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Low cost *in vitro* callus Production and Identification of Secondary Metabolites in *Tylophora indica* (Burm. F.) Merrill.

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Abstract

The low cost medium was standardize using several MS medium alternatives *viz.* macro, micro, iron, vitamin, solidiflying agent, carbon source, growth regulators for the production of callus induction in *Tylophora indica* leaf explants. The low cost medium supplemented with different concentration of Kin (0.1- 0.5 mg/l) with fixed concentration of IAA + 2,4-D. In the combination high frequency callus production from IAA+ 2,4-D (2.5 + 2.0 mg/l) plus Kin 0.2 mg/l, (84.4± 3.6), BAP 0.1mg/l (94.6± 2.7) *Tylophora indica*. Among the different solvent methanol extract was analyzed using Gas chromatography mass spectrometry (GC-MS) analysis revealed the presence of 17 compounds. In GC-MS analysis, some of the phytocomponents were screened they are viz. Propanone, Dioxolane, Tetradecane, Butane, Ethanone, Hexadecanoic acid, Heptane. Many of them are used in Pharmaceutical industry for various applications like flavor, antioxidant, anti-inflammatory, antimicrobial, pesticide and cancer preventive.

Keywords: *Tylophora indica*, callus, Primary and Secondary metabolites, GC-MS.

Introduction

Tylophora indica (Burm f.) Merrill. (Family: Asclepidaceae) commonly known as Antmul is a twining perennial plant (Fig-1) distributed throughout southern and eastern part of India in plains, forests, and hilly places (Kirtikar, 1991). Low cost alternative are needed to reduce cost of production of tissue cultured plants (George, 1993). Low cost options should lower the cost of production without compromising the quality of the micropropagules and plants (Anonymous, 2004). The cost of medium preparation can account 30-35% of the cost of micro propagation of the plants (Brink, *et al*., 1998). Thus the plant is in great demand for the production of traditional and modern medicines.

In this plant the leaves have been used for the treatment of asthma as well as bronchitis, rheumatism, jaundice, inflammation and dysentery (Chopra, 1986; Sherry and Chemler, 2009). These secondary metabolites are an important source with a variety of structural arrangements and properties (de-Fatima *et al*., 2006) for identification medicinal compounds. Chromatography is the term used to describe a separation technique in which a mobile phase carrying a mixture is caused to move in contact with a selectively absorbent stationary phase. It also plays a fundamental role as an analytical technique for quality control and standardization of phytotherapeuticals (Andrew, 2007).

A knowledge of the chemical constituents of plants is desirable not only for the discovery of therapeutic agents, but also because such information may be of great value in disclosing new sources of economic phytocompounds for the synthesis of complex chemical substances and for discovering the actual significance of folk remedies (Milne *et al.*, 1993).

In the present study is aimed to extract the secondary metabolites of this plant using solvent extract and identify using GC-MS. Therefore, the availability of a reliable, *in vitro* callus propagation system would provide a low cost methods of propagation to meet the pharmaceutical needs for effective conservation of this important plant species. Low cost methods using different carbon source reported on *Tylophora indica* (Rajavel and Stephan, 2014).

Materials and Methods

Plant material

In the present study the plant was collected in Lalgudi area (near Tiruchirappalli Dt) and maintained in the college medicinal garden. PG and Research Department of Botany, Government Arts College, Ariyalur. Fresh explants were collected from the garden this was used for low cost tissue culture propagation (Fig-1).

Fig- 1: Habit of *Tylophora indica* (Burm. f).

Low cost callus induction medium:

In the present study conventional MS (Murashige and Skoog, 1962) medium was replaced by low cost alternatives (Table- 1). The different low cost alternatives (Table-1) are standardize using different concentration supplemented with *Tylophora indica* morphogenic response medium. The standardize low cost medium was supplemented with 30 g/L of table sugar and 8 g/L agar agar (AR grade) and different concentration of growth regulators IAA (2.0 mg/l), 2,4-D (1.0 mg/l), BAP (0.1 to 0.5 mg/l) and Kin (0.1 to 0.5 mg/l) (Table- 2). The volume of all the nutrients was made to 1 liter of culture media. The pH of media was adjusted to 5.8, using 1N NaOH and 1N HCl, and the media was dispensed into glass bottles, test tube. The test tube containing media were sterilized by pressurized steam at a temperature of 121°C and 15 pounds of pressure per square inch for 15 minutes in the pressure cooker. The sterile media were kept in the transfer room under sterile conditions until use.

Table- 1: Low cost medium and conventional medium composition

Conventional MS medium (Murashige & Skoog media 1962)	(mg/l)	Low cost Alternative medium	(mg/l)
Macro nutrients		***Macro nutrients***	
Ammonium Nitrate (NH_4NO_3)	1650	Ammonium nitrate fertilizer	6.0g
Calcium chloride ($CaCl_2$)	440	Calcium Chloride	0.6g
Potassium Nitrate (KNO_3)	1900	fertilizer	
Magnesium Sulphate ($MgSO_4$)	370	Potassium Nitrate fertilizer	10.0g
Potassium dihydrogen Phosphate (KH_2PO_4)	170	Magnesium Sulphate fertilizer	0.6 g
Micro nutrients		Single super Phosphate	1.0g
Potassium iodide (KI)	0.83	***Micro nutrients***	
Boric oxide (H_3BO_3)	6.2	Potassium Iodide(LR)	1.5
Manganese Sulphate ($MnSO_4.4H_2O$)	22.3	Power B-boran, Boric powder	15.0
Zinc Sulphate ($ZnSO_4.7H_2O$)	8.6	Manganese Sulphate	30.0
Sodium Molybdate ($Na_2\ MOO_4.2H_2O$)	0.25	fertilizer	
		Zinc Sulphate fertilizer	10.0
Copper Sulphate ($CuSO_4.5H_2O$)	0.025	Adbor powder	0.50
		Chelated fertilizer	0.1
Cobalt chloride ($COCl_2$)	0.025	Grandular/ powder	0.1
Iron Nutrient		***Iron Nutrient***	
Ethylene diamine tetra acetic acid (EDTA)	1.9	Ethylene diamine tetra acetic acid (EDTA)	0.50
Ferrous Sulphate ($FeSO_4.7H_2O$)	1.39	Ferrous Sulphate fertilizer	0.4
Vitamins		***Vitamins***	
Myo Inositol	100	Becosules B-complex	10
Glycine	2	Tablets Thiamine	10
ThiamineHcl	0.1	Riboflavin	3
Nicotinic acid	0.5	Pyridoxine HCl	100
Pyridoxine Hcl	0.5	Ascorbic acid	150
		Biotin	1.5
Growth regulators		Folic acid	50
IAA	0.1	Calcium pantothenate	100
2, 4-D	0.1	Niacinamide	
NAA	0.1	***Growth regulators***	
IBA	0.1	IAA	0.1
Kinetin	0.1	2, 4-D	0.1
BAP	0.1	NAA	0.1
GA3	0.1	IBA	0.1
		Kinetin	0.1
Carbon source		BAP	0.1
Sucrose	30	GA3	0.1
		Carbon source	
Solidifying agent		White refined sugar	
Agar - Agar	8	(Table sugar)	30
		Solidifying agent	
		Agar Agar (A)	8

Solvent extraction

The calli were dry at 35° C in a hot air oven. The dry calli was made into fine powder (500g) for further analysis. The leaf powder (Control) and calli powder was subjected extraction of secondary metabolites using Soxhlet apparatus. Using organic solvents of Methanol and ethanol were used for the extraction.

The specific solvent extraction was dried and extracted with 1 liter of the same solvent (80-90°c) by continuous hot percolation until the extraction was completed. After the completion of the extraction, the extract was filtered and the solvent was removed by distillation under reduced pressure. Finally, dark green and light green colored residues were stored in screw capped test tubes for further analysis.

Gas chromatography analysis was carried out (Fig- 3) at the South India Textile Research Association, Coimbatore. Equipment using THERMO GC - TRACE ULTRA VER: 5.0, THERMO MS DSQ II, and Column using DB 35 - MS CAPILLARY STANDARD NON - POLAR COLUMN. This analysis dimension - 30 Mts, ID : 0.25 mm, FILM : 0.25 µm, Temperature as 70 C raised to 260 C at 6 C /min, Injection volume 1 micro liter. It is one of the key techniques, generally used for screening/identification of different groups of plant phytochemicals. The high attainable separation power in combination with wide range of the detectors employing various detection principles to which it can be coupled makes GC an important, often irreplaceable tool in the analysis at trace level of plant phytochemical compounds.

Gas chromatography analysis GC-MS

Identification was based on the molecular structure, molecular mass and calculated fragments. Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The name, molecular weight and structure of the components of the test materials were ascertained. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The spectrum of the unknown component was compared with the spectrum of the component stored in the NIST library version (2005), software, Turbomas 5.2.

Results

Low cost medium

The low-cost medium was standardize (Table-1) viz. macro, micro, vitamin, carbon source, iron source, solidifying agent and other salts as equivalent to the MS medium composition.

Effect of low cost medium on callus induction from leaf explant

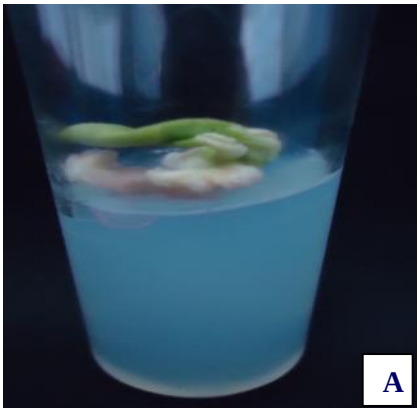
Low cost medium with various concentration of Kin, BAP, (0.1.-0.5 mg/l) with IAA + 2,4-D (2.5 + 2.0 mg/l) in all combination resulted callus formation from leaf explants. The callus response was slow at the initial stages but the callus exhibited good growth within 28 days and covered the entire explants. Low cost medium with 0.1 mg/l of BAP showed 94% callus induction (Table - 2). In all leaf explants, the callus was developed from the adaxial as well as abaxial surface. Among the different combination the optimum concentration of IAA+ 2,4-D favored the on callus development (Fig-2). The leaf callus combination was also further with elicitor treatment for the enrichment of secondary metabolite in *Tylophora indica*.

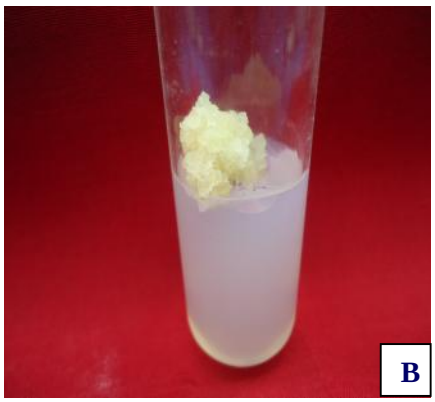
Table – 2 Effect of Low cost media supplemented with different concentration of Kin, and BAP combination of IAA+2,4-D (2.0+1.0 mg/l) on Callus induction from *Tylophora indica* Leaf explant.

Plant growth regulators IAA+2,4-D (2.5+2.0 mg/l)	Mass Callus developed Leaf explants (Mean ± SD)
Kin	**Leaf explants**
0.1	60.5± 2.2
0.2	**86.4±3.6**
0.3	78.2±4.5
0.4	64.7±6.8
0.5	56.4±3.7
BAP	
0.1	**94.6±2.7**
0.2	82.4±4.6
0.3	74.2±2.5
0.4	66.4±5.8
0.5	58.6±3.5

Data presented as the mean value ± standard error after 30 days of culture from four independent experiments each with 10 replicates.

Fig- 2: Low cost medium supplemented with different growth regulators on *Tylophora indica* callus induction using leaf explant . (A. callus induction, B. callus)





GC-MS Analysis *in vitro* callus leaf methanolic extract

GC-MS chromatogram of the methanolic *in vitro* callus extract of *Tylophora indica* showed 17 compounds. The chemical compounds identified in the methanolic extract of the *in vitro* callus leaf of *Tylophora indica* presented in (Table- 3).The first compound identified with less retention time (3.69min) was 2-Propanone (CAS), whereas Azetidine-di was the last compound which took longest retention time (40.51min) to identify. The phytochemicals identified through GC-MS analysis showed many biological activities relevant to this study are listed in (Table- 4). Among the biological activity viz. Antimicrobial activity for (2S)-2,3-Dihydroxy-4-pentene, 1,3-Dioxolane,2-(1-methylethyl 1,3-Dioxolane-4-methanol,2,2-dimethyl-(CAS) presence of chemicals, 1-Formyl 1-9 methylcarbazole, Butane,2,2-dimethyl-(CAS). Antioxidant. effect on (2S)-2,3-Dihydroxy-4-pentene, Cis-1-Bromo-2phenyleyclopropane, Hexadecanoic acid. anti-inflammatory Cis-1-methyl1-2,3-dihydro-1H-indene-2-carboxylate. Cancer preventive 2-(Methoxycalbonyl) ethyl-4- chloromethylfulan ,8-Azabicyclo[3.2.1]octan -3-Dne . phyto constituents using cure various disease and infection against pathogenic organism. (Table-5., Fig- 3).

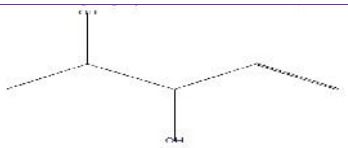
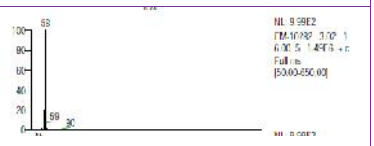
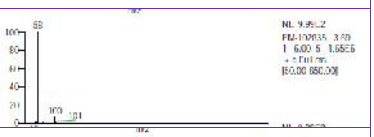
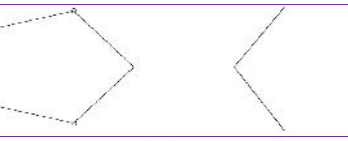
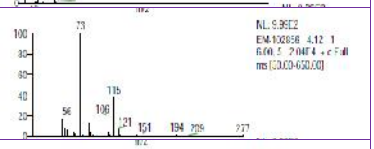
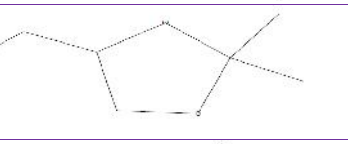
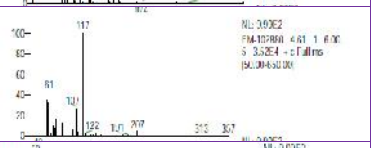
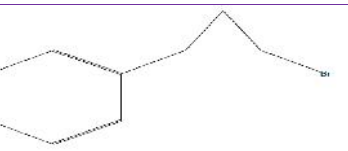
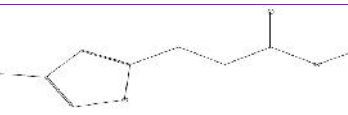
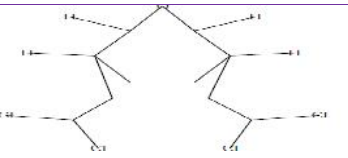
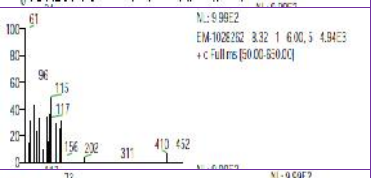
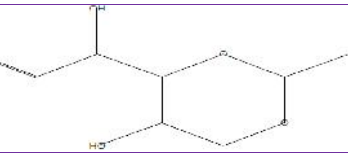
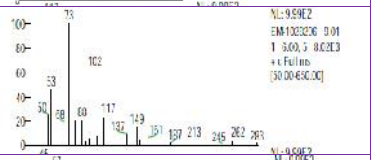
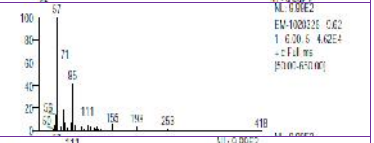
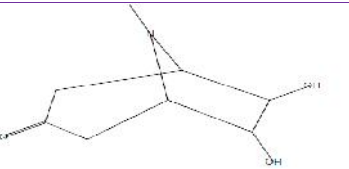
Table: 3 GC- MS analysis revealed the presence of phytochemical components present in methanolic extract of *Tylophora indica* L.

S.no	Run Time	Name of the Compound	Molecular formula	MW	Peak Area %
1	3.02	(2S)-2,3-Dihydroxy-4-pentene	$C_5H_{10}O_2$	102	1.81
2	3.69	2-propanone(CAS)	C_3H_6O	58	3.87
3	4.12	1,3-Dioxolane,2-(1-methylethyl	$C_6H_{12}O_2$	116	3.10
4	4.61	1,3-Dioxolane-4-methanol,2,2-dimethyl-(CAS)	$C_6H_{12}O_3$	132	0.62
5	5.37	Cis-1-Bromo-2phenyleyclopropane	C_9H_9BR	196	0.64
6	6.79	2-(Methoxycalbonyl)ethyl-4-chloromethylfulan	$C_9H_{11}CI_{03}$	202	11.74
7	8.32	Zendo,3 exo-Bis(dichloromethyl)-5 exo,6exo-and -5 endo,6 endo-epoxybicycloe[2.2.2]oetans	$C_{10}H_{12}CI_{40}$	288	1.19
8	9.01	3.5-0-Ethylidene –D-lyxose	$C_7H_{12}O_5$	176	2.34
9	9.62	Tetradecane(CAS)	$C_{14}H_30$	198	3.15
10	12.54	8-Azabicyclo[3.2.1]octan -3-Dne	$C_8H_{13}NO_3$	171	1.67
11	13.31	Butane,2,2-dimethyl-(CAS)	C_6H_{14}	86	0.69
12	14.82	1-Formyl 1-9 methylcarbazole	$C_{14}H_{11}NO$	209	1.99
13	15.21	Ethanone,1-(2-hydroxyphenyl)-(CAS)	$C_8H_8O_2$	136	9.05
14	21.73	Hexadecanoic acid, methyl ester(CAS)	$C_{17}H_{34}O_2$	270	1.52
15	16.88	4-Di-Heptane	$C_7H_{15}D$	100	1.06
16	17.28	5-0-Acetyl,Th10-octyl-a-L-Rhamnofura Noside	$C_{16}H_{30}O_5S$	334	4.19
17	17.92	Cis-1-methyl1-2,3-dihydro-1H-indene-2-carboxylate	$C_{12}H_{1402}$	190	1.18

Table – 4 : GC-MS analyzed phytochemical compounds and their biological activates of methanolic extract of *Tylophora indica*.

S.no	Run Time	Peak Area %	PROBILITY	Name of the Compound	Biological activates
1	3.02	1.81	11.31	(2S)-2,3-Dihydroxy-4-pentene	Antimicrobial,antioxidant.
2	3.69	3.87	8.38	2-propanone(CAS)	Flovor,hypocholesterolemic
3	4.12	3.10	23.78	1,3-Dioxolane,2-(1-methylethyl	Antimicrobial
4	4.61	0.62	8.80	1,3-Dioxolane-4-methanol,2,2-dimethyl-(CAS)	Antimicrobial.anti-inflammatory
5	5.37	0.64	23.90	Cis-1-Bromo-2phenyleyclopropane	Antioxidant,hypocholestreolemic
6	6.79	11.74	30.48	2-(Methoxycalbonyl)ethyl-4-chloromethylfulan	Cancer-preventive
7	8.32	1.19	42.54	Zendo,3 exo-Bis(dichloromethyl)-5 exo,6exo-and -5 endo,6 endo-poxybicycloe[2.2.2]oetans	Antitumor,immunostimulant,perfumery,pesticide
8	9.01	2.34	52.18	3.5-0-Ethylidene –D-lyxose	Preservative
9	9.62	3.15	19.86	Tetradecane(CAS)	Cosmetic,flovor,perfumery
10	12.54	1.67	23.99	8-Azabicyclo[3.2.1]octan -3-Dne	Cancer preventive,nematicide
11	13.31	0.69	10.06	Butane,2,2-dimethyl-(CAS)	Antimicrobial
12	14.82	1.99	84.54	1-Formyl 1-9 methylcarbazole	Antimicrobial,preservative
13	15.21	9.05	36.70	Ethanone,1-(2-hydroxyphenyl)-(CAS)	Lipoxygenase-inhibitor,pesticide
14	21.73	1.52	40.45	Hexadecanoic acid, methyl ester(CAS)	Antioxidant,Antitumor,Hypocholesterolemic
15	16.88	1.06	8.55	4-Di-Heptane	Flovor,lubricant
16	17.28	4.19	18.74	5-0-Acetyl,Th10-octyl-a-L-Rhamnofura Noside	Cadioprotective,Hypocholesterolemic
17	17.92	1.18	19.76	Cis-1-methyl1-2,3-dihydro-1H-indene-2-carboxylate	Lubricant,used to produce dietary supplements and anti-inlammatory.

Table – 5 GC-MS analysed phytochemical compounds structure and mass spectrum of *Tylophora indica* callus extract.

S.No	Name of the Compound	Chemical Structure	Mass Spectrum
1	(2S)-2,3-Dihydroxy-4-pentene		
2	2-propanone(CAS)		
3	1,3-Dioxolane,2-(1-methylethyl		
4	1,3-Dioxolane-4-methanol,2,2-dimethyl-(CAS)		
5	Cis-1-Bromo-2phenyleyclopropane		
6	2-(Methoxycalbonyl)ethyl-4-chloromethylfulan		
7	Zendo,3 exo-Bis(dichloromethyl)-5 exo,6exo-and -5 endo,6 endo-poxybicycloe[2.2.2]oetans		
8	3.5-0-Ethylidene –D-lyxose		
9	Tetradecane(CAS)		
10	8-Azabicyclo[3.2.1]octan -3-Dne		

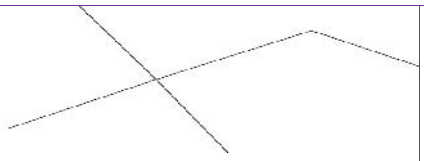
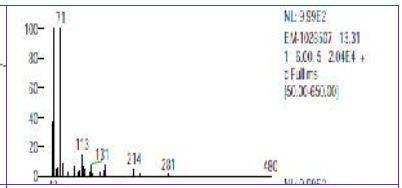
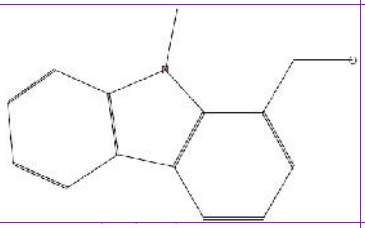
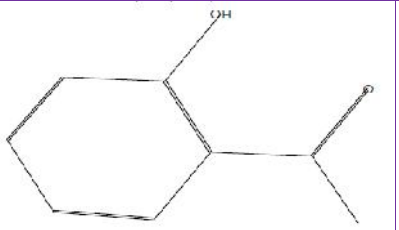
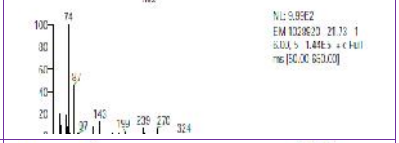
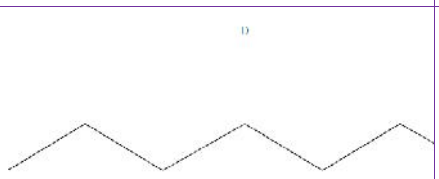
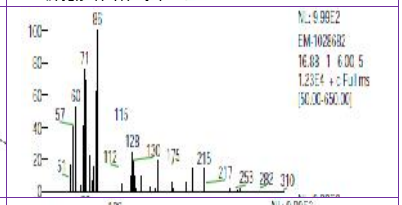
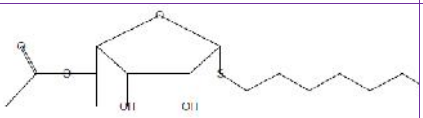
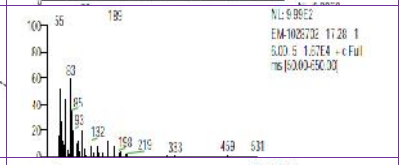
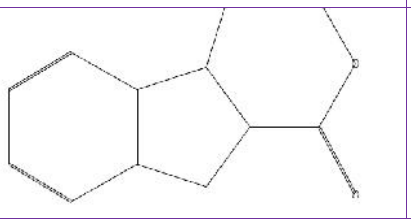
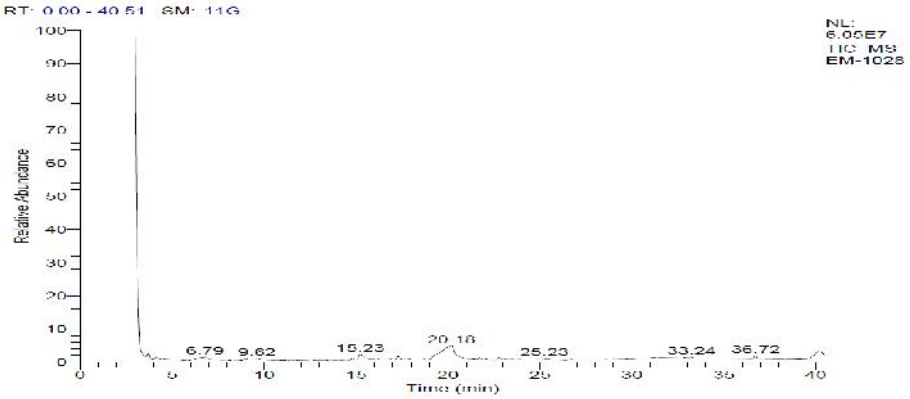
11	Butane,2,2-dimethyl-(CAS)		
12	1-Formyl 1-9 methylcarbazole		
13	Ethanone,1-(2-hydroxyphenyl)-(CAS)		
14	Hexadecanoic acid, methyl ester(CAS)		
15	4-Di-Heptane		
16	5-0-Acetyl,Th10-octyl-a-L-Rhamnofura Noside		
17	Cis-1-methyl1-2,3-dihydro-1H-indene-2-carboxylate		

Fig-3 : GC-MS analysed mass spectra for *Tylophora indica In vitro* leaf callus methonalic extract



GC-MS is one of the best techniques to identify the constituents of volatile matter, long chain, branched chain hydrocarbons, alcohols acids, esters etc. The GC-MS analysis of *Tylophora indica* in methanolic extract revealed the presence of compounds (phytochemical constituents) that could contribute the medicinal quality of the plant. The identification of the phytochemical compounds was confirmed based on the peak area, retention time and molecular formula. The active principles with their Retention time (RT), Molecular formula, peak area and chemical formula are presented in (Table -3, 4, 5; Fig- 3). The GC-MS method is a direct and fast analytical approach for identification of terpenoids and steroids and only few grams of plant material is required.

Discussion

In the present study low cost medium was used to produce callus in *Tylophora indica.*

Similarly low cost tissue culture means in which cost reduction is achieved by improving process efficiency and better utilization of resources (Savangikar, 2002). The low cost medium containing the nutrient viz., carbon sources, gelling agents, inorganic, organic supplements, and growth regulators are the alternative of the conventional medium. In plant production through micropropagation, media chemicals cost a little less than 15% of the total cost (Prakash *et al*., 2004). Similarly out of all components used in a media, gelling agents such as agar contribute 70% to the total cost of media (Gaur and Kant, 2011). Several authors are discussed about the low cost medium components (Chaudhuri *et al*., 2004; Thomas and Philip, 2005; Sivakumar *et al*., 2006)

In the present results the effect benzyladenine for shoot organogenesis from leaf callus was also advocated by Faisal and Anis, 2003 and Sahai *et al*., 2010.In earlier reports, either indole 3 butyric acid or indole 3 acetic acid has been reported to be optimum for rooting in regenerated shoots of *Tylophora indica* (Bera and Roy ,1993 , Thomas and Philip, 2005).

Likewise extraction and analysis of secondary metabolite in *Tylophora indica*. The more precise information in qualitative analysis can be obtained by gas-chromatography coupled with mass spectrometry (GC-MS) (Cong *et al*., 2007). For quantitative determination, gas-chromatography with flame ionization detector (GC-FID) and GC-MS are preferred (Haznagy-Radnal *et al*., 2007).

The GC-MS analysis of *Tylophora indica* leaves revealed the presence of 17 compounds. The identified compounds possess many biological properties. In is an Antitumor, immunostimulant, perfumery, pesticide as antimicrobial, anticancer, anti-inflammatory and diuretic agent (Praveen kumar *et al*., 2010). 9, 12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z) -, n-Hexadecanoic acid, 1,2-Benzenedicarboxylic acid and di-isooctyl ester were present in *Caesalpinia sappan* ethanol extract (Sarumathy *et al*., 2011). Similar types of compounds were identified among the seventeen compounds of this present study.

Phytol is one among the seventeen compounds of the present study. Similarly Maria Jancy Rani *et al.* (2011) observed the presence of phytol in the leaves of *Lantana camara* and Sridharan *et al.* (2011) in *Mimosa pudica* leaves. Similar result was also observed in the leaves of *Lantana camara* (Sathish kumar and Manimegalai, 2008). Phytol, Phenol, 2, 4-bis (1-phenylethyl) - which are all have medicinal properties. Mangunwidjaja *et al*. (2006) reported the main components of 9, 12 octadecadienoic acid, Octadec- 9enoic acid and 9, 12-actadecadienoic acid present in *Croton tiglium* seed. These compounds were found to have potential antioxidant and anticancer activities.

Hexadenoic acid has earlier been reported as a component in alcohol extract of the leaves of *Kigelia pinnata* (Grace *et al*., 2002). Parasuraman *et al*. (2009) identified 17 compounds with n-Hexadecanoic acid and Octadecanoic acid as the major compounds in the leaves of *Cleistanthus collinus.* GC-MS analysis of ethyl acetate extract of *Goniothalamus umbrosus* revealed the presence of n-Hexadecanoic acid (Siddig Ibrahim *et al.*, 2009). n-hexadecanoic acid, Hexadecanoic acid, Phytol, 9, 12 - Octadecadienoic acid, 9, 12, 15-Octadecatrienoic acid and Squalene were identified in the ethanol leaf extract of *Aloe vera* (Arunkumar and Muthu selvam, 2009). Squalene is used in cosmetics as a natural moisturizer**.** Devi *et al.* (2009) reported that *Euphorbia longan* leaves mainly contained n-hexadecanoic acid and 9, 12-Octadecadienoic acid. These reports are in accordance with the result of this study.

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