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PHYTOCHEMICAL PROFILING OF *MACROTYLOMA UNIFLORUM* SEEDS : A COMPARATIVE SOLVENT EXTRACTION STUDY BY GCMS ANALYSIS

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ABSTRACT

Macrotyloma uniflorum commonly known as horse gram (Kollu in Tamil) is a legume with high nutritive and therapeutic value but remains scientifically underexplored. The present study investigates the phytochemical profile of *M. uniflorum* seeds (MUS) by identifying bioactive compounds using different solvent extraction systems viz. methanolic, ethanolic, chloroform, aqueous and hydro-alcoholic by GCMS analysis. Of these, methanolic extraction was found to be most effective resulting in identification of 31 different phytoconstituents at a higher proportion. Major phytochemicals identified include 3-O-Methyl-D-glucose with peak area of 12.47-25.30% followed by hexadecanoic acid and stigmaterol, which were detected in all the solvent extraction systems except hydro-alcoholic MUS extract. Compounds such as γ -Sitosterol, 2,4-Di-tert-butylphenol, β -Amyrin, 2-hydroxy-1-(hydroxymethyl) ethyl ester and 17-Pentatriacontene were found in all the solvent extracts. This is the first study of its kind to report the presence of certain (six) phytochemicals with diverse biological activities in all the MUS extracts. The study thus highlights the effectiveness of methanolic extract in extracting a diverse array of bioactive metabolites, supporting the therapeutic potential of *M. uniflorum* seeds.

Keywords : *Macrotyloma uniflorum* seeds, phytochemicals, solvent extraction, GCMS

Introduction

Identification of plant bioactive compounds is essential in deciphering the medicinal properties of plants that have been documented in Indian traditional medicine system with varied therapeutic role. These phytochemicals provide an avenue for new drug development. Nearly, half of the FDA approved drugs that were released over the past for the treatment of human diseases were derived from/ based on such natural products (Zhang *et al.*, 2018). *Macrotyloma uniflorum* (horse gram) is one such legume of Fabaceae family with significant medicinal potential as emphasized in Charak Samhita (renowned text on Indian medicinal system). Medicinal preparations from the seed decoctions of *M. uniflorum* like Kulatthadi Pralepa (paste), Kulattha Yusha, Kulatthadi Gruta

(ghee), Dantimuladi Kwatha and Dhanyamla (sour gruel) were mainly used in traditional medicine as a tonic, diuretic, astringent and for managing several other disorders (Ranasinghe & Ediriweera, 2017). Various studies have documented the anti-hypercholesterolemic effect, anti-microbial activity, anthelmintic activity, anti-inflammatory, anti-diabetic, antioxidant and anti-urolithiasis activity of *M. uniflorum* seeds (Mohanraj, 2021). These properties of a plant may be attributed to its diverse phytochemical compounds such as alkaloids, flavonoids, terpenoids, phenols, glycosides, etc.

As these phytoconstituents are usually present at a lower concentration in plants, their extraction with appropriate solvent system is very crucial. Therefore, selection of solvent and optimization of the

methodology is important to maximize the yield of such bioactive compounds. The solvents used for extraction may vary in their polarity and hence may selectively solubilize a specific group/class of chemical compounds. More polar solvents like water are known for their ability to extract a diverse range of bioactive compounds followed by moderately polar solvents like methanol and ethanol. Non-polar solvents such as chloroform and ether are effectively used for extraction of hydrophobic substances (Sasidharan *et al.*, 2011).

Elucidation of the chemical structure of the extracted plant compound is essential for further studies on their mechanism of biological action and for synthesis of the bioactive compound. Spectrometric and chromatographic methods are widely used for the initial screening of the medicinal plants for the identification of pharmacologically active compounds. Recently, advanced analytical techniques like Fourier-transform infrared (FTIR) and gas chromatography-mass spectrometry (GC-MS) are employed for detection of functional groups of various bioactive compounds. Of these, GCMS is considered the best, fast and accurate technique for identification of compounds such as alcohols, alkaloids, nitro compounds, long chain hydrocarbons, organic acids, steroids, esters, amino acids, etc. and requires only a small volume of plant extracts for analysis (Konappa *et al.*, 2020).

The seeds of *M. uniflorum* are the most studied and are considered the richest in bioactive phytochemicals and medicinal properties. A study by Rao *et al.* (2019) showed that among the extracts isolated from different parts of *M. uniflorum* highest antibacterial activity, antioxidant property and anticancer potential were found in the seed extract. Raw horse gram seeds are reported to possess significant antioxidant activity, with the seed coat containing the highest concentration of antioxidants among the various parts of the plant (Ranasinghe & Ediriweera, 2017). Hence, the present study was taken up to investigate the phytochemicals / bioactive compounds present in the seeds of *M. uniflorum*, extracted using solvents of different polarity. The study would thus document the major bioactive compounds and their pharmacological potential thereby contributing to the development of *M. uniflorum* as therapeutic and nutraceutical legume.

Materials and Methods

Chemicals and Instruments

All the solvents (methanol, ethanol, chloroform) used in this study were procured from M/s. Merck Lifesciences Pvt. Ltd., and were of GC Analytical grade (EMSURE[®], ≥99.9%). The Soxhlet extraction

apparatus (Make: Guna), Rotary evaporator (Make: Superfit Continental) were used for the MUS extraction process and for GCMS study, the GC System (Agilent 8890) with Single Quadrupole Mass Spectrometer (5977B MSD) analyzer was used for the phytochemical analysis.

Preparation of *Macrotyloma uniflorum* seed extract

Certified seeds of *Macrotyloma uniflorum* (horse gram, Paiyur-2 variety) were obtained from the Krishi Vigyan Kendra, Tamil Nadu Agricultural University (TNAU), Papparapatty, Dharmapuri (Dist.), Tamil Nadu. After cleaning, the seeds were ground into a coarse powder, and 20 g of this powder was extracted using five different solvents *viz.*, methanol, ethanol, chloroform, water, and a hydro-alcoholic solution. The extraction process was carried out in a Soxhlet apparatus using 200 ml of each solvent over a period of 6 hours. The extracts were concentrated by evaporation at 60°C and subsequently dried. The final *Macrotyloma uniflorum* seed (MUS) extracts were stored at room temperature for future phytochemical analysis and extraction yield was calculated with the following formula:

$$\text{Extract yield (\%)} = \frac{\text{Mass of extract (g)}}{\text{Mass of dry leaves sample (g)}} \times 100\%$$

Quantitative analysis of the phytochemicals by GCMS

GCMS analysis of all the five extracts of *M. uniflorum* seeds were performed using Agilent 8890 at Sophisticated Analytical Instrument Facility (SAIF), IIT Madras, Chennai. For GC-MS detection, a carrier gas (helium) at a constant flow rate of 1.2 ml/min, and an injection volume of 1µl was employed. The ion-source temperature was 230°C, and the oven temperature was programmed from 60°C to 350°C. The oven temperature was maintained at 50°C isothermal at 280°C Mass Spectra transfer line temperature. The compounds were detected in the range of 50- 600 amu by NIST library search.

Results and Discussion

Macrotyloma uniflorum seed extract

Biologically active compounds in plants are often present in low concentrations, necessitating extraction techniques that maximize yield while maintaining the functional integrity of the extract (Dhanani *et al.*, 2017). In this study, extraction yields varied based on the solvent used (Fig.1), with the aqueous extract (MUS-AE) yielding the highest at 20.3%, followed by methanolic (MUS-ME, 19.54%), hydro-alcoholic (MUS-HAE, 17.15%), ethanolic (MUS-EE, 9.37%),

and chloroform (MUS, CE, 2.19%) extracts. Various studies have highlighted the polarity-dependent increase in extraction yield of compounds in plant material (Nawaz *et al.*, 2020; Ghaffar & Perveen, 2024).

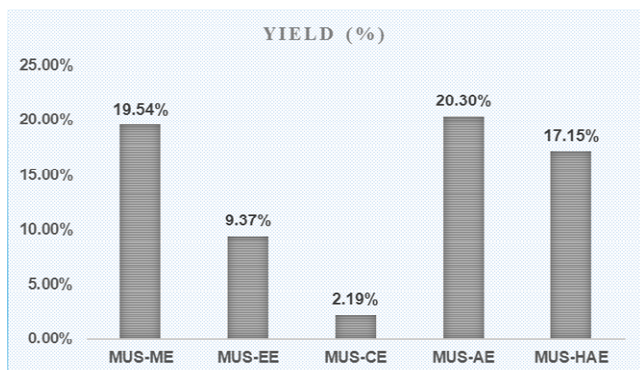


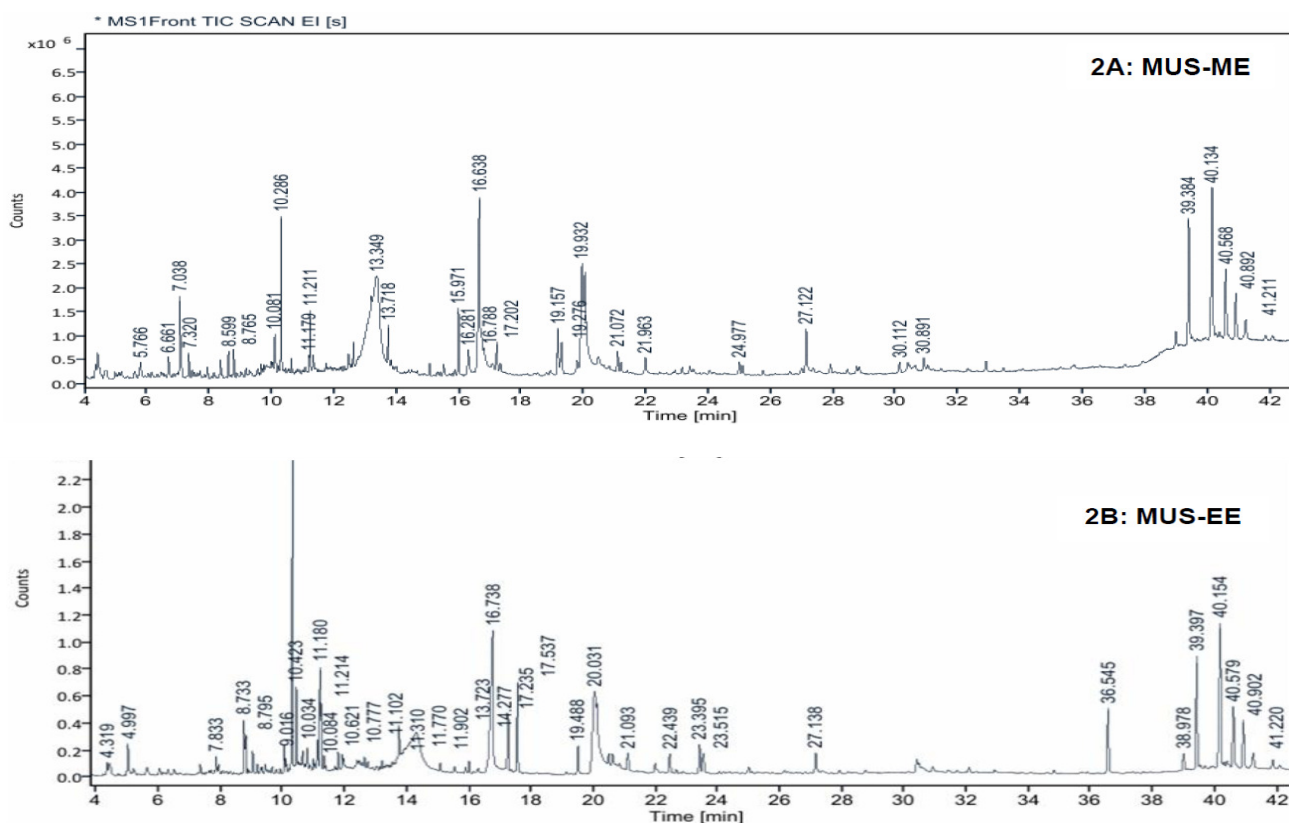
Fig. 1 : Percentage yield of different solvent extracts of MUS

Quantitative analysis of phytochemicals by GCMS

Phytochemical screening of *M. uniflorum* extracts using GC-MS identified a diverse range of metabolites (Fig. 2). The active compounds, along with their retention times (RT) and peak area percentages present in the five different MUS extract systems are summarized in Table 1 (1A and 1B). Among the extracts, the hydro-alcoholic extract showed the

highest diversity, yielding 47 phytoconstituents, followed by methanolic (31), ethanolic (30), chloroform (24), and aqueous (15) extracts. The compounds detected were alkaloids (2-aminoquinoline, apraclonidine), flavonoid (malvidin 3-O-galacto-side cation), triterpenoids/steroids (β -amyrin, γ -sitosterol, stigmasterol, estra-1,3,5(10)-trien-17 β -ol), Phenols (2,4-di-tert-butylphenol, 2-phenylpropenal, benzene,1,3-bis (1,1-dimethyl-ethyl) and glycoside (methylglycocholate 3TMS derivatives). Notably, several phytoconstituents were consistently detected across multiple solvent extracts (Table 2); but for the exception of hexadecanoic acid and stigmasterol, all the other six phytoconstituents *viz.* 3-O-Methyl-d-glucose, γ -Sitosterol, 2,4-Di-tert-butylphenol, β -Amyrin, 2-hydroxy-1-(hydroxymethyl) ethyl ester and 17-Pentatriacontene are being reported for the first time in MUS extracts.

Although hydroalcoholic extract yielded a greater number of compounds, the methanolic extraction of phytochemicals from *M. uniflorum* seeds emerged as the most effective system, extracting a broader range of compounds and yielding them in higher proportions than HAE extract. The moderate polarity of methanol facilitates the dissolution of both polar and semi-polar compounds, making it particularly suitable for capturing diverse bioactive metabolites.



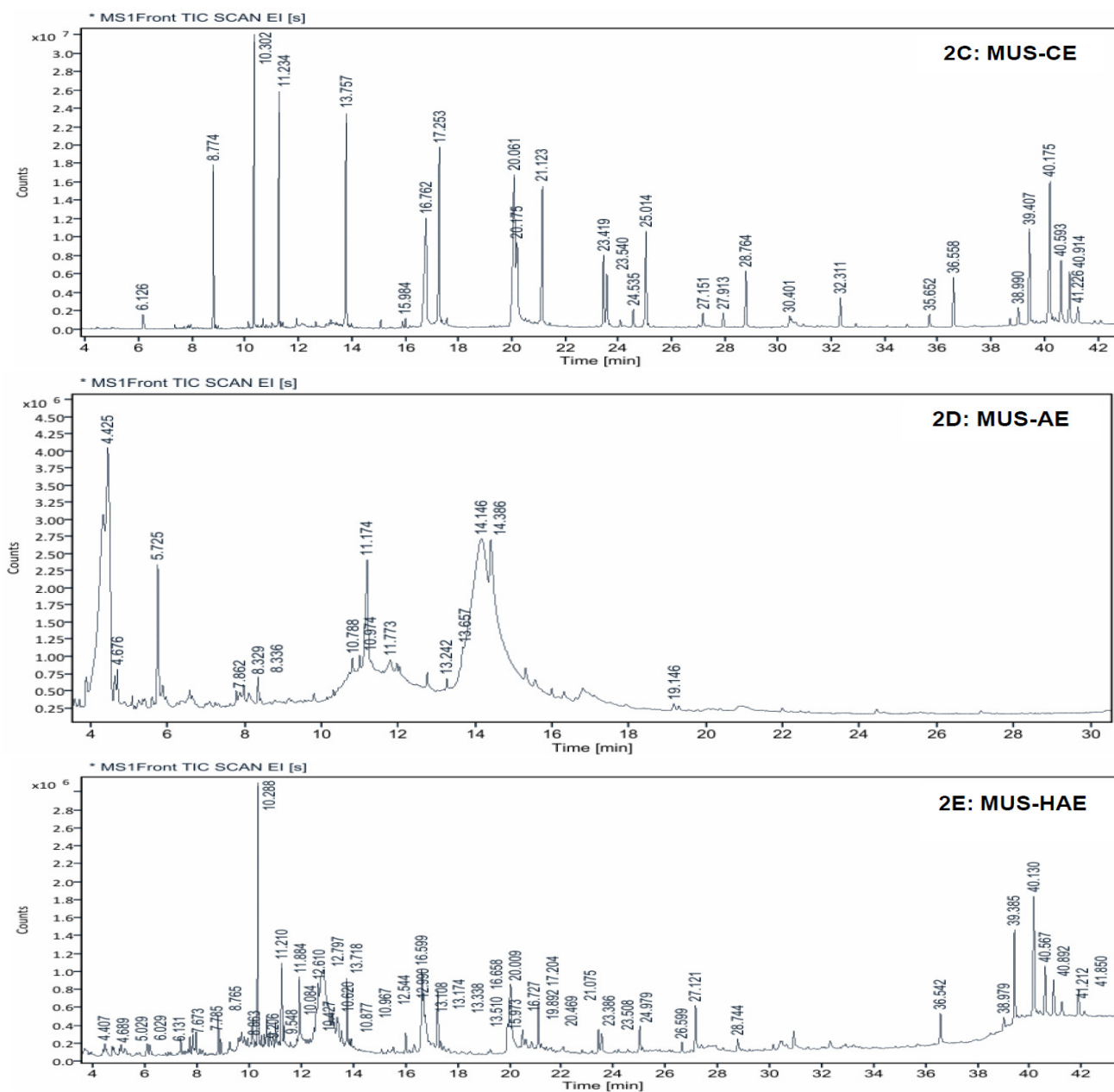


Fig. 2 : GCMS chromatogram of *M. uniflorum* seed extract (2A- methanolic extract, MUS-ME, 2B- ethanolic extract, MUS-EE, 2C- chloroform, MUS-CE, 2D- aqueous, MUS-AE and 2E- hydro-alcoholic, MUS-HAE)

The major phytochemicals identified in *M. uniflorum* (MUS) seeds include 3-O-Methyl-D-glucose, Hexadecanoic acid, γ -Sitosterol, 2,4-Di-tert-butylphenol, Stigmasterol, β -Amyrin, 2-hydroxy-1-

(hydroxymethyl)ethyl ester, and 17-Pentatriacontene. These phytochemicals have been reported to have varied biological activities.

Table 1: Phytochemical profile of *Macrotyloma uniflorum* extracts: Retention time and peak area percentages from GC-MS Analysis

Table 1A: Phytochemicals detected in methanolic, ethanolic and hydro-alcoholic extracts of MUS

Sl. No	Methanolic extract of MUS			Ethanolic extract of MUS			Hydro-alcoholic extract of MUS		
	RT (min)	Area %	Compound	RT (min)	Area %	Compound	RT (min)	Area %	Compound
1	13.349	25.3	3-O-Methylglucose	16.738	12.97	n-Hexadecanoic acid	12.797	12.47	3-O-Methyl-d-glucose
2	19.932	12.84	9,12-Octadecadienoic acid	10.294	10.58	2,4-Di-tert-butylphenol	10.288	7.27	2,4-Di-tert-butylphenol
3	16.638	11.34	n-Hexadecanoic acid	40.154	8.32	γ -Sitosterol	20.009	7.07	12-Methyl-E,E-2,13-octadecadien-1-ol
4	40.134	8.33	γ -Sitosterol	14.277	6.29	3-O-Methyl-d-glucose	40.13	6.84	γ -Sitosterol
5	39.384	5.53	Stigmasterol	39.397	5.08	Stigmasterol	39.385	4.52	Stigmasterol
6	40.568	3.48	β -Amyrin	40.579	3.37	β -Amyrin	15.973	4.12	1-Tetracosene
7	10.286	3.33	2,4-Di-tert-butylphenol	36.545	3.32	γ -Tocopherol	11.21	3.2	Cetene
8	7.038	2.76	Benzene,1,3-bis(1,1-dimethyl-ethyl)-	40.902	2.58	Stigmast-7-en-3-ol, (3 β ,5 α)	11.884	2.84	4-Methyl(trimethylene)silyloxyoctane
9	15.971	2.52	Hexadecanoic acid methyl ester	11.18	2.54	3-Methyl-4-phenyl-1H-pyrrole	40.567	2.76	β -Amyrin
10	40.892	2.42	γ -Sitosterol	17.235	2.51	Hexadecanoic acid, ethyl ester	16.658	2.72	n-Hexadecanoic acid
11	27.122	2.24	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	10.419	2.03	o-Hydroxy biphenyl	17.204	2.64	1-Eicosene
12	19.157	1.84	9,12-Octadecadienoic acid, methyl ester	11.214	1.62	Cetene	13.718	2.68	1-Hexadecanol, 2-methyl
13	19.276	1.75	10-Octadecenoic acid, methyl ester	8.733	1.52	Biphenyl	27.121	2.57	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
14	16.788	1.55	Estra-1,3,5(10)-trien-17 β -ol	4.997	1.33	Benzene ethanamine	12.61	2.5	Desulphosinigrin
15	16.281	1.48	Benzothiazole, 2(2-hydroxyethylthiol)-	27.135	1.25	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	21.075	2.14	1-Tricosanol
16	11.211	1.34	1-Hexadecanol,2-methyl	13.723	1.2	1-Hexadecanol, 2-methyl	16.727	1.83	N,N'-Bis(Carbobenzyloxy)-lysine methyl ester
17	41.211	1.18	Methyl glycocholate 3TMS derivatives	8.795	1	Benzene acetaldehyde, α -methyl	36.542	1.7	Arg-Leu-Lys
18	13.718	1.15	1-Hexadecanol,2-methyl	41.22	0.97	α -Amyrin	19.892	1.48	9,12-Octadecadienoic acid (Z,Z)
19	10.081	1.07	Tetradecane 26,10- trimethyl	38.978	0.92	Campesterol	24.979	1.39	17-Pentatriacontene
20	6.661	0.98	2-Aminoquinolin	11.102	0.85	1,1'-Biphenyl, 2-chloro	23.508	1.37	Oxazole, 2-(8Z)-8-heptadecen-1-yl-4,5-dihydro
21	17.202	0.98	1-Eicosene	10.034	0.74	L-Phenylalanine, ethyl ester	8.765	1.27	1-Tetradecene
22	21.963	0.84	2,6-Diphenylpyridine	9.016	0.66	Diphenyl ether	12.99	1.2	β -D-Mannofuranoside, 1-O-(10-undecenyl)
23	21.072	0.82	1-Tricosanol	7.833	0.62	p-Cymen-7-ol	10.084	1.17	Tetradecane, 2,6,10-trimethyl
24	7.32	0.78	Pentadecane	11.77	0.56	3-Methyl-4-phenyl-1H-pyrrole	23.386	1.17	2-((8Z,11Z)-Heptadeca-8,11-dien-1-yl)-4,5-dihydrooxazole
25	5.766	0.75	2-phenylpropenal	10.777	0.55	1-Naphthalenaminate	10.62	1.12	Tetradecane, 2,6,10-trimethyl
26	8.765	0.74	4-Trifluoro-acetoxy tetradecane	11.902	0.5	Phenol, 2,4,6-tribromo	10.967	1.06	Hexadecane, 1,1-bis(dodecyloxy)
27	8.599	0.71	Apraclonidine	10.084	0.45	Tetradecane, 2,6,10-trimethyl	12.544	1	β -D-Glucopyranose, 4-O- β -D-galactopyranosyl
28	30.891	0.59	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	11.31	0.44	Naphthol [2,3-c] thiophene, 1,3-dihydro	20.469	0.95	Linoleic acid ethyl ester
29	30.112	0.52	Malvidin 3-O-galactoside cation	10.62	0.37	Tetradecane, 2,6,10-trimethyl	16.599	0.91	n-Hexadecanoic acid
30	24.977	0.5	17-Pentatriacontene	4.319	0.34	Benzene acetaldehyde	28.744	0.86	17-Pentatriacontene
31	11.179	0.33	3-Methyl-4-phenyl-1-H pyrrole	-	-	-	6.029	0.78	N-methyliminopropylbenzene
32	-	-	-	-	-	-	38.979	0.71	Ethyl iso-allocholate
33	-	-	-	-	-	-	7.785	0.69	1-Hexadecanol, 2-methyl

34	-	-	-	-	-	-	4.407	0.62	Benzeneacetic acid, 3-tetradecyl ester
35	-	-	-	-	-	-	9.548	0.61	Spermine
36	-	-	-	-	-	-	13.174	0.61	Heptacosane
37	-	-	-	-	-	-	7.673	0.57	1-Dodecanol, 3,7,11-trimethyl
38	-	-	-	-	-	-	9.206	0.57	Falcarinol
39	-	-	-	-	-	-	6.131	0.56	3-Trifluoroacetyoxydodecane
40	-	-	-	-	-	-	10.877	0.56	1-Hexadecanol, 2-methyl
41	-	-	-	-	-	-	26.599	0.56	3-Hydroxypropyl palmitate derivative
42	-	-	-	-	-	-	8.863	0.54	Tetradecane
43	-	-	-	-	-	-	5.029	0.51	Benzeneacetic acid, 4-tetradecyl ester
44	-	-	-	-	-	-	13.338	0.44	cis-4-(2-Bromophenyl)-3-phthalimidoazetidion-2-one
45	-	-	-	-	-	-	13.108	0.36	1,2-Propanediol, 3-(tetradecyloxy)
46	-	-	-	-	-	-	4.689	0.34	2-Cyclopenten-1-one, 3-ethyl-2-hydroxy
47	-	-	-	-	-	-	10.427	0.34	1-Dodecanol, 3,7,11-trimethyl

Table 1B: Phytochemicals detected in chloroform and aqueous MUS extract

Sl. No.	Chloroform extract of MUS			Aqueous extract of MUS		
	RT (min)	Area %	Compound	RT (min)	Area %	Compound
1	15.984	33.04	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	14.146	34.43	3-O-Methyl-d-glucose
2	20.061	11.33	(Z)-18-Octadec-9-enolide	4.425	33.89	Benzeneacetaldehyde
3	16.762	8.51	n-Hexadecanoic acid	14.386	17.82	2-Methyl-d-glucose
4	17.253	7.39	1-Nonadecene	11.174	5.36	3-Methyl-4-phenyl-1H-pyrrole
5	21.123	5.92	Behenic alcohol	5.725	3.41	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl
6	20.175	5.62	cis-Vaccenic acid	13.657	2.09	Pyrrolizin-1,7-dione-6-carboxylic acid, methyl(ester)
7	25.014	4.17	n-Tetracosanol-1	11.773	0.53	Salicylidene solanocapsine
8	39.407	3.80	Stigmasterol	7.862	0.51	Ascaridole epoxide
9	23.419	2.95	2-((8Z,11Z)-Heptadeca-8,11-dien-1-yl)-4,5-dihydrooxazole	4.676	0.46	Cyclo-trisiloxane, hexamethyl
10	23.54	2.46	Oxazole, 2-(8Z)-8-heptadecen-1-yl-4,5-dihydro	10.974	0.34	3,6-Diazahomoadamantan-9-one Hydrazone
11	28.764	2.38	1-Methoxyhexacosane	10.788	0.28	5-Methyluracil, 1-(2-hydroxymethyl-3-ethylaminotetrahydrofuran-5-yl)
12	36.558	2.13	γ -Tocopherol	13.242	0.23	Pyrazole[4,5-b]imidazole, 1-formyl-3-ethyl-6- β -d-ribofuranosyl
13	32.311	1.28	Octacosanol	8.329	0.22	Phenol, 2,6-dimethoxy
14	38.99	0.75	Campesterol	8.336	0.22	Phenol, 2,6-dimethoxy
15	24.535	0.71	[1,1'-Biphenyl]-2,3'-diol, 3,4',5,6'-tetrakis(1,1-dimethylethyl)	19.146	0.22	3-Methyl-6,7-benzoisoquinoline
16	27.151	0.70	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	-	-	-
17	27.913	0.59	Bis(2-ethylhexyl) phthalate	-	-	-
18	35.652	0.57	17-Pentatriacontene	-	-	-
19	30.401	0.49	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	-	-	-
20	7.64	0.14	E-15-Heptadecenal	-	-	-
21	5.83	0.11	Cetene	-	-	-
22	7.5	0.10	2,4-Di-tert-butylphenol	-	-	-
23	3.58	0.08	1-Tetradecene	-	-	-
24	0.46	0.06	Dodecene	-	-	-

3-O-Methyl-D-glucose (3-OMG), constituting 25.3% of the methanolic extract (MUS-ME), is a non-toxic, non-metabolizable glucose analogue detected in

all solvent extracts except the hydroalcoholic extract. It has been reported to mitigate streptozotocin-induced toxicity and protect pancreatic beta cells from the

harmful effects of alloxan, indicating its potential in diabetes management (Isam *et al.*, 2019). Furthermore, n-Hexadecanoic acid (palmitic acid) exhibits antioxidant, lubricant, antiandrogenic properties and acts as a 5-alpha reductase inhibitor with hemolytic activity (Kumar *et al.*, 2010). Compounds such as γ -sitosterol and stigmasterol have hypolipidemic effects

(Feng *et al.*, 2018), while 2,4-Di-tert-butylphenol is recognized for its antioxidant and antifungal activities (Varsha *et al.*, 2015). A study by Viet *et al.* (2021) on α -Amyrin and β -amyrin demonstrated their anti-tyrosinase activity, suggesting their utility as therapeutic agents for managing skin hyperpigmentation.

Table 2: Phytochemicals detected commonly across different solvent extract systems from MUS

Sl. No.	Compound	Retention Time (min), range	Peak Area %, range	Found In Extracts
1.	3-O-Methyl-d-glucose	12.797-13.349	12.47-25.30	MUS-ME, MUS-EE, MUS-CE, MUS-AE
2.	Hexadecanoic acid	16.638-16.599	8.51-11.34	MUS-ME, MUS-EE, MUS-CE, MUS-AE
3.	γ -Sitosterol	40.13-40.134	6.84-8.33	MUS-ME, MUS-EE, MUS-CE,
4.	2,4-Di-tert-butylphenol	10.286-10.288	3.33-7.27	MUS-ME, MUS-EE, MUS-AE
5.	Stigmasterol	39.384-39.385	3.80-5.53	MUS-ME, MUS-EE, MUS-CE, MUS-AE
6.	β -Amyrin	40.567-40.568	2.76-3.48	MUS-ME, MUS-EE
7.	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	27.121-27.122	2.24-2.57	MUS-ME, MUS-EE
8.	17-Pentatriacontene	24.977-24.979	0.5-1.39	MUS-ME, MUS-CE

Some of these compounds, such as hexadecanoic acid and stigmasterol, have previously been reported in the ethanolic extract of MUS (Das *et al.*, 2014). The hydroalcoholic extract of MUS has been shown to contain γ -Sitosterol (Priyadarshini, F.C., 2022). Other compounds, including 3-O-Methyl-D-glucose, β -Amyrin, 2-hydroxy-1-(hydroxymethyl) ethyl ester, 2,4-Di-tert-butylphenol and 17-Pentatriacontene, have been reported in extracts from other plants such as *B. aegyptiaca* (Isam *et al.*, 2019), *C. hindsii* (Viet *et al.*, 2021), *L. camara*, *A. sikkimensis* and *C. iberica* (<https://neist.res.in/osadhi/>) respectively.

Conclusion

This is the first study to report the presence of the following six bioactive phytoconstitueunts in *M. uniflorum* seed extracts, viz. 3-O-Methyl-d-glucose, γ -Sitosterol, 2,4-Di-tert-butylphenol, β -Amyrin, 2-hydroxy-1-(hydroxymethyl) ethyl ester and 17-Pentatriacontene. Moreover, of the different solvents used for the extraction of phytoconstituents from the seeds of *M. uniflorum*, methanolic extract was found to be effective in extracting most of the bioactive compounds at a higher proportion. Further *in vitro* and *in vivo* studies on the therapeutic role of these individual phytoconstituents may prove useful in validating the medicinal properties of *Macrotyloma uniflorum*.

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