported by Cejka (1985). Both sugars and organic acids are used to synthesize mevalonic acid, which is the primary precursor of the triterpenoid saponins (Bhalsing and Maheshwari, 1998). Continuous provision of these nutrients from malt extract due to frequent subculturing, could have stimulated the mevalonic acid pathway and thus may have resulted in continuous increase in saponin synthesis with increase in age

5.4.2.2 Yield of saponins extracted from the cultures

From the cell suspensions, saponins were detected from ABA, yeast extract, malt extract, activated charcoal, sucrose and mannitol containing cultures. Among them, mannitol (3 %) yielded highest quantity of saponins, followed by ABA (0.5 mg l^{-1}) and yeast extract (1%).

Funk *et al.* (1987) cultivated *Glycine max* and *Thalictrum rugosum* cultures with yeast extract to act as an elicitor for enhancing secondary metabolite production. They found that production of glyceollin, from *Glycine max* increased till 10 days and later dropped sharply, whereas berberine production from *T. rugosum* showed uniform increase till 16 days of culturing. In this study on *Gymnema*, yeast extract containing medium showed high saponin content in the initial stages, followed by a sharp drop and again an increase, with increase in age (Table No.24) Presence of yeast extract acted as an elicitor and increased the production of saponins in the initial stages. But, as the cultures started to grow, the secondary products were metabolized and so the saponin contents registered a decline. But again as the cultures gained maturity secondary products were resynthesized and so saponin production was found to increase.

5.4.2.3 Influence on the types of saponins produced by *in vitro* cultures

Most of the cell suspension cultures uniformly showed only one spot of saponins in between R_f values 0.85 to 0.95. These were blue in colour. In contrast to this, the calli

cultures produced spots of different colours at 2 to 3 different R_f values. So, it can be presumed that the structures of cells are different in cell suspension cultures than in calli cultures.

This meant, that the cell suspension cultures could produce only a limited fraction of saponins than produced by the calli. These may be oleanane-type saponins due to their blue colour (Yoshikawa *et al.*, 1992a). Since their R_f value was higher (0.85 to 0.95) than that of the standard sample (R_f 0.5), they were less polar than the standard sample.

Phloroglucinol containing medium showed an additional spot at R_f 0.7 of purple colour. Mannitol (3 %) containing medium showed purple and red spots at R_f values of 0.05 and 0.4. These saponins could be of dammarane-type (due to red and purple colour) and more polar than standard (due to lesser R_f value than that of the standard). So, it could be said that phloroglucinol and mannitol responded differently than other additives by producing a different group of saponins.

5.5 Standardization of techniques for estimation of saponins from in vitro cultures of *Gymnema sylvestre*

5.5.1 Selection of appropriate solvent to extract saponins from callus

Chakravarti and Debnath (1981) had used petroleum ether, chloroform, ethyl acetate and ethanol for extracting saponins from the leaves of *Gymnema sylvestre*. They found that ethanol yielded maximum saponins followed by chloroform. Ethyl acetate could extract very little amounts of saponin while petroleum ether did not extract saponins. Hence, in this study, ethanol was used as the solvent to extract saponins from *in vitro* samples.

Ethanol, in different strengths, was evaluated for ability to extract saponins. Among them, 100 per cent ethanol showed minimum solid matter content in extract. Mukherjee *et al.* (1996) had also prepared tinctures from leaves of *G. sylvestre* in various alcohol

strengths and found that 100 per cent alcohol extracted minimum dry matter than aqueous alcohol solutions. They found 30 per cent alcohol to extract maximum dry matter. But no trend was noticed with decreasing concentrations of alcohol. Similarly, the results from *Gymnema* calli also failed to produce any trend of uniform rise or decline in dry matter extraction with respect to aqueous alcohol strengths.

Mukherjee *et al.* (1996) found that 30 and 50 per cent alcohol tinctures produced brownish colour, while 80 and 100 per cent alcohol tinctures had greenish yellow colour. Calli lacked green and brown colour and all uniformly produced yellow colour of varying intensities. This may be due to the pigment and resin content in calli, which rendered green and brown colours, was quite lower than that of leaves.

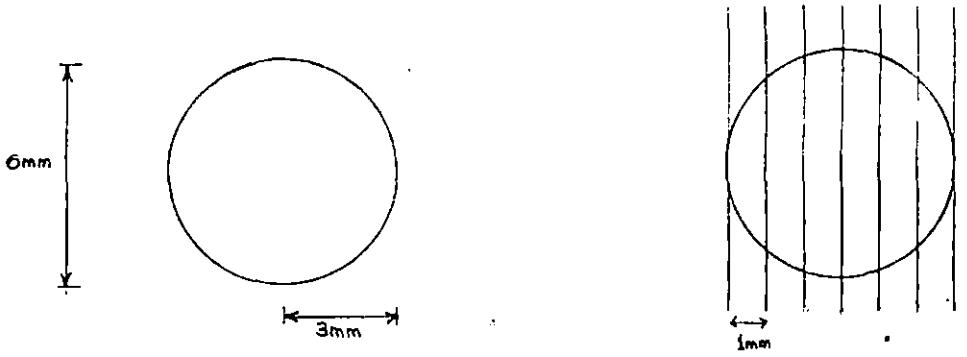
The densitometric method recorded high quantity of saponins. Interference of non-saponin compounds, which had affinity towards saponins and were located at same R_f values as that of saponins were also recorded as total saponins. These could not be eliminated while scanning. So, during quantification, they too were accounted for. This raised the measured quantity of saponins quite higher than the actual.

Owing to such interference, extraction of calli by aqueous ethanol solutions was rendered ineffective for densitometric quantification. So, it was imperative that saponins had to be separated from non-saponin compounds. Saponins are conventionally separated by either successive extraction with solvents of increasing polarity (Sahu *et al.*, 1996) or by column chromatography (Yoshikawa *et al.*, 1993). Varshney *et al.* (1984) defatted the leaf samples with ether and extracted successively with carbon tetrachloride, chloroform, ethyl acetate, acetone and finally with alcohol. This provided saponins with much less impurities. Maeda *et al.* (1984) and Yoshikawa *et al.* (1993) used column chromatography for the same purpose. But, successive extraction and column chromatography needed large quantity of sample to start with. Saponins being secondary

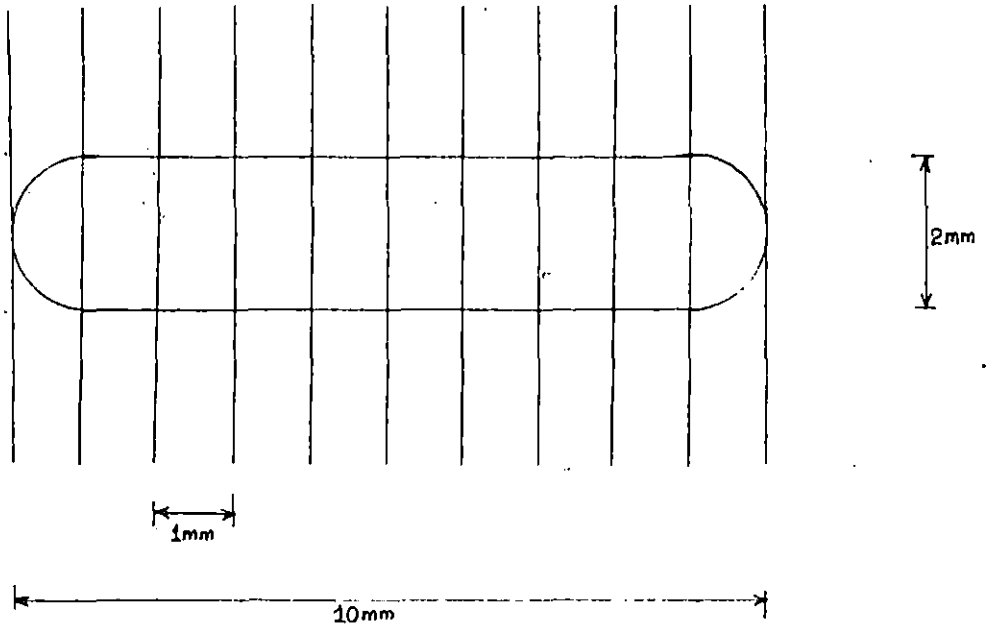
metabolites, their yields are very low, and so large quantities of samples were used in all the previous studies (1 to 4 kg fresh weight of leaves). In this study, not more than 10 grams (fresh weight) per sample was available. Owing to small sample size of calli, these methods could not be used. Another method, specially suited for *in vitro* cultures, called as the 'fractionation method', was evaluated for the same and was found quite suitable. It is described in section 3.6.1.1.*(A)*. It was quite successful in separating saponins from non-saponins.

The comparison of the extracts from ethanol with the fractionation method revealed that, though the chloroform extract had lesser yield of dry matter, it showed a clear spot of saponins, which no other solvent could exhibit. Also, the dry weight of chloroform extract was quite lower than that of the aqueous extract. Despite this, it showed a clear spot of saponins while the aqueous extract failed to do so. This meant that the chloroform extract had mostly saponins in it along with little amounts of other compounds, while the aqueous extract contained only non-saponin compounds. Thus, the fractionation method was quite successful in isolating the saponins into the chloroform fraction and the other unwanted chemicals into the aqueous fraction.

Since the saponin spot in the aqueous extracts was not clearly visible, for densitometric quantification, a circular area was marked on the scanned image of the chromatogram at R_f 0.5 for each of them. This marked area was quantified. Considering the visibility pattern of the chromatograms, it is quite evident that all the alcohol extracts (Plate 2 (A)-no. 1 to 4) had many compounds other than saponins at R_f 0.5 and all these were included in the marked area. These also contributed to the crude saponin yields during quantification. Due to the inherent limitations of this quantification procedure, the effect of these compounds could not be eliminated. Hence the computed data was not specific to



a. Application of sample in circular spot



b. Application of sample in horizontal streak

Figure 5.7 Application of sample by different methods.

pure saponins. The data reflected not on their ability to extract saponins but on their ability to extract more quantity of compounds. Moreover, the data also suggested that the actual saponin content was quite low, comparable to 3.46 $\mu g\ g^{-1}$, as obtained from the chloroform fraction, but the interfering compounds increased it to as high as 23.68 $\mu g\ g^{-1}$ in case of 100 per cent ethanol extract. This further stressed the need to separate the saponins as they could not be quantified from the chromatograms developed from mere ethanol extracts. Based on all these observations, the fractionation method was considered to be the best one. The chloroform extract was quantified for saponin estimation.

5.5.2 Method of application of samples on the TLC plates

When sample was less concentrated, application in form of circular spots gave better results. When in high concentrations, application in horizontal streaks was found to give better results.

As a generalised rule, Hamilton and Hamilton (1987) have reported that, during elution of a chromatogram, different compounds elute vertically upwards and accumulate in form of spots at their characteristic R_f values. Chemicals with similar elutropic abilities accumulate at adjacent R_f values. When concentration of applied sample is high, the amount of each compound in the sample is also high. So the quantity of organic compounds accumulating at any particular R_f also increases proportionately. This causes the spots on the chromatograms to spread beyond their respective R_f values. Further, this causes mixing of adjacent spots, thus reducing the clarity of the chromatogram (Hamilton and Hamilton, 1987). When concentration of the sample increases even more, all the spots enlarge and merge with respective neighbouring spots, to form a big smear of compuonds spreading over large distances on the chromatogram. This results in very poor visibility of individual spots and is not at all suitable for quantification by densitometry.

The reasoning behind this phenomenon is explained in Figure 7 : shows that the difference in the mode of application of the sample. In this experiment, 20 µl of sample was applied in circular spots of radius of 3 mm, and in horizontal streaks of one cm length and 2 mm breadth. So, 20 µl of the sample was divided in 28.26 mm^2 area of the circular spot (area of the spot = $\pi \times 3^2$), while it was divided into 20 mm^2 area of the streak (10 mm x 2 mm). Touchstone and Dobbins (1978) have reported that elution of the sample is in the vertical direction. When the running solvent system moves vertically upwards, it elutes all the compounds that come in its way. So, the clarity of the chromatograms is determined by the concentration of compounds present in every vertical column. To find this, both these modes of application were divided into vertical columns of one mm breadth. The figure shows that, there were 6 vertical columns in the circular spot, while 10 in the streak. So, the concentration of the sample per unit vertical column was 4.71 µl for the circular spot (28.26 / 6), while it was 2.82 µl for streak application (28.26 / 10). This showed that the quantity of sample per unit vertical column was quite high in case of the circular spot than the streak, hence, the clarity of chromatogram was better when samples were applied in streaks.

5.5.3 Selection of suitable running solvent systems to elute saponins from *Gymnema sylvestre*

5.5.3.1 Elution of saponins using single solvents

Hamilton and Hamilton (1987) have recommended that the search for a good running solvent system should begin with the evaluation of single solvents for their elutropic abilities. If single solvents fail to give proper elution, then mixtures of different running solvents can be experimented on. They have reported that the solvent strength parameter denoted by ϵ°, differs for each solvent. The solvents are graded according to their ϵ° values (Touchstone and Dobbins, 1978 and Hamilton and Hamilton, 1987). More

the ϵ° value, more is the ability of that solvent to elute any compound to higher R_f. In short, ϵ° determines the carrying capacity of the solvent. Solvents with lower polarity have lower ϵ° values and vice versa. The solvents used in this study are listed in increasing elutropic abilities, viz. hexane, chloroform, ethyl acetate, acetone, methanol and finally water. Hexane has least value of ϵ°, while water has the highest.

The results of elution are in accordance with the elutropic potentials and ϵ° values of the solvents. Plate 2B reveals that saponins were at R_f values of 0.1 and 0.2 when eluted in chloroform and ethyl acetate respectively. Since their ϵ° values are poor, the saponins were not eluted beyond R_f value of 0.2. Also, due to poor carrying capacity of these solvents, much of the sample was not eluted and remained at the point of application.

In contrast, methanol and water are at the other extreme in the elutropic series of the solvents (Touchstone and Dobbins, 1987) and hence have very high ϵ° values. So they could elute the saponins to R_f value beyond 0.9. Also these solvents have eluted more compounds from the spot of application leading to clustering at the solvent front. The elution patterns of chloroform and methanol show opposite performances as they exist at opposite extremes in the elutropic series.

In order to strike balance between the extreme performances of chloroform and methanol, successive elution was done, firstly in methanol, followed by chloroform. The first elution helped to elute most of the applied sample. The second elution helped in moving it further up and fractionating it. Due to a lower ϵ° value, chloroform helped to fractionate the already eluted sample, resulting in four spots above Rf 0.5.

5.5.3.2 Selection of solvents based on R_f values

Touchstone and Dobbins (1978) have stated that, the R_f value of any spot depends on the polarity of the sample itself, the eluting solvent and the adsorbent (silica gel in this case). The theory states that, while moving upwards, the running solvents dissolve the

compounds applied in the sample and carry them upward along with themselves. A competition arises between the silica gel on the TLC plate and the running solvent for retention of the eluting sample. Though both of them try to retain the sample, only the one having a better ability to adhere the sample retains it by electrostatic force or covalent bonding. Adhesion of the sample can also occur due to the attraction between the ions on the sample with those on the solvent or silica (polarity).

Solvents with lower polarity have a better adhesion to samples with lower polarity, than for highly polar samples. So, when a competition occurs in between silica gel and the solvent, the low-polarity solvents retain the low-polarity samples while loose the highly polar solvents to the silica gel. So, solvents of lower polarity (ϵ° value) carry samples of lower polarity along with them to greater R_f values. But samples of higher polarity having more affinity for silica gel adhere to the plate without much elution. Thus a solvent of low polarity produces low R_f values for highly polar samples and high R_f values for low-polarity samples.

Polar solvents have high affinity for both non-polar as well as polar samples. So, they elute all the samples to higher R_f values. Solvents with high ϵ° move the samples up with them and so produce spots at higher R_f values.

5.5.3.3 Selection of solvents for fractionating saponins into various spots

One of the objectives of selecting the running solvent system was to fractionate the mixture of saponins present in *G. sylvestre*. Saponins in *G. sylvestre* are highly polar. Usually, triterpenes have lower polarity, but when they attach to sugars to form saponins, their polarity greatly increases (Robinson, 1975). Touchstone and Dobbins (1978) have reported that the solvents with different ϵ° values when mixed in certain proportion cause the mixtures of chemicals in any sample to separate. In a mixture consisting of a polar and a non-polar solvents, the non-polar solvent (one with lower ϵ°) looses some polar saponins

(polar compounds) to silica producing spots at low R_f values. While the polar solvent (one with higher ϵ°) tries to retain other saponins and elutes them to higher R_f values. As a result, the mixture of saponins breaks up into different components and is distributed at different R_f values. Hence a pattern of numerous bands of saponins is obtained. The extent of fractionation of any group of chemicals depends on the polarity of the solvents as well as their relative proportions in the solvent mixture. The proportion of solvents is quite critical and is specific to each group of chemicals. In this study, different solvent system combinations were experimented to fractionate triterpene saponins.

Sinsheimer *et al.* (1970) used chloroform : acetone : methanol (5 : 1 : 1) to elute genins from saponins of *Gymnema sylvestre*. Rao and Sinsheimer (1971) used chloroform : methanol (9 : 1) and chloroform : ethyl acetate : methanol (97 : 2 : 1) to elute sapogenins. Kesselmeir and Ruppel (1979) eluted saponins from *Avena sativa* L. in chloroform : methanol : water (70 : 30 : 4), while Drapeau *et al.* (1986) eluted saponins from *Dioscorea deltoidea* in chloroform : methanol : water (65 : 35 : 10). These running solvent systems were tested for fractionating saponins from *G. sylvestre* and some modifications were made in them to get desired results.

In this study, hexane : ethyl acetate (3 : 1) solvent system separated the saponins into seven spots, but much of the applied sample remained uneluted. It was because of the lesser difference in ϵ° values of hexane and ethyl acetate. Also, both have lower polarities. More over hexane amounted to 75 per cent (v/v) and so the overall ϵ° value of the solvent mixture became quite less. Hence, separation as well as elution of saponins was not very good.

Chloroform and methanol mixture gave more spots as the difference in individual ϵ ϵ° values was more. Hence, separation of saponin spots was more. As the percentage of chloroform reduced and that of methanol increased, in the mixture, the amount of uneluted

sample decreased. Also the R_f values of saponins increased and accumulation of compounds at solvent front was found to increase. This was because, increase in methanol proportion increased the ϵ° value of the solvent mixture.

To get an even better separation of saponins, the same TLC plate was repeatedly eluted in different solvent systems. Plates were initially eluted till 1/3rd height in one solvent system and later on again eluted from bottom to 2/3 rd height in another system. The first solvent system eluted the sample upwards, while second separated the saponins in distinct locations. A mixture of chloroform : methanol when eluted successively in ratios of 80 : 20 followed by 98 : 2, gave very impressive results. It separated the mixture of saponins into 15 spots. When second elution was in 95 : 5 ratio (solvent no. 14), fractionation was equally good, but more compounds accumulated at the solvent front. This was due to increased proportion of methanol in the running solvent system. So, the former combination (solvent no. 13) was found to be the best for separating the saponins in *G. sylvestre*. A two dimensional chromatogram was developed using these systems, which enhanced the number of saponin spots to beyond 20.

When water was added to chloroform and methanol, the ϵ° value of solvent mixture increased. This increased R_f values of saponins and also the accumulation of compounds at the solvent front. This was in perfect unison with the theory that high ϵ° solvents (water) increase elution (Touchstone and Dobbins, 1978).

The solvent mixture of chloroform :ethyl acetate: methanol (97 : 2 : 1) gave poor elution and poor separation as the percentage of chloroform was too high and it dominated the effects of methanol and ethyl acetate by reducing the overall ϵ° value of the solvent mixture.

5.5.3.4 Selection of solvents to condense all the saponins into one spot

For densitometric quantification, it was expected to condense all the saponins into one spot. The combination of chloroform : acetone : methanol gave a very good single condensed spot of all saponins. The R_f value of this spot varied as per the ϵ° value of system. When in ratio of 10 : 1 : 1, the R_f of saponin spot was 0.2 and it increased as the proportion of chloroform decreased in the solvent system. The ratio of 5 : 1 : 1.5 gave the best R_f value for the saponin spot, which was at 0.5. Hamilton and Hamilton (1987) have suggested that a R_f value between 0.3 and 0.7 is better as it is not disturbed by either the less eluted samples usually lying below R_f 0.3 and due to fluctuations in solvent front, which occur normally above R_f 0.7.

When ethyl acetate was added to this combination, the ϵ° value dropped and elution was poor owing to lesser elutropic ability of ethyl acetate. As a result, the separation was poor. The combination of ethyl acetate and methanol gave poor results and was not as impressive as the chloroform and methanol mixture. This difference in ϵ° values of ethyl acetate and methanol is lower than the difference in the ϵ° values of chloroform methanol. More the difference in the ϵ° values of solvents in a mixture, more is the separation of saponins.

When water was added to ethyl acetate and methanol, it increased the efficiency of elution of sample. These mixtures in various proportions gave either many saponin spots, which became different to quantity or else, the saponins remain mixed with other compounds. As a result, these combinations were not found successful.

Touchstone and Dobbins (1978) have reported that, repeated elution in same solvent system gave better separation of adjacent spots. Using this principle, a sample was eluted in chloroform : methanol (90 : 10) ratio for three consecutive times and the resultant was a better separated chromatogram (solvent no. 40 a, b and c). Successive elution

showed enhanced separation. This could be used to separate a mixture of closely related saponins having very close R_f values.

So the best solvent system to get a condensed spot was determined as chloroform : acetone : methanol (5 : 1 : 1.5) and getting maximum separation of saponins was chloroform : methanol mixture in 80 : 20 ratio followed by elution in 98 : 2 ratio.

5.5.4 Selection of suitable spray reagent

The saponins in *Gymnema* contain triterpenoid structures linked to sugars and organic acids through oxygen atoms (Yoshikawa *et al.*, 1989a). So spray reagents used to detect these saponins can act on either the triterpenes or the sugars or the oxygen mediated bonds, to produce colours. Sinsheimer *et al.* (1970) used benzoyl chloride-sulphuric acid reagent to detect saponins from *G. sylvestre*. Rao and Sinsheimer (1971) used Leibermann-Burchard reagent for the same. Touchstone and Dobbins (1978) have suggested the use of vanillin phosphoric acid, vanillin sulphuric acid, anisaldehyde sulphuric acid, antimony trichloride in chloroform, ceric sulphate-sulphuric acid reagents to detect saponins on TLC plates. Blood reagent is quite popularly used as reported by Wagner *et al.* (1984).

From the above mentioned methods, vanillin-sulphuric acid and anisaldehyde-sulphuric acid reagents were evaluated to visualise saponins.

Free and bound sugars are detected by *p*-anisaldehyde. So, it also detects the sugars bound by glycosidic linkages in the triterpenoid saponins as purple and blue spots. All the more, it detected the free sugars as blue and green spots. This added to confusion in identifying the exact coloured spot for saponin.

Vanillin-sulphuric acid reagent was found to be quite specific to saponins. It exhibited saponins by purple and blue coloured spots while, all other chemicals were exhibited as only charred brown material. So, identifying saponins by vanillin-sulphuric acid reagent was found to be more suitable due to its specificity towards saponins.

Some calli had very less saponin content, which could not be detected by normal amounts of vanillin in vanillin-sulphuric acid reagent. When, the vanillin concentration was increased, the intensity of spots increased, thus improving the visibility. Beyond three per cent concentration of vanillin, the visibility remained same. This suggested that the quantity of vanillin available to react with saponin might be sparse in 1% concentration. When concentration was increased, the chances of vanillin reacting with saponins increased and thus the visibility increased. Since the content of saponin in a specific sample was constant, once a threshold level of vanillin was available, all the saponins reacted to show colour. This created spots of desired colour intensity. Even when vanillin concentration was raised beyond this threshold level, the quantity of saponins available for reaction being constant, no further reaction occured with vanillin. Thus no further increase in colour intensity was noticed. The threshold level was at three per cent vanillin concentration in this study.

Increasing concentration of sulphuric acid lead to increased charring of the organic compounds. This reduced the visibility.

So three per cent vanillin with five per cent sulphuric acid in ethanol was found to be the best spray reagent for detecting saponins from *G. sylvestre* calli.

SUMMARY

SUMMARY

The study entitled "*In vitro* callus induction in Gurmar (*Gymnema sylvestre* R.Br.) for secondary metabolite synthesis" was carried out at the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara, from May, 1998 to November, 1999. The salient findings of the study are stated below:

1. Among the several auxins tried to initiate and proliferate calli, 2,4-D showed the best performance followed by NAA.
2. IAA and IBA showed poor equivalent to MS medium without phytohormones.
3. Among auxins, maximum callusing in minimum time was achieved by addition of 2,4-D (faster growth rate).
4. Incorporation of 2,4-D in the medium showed minimum percentage of tubes without callus initiation and maximum percentage of tube producing full-tube callus, than any other auxin.
5. Among the explants, internode was the most potent in initiating and proliferating calli. Following it were petiole and nodal explants. Root explant failed to give satisfactory callusing.
6. The stem and leaf explants preferred 2,4-D as the source of auxin to enhance callusing.
7. Combination of cytokinins with auxins gave better callusing than, when only auxins were incorporated in the MS medium.
8. Combination of 2,4-D with BA produced maximum callus followed by that of 2,4-D with kinetin.
9. Combination of 2,4-D with BA showed highest callus initiation, maximum proliferation and also fastest growth rate.
10. Most of the treatments produced friable calli.

11. Green was the most dominant colour of callus. In some instances, incorporation of 2,4-D in media produced white colour, while NAA produced yellow colour in the callus.
12. Stem explants (node and internode) exhibited mostly green coloured calli but leaf explants (lamina and petiole) produced white, yellow and green colours depending on auxins incorporated in the media.
13. Among the various combinations of auxins and cytokinins, fastest callus growth was seen in MS + 2,4-D (2 mg l^{-1}) + BA (1 mg l^{1}) combination, amounting of 58.1 μg $(day)^{-1}$ $(tube)^{-1}$, followed by MS + 2,4-D (2 mg l^{-1}) + kinetin (1 mg l^{-1}) combination, producing 53.1 g $(day)^{-1}$ $(tube)^{-1}$.
14. Production of saponins at two months age of callus was highest in MS + 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}), yielding 270 μg g^{-1} of callus weight.
15. When media were supplemented with only phytohormones, the fastest rate of saponin production of 15.68 μg $(day)^{-1}$ $(tube)^{-1}$ was obtained from MS + 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}) medium. Following it was MS + 2,4-D (2 mg l^{-1}) + kinetin (1 mg l^{-1}) with a saponin yield index of 12.59 μg $(tube)^{-1}$ $(day)^{-1}$.
16. Among the various additives tried to enhance saponin yield, malt extract (1%) produced maximum callus, followed by yeast extract (1%).
17. Among stress inducing compounds, addition of activated charcoal (0.1 %) increased saponin yield to the highest value of 725.58 μg g^{-1}.
18. Based on the overall performance, the best medium combination to produce maximum saponins within minimum time was MS + 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}) + malt extract (1%), which produced saponins at the rate of 32.75 μg $(day)^{-1}$ $(tube)^{-1}$.

19. Stress induced by cool temperature (6 to 7 °C) and by increasing agar to 0.9 % showed little increase in callusing over culturing at room temperature and with agar at 0.75 %. These stresses could not increase the saponin yield.

20. Most stress inducing chemocals showed the presence of saponins that are absent in leaves of *G. sylvestre*. A violet coloured spot at R_f 0.7 and a purple spot at R_f 0.3 in TLC represented *de novo* synthesized saponins in the *in vitro* cultures which were not present in the leaves of *Gymnema sylvestre*, due to the difference in their R_f values with those obtained from the leaf extract.

21. In cell suspension cultures, cell viability, cell count and per cent packed cell volume were found to decrease with increase in age of cultures.

22. From cell suspension cultures, maximum saponin yield of 1250 μg ml^{-1} $(week)^{-1}$ was reported from liquid cultures grown on MS + 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}) + phloroglucinol (50 mg l^{-1}).

23. Most of the cell suspension cultures showed presence of newly synthesized saponins, which were different from those produced in leaves. Highly polar saponins showing blue spots above R_f value of 0.85 were the *de novo* synthesized saponins.

24. Incorporation of mannitol produced red and purple spots of *de novo* synthesized saponins at R_f values of 0.3 in calli cultures and at 0.05 and 0.4 in cell suspension cultures.

25. Regarding the standardization of procedure to estimate saponins from *in vitro* cultures, extraction with 60 per cent ethanol, followed by fractionation with 1:1 ratio of chloroform and methanol mixture was found to be the best. The chloroform fraction, which contained the saponins, was used for estimation.

26. Application of sample in circular spots of 3 mm radius was better for less concentrated samples. For highly concentrated samples, application of sample in horizontal streaks of 1 cm length and 2 mm width gave maximum clarity.

27. For eluting saponins into a single condensed spot, the running solvent system comprising of chloroform : acetone : methanol (5 : 1 : 1.5) was found to be the best.
28. To fractionate saponins into maximum possible distinct spots, the best results were obtained by eluting the TLC plate upto 1/3rd length in chloroform : methanol (80:20), followed by elution in chloroform : methanol (98 : 2) solvent systems.
29. To detect saponins from *G. sylvestre* on TLC plates, even when present in weak concentration, vanillin sulphuric acid reagent was found to be the best spray reagent, with vanillin at 3 per cent and sulphuric acid at 5 per cent concentrations.

REFERENCES

REFERENCES

Akashi, T., Furuno, T., Takahashi, T. and Ayabe, S. 1994. Biosynthesis of triterpenoids in cultured cells and regenerated and wild plant organs of *Taraxacum officinale* *Phytochemistry* **36**(2): 303-308

Anonymous. 1996. *Phytotherapy Res.* **10**: 53-55

Anonymous. 1997a. Hypoglycemic plants from the Canary islands. *Current R&D Highlights.* July-Sept: 37-39

Anonymous. 1997b. Hypoglycemic effect of rhizomes of *Similax glabra* in normal and diabetic mice. *Curr. R&D Highlights* (3): 37

Anonymous. 1997c. Hypolglycemic effects of *Opuntia ficus-indica* Mill., *Opuntia lindheimeri* Engelm and *Opuntia robusta* Wendl. in streptozotocin induced diabetic rats. *Curr. R&D Highlights* (3): 40

Anonymous. 1998. Minimum value norms for herbal drugs to be specified. *Industry Highlights* June: 41

Anu, K.I 1993. Standardization of *in vitro* propagation technique in *Gymnema sylvestre* R.Br. M.Sc. (Horticulture) thesis, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala, p. 54-78

Anu, K.I., Nazeem, P.A., Joseph, L. and Vijayakumar, N.K. 1994. Response of 'Gurmar' (*Gymnema sylvestre* R.Br.) for *in vitro* propagation. *South Indian Hort.* **42**(6): 365-368

Bell, J.I. and Hockday, T.D.R. 1996. Diabetes mellitus. *Oxford Textbook of Medicine* (Ed. Weatherall, D.J., Ledingham, J.G.G. and Warrel, D.A.), 3rd edition, Oxford University Press, Oxford, U.K., p. 1448-1505

Bever, B. and Zahnd, G.R. 1979. Plants with oral hypoglycemic action. *Quarterly J. Crude Drug Res.* **17**: 139-196

Bhalsing, S.R. and Maheshwari, V.L. 1998. Plant tissue culture - a potential source of medicinal compounds. *J. Sci. ind. Res.* **57**: 703-708

Bhojwani, S.S. and Razdan, M.K. 1983. *Plant Tissue Culture. Theory and Practice.* Elsevier Publications, Amsterdam, The Netherlands, p. 43-70

Burnouf, R.M. and Paupardin, C. 1983. Triterpenoid saponins from plant organs and *in vitro* tissue cultures of *Chenopodium quinoa* Willd. *C. R. Seances Acad. Sci. Ser.3* **296**(8): 429-432

Cejka, A. 1985. Preparation of media. *Biotechnology volume 2. Fundamentals of Biochemical Engineering* (ed. Rehm, H.J., Reed, J. and Brauer, H.) VCH, Weinheim, p. 640-641

Chakravarti, D. and Debnath, N.B. 1981. isolation of gymnemagenin, the sapogenin from *Gymnema sylvestre* R.Br.(Asclepiadaceae). *J. Inst. Chemists (India)* **53:** 155-158

Cheng, K.C. and Liang, C. 1981. Callus cultures of three well known Chinese herbs and their medicinal contents. *Proc. Symp. Pl. Tiss. Cult.* Kumming Institute of Botany, Peking, China, p. 469-479

Chopra, R.N., Chopra, I.C., Handa, K.L. and Kapoor, L.D. 1958. *Indigenous Drugs of India.* 2nd edition, U.N. Dhur and Sons Pvt. Ltd., Calcutta, p. 336-339

Dateo Jr., G.P. and Long Jr., L. 1973. Gymnemic acid, the antisaccharine principle of *Gymnema sylvestre*. Studies on the isolation and heterogeneity of gymnemic acid A_1. *J. Agri. Fd. Chem.* **21**(5): 899-903

Dougall, D.K. 1980. Nutrition and metabolism. *Plant Tissue Culture as a Source of Biochemicals* (ed. Staba J E), CRC Press, Florida, p. 28-29

Drapeau, D., Sauvaire, V., Blanch, H.W. and Wilke, C.R. 1986. Improvement of diosgenin yield from *Dioscorea deltoidea* plant cell cultures by use of a non-traditional hydrolysis method. *Pl. Med.* **51**: 474-478

Fowler, M.W. 1983. Commercial applications and economic aspects of mass plant cell culture. *Plant Biotechnology* (ed. Mantell, S.H. and Smith, H), Cambridge University Press, Cambridge, p. 3-37

Fowler, M.W. and Stepan-Sarkissian, G. 1985. Carbohydrate source, biomass productivity and natural product yield in cell suspension cultures. *Primary and Secondary Metabolism of Plant Cell Cultures* (ed. Neumann, K.H., Barz, W. and Reinhard, E.), Springer-Verlag, Berlin, p. 66-73

Fridborg, G. and Eriksson, T. 1975. Effects of activated charcoal on growth and morphogenesis in cell cultures. *Physiol. Pl.* **34**: 306-308

FRLHT. 1997. *Medicinal plants of India. Guidelines for National Policy and Conservation Programmes.* Foundation for Revitalisation of Local Health Traditions, Bangalore, p. 11

Fujioka, N., Kohda, H., Yamasaki, K., Kasai, R., Tanaka, O., Shoyama, Y. and Nishioka, I. 1989. Production of oleanane saponins by callus tissue of *Panax japonicus. Pl. Med.* **55**: 576-577

Fujita, T. 1988. Shikonin: Production by plant (*Lithospermum erythrorhizon*) cell cultures. *Biotechnology in Agriculture and Forestry, Vol 4. Medicinal and Aromatic Plants I* [Ed. Bajaj, Y.P.S.], Springer-Verlag, Berlin, p.236

Funk., C., Gugler, K. and Brodelius, P. 1987. Increased secondary product formation in plant cell suspension cultures after treatment with a yeast carbohydrate preparation (elicitor). *Phytochemistry* **26**(2): 401-405

Furuya, T., Yoshikawa, T., Orihara, Y. and Oda, H. 1983. Saponin production in cell suspension cultures of *Panax ginseng. Pl. Med.* **48**: 83-87

Gamborg, O.L. and Shyluk, J.P. 1981. Nutrition, media and characteristics of plant cell and tissue cultures. *Plant Tissue Culture Methods and Applications in Agriculture.* (ed. Thorpe, T.A.) Academic Press, New York, p. 21-24

Goldsmith, M.H.M. 1977. The polar transport of auxins. *Annual Rev. Pl. Physiol* **28**: 439

Gupta, P.K.1995. *Elements of Biotechnology.* Rastogi and Company, Meerut, p. 267-271

Hamilton, R. and Hamilton, S. 1987. *Thin Layer Chromatography.* John Wiley and Sons, London, p. 129

Hayashi, H., Fukui, H. and Tabata, M. 1988. Examination of triterpenoids produced by callus and cell suspension cultures of *Glycirrhiza glabra. Pl. Cell Rep.* 7: 508-511

Henry, M. and Guignard, J.L. 1982. Growth and quillaic acid production of *Saponaria officinalis* L. cell suspension culture. *Pl. Tiss. Cult.* 5: 299

*Hooper, D. 1887. *Pharm. J.* **17:** 867

Hossain, M., Islam, R., Karim, M.R., Rahman, S.M.,and Joarder, O.I. 1994. Production of plantlets from *Aegle marmelos* nucellar callus. *Pl. Cell Rep.* **13**(10): 570-573

http://www.harvard/edu/china/asclepiadaceae/html

Hunter, C.S.1979. *In vitro* culture of *Cinchona ledgeriana* L. *J. hort. Sci.* **54**(2): 111-114

Ilahi, I. and Ghauri, E.G. 1994. Regeneration in cultures of *Papaver bracteatum* as influenced by growth hormones and temperatures. *Pl. Cell. Tiss. Org. Cult.* **38**(1): 81-83

Imoto, T., Miyasaka, A., Ishima, R. and Akasaka, K. 1991. A novel peptide isolated from the leaves of *Gymnema sylvestre* -I. Characterisation and its suppressive effect on the neural responses to sweet taste stimuli in rat *Comparative Biochem. Physiol.- A. Comparative Physiol.* **100**(2): 309-314.

Ionkova, I and Kartnig, T. 1997. Densitometric determination of cycloartane triterpene saponins for transformed root cultures of *Astragalus mongholicus* Bge. *Pharmazie* **52**(3): 218-220

Ivorra, M.D., Paya, M. and Villar, A. 1989. A review of natural products and plants as potential antidiabetic drugs. *J. Ethnopharm.* **27**: 243-275

Jeon, H.H., Sung, S.H., Hun, H. and Kim, Y.C. 1995. Ginkgolide B production in cultured cells derived from *Ginkgo biloba* L. leaves. *Pl. Cell Rep.* **14**: 501-504

John, S.A. 1996. Standardisation of *in vitro* techniques for rapid multiplication of *Holostemma annulare* K. Schum. M.Sc. (Horticulture) thesis, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala, p. 242-248

Joseph, M. 1997. Indirect organogenesis and embryogenesis in *Kaempferia galanga* L. M.Sc. (Horticulture) thesis, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala, p. 67

Kako, M., Miura, T., Nishiyama, Y., Ichimaru, M., Moriyasu, M. and Kato, A. 1997. Hypoglycemic activity of some triterperiod glycosides. *J. Nat. Prod.* **60**: 604-605

KAU. 1998. *Annual Report*. Kerala Agricultural University, Vellanikkara, Thrissur, Kerala, p.21-23

Kamei, K., Takano, R., Miyasaka, A., Imoto, T. and Hara, S. 1992. Amino acid sequence of sweet taste suppressing peptide (gurmarin) from leaves of *Gymnema sylvestre*. *J. Biochem. Tokyo* **111**(1): 109-112

Kar, D.K. and Sen, S. 1984. *In vitro* synthesis of sapogenin in *Asparagus racemosus* Willd. *Appl. Biotechnol. Med. Aromatic Timber Pl.* : 62-69

Kar, D.K. and Sen, S. 1985. Sarasapogenin in callus culture of *Asparagus racemosus*. *Curr. Sci.* **54**(12): 585

Kesselmeier, J. and Ruppel, H.G. 1979. Building units of prolamellar bodies from etioplasts of *Avena sativa* L.: Saponin content and reaggregation experiments. *Advances in the Biochemistry and Physiology of Plant Lipids* (ed. Appelquist, L. and Liljenberg, C.), Elsevier, Amsterdam, p. 187-191

Komalavalli, N. and Rao, M.V. 1997. *In vitro* micropropagation of *Gymnema elegans* W & A - a rare medicinal plant. *Indian J. expt. Biol.* **35**: 1088-1092

Liu, H., Kiuchi, F. and Tsuda, Y. 1992. Isolation and structure eluadation of gymnemic acids, antisweet principles of *Gymnema sylvestre*. *Chem. pharm. Bull.* **40**(6): 1366-1375

Maeda, M., Iwashita, T. and Kurihara, Y. 1989. Studies on taste modifiers. II. Purification and structure determination of gymnemic acids, antisweet active principle from *Gymnema sylvestre* leaves. *Tetrahedron Lett.* **30**(12): 1547-1550

Mathur, A., Shukla, Y.N., Pal, M., Ahuja, P.S. and Uniyal, G.C. 1994. Saponin production in callus and cell suspension cultures of *Panax quinquefolium*. *Phytochemistry* **35**(5): 1221-1225

*Mhaskar, K.S. and Caius, J.F. 1930. *Indian Med. Res. Mem.* **16:** 1

Miyatake, K., Takenaka, S., Fujimoto, T., Kensho, G., Upadhaya, S.P., Kirihata, M., Ichimoto, I. and Nakano, Y. 1993. Isolation of conduritol-A from *Gymnema sylvestre* and its effects against intestinal glucose absorption in rats. *Biosci. Biotech. Biochem.* **57**(12): 2184-2185

Mizukami, H., Konoshima, M. and Tabata, M. 1977. Effect of nutritional factors on shikonin derivative formation in *Lithospermum* callus cultures. *Phytochemistry* **16**: 1183-1186

Mohan, V. 1999. Controlling diabetes. *The Hindu-Magazine*, November 7, p. 7

Mukherjee, P.K., Kumar, M.R., Saha, K., Giri, S.N., Pal, M. and Saha, B.P. 1996. Preparation and evaluation of tincture of *Gymnema sylvestre* (Fam. Asclepiadaceae) by physico-chemical, thin-layer chromatographic and spectroscopic characteristics. *J. Sci. ind. Res.* **55**: 178-181

Mumtaz, N., Choudhary, Q.F. and Quaraishi, A. 1990. Tissue culture studies on *Catharanthus roseus. J. Agric.* **6**(5): 467-470

Murashige, T. 1974. Plant propagation through tissue culture. *Annual Rev. Pl. Physiol.* **25**: 135-166

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Pl.* **15**: 473-497

Nahar, N. 1993. Medicinal plants in the treatment of diabetes. *Traditional Medicine. Proc. Intl. Seminar* (ed. Mukherjee, B., Pata, A., Tripathi, S.K., Bhunia, S.K. and Mukherjee, B.), Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, p. 205-209

Narayanaswamy, S. 1977. Regeneration of plants from tissue cultures. *Applied and Fundamental Aspects of Plants Cell, Tissue and Organ Culture* (Ed. Reinert, J. and Bajaj, Y.P.S.) Springer-Verlag, Berlin (Indian Reprint, Narosa Publishing House, New Delhi) p. 179-204

Nazeem, P.A., Sudhadevi, P.K., Joseph, L. and Geetha, C.K. 1991. *In vitro* callus production from leaves of *Gymnema sylvestre* R.Br. *Ancient Sci. Life* **11 (1&2)**: 43-45

Nissen, S.J. and Sutter, E.G. 1990. Stability of IAA and IBA in nutrient medium to several tissue culture procedures. *HortScience* **25**(7): 800-802

O'Dowd, N.A., Mc Cawley, P.G., Richardson, G.H.S. and Wilson, G. 1993. Callus production, suspension culture and *in vitro* alkaloid yields of ephedrine. *Pl. Cell. Tiss. Cult.* **34**: 149-155

Ota, M. and Ariyoshi, Y. 1995. Location of disulfide bonds of the sweetness suppressing polypeptide gurmarin. *Biosci. Biotech. Biochem.* **59**(10): 1956-1957

Pauthe-Dayde, D., Rochd, M. and Henry, M. 1990. Triterpenoid saponin production in callus and multiple shoot cultures of *Gypsophila* spp. *Phytochemistry* **29**(2): 483-487

*Power, F.B. and Tutin, F. 1904. *Pharm. J.* **19:** 234

Rahman, A.U. and Zaman, K. 1989. Medicinal plants with hypoglycemic activity. *J. Ethnopharm.* **26**: 1-55

Rao, G.S. and Sinsheimer, J.E. 1968. Structure of gymnemagenin. *Chem. Communications* 1681-1682

Rao, G.S. and Sinsheimer, J.E. 1971. Constituents from *Gymnema sylvestre* leaves VIII : Isolation, chemistry and derivatives of gymnemagenin and gymnestrogenin. *J. pharm. Sci.* **60**(2): 190-193

Rao, P. 1998. Cure for diabetes. *The Week*, June 21, p. 32-41

Reddy, P.S., Gopal, G.R. and Sita, G.L. 1998. *In vitro* multiplication of *Gymnema sylvestre* R.Br. - an important medicinal plant. *Curr. Sci.* 75(8): 843-845

Robinson, T. 1975. *The Organic Constituents of Higher Plants. Their Chemistry and Interrelationship.* 3rd edition, Cordus Press, North Amherst, Massachusetts, USA, p. 133-189

Rudge, K. and Morris, P. 1986. The effect of osmotic stress on growth and alkaloid accumulation in *Catharanthus roseus. Secondary Metabolism in Plant Cell Cultures* (ed. Morris, P., Scragg, A.H., Stafford, A. and Fowler, M.W.), Cambridge University Press, London, p. 75-81

Sahu, N.P., Mahato, S.B., Sarkar, S.K. and Poddar, G. 1996. Triterpenoid saponins from *Gymnema sylvestre. Phytochemistry* **41**(4): 1181-1185

Sandhya, P. and Vilasini, G. 1996. A rapid multiplication programme by *in vitro* culture for *Gymnema sylvestre* R.Br. *Proc. Eighth Kerala Sci. Cong.*, Kochi, p. 555

Sankar, A.M. 1998. *In vivo* and *in vitro* screening of *Sida* spp. for ephedrine content. Ph.D. thesis, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala, p. 298

Satheeshkumar, K. and Bhavanandan, K. 1989. Regeneration of plants from leaf callus of *Plumbago rosea* Linn. *Indian J. exp. Biol.* **27**(4): 368-369

Sehgal, C.B. and Khurana, S. 1985. Morphogenesis and plant regeneration from cultured endosperm of *Emblica officinalis*. Gaertn. *Pl. Cell Rep.* **4**(5): 263-266

Shanmugasundaram, K.R., Panneerselvam, C., Lalitha, T., Ranibai, A.J., Samudram, P. and Shanmugasundaram, E.R.B. 1981. Studies on the hypoglycemic action of *Gymnema sylvestre* R. Br. in diabetes mellitus. *Arogya J. Health Sci.* 7: 38-60

Shanmugasundaram, K.R., Panneerselvam, C., Samudram, P. and Shanmugasundaram, E.R.B. 1983. Enzyme changes and glucose ulilization in diabetic rabbits. The effect of *Gymnema sylvestre* R.Br. *J. Ethnopharm.* 7: 205-234

Shemeryankina, M.I. 1986. Analysis of triterpene glycosides from *Astragalus dasyanthus* Pall. *Khim. Farm. Zh.* **20**(1): 63-65

Sindhu, M. 1999. *In vitro* callus induction and its exploitation in *Coscinium fenestratum* (Gaetrn.) Colebr. M.Sc. (Horticulture) thesis, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala, p. 130

Singh, A.K. and Kumar, S. 1998. Conservation and domestication of medicinal plants. *J. med. aromatic Pl.* **20:** 1-2

Sinsheimer, J.E. and Rao, G.S. 1970. Constituents from *Gymnema sylvestre* leaves VI : Acylated genins of the gymnemic acids - Isolation and preliminary characterisation. *J. pharm Sci.* **59**(5): 629-632

Sinsheimer, J.E., Rao, G.S. and Mc Ilhenny, H.M. 1970. Constituents from *Gymnema sylvestre* leaves V : Isolation and preliminary characterisation of the gymnemic acids. *J. pharm. Sci.* **59**(5): 622-628

Song, W.X., Dongbo, H. and Zhan, Q. 1994. Callus induction and plant regeneration of *Eucommia ulmoides. J. North-West Forestry College* **9**(4): 32-35

Stepanova, E.F. 1985. Study of fermentation process of *Glycirrhiza glabra* L. herb. *Farmatsiya* **34**(4): 20-22

Suttisri, R., Lee, I. and Kinghorn, A.D. 1995. Plant-derived triterpenoid sweetness inhibitors. *J. Ethnopharm.* **47**: 9-26

Takemoto, T., Arihara, S., Yoshikawa, K., Hino, K., Nakajima, T. and Okuhira, M. 1984a. Studies on the constituents of cucurbitaceae plants. XII : On the saponin constituents of *Gynostemma pentaphyllum* Makino. *J. Pharm. Soc. Japan* **104**(11): 1155-1162

Takemoto, T., Arihara, S., Yoshikawa, K., Kawasaki, J., Nakajima, T. and Okuhira, M. 1984c. Studies on the constituents of cucurbitaceae plants. XI. On the saponin constituents of *Gynostemma pentaphyllum* Makino. *J. Pharm. Soc. Japan* **104**(10): 1043-1049

Takemoto, T., Arihara, S., Yoshikawa, K., Nakajima, T. and Okuhira, M. 1984b. Studies on the constituents of cucurbitaceae plants. VII. On the saponin constituents of *Gynostemma pentaphyllum* Makino. *J. Pharm. Soc. Japan* **104**(4): 325-331

Thamburaj, S., Subbaraj, D., Kasthuri, S. and Vijayakumar, M. 1996. Evaluation of germplasm accessions of *Gymnema sylvestre* R.Br. *South Indian Hort.* **44**(5 & 6): 174-176

Touchstone, J.C. and Dobbins, M.F. 1978. *Practice of Thin Layer Chromatography.* John Wiley & Sons, New York, p. 383

Valk van der, P., Ruis, F., Schrier, A.M.T. and Velde van der, C.M. 1995. Optimising plant regeneration from seed derived callus cultures of Kentucky blue grass: the effect of benzyladenine. *Pl. Cell Tiss. Org. Cult.* **40**: 101-103

Vanhaelen, M. and Vanhaelen-Fastre, R. 1984. Quantitative determination of biologically active constituents in crude extracts of medicinal plants by thin-layer chromatography-densitometry. *J. Chromatography* **312:** 497-503

Varshney, I.P., Jain, D.C. and Srivastava, H.C. 1984. Saponins from *Trigonella foenum-graecum* leaves. *J. nat. Prod.* **47**(1): 44-46

Wagner, H. 1984. Plant Drug Analysis. Springer-Verlag, Berlin, p.282

Warrier, P.K., Nambiar, V.P.K. and Ramankutty, C. 1995. *Indian Medicinal Plants: A compendium of 500 species. Vol. 3.* Orient Longman, Hyderabad, p. 107-109

Yeoman, M.M., Miedzybrodzka, M.B., Lindsey, K. and Mc Lauchlan, W.R. 1980. The synthetic potential of cultured plant cells. *Plant Cell Cultures: Results and Perspectives* (ed. Sala, F., Parisi, R., Cella, R. and Ciferri, O.), Elsevier/North-Holland Biomedical Press, Holland, p. 327-343

Yoshikawa, K., Amimoto, K., Arihara, S. and Matsuura, K. 1989a. Structure studies of new antisweet constituents from *Gymnema sylvestre. Tetrahedron Lett.* **30**(9): 1103-1106

Yoshikawa, K., Amimoto, K., Arihara, S. and Matsuura, K. 1989b. Gymnemic acid V, VI and VII from Gur-ma, the leaves of *Gymnema sylvestre* R. Br. *Chem. pharm. Bull.* **37**(3): 852-854

Yoshikawa, K., Arihara, S. and Matsuura, K. 1991. A new type of antisweet principle occuring in *Gymnema sylvestre. Tetrahedron Lett.* **32**(6): 789-792

Yoshikawa, K., Arihara, S., Matsuura, K. and Miyase, T. 1992a. Dammarane saponins from *Gymnema sylvestre. Phytochemistry* **31**(4): 237-241

Yoshikawa, K., Kondo, Y., Arihara, S. and Matsuura, K. 1993. Antisweet natural products. IX. Structures of gymnemic acids XV - XVIII from *Gymnema sylvestre* R. Br. *Chem. pharm. Bull.* **41**(10): 1730-1732

Yoshikawa, M., Murakami, T. and Matsuuda, M. 1997a. Medicinal foodstuffs. X. Structures of new triterpene glycosides, gymnemosides -c, -d, -e and -f, from leaves of *Gymnema sylvestre* R.Br.: Influence of Gymnema glycosides on glucose uptake in rat small intestine fragments. *Chem. pharm. Bull.* **45**(12): 2034-2038

Yoshikawa, M., Murakami, T., Kadoya, M., Li, Y., Murakami, N., Yamahara, J. and Matsuda, H. 1997b. Medicinal foodstuffs. IX. The inhibitors of glucose absorption from the leaves of *Gymnema sylvestre* R.Br. (Asclepiadaceae): Structure of gymnemosides a and b. *Chem. pharm. Bull.* **45**(10): 1671-1676

Yoshikawa, K., Nagakawa, M., Yamamoto, R., Arihara, S. and Matsuura, K 1992b. Antisweet Natural Products. V. Structures of gymnemic acids VIII-XII from *Gymnema sylvestre* R.Br. *Chem. pharm. Bull.* **40**(7): 1779-1782

Zhang, Y.H., Zhong, J.J. and Yu, J.T. 1995. Effect of osmotic pressure on cell growth and production of ginseng saponin and polysaccharide in suspension cultures of *Panax notoginseng. Biotechnol. Lett.* **17**(12): 1347-1350

Zhiri, A., Maciejewska, K., Jazari, H., Home, J. and Vanhaelen, M. 1995. Establishment of *Taxus baccata* callus cultures and evaluation of taxoid production. *Meded. Fac. Landbouwwet Rijksuniv Gent.* **60**(40): 2111-2114

* Originals not seen

APPENDIX

Composition of MS medium (Murashige and Skoog, 1962)

MACRO NUTRIENTS	mg l^{-1}
KNO_3	1900
NH_4NO_3	1650
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170

MICRO NUTRIENTS	mg l^{-1}
$MnSO_4.7H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$CuSO_4.5H_2O$	0.025
$Na_2MoO_4.2H_2O$	0.25
$CaCl_2.6H_2O$	0.025
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3

ORGANIC CONSTITUENTS	mg l^{-1}
Myo-inositol	100
Thiamine-HCl	0.1
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Glycine	2.0

IN VITRO CALLUS INDUCTION IN GURMAR (*Gymnema sylvestre*, R.Br.) FOR SECONDARY METABOLITE SYNTHESIS

By

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ABSTRACT OF THE THESIS

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Faculty of Agriculture

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ABSTRACT

Diabetes mellitus is a disorder affecting 100 million people round the globe. *Gymnema sylvestre* R.Br. is being used in antidiabetic therapies with good success. Depletion of forest area has reduced the supply of *Gymnema* leaves. To bridge the gap between an ever increasing demand and the dwindling supply of antidiabetic medicines from *Gymnema*, this study was undertaken to examine whether these metabolites could be produced by *in vitro* techniques.

This study was undertaken at the Department of Plantation Crops and Spices, College of Horticulture, Kerala Agricultural University, Vellanikkara during May, 1998 to November, 1999. Callus cultures were initiated in test tubes and were maintained at 26 ± 2 °C temperature and 60 to 80 per cent relative humidity for three months. Observations on growth of calli were noted at weekly intervals, while saponin production was noted after each month.

Internode, node, petiole, leaf lamina and root segments were evaluated as explants to initiate calli. Among them, internode and petiole were found to have maximum potential to initiate and proliferate calli. Root explants did not produce significant amounts of callus.

The auxins evaluated for stimulating callus production were 2,4-D, NAA, IAA and IBA. Among them, 2,4-D was the most potent in callusing followed by NAA. Both, IAA and IBA performed very poorly. The cytokinins (BA and kinetin) showed statistically similar performances. The combination of 2,4-D with BA produced maximum callus.

MS medium supplemented with 2, 4-D (2 mg l^{-1}) and BA (1 mg l^{-1}) was selected as the basal medium due to high callusing and high saponin yields. Stress inducing chemicals were added to it to examine whether they increased the production of saponins. It was found that addition of mannitol, activated charcoal, peptone and malt extract enhanced the

saponin yields. The highest saponin yield per day per tube was produced from the medium comprising of MS + 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}) + malt extract (1 %).

The cell suspensions were maintained on rotary shaker at 105 rpm for four weeks and were subcultured every seven days. Observations on growth of cultures and saponin production were recorded at weekly interval. The production of saponins was highest in the medium containing MS + 2,4-D (2 mg l^{-1}) + BA (1 mg l^{-1}) + phloroglucinol (50 mg l^{-1}). These saponins need to be identified and charachterised.

Both calli and cell suspensions produced new groups of saponins which were not present in the plant extracts, suggesting that *de novo* synthesis occurred in the *in vitro* cultures.

Saponins were extracted from *in vitro* samples by 60 per cent ethanol solution and the extract was fractionated with an equal volume of a mixture of chloroform and methanol in 1:1 proportion. The chloroform fraction was spotted on thin layer chromatograms and eluted at 29 to 30 °C temperature and 75 per cent relative humidity. Elution was done in chloroform : acetone : methanol (5 : 1 : 1.5) running solvent system. Spraying was done with three per cent vanillin and five per cent sulphuric acid in ethanol to develop purple, blue, violet and red spots for saponins. Saponins were fractionated into a profile of distinct spots by eluting in chloroform and methanol mixture in 80 : 20 ratio followed by repeated elution in 98 : 2 proportion.