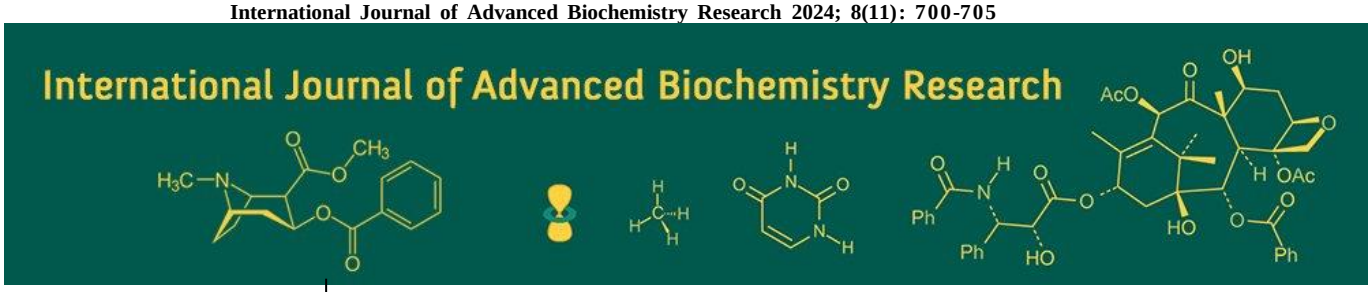

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In-silico exploration of molecular interactions between stigmasterol from the methanolic extract of *Macrotyloma uniflorum* and proteins associated with lipid metabolism

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Abstract
Macrotyloma uniflorum (horse gram or Kulthi bean) is an overlooked yet valuable legume with known medicinal properties, particularly in managing metabolic disorders like diabetes and obesity. Primarily cultivated in South Asia, with India as its largest producer, *M. uniflorum* remains underexplored scientifically. This study investigates its phytochemical profile using GC-MS analysis and evaluates the potential therapeutic effects of its bioactive compounds on lipid metabolism-related proteins through molecular docking. GC-MS analysis of methanolic extracts revealed 31 phytoconstituents, with stigmasterol as a major component (5.53% peak area). Molecular docking studies indicate that stigmasterol binds significantly to key lipid-regulating proteins, including Peroxisome proliferator-activated receptor alpha (PPAR-α) and Cholesterol 7 alpha-hydroxylase (CYP7A1), with binding energies of -8.1 and -8.7 kcal/mol, respectively. Stigmasterol exhibited favorable hydrophobic interactions and hydrogen bonding, notably involving MET 220 on PPAR-α (2.378 Å) and ASN126 on CYP7A1 (2.863 Å), suggesting a role in triglyceride reduction and cholesterol excretion. These findings propose stigmasterol in *M. uniflorum* as a promising candidate for lipid metabolism regulation and obesity management, warranting further *in vitro* and *in vivo* investigation.

Keywords: *Macrotyloma uniflorum* seeds (MUS), GCMS, Stigmasterol, molecular docking, PPAR-α and CYP7A1

Introduction
Macrotyloma uniflorum (Lam.) Verdc., commonly known as horse gram, kulthi bean, gahat, hurali, or Madras gram/ kollu, is a legume of the tropical southern Asian countries. It is cultivated for food in India, Myanmar, Nepal, Malaysia, Mauritius, and Sri Lanka and grown as fodder in Australia and Africa. It is a highly resilient crop capable of withstanding drought, saline environments, and exposure to heavy metal stress. *Macrotyloma uniflorum* contributes to around 5–10% of India's pulse production, with an annual yield of about 0.65 million tonnes. According to the food composition data from the National Institute of Nutrition (ICMR), the protein content in whole horse gram seeds, sampled from diverse regions of India, is comparable to other legumes and nearly double that of most cereal grains. Although horse gram is high in protein, its strong distinctive taste and flavor limit its popularity, making it an underutilized legume that is primarily consumed only by specific groups of people (Mohanraj, 2021) [14].

The medicinal value of horse gram seeds has deep roots in traditional Indian medicine, with Charak Samhita, a foundational Ayurvedic text, recommending them for ailments like piles, hiccups, abdominal lumps, and bronchial asthma, as well as for inducing and regulating perspiration (Pati and Bhattacharjee, 2013) [16]. In herbal medicine, seeds are used as a tonic, astringent, diuretic and as a remedy for conditions like asthma, bronchitis, urinary issues, hiccups, nasal infections, heart ailments, and neurological disorders. Various studies have further supported these uses by demonstrating *Macrotyloma uniflorum*'s pharmacological effects, including anti-hypercholesterolemic, antimicrobial, anthelmintic, anti-inflammatory, antidiabetic, antioxidant, and anti-urolithiatic properties (Chirania and Sharma, 2021) [6]. Research by Auxilia and Thangamalai (2013) [4] have identified antiviral properties, showing

that specific proteins (dolichins) from horse gram may have anti-HIV effects. Phytochemical analysis of medicinal plants can help to identify, isolate, and characterize lead compounds/active constituents possessing significant biological activities from various parts of the plant. Most of the phytochemicals are bioactive, exhibiting high efficacy and reduced toxicity. Polyphenols and polyunsaturated fatty acid in the legume have been attributed to its potential use in the treatment of metabolic disorders such as obesity, diabetes, and cardiovascular disease (Malarvizhi *et al.*, 2021) [10]. Deciphering of the mechanism of action of such bioactive compound from the legume is still underway.

Molecular docking is used to predict the optimal pose or conformation of molecules like bioactive phytoconstituents that fit effectively into the active site of a protein associated with disease progression/regression. It has become a powerful tool for *in-silico* drug design due to its efficiency in screening potential drug candidates. In recent years, several free and commercial docking programs have been developed, each utilizing distinct search algorithms and scoring functions to enhance prediction accuracy and efficiency (Achutha *et al.*, 2021) [1]. Hence, the present study was designed to elucidate the major phytochemicals in the methanolic extract of *Macrotyloma uniflorum* and to analyze their potential in exhibiting anti-obesity activity by docking with proteins associated with lipid metabolism.

Materials and Methods

Preparation of *Macrotyloma uniflorum* seed extract

Certified seeds of *Macrotyloma uniflorum* (Horse gram, Paiyur-2 variety) were obtained from Krishi Vigyan Kendra, Tamil Nadu Agricultural University (TNAU), located in Papparapatty, Dharmapuri district, Tamil Nadu. The seeds were thoroughly cleaned and ground into a coarse powder. Approximately 20 g of the dried seed powder was placed in a thimble and subjected to extraction using 200 ml of methanol in a Soxhlet apparatus for 6 hours. The extract was concentrated through evaporation at 60°C and subsequently dried. The resulting concentrated extract was stored at room temperature until further phytochemical analysis. The extraction yield was determined using the following equation:

$$\text{Extract yield (\%)} = \frac{mass\ of\ extract\ (g)}{mass\ of\ dry\ leaves\ sample\ (g)} x100\ \%$$

GCMS analysis

GC-MS analysis of methanolic extract of *M. uniflorum* seeds was performed using Agilent 8890 at Sophisticated Analytical Instrument Facility (SAIF), IIT Madras, Chennai. For GC-MS detection, a carrier gas 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. The major phytoconstituents were identified and subjected for molecular docking analysis with target proteins associated with lipid metabolism. One of the major phytoconstituent stigmasterol (peak area 5.5%) was taken further for molecular docking studies.

Protein selection and preparation

The crystallographic three-dimensional structures of the selected target protein proliferator-activated receptor alpha (PPAR-α) (PDB ID:2REW), Cholesterol 7 alpha-hydroxylase (CYP7A1) (PDB ID:3DAX), Stearoyl-CoA desaturase 1 (SCD1) (PDB ID:3DAX), and Sterol regulatory element-binding protein 1 (SREBP-1) (PDB ID:5GPD) were retrieved from Protein Data Bank database. The amino acid sequences, number of binding sites, molecular weight, etc. were analyzed using BIOVIA Discovery Studio Visualizer (version 2024) and the protein was prepared for docking using AutoDock 4 software.

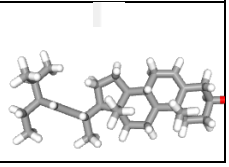
Ligand preparation

Chemical structure of the ligand stigmasterol was retrieved from Pubchem compound database (https://pubchem.ncbi.nlm.nih. gov/) in SDF format (Table 1). The SDF files were converted into PDB file format using OPEN BABEL software. The 3-Dimensional structure of the ligands were visualized and their structural analysis (viz. molecular weight, atom composition, presence of sulphur atom) were carried out using BIOVIA Discovery Studio Visualizer (version 2024) and ligand was prepared for docking using AutoDock 4 software (Molecular Graphics Laboratory, The Scripps Research Institute).

Protein-ligand docking

The docking studies were performed by using AutoDock vina version 4.2.6and the molecular interactions of target protein *viz.* peroxisome proliferator-activated receptor alpha (PPAR-α), Cholesterol 7 alpha-hydroxylase (CYP7A1), Stearoyl-CoA desaturase 1 (SCD1), and Sterol regulatory element-binding protein 1 (SREBP-1) with stigmasterol were visualized using both BIOVIA Discovery Visualizer (version 2024) and AutoDock 4 software. Among the various conformations generated for each ligand, the ligand pose with the best docking score and the least binding energy was considered for predicting the optimal ligand binding conformation. The interaction of amino acid residues at the binding site was investigated. To assess the stability of the best-docked pose for these compounds, the hydrogen bonding interactions between the protein and compounds were examined, revealing the critical amino acids involved in hydrogen bond formation. In addition to hydrogen bonding interactions, other non-bonded interactions such as hydrophobic bonding were also investigated.

Table 1: Details of the flavonols used in the study

Sl. No	Name of the Compound	Compound ID	Molecular formula	Smile	Chemical Structure
1.	Stigmasterol	PubChem CID 5280794	$C_{29}H_{48}O$	CC[C@H](/C=C/[C@@H](C)[C@H]1CC[C@@H]2[C@@]1(CC[C@H]3[C@H]2CC=C4[C@@]3(CC[C@@H](C4)O)C)C)C(C)C	

Results

GCMS analysis

The methanolic extraction of phytochemicals from *M. uniflorum* seeds (MUS) resulted in a yield of 13.19%. Quantitative analysis of the methanolic extract of *M. uniflorum* seeds by GCMS showed about thirty-one peaks which indicates the presence of thirty-one different phytochemical constituents (Fig.1). The chemical compounds, area percentage and retention time identified by NIST library search are shown in table (1). The major phytochemical constituents were: 3-O-Methylglucose, 9, 12-Octadecadienoic acid, n-Hexadecanoic acid, γ- Sitosterol and Stigmasterol.

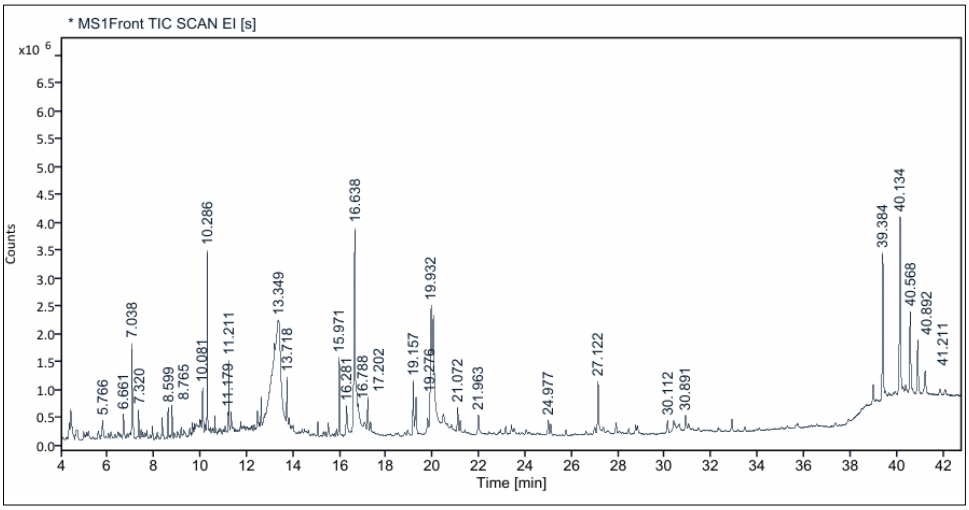


Fig 1: GCMS chromatogram of *M. uniflorum* methanolic extract

Table 2: Phytoconstituents present in *M. uniflorum* methanolic seed extract

S. No.	RT (min)	Area %	Compound	Molecular weight	Molecular formula
1	13.349	25.3	3-O-Methylglucose	194.18	C7H14O6
2	19.932	12.84	9, 12-Octadecadienoic acid	280.4472	C18H32O2
3	16.638	11.34	n-Hexadecanoic acid	256.43	C16H32O
4	40.134	8.33	γ- Sitosterol	414.7067.	C29H50O
5	39.384	5.53	Stigmasterol	412.69	$C_{29}H_{48}O$
6	40.568	3.48	β-Amyrin	426.7174	C30H50O
7	10.286	3.33	2, 4-Di-tert-butylphenol	206.3239	C14H22O
8	7.038	2.76	Benzene, 1, 3-bis (1, 1-dimethy- ethyl)-	190.3245	C14H22
9	15.971	2.52	Hexadecanoic acid methyl ester	270.45	C17H34O
10	40.892	2.42	γ- Sitosterol	414.7067	C29H50O
11	27.122	2.24	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	330.5026	C19H38O4
12	19.157	1.84	9, 12-Octadecadienoic acid, methyl ester	294.4721	C19H34O2
13	19.276	1.75	10-Octadecenoic acid, methyl ester	296.4879	C19H36O2
14	16.788	1.55	Estra-1, 3, 5(10)-trien-17β-ol	256.3826	C18H24O
15	16.281	1.48	Benzothiazole, 2(2-hydroxy ethylthiol)-	211.3	C9H9NOS2
16	11.211	1.34	1-Hexadecanol, 2-methyl	256.5	C17H36O
17	41.211	1.18	Methylglycocholate 3TMS derivatives	639.1413	C36H69NO6Si3
18	13.718	1.15	1-Hexdecanol, 2 -methyl	256.5	C17H36O
19	10.081	1.07	Tetradecane 26, 10- trimethyl	240.4677	C17H36.
20	6.661	0.98	2-Aminoquinolin	144.17	C9H8N2
21	17.202	0.98	1-Eicosene	280.5316	C20H40
22	21.963	0.84	2, 6-Diphenylpyridine	231.30	C17H13N
23	21.072	0.82	1-Tricosanol	340.626	C23H48O
24	7.32	0.78	Pentadecane	212.42	C15H32
25	5.766	0.75	2-phenylpropenal	132.1592	C9H8O
26	8.765	0.74	4- Trifluoroacetoxy tetradecane	310.39	C16H29F3O2.
27	8.599	0.71	Apraclonidine	281.5	C9H11Cl3N4
28	30.891	0.59	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	358.5558	C21H42O4
29	30.112	0.52	Malvidin 3-O-galactoside cation	528.89	C23H25ClO12
30	24.977	0.5	17-Pentatriacontene	490.9303	C35H70
31	11.179	0.33	3-Methyl-4-phenyl-1-H pyrrole	157.22	C11H11N

Molecular docking analysis

Among the different phytocompounds identified in the methanolic extract of MUS, stigmasterol attributed with cholesterol lowering effect was taken up for molecular interaction studies with four proteins associated with lipid metabolism. Stigmasterol exhibited a good binding affinity against two of the four proteins docked *viz.* PPAR-α (-8.0) and CYP7A1 (-8.7) (Table 1). Stearoyl-CoA desaturase 1 and Sterol regulatory element-binding protein 1 did not show favorable interaction. Stigmasterol was found to interact with key amino acid residues at the binding site of PPARα and CYP7A1 (Figure 2).

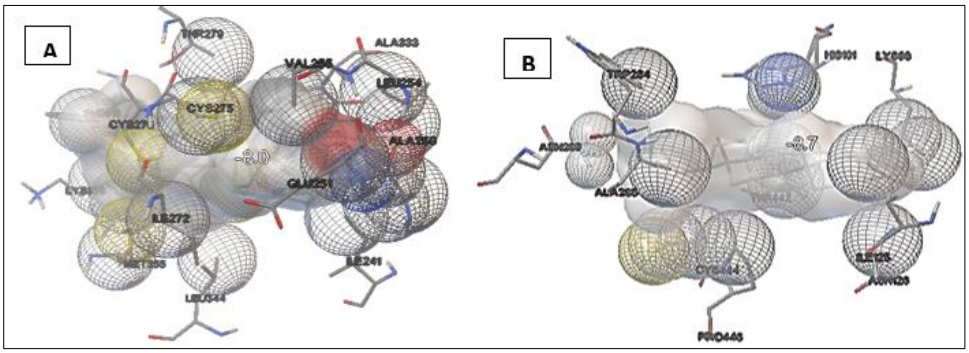


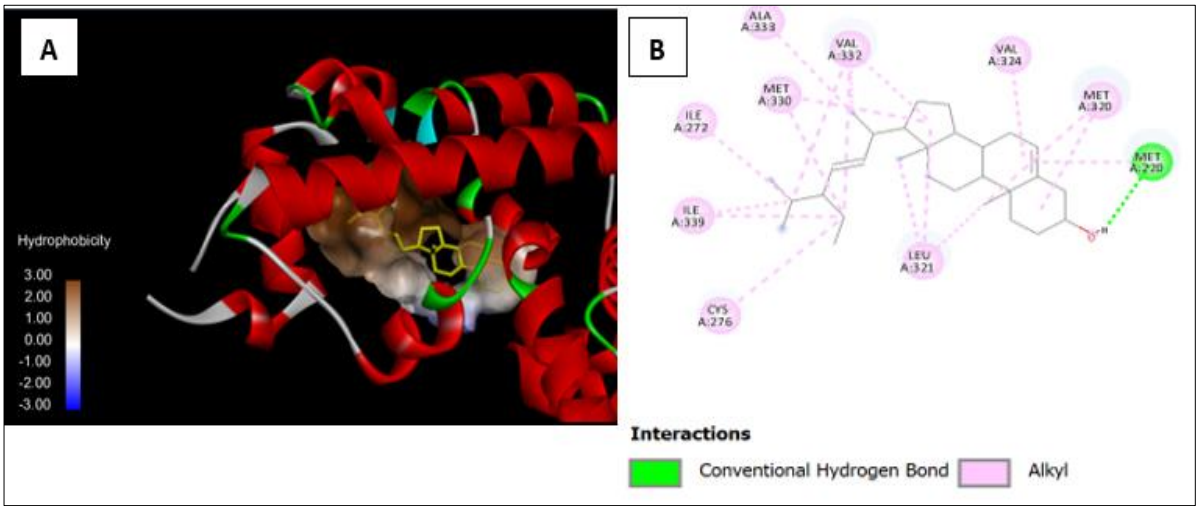
Fig 2: Binding site amino acid residues of PPAR- α and CYP7A1 interacting with the stigmasterol, A. Interacting residues of PPAR- α, B. Interacting residues of CYP7A1

The docking of stigmasterol within the binding site of PPAR-α and CYP7A1 is presented in Fig.3 and table1. It formed favorable hydrogen bond withMET220 residue at the active site of PPAR-α, highlighting its strong interaction and potential stability within the receptor's active site. Apart from covalent hydrogen bond, hydrophobic alkyl interactions were also evident (Table 2).

Table 1: Docking analysis data of the stigmasterol with the binding site of PPAR- α and CYP7A1

Protein	Binding affinity (kcal/mol)	RMSD/UB	RMSD/LB	No. of Hydrogen bonds
PPAR- α	-8.0	0.0	0.0	1
CYP7A1	-8.7	0.0	0.0	1

The docking of stigmasterol into the binding site residues of CYP7A1 is displayed in figure 2 & 3. The interaction with ASN126 residue of CYP7A1 is considered essential as it resulted in the formation of hydrogen bond involving the amide hydrogen of ASN126 and the hydroxyl group oxygen of stigmasterol. Also, various residues were involved in alkyl and pi-alkyl interaction favoring stabilization of the protein ligand complex (table 2).



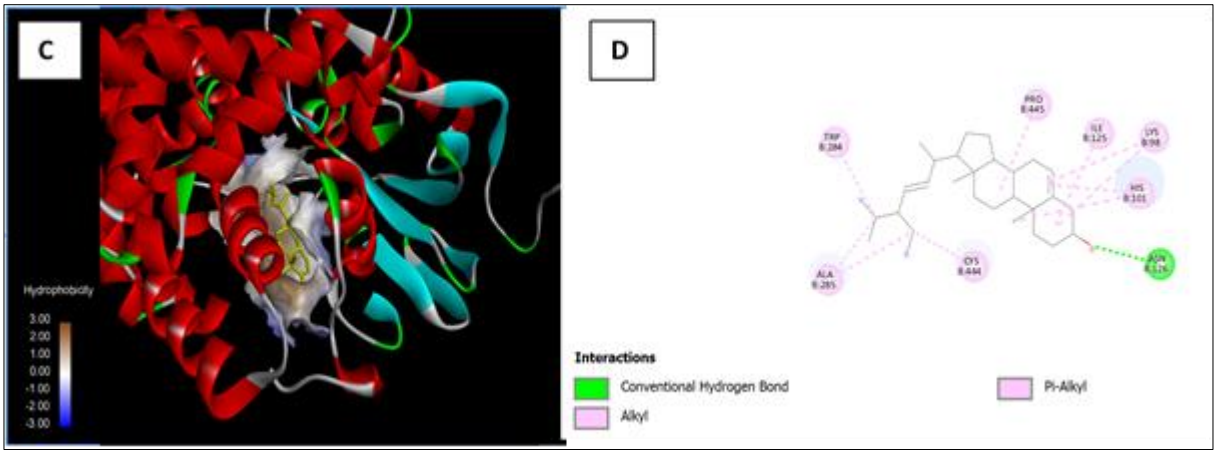


Fig 3: Molecular structural representation of the interaction of stigmasterol with PPAR- α and CYP7A1.A. 3D interaction of stigmasterol at the active binding site PPAR- α(hydrophobicity surface), B. 2D interaction plot of stigmasterol showing the interaction types, C. 3D interaction of stigmasterol at the active binding site of CYP7A1(hydrophobicity surface) and D. 2D interaction plot of stigmasterol showing the interaction types

Table 2: Interaction types and amino acids involved in the interaction of stigmasterol with PPAR- α and CYP7A.

Protein	No. of Hydrogen bonds	H donor	H acceptor	Bond Distance	Alkyl interaction/ Pi-alkyl interaction
PPAR- α	1	Stigmasterol: H (3rd carbon -OH group)	MET 220: O	2.378	ALA333, VAL332, LEU331, VAL324, MET330, MET320, CYS275, CYS276, ILE272, THR279, PHE218, LEU321
CYP7A1	1	ASN126: H (-NH group)	Stigmasterol: O (3rd carbon -OH group)	2.863	LYS 98, HIS101, ILE125, ASN126, THR442, CYS444, PRO445, TRY284, ALA285, ASN289

Discussion

The GCMS analysis of methanolic extract of *M. uniflorum* seeds revealed presence of many phytoconstituents with varied biological activities such as n-Hexadecanoic acid reported to be an antioxidant, lubricant, antiandrogenic, hemolytic 5-Alpha reductase inhibitor (Kumar *et al.*, 2010) [9]; 9, 12-Octadecadienoic acid with antihypertensive, anti-inflammatory, cancer preventive, hepatoprotective, antihistaminic activities (Senyilmaz-Tiebe *et al.*, 2018; Abdullah *et al.*, 2020) [2, 17], γ- Sitosterol and Stigmasterol with hypolipidemic activity (Feng *et al.*, 2018) [7], 4-Di-tert-butylphenol with antioxidant & antifungal activity (Varsha *et al.*, 2015) [19], β-Amyrin with antimicrobial, antidepressant, anti-inflammatory, antinociceptive, and gastroprotective activities (Thirupathi *et al.*, 2017) [18] and Malvidin 3-O-galactoside with antioxidant and anti-inflammatory effects (Merecz-Sadowska *et al.*, 2023) [11]. Among the compounds identified, stigmasterol was found to be one of the major phytoconstituent with peak percentage of 5.53%. Such plant-based phytosterols are structurally like cholesterol and have been known to reduce cholesterol levels by either competing with cholesterol for absorption in the intestines or by modulating cholesterol metabolism-related proteins. A study by Feng *et al.* (2018) [7] showed that its supplementation prevents weight gain, reduces liver fat accumulation, and improves non-alcoholic fatty liver disease (NAFLD) and suggested that further studies are needed to determine the potential use of phytosterols as preventive and adjuvant therapeutic agents for NAFLD.

Molecular docking analysis of stigmasterol showed good binding affinity with biological targets related to lipid metabolism *viz.*, PPARα and CYP7A1. PPARα is a key therapeutic target in the treatment of hyperlipidemia due to its central role in lipid metabolism (Araki *et al.*, 2018; Bougarne *et al.*, 2018) [3, 5]. Activation of PPARα, primarily expressed in tissues involved in fatty acid oxidation like the liver, heart, and muscle, triggers a cascade of metabolic processes that lower plasma triacylglycerol and increase HDL cholesterol (Araki *et al.*, 2018) [3]. Interactions of stigmasterol was found to occur through hydrophobic interactions and formation of hydrogen bond with MET220 residue of PPARα. Although methionine is considered hydrophobic, its dipole strength enables it to act as an effective hydrogen bond acceptor. Computational studies by Pal and Chakrabarti (2001) [14] demonstrated that Met residues in protein structures often interact closely with carbonyl and even carboxylate oxygen atoms. These interactions suggest methionine's significant role in molecular recognition, as it often appears in protein binding sites, aiding in the stabilization of protein-ligand complexes

There was significant interaction of stigmasterol with CYP7A1 protein. The asparagine residue in CYP7A1 participated as a hydrogen donor in this interaction. The role of asparagine in this interaction is critical, as its amino group (NH_2) can act as a hydrogen bond donor, while the carbonyl group (C=O) may function as a hydrogen bond acceptor. This dual capability allows asparagine to facilitate specific molecular interactions, particularly with substrates, enhancing the stability and specificity of the binding event. Thus, it can be postulated that interaction of stigmasterol may modulate the enzymatic activity of CYP7A1. Phytosterols such as Stigmasterol and β-sitosterol have been reported to decrease the levels of intestinal bile acids, accompanied by markedly increased fecal lipid levels (Feng *et al.*, 2018) [7]. Another study by Zhang *et al.* (2022) [19] showed that treatment of HFD rats with stigmasterol lead to an increase in the hepatic mRNA and protein expression of CYP7A1.

Conclusion

Stigmasterol found in the methanolic extract of *Macrotyloma uniflorum* interacts with key amino acid

residues at the binding site of both PPAR- α and CYP7A1. This interaction could promote fatty acid oxidation and reduce the plasma triglycerides levels and can favor catabolism of cholesterol and enhance its excretion thereby conferring hypolipidemic effect. Further *in vitro* and *in vivo* studies on stigmasterol and other major phytoconstituents of *Macrotyloma uniflorum* could prove useful in elucidating and validating the anti-obesity property of the plant.

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