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GC-MS Analysis of the *in vitro* leaf derived Callus Extracts of *Ichnocarpus frutescens* (L)W.T.Aiton

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Abstract: Medicinal plants are the treasure house of secondary metabolites. Production of seconmdary metabolites through micro propagation technology has immense application in new drug discoveries. *Ichnocarpus frutescens* (L.) W.T. Aiton - is a latex secreting medicinal herb commonly known as black creeper or Krishna Sariva belonging to the family Apocyanaceae.The present study was aimed at induction of callus from the leaf explants of *I. frutescens* using Murashige and Skoog (MS) medium supplemented with various combinations of plant growth regulators. Maximum amount of green compact mass of callus was proliferated from leaf explants on MS medium fortified with 3mg/L NAA + 2mg/L BAP. The main objective of the present study was to investigate the secondary metabolites in different extracts of callus by GC-MS analysis. To our best knowledge, no literature is available and this is the first report on GC–MS analysis of leaf derived callus extracts, which showed the presence of different phytocompounds out of which, Stigmasterol, Hexadecanoic acid, Beta-Sitosterol, Stigmasta-3,5-dien-7-one and Heptacosane are the major compounds that found to possess a wide range of biological activities. This protocol could be used to identify the secondary metabolites from leaf derived callus of the *I. frutescens* and further studies have to be carried out for the isolation and characterization of novel compounds for the formulation of new pharmaceutical drugs and also for its commercial production which will be used to cure some important ailments.

Key words: *Ichnocarpus frutescens,* MS media, Callus, GC-MS, Stigmasterol.

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1. INTRODUCTION

Ichnocarpus frutescens is an evergreen latex secreting medicinal herb with climbing branches, smooth and rust-colored stem, because of which the creeper earned the name black creeper. The different parts of *I. frutescens* (root, stem, flowers and leaves) were used for various curative illness such as, demulcent, syphilis, loss of sensation, hemiplegia, headaches, fever and wounds between fingers[1,2]. It is also useful in curing of night blindness, bleeding of gums, enlargement of spleen, rheumatism, asthma, cholera, atrophy, smallpox, ulcer, dysentery, snake bite, hematuria, cough, abdominal and glandular tumors[3,4]. Kol, Mawasi and Gond tribal communities of Chitrakoot, Madhya Pradesh, uses the leaf paste on cut wounds to stop bleeding[5]. Kurichyas tribes of Kannur district in Western Ghats of Kerala used root and leaf extract of this plant for preventing of vomiting and stomach pain[6]. The roots of *I. frutescens* are used as a substitute for *Hemidesmus indicus* (Indian sarsaparilla)[7]. Nandagopalan *et al., (*2016)[8, 9] reported the therapeutic properties of this medicinal plant due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, amino acids, coumarins, sterols and pentacyclic triterpenoids i.e. 12-dehydrolupanyl-3β-palmitate, friedelin, friedelinol lupeol acetate, 12-dehydrolupeol, oleanolic acid, , dotriacontanoic acid, β-sitosterol, sitosterol palmitate , α-amyrin and its acetates. Recent studies have revealed that the root, stem and leaf extracts of *I. frutescens* are known to have anticonvulsant[10], antioxidant and anti-inflammatory[11], anticancer[12], antimicrobial[13], anti-pyretic[14], anti diabetic[15] and analgesic activities[16]. Because of enormous medicinal properties of this plant as mentioned above, it leads to over exploitation and can affect the plant population in their natural habitat. Plant tissue culture technique is an alternative, quick and efficient method for rapid multiplication of clones which provides regular supply regenerated plants at any time in any season in a large quantity under *in vitro* conditions within a short duration of time and also it helps to meet the growing demand of medicinal plants[17,18].GC-MS analysis is one of the best, rapid, accurate and authentic method which requires a small quantity of plant extracts to detect various compounds including phenols, alcohols, alkaloids, long chain hydrocarbons, nitro compounds, glycosides, organic acids, amino acids, steroids and esters at molecular level (revealed the various types of molecular weight compounds) based on the chromatographic retention times and spectra to validate the phytochemicals [19, 20]. Hence, the present work was aimed to standardize the protocols for callus induction from leaf explants and to analyze the phytochemicals from different extracts of *in vitro* leaf derived callus of *I. frutescens*.

2. MATERIALS AND METHODS

2.1 Collection of I. frutescens

The plants were collected from Doresani Palaya Forest Campus, Bengaluru and authenticated by the Botanical Survey of India (BSI), Western Regional Centre, Pune, India(plant identification letter No.BSI/WRC/IDEN, CER, /2018/H_2/56, voucher specimen number: 136265). The plants were maintained in the Department of Botany, Bangalore University, Bangalore for the further studies.

2.2 Culture media preparation for Callus induction

Murashige and Skoog (1962)[21] medium was used as basal medium. The MS medium consists of various macronutrients, micronutrients, vitamins, organic acids, fortified with 3% sucrose and agar 0.8%. For *in vitro* callus induction, fresh leaves were excised and thoroughly washed under running tap water for 10 min. Then the leaves were treated with tween 20 (mild detergent) for 5 min by continuous shaking and later washed thrice using distilled water and further surface sterilized by using bavistin (fungicide 0.1% w/v) for 60 sec. and finally washed thrice with distilled water. The surface sterilized explants were brought in to Laminar air flow and again surface sterilized using 70% ethanol for 30 sec and finally treated with 0.1%(w/v) mercuric chloride solution for 1 min and then rinsed thrice with sterile distilled water. The surface sterilized leaves explants were cut into small pieces of size approximately 5 mm and cultured on sterilized MS media fortified with plant growth regulators like 2, 4-D (2,4-dichlorophenoxyacetic acid), IBA (Indole-3-Butyric Acid), IAA (Indole Acetic Acid), NAA (Naphthalene Acetic Acid), BAP (Benzyl Amino Purine), Kn (Kinetin) alone and in combinations with various concentration of hormones viz., 0.5 to 6.0 mg/l. Cultured bottles were incubated for 20 days in the culture room under suitable *in vitro* conditions maintained at 16 h photoperiod and 25±2 °C temperature. After 20 days of incubation, the data were recorded. The present investigation was carried out to develop a protocol for *in vitro* callus induction from leaf explant of *I. frutescens.*

2.3 Preparation of extract for GC-MS profiling

The green compact mass of callus was collected from the cultured bottles, and was air dried at 25°C for 5 days under shade. After complete dryness, the callus was powdered well using grinder and stored in an airtight container.10 g callus powder was taken and extracted separately using 150ml of solvent viz., chloroform, ethyl acetate and ethanol for 72 hr using Soxhlet apparatus. All the three concentrated crude extracts were allowed for drying and stored at 4° C used for further analysis.

2.4 GC-MS Analysis of Callus Extracts

Various solvent extracts of *in vitro* callus of the *I. frutescens* were subjected to GC-MS analysis at VIT University, Vellore using GC Clarus 680 system employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethyl polysiloxane, 30m x 0.25mm ID x 250 µm df) and helium used as carrier gas at a constant flow of 1ml/min which separated the components. The injector temperature was set at 260° C during the chromatographic run and the 1 µL of extract sample injected into the instrument, the oven temperature was programmed from 60° C (2 min) to 300°C at the rate of 10°C min-1 and 300°C where it was held for 6 min. The GC MS detector conditions were set as: transfer line temperature 230° C, ion source temperature 230°C and Mass spectra were taken at 70eV (ionization mode electron), along with a scan time of 0.2 sec and scan intervals of 0.1 sec. Based on the types of solvent used for extraction, these different temperature programs were selected. The mass of

the components and fragments obtained from 40 to 600 Da using Software Turbo Mass version 5.42. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library[22].

3. RESULTS AND DISCUSSION

3.1 In vitro callus induction from leaf explants

The present study was designed to investigate the effect of different concentrations of plant growth regulators for the *in vitro* callus induction and to identify its bioactive compounds from different extracts of callus by GC MS analysis. Initiation of callus was observed in cut surface of leaf explants (Fig. 1A) and after that profuse growth of callus was noted on MS media fortified with different concentrations of hormones viz., Auxins (2, 4-D, IAA, NAA), and Cytokinin (BAP, Kn) individually as well as in combination). Among the different concentrations of auxin, callus proliferation was achieved at 1.0mg/L of 2,4-D in short duration (Fig. 1A) (15days) showing creamy white color. Greenish white color callus was observed in leaf explants after 25 days of culture on MS medium fortified with 3.0 mg/L NAA (Fig. 1B). Whereas in IAA at lowest concentration 1 mg/L which initiated callus but did not proliferated further. But in different concentrations of IBA, BAP, Kn did not induce callus from the leaf explants and also in hormone-free MS medium. Similar results were recorded in *Mandevilla guanabarica,* 40 µM/L 2, 4-dichloro phenoxy acetic acid (2, 4-D) induced compact creamish color, non-morphogenic callus from leaf explants on MS media[23]. The embryogenic callus was formed from mature embryos of winter Wheat on MS media fortified with 2,4-D as a effective growth regulator for higher callus induction than NAA hormone[24]. In *Vigna radiata,* callus was induced on MS media with a combination of 9.05 µM/L 2, 4-D and 10.72 µM/L NAA which supported the callus formation but highest callus proliferation was achieved in combination with Kinetin[25]. Further, callus induction on MS medium fortified with various combinations of auxin and cytokinin like 2.4-D + NAA, 2.4-D + IAA,2.4-D + IBA, 2.4-D + BAP, 2.4-D+Kn, NAA + IAA, NAA + IBA, NAA + Kn, NAA+ BAP, BAP + IAA, BAP +IBA, BAP + Kn, Kn + IAA and Kn + IBA at different concentrations viz 0.5 mg/L to 6 mg/L and vice versa were used. Among combinational concentrations, 2,4-D+BAP (0.5+2.0, 1.0+2.0, 2.0+ 2.0, 3.0+2.0, 4.0+2.0 mg/L) and NAA+BAP (0.5+2.0,1.0+2.0, 2.0+2.0, 3.0+2.0, 4.0+2.0 mg/L) were the most effective combinations in which leaf explants induced callus. In other combination of hormones even at higher concentrations viz., 2.4-D with IAA and IBA which initiated the callus from leaf explants but further proliferation was not observed even in longer period of incubation. While 2,4-D (0.5mg /L) +BAP (2.0 mg/L) (Fig. 1 C) combination at lowest concentration induced friable creamy whitish colored mass of callus, but it did not produced green colored callus and even at higher concentration, it turned to light brownish white color (Table 1). The highest percentage of green compact mass of callus was obtained from leaf explants at the concentration of 3mg/l NAA+2mg/l BAP (Fig. 1D) after 20 days period of incubation and the results were represented in table 2 and Fig.1.There was no callus induction on MS basal medium. Many plant species in the family Apocyanaceae have been reported to exhibit successful callus induction on MS media with different concentrations of growth hormones. Similar results were observed in *Decalepis arayalpathra* that the maximum amount of callus was obtained on MS medium supplemented with combination of BAP (1 mg/L) + NAA (0.5 mg/L) and BAP (0.5 mg/L) +2, 4-D (1 mg/L) from the leaf explants[26]. Negi (2011)[27], reported that MS medium supplemented with different concentration of combinational hormones like BAP (2mg/L) and 2, 4-D (1mg/L) induces light green and resin secreting callus from leaf explants of *Catharanthus roseus***.** In *Decalepis hamiltonii,* callus induction was observed on MS media supplemented with 2mg /L 2, 4-D alone and also in combination of NAA + BAP (0.5mg/L + 1mg/L) from stem explants[28]. Callus formation was achieved in *Matthiola incana* on MS media fortified with 0.5 mg/L Kn and 1 mg/L NAA after 4 weeks of culture under from the leaf explants[29]. Guru, *et al.,* (2014)[30] reported that highest callus was achieved from leaf explants of *I. frutescens* on MS medium supplemented with the concentration 3mg/L BAP+ 1 mg/L NAA.[13] However, no other work has been done on the induction of callus and also phytochemical analysis on *I. frutescens. These* results are in conformity with the previous report of Guru *et al.,* (2014)[30] that the callus formation was achieved from leaf explants on MS media supplemented with BAP and NAA.

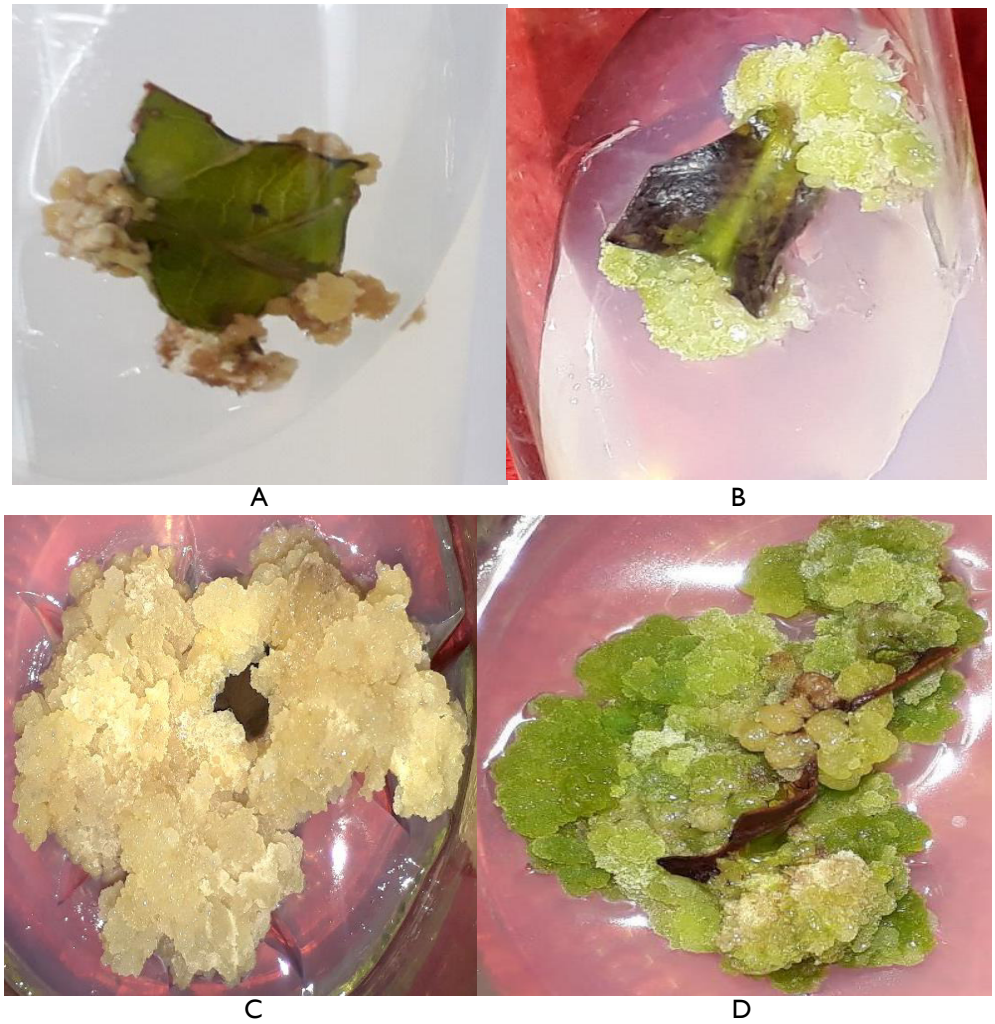

Fig. 1: A-D. *In vitro* callusing from leaf explants of *I. frutescens*. A: MS+2, 4-D (1 mg/L), B: MS+ NAA (3 mg/L), C: MS+2, 4-D (0.5 mg/L) + BAP (2 mg/L) D: MS+NAA (3 mg/L) +BAP (2 mg/L)

Table 1: *In vitro* callus induction from the leaf explants of *I. frutescens* on MS medium fortified with different concentration of 2,4-D+BAP

Conc. in mg/L 2,4-D+BAP	Percentage of Callus induction ± S.D*	Time taken for callus response in days	Callus color	Texture of callus
0.5+2.0	23.33 ± 5.16[a]	17	Cream white	Friable
1.0+2.0	43.33 ± 5.16[b]	15	Cream white	Friable
2.0+2.0	60.00 ± 8.94[c]	14	White	Friable
3.0+2.0	76.67 ± 5.16[d]	13	White	Friable
4.0+2.0	83.33 ± 5.16[e]	13	White	Friable
5.0+2.0	90.00 ± 0.00[f]	12	White	Friable
6.0+2.0	93.33 ± 5.16[g]	11	White	Friable

Table 2: *In vitro* callus induction from the leaf explants of *I. frutescens* on MS medium fortified with different concentration of NAA+BAP

Conc. in mg/L NAA+BAP	Percentage of Callus induction ± S.D*	Time taken for callus response in days	Callus color	Texture of callus
0.5+2.0	53.33 ± 5.16[a]	25	Greenish white	Friable
1.0+2.0	60.00 ± 8.94[b]	23	Greenish white	Friable
2.0+2.0	76.67 ± 5.16[c]	22	Pale green	Compact
3.0+2.0	96.67 ± 5.16[g]	20	Green	Compact
4.0+2.0	86.67 ± 5.16[f]	18	Pale green	Compact
5.0+2.0	83.33 ± 5.16[e]	15	Pale green	Soft Friable
6.0+2.0	80.00 ± 8.94[d]	12	Greenish white	Friable

**Values are mean ± SD. Values in columns with the same superscripts do not differ significantly (p>0.05).*

3.2 Phytocompounds found in the in vitro callus extracts of I. frutescens evaluated by GC-MS

The gas chromatography-mass spectrometry (GC-MS) analysis of chloroform, ethyl acetate and ethanolic leaf derived callus extracts led to identification of the phytocompounds based on the molecular formula, molecular weight and its structure. The mass spectrum of *I. frutescens* showed numerous prominent peaks indicating the presence of bioactive compounds. Major compounds were identified in all the three extracts with their retention time (RT), molecular formula, molecular weight (MW) and area percentage were represented in Table 3, 4, 5.The spectrums of the components (Fig. 2, 3, 4) were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library. Nine compounds were identified in chloroform extract; seven each in ethyl acetate and ethanolic callus extracts. Among these, Stigmasterol, Hexadecanoic acid, Beta-Sitosterol, Stigmasta-3,5-dien-7-one and Heptacosane are the important compounds recorded from leaf derived callus by GC MS analysis and possess a wide range of biological activities. Stigmasterol a phytosterol, found in methanolic callus extract of *Dipterygium glaucum* was used as a precursor for manufacturing of semi synthetic progesterone and its applications in anti-peroxidative, thyroid inhibitory, anti-inflammatory, anti-osteoarthritic, antiviral, anti-hepatotoxic, cancer- preventive[31] activities**.** Stigmasterol isolated from the stem bark of *Neocarya macrophylla* showed the antimicrobial activity against human pathogens like methicillin-resistant *Staphylococcus aureus* (MRSA), *Vancomycin-resistant enterococci (VRE), Staphylococcus aureus, Klebsiella pneumoniae Streptococcus faecalis, Salmonella typhimurium, Escherichia coli, Pseudomonas fluorescens, Candida krusei* and *Candida albicans*[32]. 15 different phytoconstituents were identified by the GC-MS analysis and N-hexadecanoic acid was isolated from chloroform leaf extract of *Kigelia pinnata* and was subjected to anticancer activity which showed significant IC50 value of 0.8 µg/mL against HCT-116 cells[33]. The evidence suggests that n-hexadecanoic acid has anti-inflammatory properties which was reported based on the structural and kinetic studies of n-hexadecanoic[34]. Beta-Sitosterol is a phytosterol bioactive compound possess biological activity viz., analgesic, immune-modulatory, antimicrobial, anticancer, anxiolytic, anti-inflammatory, hepatoprotective, wound healing, lipid lowering effect, antioxidant and anti-diabetic activities[35]. The anti-obesity effect of the active sub-fraction with gamma-sitosterol isolated by GC-MS of leaves of the *I. frutescens* using *in vitro* and *in vivo* models showed decrease in adipose mass and liver lipid accumulation which control the obesity through adipocyte apoptosis[36]**.**

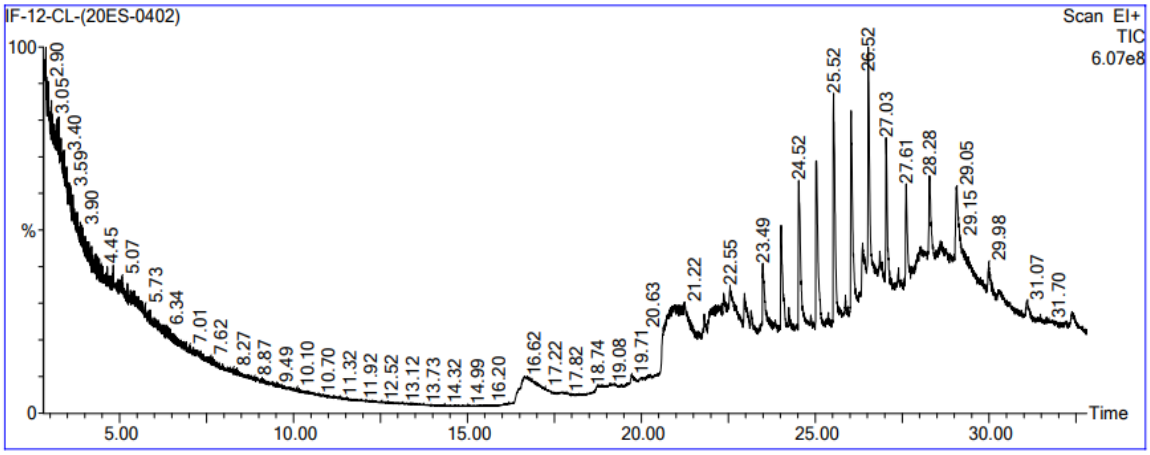

Fig.2: GCMS chromatogram of chloroform leaf derived callus extract of *I. frutescens*

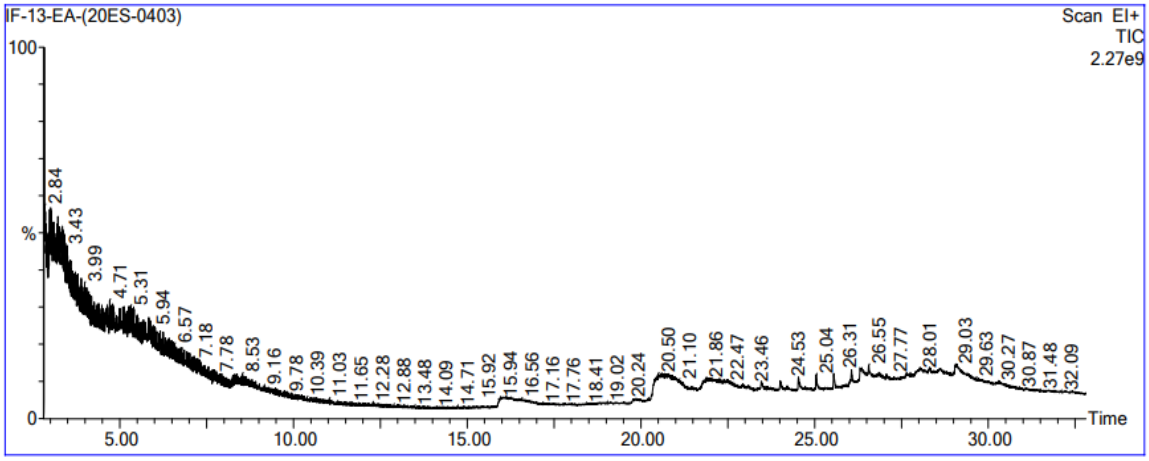

Fig.3: GCMS chromatogram of ethyl acetate leaf derived callus extract of the *I. frutescens*

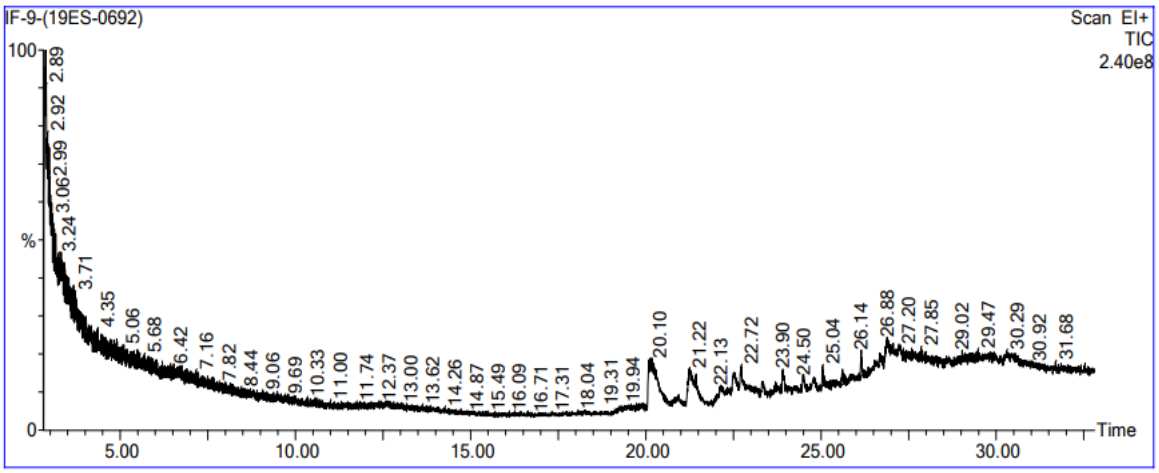

Fig.4: GCMS chromatogram of ethanolic leaf derived callus extract of the *I. frutescens*

Table 3: Phytocompounds of chloroform leaf derived callus extract of *I. frutescens*

Sl. No	RT	Area %	Name of the compound	MF	MW
1.	20.966	10.738	N-Hexadecanoic acid	C16H32O2	256
2.	21.801	1.781	Eicosanoic acid	C20H40O2	312
3.	22.546	3.716	Pentadecanoic acid	C15H30O2	242
4.	24.517	3.477	Heptacosane	C27H56	380
5.	26.358	3.124	Tetracosane, 1-bromo-	C24H49Br	416
6.	27.028	4.992	Triacontane, 11,20-didecyl-	C50H102	702
7.	27.609	3.627	Heptacosane, 1-chloro-	C27H55Cl	414
8.	28.024	7.202	Urs-12-en-28-ol	C30H50O	426
9.	28.594	8.054	Beta.-Sitosterol	C29H50O	414

Table 4: Phytocompounds of ethyl acetate leaf derived callus extract of *I. frutescens*

Sl. No	RT	Area %	Name of the compound	MF	MW
1.	20.596	24.402	N-Hexadecanoic acid	C16H32O2	256
2.	26.058	3.607	Octadecane, 1-chloro-	C18H37Cl	288
3.	26.843	5.847	2(3h)-Furanone, 3-(15 hexadecynylidene)dihydro-4-hydroxy-5-methyl	C21H34O3	334
4.	27.058	2.310	Bicyclo[3.2.1]oct-3-en-2-one, 3,8-dihydroxy-1-methoxy-7-(7-methoxy-1,3	C21H24O7	388
5.	28.304	3.225	4-Cholesten-3-ol, acetate	C29H48O2	428
6.	28.599	11.640	Cholest-5-en-7-one, 3-(acetyloxy)-, (3.beta.)-	C29H46O3	442
7.	29.094	15.398	Stigmasta-3,5-dien-7-one	C29H46O	410

Table 5: Phytocompounds of ethanolic leaf derived callus extract of *I. frutescens*

Sl. No	RT	Area %	Name of the compound	MF	MW
1.	20.165	19.497	octadecanoic acid, ethyl ester	C20H40O2	312
2.	20.210	20.768	2-tridecene, (z)-	C13H26	182
3.	21.246	9.173	pentadecanoic acid, 2,6,10,14-tetramethyl-, methyl ester	C20H40O2	312
4.	21.281	8.635	ethyl 14-methyl-hexadecanoate	C19H38O2	298
5.	26.878	4.270	cholest-8-en-3-ol,14-methyl-, (3.beta.,5.alpha.)	C28H48O	400
6.	26.928	2.913	Stigmasterol	C29H48O	412
7.	27.253	4.080	7-hydroxy-3-(1,1-dimethylprop-2-enyl) coumarin	C14H14O3	230

RT- Retention Time, MF- Molecular Formula, MW- Molecular Weight

Abubakar *et al.,* (2016)[37] reported the Stigmasta-3,5-dien-7-one have the free radical scavenging, anti-diabetic and anticancer property. Whereas, Konovalova, *et al.,* (2013)[38] reported Heptacosane found to possess antibacterial property. In the present investigation also GC-MS analysis of *I. frutescens* callus extracts revealed the presence of Stigmasterol, hexadecanoic acid, Beta-Sitosterol, Stigmasta-3,5-dien-7-one and Heptacosane which are responsible for many biological activities. Therefore, these results confirmed the presence of various compounds which were also reported by Choudhary, *et al.,* (2019)[31], Mailafiya et al., (2018)[32], Ravi et al., (2017)[33], Aparna, *et al.,* (2012)[34], Babu, *et al.,* (2020)[35], Abubakar et al., (2016)[37], Konovalova, *et al.,* (2013)[38]. Hence, *I. frutescens* callus extracts will be used for the formulation of therapeutic drugs to cure various ailments.

4. STATISTICAL ANALYSIS

All the experiments were conducted in triplicates (n=3). The results were expressed as mean ± standard deviation (SD). Statistica software (6th Version, Statsoft. Inc) was used for

data analysis and the significance difference between samples was analyzed by ANOVA performed using Duncan's multiple range test. Probability value (P) of less than 0.05 was considered statistically significant.

5. CONCLUSION

The present investigation was carried out to develop an effective protocol for *in vitro* callus induction and to analyze the secondary metabolites from the leaf derived callus extract of *I. frutescens.* This is the first report on GC-MS analysis of chloroform, ethyl acetate and ethanolic extract of callus. It revealed the presence of 23 phytocompounds which are acting as precursor material for preparation of novel drugs. Further research is required for detailed phytochemical investigation on separation, isolation and characterization of novel compounds from the callus extract and to study various therapeutically useful properties of this plant.

6. ACKNOWLEDGEMENT

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7. AUTHORS CONTRIBUTION STATEMENT

Prof. L. Rajanna was involved in supervision project administration, data interpretation, writing, editing and reviewing the manuscript. Ashwini was responsible for methodology, conducting the experiment, writing the original draft manuscript and funding acquisition.

8. CONFLICT OF INTEREST

The authors declare no conflict of interests.

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