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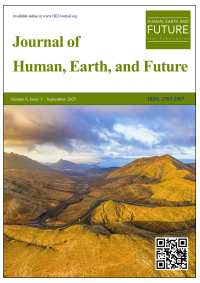

Antioxidant and Molecular Docking of *Morinda citrifolia* Leaves Extract as an Alternative Infertility Treatment

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Abstract

The male reproductive system shows heightened sensitivity to oxidative stress from reactive oxygen species. However, the effects of free radical exposure can be mitigated by antioxidant compounds. This investigation sought to characterize the active compounds from secondary metabolites that contribute to the activity of *Morinda citrifolia* leaves. This study began by extracting *M. citrifolia* leaves using n-hexane, ethyl acetate, and methanol solvents. Following extraction, the samples underwent phytochemical profiling and GC-MS analysis to determine the active compounds in the *M. citrifolia* leaf extract. The DPPH method was employed for testing antioxidant activity and analyzed using ultraviolet-visible (UV-VIS) spectrophotometry. Molecular docking analysis was carried out on five major compounds from the *M. citrifolia* leaf extract against three selected proteins: the FSH (PDB ID: 1XWD), testosterone (PDB ID: 1I9J), and androgen (PDB ID: 1E3G) receptors. The analytical results demonstrated that the polar extract (methanol) of *M. citrifolia* leaf exhibited the highest antioxidant activity, indicated by a lower IC50 (13.1), than those found in the n-hexane and ethyl acetate extracts. The active compounds contributing to this activity were squalene (n-hexane), phytol (ethyl acetate), and 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (methanol). Docking results showed that the ethyl acetate extract exhibited higher binding affinity through tocopherol and stigmasterol compounds than the n-hexane extract. These findings collectively demonstrate that *M. citrifolia* leaves demonstrate significant antioxidant activity across all levels and potential as agents against infertility due to their high binding affinities to the target receptors.

Keywords: Antioxidant; Extraction; Gas Chromatography-Mass Spectroscopy; Molecular Docking; *Morinda citrifolia*.

1. Introduction

Infertility is clinically defined as the failure to conceive following twelve months of regular unprotected sexual intercourse [1]. This condition impacts countless couples globally, with male factors contributing to approximately half of all cases, either partially or entirely [2]. Male infertility arises from various causes, including hormonal imbalances (e.g., hypothyroidism, hyperprolactinemia), anatomical abnormalities (e.g., varicocele, obstructive azoospermia), lifestyle factors (e.g., smoking, alcohol abuse), environmental exposures (e.g., radiation, endocrine disruptors), and psychological stress, all of which impair sperm quality and function [3].

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Pharmacological interventions, such as clomiphene citrate, have been widely used to treat male infertility by stimulating gonadotropin secretion and enhancing spermatogenesis [4]. Unlike exogenous testosterone therapy, which suppresses endogenous spermatogenesis [5], clomiphene maintains or improves sperm production in hypogonadal men [6, 7]. However, long-term use of synthetic drugs carries risks encompassing mood alterations, visual disturbances, and mammary gland sensitivity [8], alters the hormonal homoeostasis [9] which prompting the need for safer of natural alternatives. This has led researchers to explore alternative strategies, particularly antioxidants, which address a key underlying cause of infertility such as oxidative stress, while minimizing synthetic drug-related risks.

Oxidative stress resulting from ROS overproduction significantly contributes to impaired spermatogenesis and male subfertility [10], damaging sperm DNA, lipids, and proteins and impairing sperm function [11]. Antioxidants, such as vitamin C and flavonoids, counteract ROS, protecting sperm integrity and improving motility, morphology, and count [12]. Other studies show that antioxidant supplementation not only enhances sperm quality but also boosts testosterone levels [12, 13], offering a natural and potentially safer complement or alternative to pharmacological treatments like clomiphene. By targeting oxidative stress, antioxidants provide a promising therapeutic pathway that aligns with the goal of improving fertility while reducing reliance on synthetic drugs.

Among natural sources, *Morinda citrifolia* (*M. citrifolia*) has gained attention for its diverse array of bioactive constituents, particularly flavonoid derivatives, alkaloid compounds, and polyphenolic substances, which exhibit potent antioxidant properties [14, 15]. This plant, characterized by its small tree structure and shiny, oval leaves with prominent veins [15]. While previous studies have focused on *M. citrifolia* fruit, recent findings suggest that its leaves possess even higher antioxidant activity due to their flavonoid and phenolic content [16, 17]. However, most research has been limited to crude extracts, with little investigation into the polar-specific distribution of bioactive compounds or their mechanistic effects on male fertility.

The leaves of *M. citrifolia* are particularly rich in alkaloids, tannins, flavonoids [14, 18, 19], anthraquinones, sterols [20], terpenoids, ascorbic acid, beta-carotene, and proxeronine [21]. Studies by Puruhita et al. [22] and Budianta et al. [23] have demonstrated that extracts from these leaves contain flavonoids that serve essential biological functions in their antioxidant capacity. *M. citrifolia* leaves may exhibit superior antioxidant activity compared to the fruit and roots, reinforcing their potential as a potent natural antioxidant agent [24]. These findings underscore the need for deeper exploration into the specific bioactive fractions of *M. citrifolia* leaves and their therapeutic applications, particularly in addressing oxidative stress-related conditions such as male infertility.

Although numerous studies have established the antioxidant properties of *M. citrifolia* leaves [20, 21, 25], significant knowledge gaps remain regarding solvent-specific extraction effects on its phytochemical profile and biological activity. Zin et al.'s [26] demonstration of polarity-dependent phytochemical extraction (using n-hexane, ethyl acetate, and dichloromethane) was restricted to single-step maceration, leaving the optimization potential of sequential extraction un-investigated. Furthermore, while flavonoids and phenolics are well-established as potent antioxidants [27], the particular solvent-extracted bioactive compounds responsible for *M. citrifolia*'s therapeutic effects remain inadequately characterized in the scientific literature.

Beyond its antioxidant properties, *Morinda citrifolia (M. citrifolia)* has demonstrated promising effects on reproductive health in preclinical studies. For instance, in vivo experiments showed that fresh *M. citrifolia* juice administered to adult male rats at 10 mL/kg body weight for 60 days enhanced fertility markers, including increased testosterone, follicle-stimulating hormone (FSH) levels, and improved semen [28]. Similarly, in mice, a 15-day treatment with *M. citrifolia* extract (0.3 g/kg body weight) demonstrated significant enhancement in both sperm morphological parameters and motility characteristics [29]. Despite these findings, clinical trials investigating the hormonal regulatory effects of *M. citrifolia* leaf extracts are still lacking. Most importantly, despite the growing use of computational tools in drug discovery, no studies have investigated the interactions between *M. citrifolia* leaf phytochemicals and fertility-related targets such as FSH, testosterone, and androgen receptors that have a vital function in spermatogenesis and its hormonal regulation [30, 31]. Addressing these gaps could unlock the mechanistic basis for *M. citrifolia*'s therapeutic effects and pave the way for its development as a natural treatment for male infertility.

The present study aims to characterize secondary metabolites in *M. citrifolia* leaves through sequential extraction using solvents of varying polarity (n-hexane, ethyl acetate, and methanol) followed by GC-MS analysis, and assess the antioxidant capacity of each extract to correlate bioactivity with chemical composition. Additionally, molecular docking was also conducted using in silico methods to predict interactions between the dominant phytocompounds and key fertility-related targets (FSH, testosterone, and androgen receptors) to elucidate potential mechanisms of action.

2. Material and Methods

M. citrifolia leaves were gathered from the Ulee Lheu area, Banda Aceh, Indonesia. The plant materials were taxonomically authenticated at the Biosystematics Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala, with the registration number 209/UN11.1.8.4/TA.00.03/2023.

Fresh *M. citrifolia* leaves were thoroughly rinsed under running water to eliminate any adhering dirt. After washing, the samples were air-dried in a shaded area to avoid direct sunlight exposure. Once completely dry, the leaves were blended into a fine powder, sieved to ensure uniformity, and then accurately weighed.

2.1. Extraction of *M. citrifolia*

The extraction method used was maceration using three solvents with increasing polarity: non-polar n-hexane, semi polar ethyl acetate, and highly polar methanol. The dried and coarsely ground samples were first macerated in n-hexane for 3×24 hours until fully submerged. The ratio for sample and solvents was 1:10 (W/V). During the maceration process, the samples were stirred twice daily. After three days, the macerated samples were filtered using filter paper to obtain the extract and the residue. The residue was then re-macerated using the same method. This process was repeated three times. The resulting extracts were combined and evaporated using a rotary evaporator at 50°C to obtain the concentrated n-hexane extract.

After the n-hexane maceration process, the sample residue was macerated using the same procedure with ethyl acetate solvent to obtain the ethyl acetate extract. The residue obtained with ethyl acetate was then processed further with methanol solvent to obtain the methanol extract.

2.2. Phytochemical Analysis

Phytochemical screening was conducted based on established protocols [32] to detect secondary metabolites in the crude extracts through qualitative chemical tests.

– *Alkaloid Screening*

The screening for alkaloids was conducted by reacting *M. citrifolia* leaves extract with several reagents, including Mayer's, Dragendorff's, and Bouchardat's reagents. The filtrate underwent color change and precipitate formation upon the addition of these reagents, confirming the presence of alkaloids. Alkaloid detection assays yielded characteristic precipitates: Mayer's reagent (white/yellow), Dragendorff's reagent (producing an orange-yellow color) and Bouchardat's reagent (yielding a brown-black color), confirming the presence of these secondary metabolites.

– *Flavonoid Screening*

Flavonoid identification was performed using the magnesium-hydrochloric acid reduction test. Briefly, 500 mg of *M. citrifolia* extract was diluted in 2 mL ethanol, heated at 60°C for 5 minutes with constant agitation, and filtered. The filtrate was treated with 0.2 g magnesium powder and 3 drops of concentrated HCl (12N), with flavonoid presence indicated by development of red, orange, or green coloration in the ethanolic phase.

– *Steroid and Terpenoid Screening*

The Liebermann-Burchard test was performed by combining 2 mL *M. citrifolia* extract with 10 drops of anhydrous acetic acid followed by 3 drops of concentrated sulfuric acid. Steroids were identified by blue-green color, while terpenoids produced characteristic orange-purple coloration upon reaction.

– *Tannin Screening*

The tannin screening test was performed by heating 2 mL *M. citrifolia* leaf extract with 1 mL distilled water at temperature of 80°C for 15 minutes. After cooling to room temperature, addition of 1% aqueous ferric chloride solution ($FeCl_3$) produced a greenish-brown chromogenic complex, confirming phenolic tannin presence.

– *Saponin Screening*

The saponin test was conducted by mixing 2 mL of *M. citrifolia* leaf extract with 2 mL of 25% sodium hydroxide solution and heating in a water bath (80°C) with 20 mL distilled water. After filtration and 15-minute standing, persistent foam formation (≥1 cm height for 15 min) indicated triterpenoid saponin presence.

2.3. Antioxidant Analysis of *M. citrifolia* Leaf. Extract

Concentration of 1000 ppm was prepared by dissolving *M. citrifolia* leaf extract in methanol using a 25 mL volumetric flask, with sonication for 5 minutes to ensure complete homogenization. Serial dilutions were prepared by transferring 0.1-0.4 mL aliquots of the 1000 ppm stock solution into separate 25 mL volumetric flasks, yielding final concentrations of 4-16 ppm. Each test solution was reacted with 5 mL of 0.5 mM DPPH methanolic solution. A blank control was set by combining 5 mL DPPH solution with methanol in a 25 mL volumetric flask. The absorbance of DPPH solution was measured using a visible light spectrometer with wavelength set at 515 nm at 5-minute intervals from 0 to 30 minutes. The antioxidant capacity was evaluated by assessing the reduction in absorbance of the DPPH solution upon adding the extract sample. The percentage inhibition (% inhibition) was computated using following Equation:

$$\% \text{ Inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100\% \quad (1)$$

The results were then plotted into a regression equation, with the x-axis representing the extract concentration (ppm) and the y-axis representing the IC_{50} value.

2.4. GC-MS Analysis

Phytochemical profiling was conducted using an ISQ™ 7000 Single Quadrupole GC-MS system (Thermo Fisher Scientific, USA) equipped with a TraceGOLD™ TG-5MS capillary column (30 m length × 0.25 mm internal diameter × 0.25 µm film thickness) featuring a split/splitless injector. The analysis was performed in constant flow mode with ultra-high-purity helium as the carrier gas at a flow rate of 1.2 mL/min. The temperature protocol began with an initial oven temperature of 35°C (no hold time), followed by a temperature ramp of 18°C/min to 100°C (held for 2 minutes), and a final increase at 20°C/min to 250°C (held for 1 minute). The injector port was maintained at 250°C for optimal sample volatilization, with a 5 µL injection volume. Electron ionization (EI) was carried out at 70 eV with the ion source temperature set to 300°C to ensure efficient fragmentation. Tentative identification of compounds was accomplished by cross-referencing acquired mass spectra and retention indices against the NIST 2020 and Wiley 11th Edition mass spectral libraries.

2.5. In Silico Analysis

Computational docking studies were conducted to evaluate the binding interactions between predominant bioactive compounds from *M. citrifolia* leaves and target proteins. The molecular docking simulations were executed using AutoDock Vina integrated within the PyRx virtual screening platform, with subsequent three-dimensional visualization and interaction analysis performed using BIOVIA Discovery Studio Visualizer 2021.The target proteins used included FSH (PDB ID: 1XWD), testosterone (PDB ID: 1I9J), and androgen receptor (PDB ID: 1E3G), which were obtained from the Protein Data Bank (PDB) at https://www.rcsb.org/. The downloaded proteins were processed by removing water molecules and optimizing their structures. The identified phytochemical ligands were computationally docked into the binding sites of target proteins, with binding affinities and interaction patterns benchmarked against clomiphene citrate (positive control) using equivalent docking parameters. The control ligand used was a standard drug commonly used to address infertility issues. The research workflow design is presented in Figure 1.

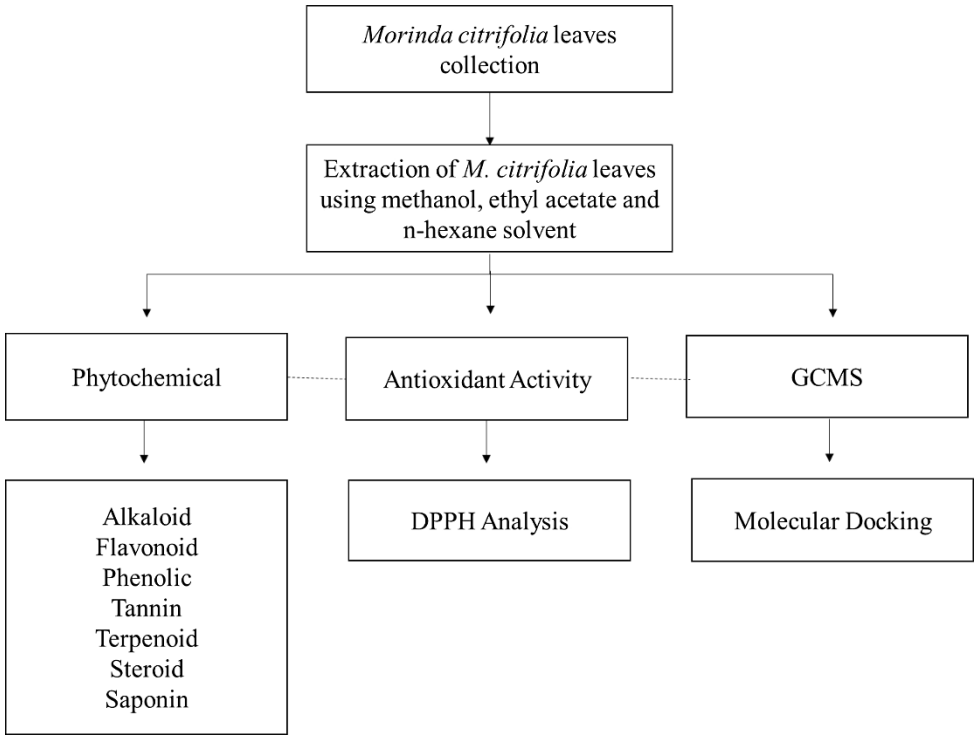


Figure 1. Flowchart of the Research Methodology

3. Results

3.1. Phytochemical Profile of M. *citrifolia* Leaves

The findings from the sequential extraction of *M. citrifolia* using n-hexane, ethyl acetate, and methanol as solvents revealed that the methanol extract contained a more comprehensive range of active phytochemical compounds than n-hexane extract and ethyl acetate extract. Table 1 shows that methanol extract was positive for all phytochemical compounds analysed, which included alkaloid, flavonoid, phenolic, tannin, terpenoid, steroid and saponin compounds.

the ethyl acetate fraction showed absence of alkaloids and saponins, whereas the n-hexane fraction exclusively contained terpenoid and steroid classes.

Table 1. Phytochemical profile of M. *citrifolia* leaf extract

Active Compounds	*M. citrifolia* Leaf Extract		
	n-Hexane	Ethyl Acetate	Methanol
Alkaloid			
- Mayer	-	-	+
- Dragendorff	-	-	+
- Wagner	-	-	+
Flavonoid	-	+	+
Phenolic	-	+	+
Tanin	-	+	+
Terpenoid	+	+	+
Steroid	+	+	+
Saponin	-	-	+

3.2. Antioxidant Content of M. *citrifolia*

Antioxidant analysis revealed that *M. citrifolia* leaf extracts have the potential to inhibit DPPH activity due to the high antioxidant activity in the extracts. The absorption activity of the DPPH solution was recorded as % inhibition (IC_{50}), as presented in Table 2. *Morindra citrifolia* leaf extracted using methanol exhibited higher antioxidant activity than those extracted using n-hexane and ethyl acetate. However, the results for n-hexane and ethyl acetate extracts of *M. citrifolia* leaves also showed significant antioxidant activity (32.13 ppm and 57.20 ppm, respectively).

Table 2. Antioxidant activity of M. *citrifolia* leaf extract

Concentration (ppm)	Absorbance	% Inhibition	Slope	Intercept	IC50
n-Hexane Extract					
50	0.300	62.59			
40	0.337	57.98			
30	0.396	50.62	0.82	23.64	32.13
20	0.489	39.03			
10	0.553	31.05			
Ethyl Acetate Extract					
50	0.428	46.6			
40	0.504	37.2			
30	0.531	33.8	0.6	13.8	57.2
20	0.597	25.6			
10	0.635	20.8			
Methanol Extract					
50	0.083	89.7			
40	0.108	86.5			
30	0.182	77.3	1.2	33.8	13.1
20	0.329	59.0			
10	0.468	41.6			

3.3. GC-MS Profiling of Phytoconstituents in *M. citrifolia* Leaf Extracts

GC-MS characterization of the n-hexane fraction from *M. citrifolia* leaves identified 31 phytochemically significant compounds, including fatty acids, terpenoids, sterols, and other phytochemicals with potential biological significance. The most abundant compound was squalene (54.03%), a well-known triterpene with antioxidant, anticancer, and skin-protective properties. Vitamin E (α-tocopherol) was also detected in a considerable amount (10.52%), contributing to

the extract's antioxidant capacity. Additionally, phytol (8.49%), a diterpene alcohol with antimicrobial and anti-inflammatory effects, was identified as a major constituent.

Several fatty acids were present, including n- hexadecanoic acid (known as palmitic acid, 2.40%) and 9,12,15-octadecatrienoic acid (linolenic acid, 0.47%), which are recognized for their role in anti-inflammatory and metabolic regulation. Sterols such as β-sitosterol (2.95%), stigmasterol (2.04%), and campesterol (1.35%) were also identified, suggesting potential cholesterol-lowering and immunomodulatory effects. Other notable compounds were neophytadiene (0.65%), a diterpene with reported antimicrobial activity, and fumaric acid derivatives (0.38%), which contribute to the extract's pharmacological properties. Minor constituents such as α-tocospiro B (0.15%) and androstene derivatives (1.56%) were also detected.

Table 3. Chemical characterization of n-hexane-soluble bioactive constituents in *M. citrifolia* leaves

No.	Compound	Retention Time (min)	Peak Area (%)
1	E-11,13-Tetradecadien-1-ol	19.788	0.43
2	E-14-Hexadecenal	24.669	0.50
3	E-15-Heptadecenal	29.070	0.85
4	Neophytadiene	30.032	0.65
5	n-Hexadecanoic acid	32.634	2.40
6	1-Heneicosanol	33.063	0.90
7	Phytol	35.342	8.49
8	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	35.903	0.47
9	Nonacos -1- ene	36.716	0.73
10	Nonacos -1- ene	40.062	0.58
11	Phthalic acid, di (2-propylpentyl) ester	42.504	0.45
12	9-octadecenoic acid, 2,2,2-trifluoroethyl, ester	46.089	0.58
13	Squalene	46.681	54.03
14	a-Tocospiro B	47.239	0.15
15	Tetradecane, 2,6,10-trimethyl-	47.463	0.71
16	1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-, (all-E)-(±)	47.983	1.13
17	Oxirane, 2,2-dimethyl-3-(3,7,12,16,20pentamethyl-3,7,11,15,19heneicosapentaenyl)-,	48.133	0.66
18	Nonacos-1-ene	48.739	0.65
19	1, 6, 10, 14, 18, 22-Tetracosahexaen-3-ol, 2, 6, 10, 15, 19, 23-hexamethyl-, (all-E)-(±)	49.068	0.46
20	?-Tocopherol	49.562	0.72
21	Tetratriacontane	50.082	2.55
22	Fumaric acid, 4-octyl dodec – 2 – en -1- yl ester	50.439	0.38
23	Vitamin E	50.664	10.52
24	Campesterol	51.823	1.35
25	Stigmasterol	52.228	2.04
26	?-Sitosterol	52.983	2.95
27	1-Heptatriacotanol	53.286	0.49
28	ß-Acorenol	53.752	0.45
29	9, 19 -Cyclolanost - 24- en-3 -ol, (3ß)-	54.014	0.97
30	Phytyl decanoate	56.452	1.21
31	Androst - 7- ene -6, 17- dione, 2,3, 14 -trihydroxy, (2ß,3ß,5a)	57.670	1.56

The chemical constituents identified by GC-MS in the ethyl acetate extract are summarized in Table 4. It's revealed 26 distinct bioactive compounds, primarily consisting of fatty acids, terpenoids, sterols, and phenolic compounds with significant pharmacological potential. The most abundant compounds were phytol (28.86%) and squalene (28.61%), both known for their antioxidant, anticancer properties, and anti-inflammatory. Another significant compound was n-hexadecanoic acid (palmitic acid, 5.21%), a saturated fatty acid with reported antimicrobial and anti-inflammatory activities.

Table 4. Chemical characterization of ethyl acetate soluble bioactive constituents in *M. citrifolia* leaves

No.	Compound	Retention Time (min)	Peak Area (%)
1	1, 2, 3 -Propanetriol, 1 -acetate	11.959	1.61
2	1, 2, 3 -Propanetriol, 1 -acetate	15.996	2.39
3	Eugenol	18.972	1.41
4	2-Cyclohexen-1-one, 4-(3-hydroxybutyl)3,5,5 – trimethyl	27.264	0.59
5	6 -Hydroxy-4, 4, 7 a-trimethyl-5, 6, 7,7 atetrahydrobenzofuran-2 (4H)-one	28.706	0.73
6	1 -Isobutyl- 7, 7-dimethyl- octahydroisobenzofuran- 3a -ol	29.080	2.78
7	(S,E)-4 - Hydroxy-3, 5, 5 -trimethyl-4-(3-oxobut1-en-1-yl)cyclohex-2-enone	29.240	3.36
8	Neophytadiene	30.039	0.98
9	n-Hexadecanoic acid	32.678	5.21
10	3-Trifluoroacetoxypentadecane	33.070	0.64
11	1,2,3,4-Tetrahydroisoquinolin-6-ol-1carboxylic acid	35.137	0.85
12	Phytol	35.396	28.86
13	9, 12, 15 -Octadecatrienoic acid, (Z, Z, Z)-	35.954	3.30
14	2- Hexadecen-1-ol, 3, 7, 11, 15-tetramethyl-, acetate, [R- [R*, R*-(E)]]	37.178	0.69
15	Hexadecanoic acid, 2-hydroxy-1(hydroxymethyl)ethyl ester	41.926	1.24
16	Methyl (Z)-5,11,14,17-eicosatetraenoate	44.732	0.73
17	Squalene	46.623	28.61
18	1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-, (all-E)-(±)	47.983	0.51
19	?-Tocopherol	49.565	0.61
20	Vitamin E	50.640	7.14
21	Ergost-5-en-3-ol, (3ß)-	51.820	1.09
22	Stigmasterol	52.225	1.92
23	?- Sitosterol	52.986	2.83
24	9, 19 -Cyclolanost-24 -en-3 -ol, (3ß)-	54.010	0.67
25	a-Tocopheryl acetate	54.653	0.54
26	Phytyl heptadecanoate	56.449	0.73

Several other important fatty acids were identified, including 9, 12, 15- octadecatrienoic acid (known as linolenic acid, 3.30%), an omega-3 fatty acid and methyl (Z)-5, 11, 14, 17 -eicosatetraenoate (0.73%). Additionally, Vitamin E (α-tocopherol, 7.14%) was detected, reinforcing the extract's strong antioxidant potential. The analysis also identified key sterols such as γ-sitosterol (2.83%), stigmasterol (1.92%), and ergost-5-en-3-ol (1.09%). Furthermore, eugenol (1.41%), a phenolic compound, was present, along with neophytadiene (0.98%), a diterpene with reported antimicrobial and antiplasmodial activities. Minor but potentially bioactive compounds included α-tocopheryl acetate (0.54%), a more stable form of vitamin E, and phytyl heptadecanoate (0.73%), a fatty acid ester that may enhance bioavailability. The occurrence of 1,2,3-propanetriol, 1-acetate (glycerol acetate, 1.61–2.39%) was also detected in the extract.

Table 5 displayed the chromatographic characterization of biologically active constituents in the methanol extract of *M. citrifolia* leaves. The methanol extract contained 37 bioactive compounds, predominantly consisting of sugars, furan derivatives, phenolic compounds, fatty acids, and terpenoids. The most abundant compounds were 4H- Pyran- 4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl (15.95%) and 5- hydroxymethylfurfural (14.39%), both of which are known for their antioxidant properties.

Notable sugar derivatives included 2-deoxy-D-galactose (combined 5.89%), melezitose (combined 3.12%), and various acetylated sugar compounds. The extract contained significant phenolic components such as eugenol (4.17%), a well-known antimicrobial and anti-inflammatory agent, and 2- methoxy- 4 -vinylphenol (3.78%), which possesses antioxidant properties. Fatty acid constituents included n- hexadecanoic acid (2.91%) and 9, 12, 15-octadecatrienoic acid (2.99%), both of which contribute to the extract's potential anti-inflammatory and antimicrobial activities. The terpenoid profile featured phytol (3.03%) and squalene (1.10%), compounds recognized for their antioxidant and skin-protective properties.

Table 5. Chemical characterization of methanol-soluble bioactive constituents in *M. citrifolia* leaves

No.	Name of Compound	Retention Time (min)	Peak Area (%)
1	Butyrolactone	6.732	0.74
2	Pyrazole-4-carboxaldehyde, 1-methyl-	8.017	0.90
3	2, 4-Dihydroxy-2, 5-dimethyl-3 (2H)-furan-3one	8.419	0.91
4	DL-Arabinose	11.425	0.71
5	2- Deoxy-D-galactose	12.418	3.62
6	2- Deoxy-D-galactose	12.687	2.27
7	4H -Pyran- 4-one, 2, 3-dihydro-3, 5-dihydroxy6-methyl	13.476	15.95
8	6- Acetyl-ß-d-mannose	14.268	0.56
9	1-Nitro- 2-acetamido-1, 2-dideoxy-d-mannitol	15.268	0.83
10	Benzofuran, 2, 3-dihydro-	15.432	4.66
11	5- Hydroxymethylfurfural	16.115	14.39
12	6- Acetyl-ß-d-mannose	17.707	0.47
13	2- Methoxy-4 -vinylphenol	17.867	3.78
14	Methyl 6 -oxoheptanoate	18.414	1.34
15	Eugenol	19.013	4.17
16	1- Nitro- ß-d-arabinofuranose, tetraacetate	19.472	2.84
17	1- Nitro-2 -acetamido-1, 2- dideoxy-d-mannitol	19.959	6.68
18	2- Acetonyl- 9 -[3-deoxy-ß-dribouranosyl]hypoxanthine	20.190	5.54
19	2- Acetonyl- 9- [3 -deoxy-ß-dribouranosyl]hypoxanthine	20.424	2.99
20	D-Streptamine, O -6- amino-6 -deoxy-a-Dglucopyranosyl-(1-4)-O- (3-deoxy- 4-C-methyl 3-(methylamino)-ß-L-arabinopyranosyl-(16))-2-deoxy-	22.149	0.60
21	dl- Citrulline	23.921	1.22
22	4- (2, 4, 4-Trimethyl-cyclohexa-1,5-dienyl)- but 3-en-2-one	24.506	0.54
23	Cyclopenta[1, 3] cyclopropa[1,2]cyclohepten3(3aH)-one,	25.587	1.24
24	1, 2, 3b, 6, 7, 8-hexahydro-6, 6dimethyl-2 -Cyclohexen-1-one, 4- (3-hydroxybutyl) 3, 5, 5-trimethyl	27.349	1.76
25	Melezitose	28.036	0.60
26	ß-D-Glucopyranose, 4 -O-ß -Dgalactopyranosyl	28.295	1.26
27	Melezitose	28.536	0.63
28	d-Mannose	28.812	0.86
29	[1, 1'-Bicyclopropyl]- 2 -octanoic acid, 2' -hexyl, methyl ester	29.196	2.06
30	[1, 1'-Bicyclopropyl]- 2 -octanoic acid, 2' -hexyl, methyl ester	29.376	2.55
31	Melezitose	30.550	1.89
32	n- Hexadecanoic acid	32.614	2.91
33	[1, 1'-Bicyclopropyl]- 2-octanoic acid, 2'-hexyl, methyl ester	33.468	1.16
34	Phytol	35.338	3.03
35	[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl, methyl ester	35.733	0.29
36	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	35.910	2.99
37	Squalene	46.555	1.10

3.4. Molecular Docking

The molecular docking analysis of *M. citrifolia* leaf extracts yielded significant insights into their interactions with key reproductive hormone receptors. The study focused on three critical receptors: FSH (PDB ID: 1XWD), testosterone (PDB ID: 1I9J), and androgen (PDB ID: 1E3G). These receptors were selected due to their central roles in reproductive physiology and their potential as therapeutic targets. Binding affinity results (Table 6) revealed that several *M. citrifolia* compounds outperformed the control ligand clomiphene. Campesterol from the n-hexane extract exhibited exceptional binding to the FSH receptor with an affinity of -8.4 kcal/mol. This value significantly exceeds clomiphene's binding affinity of -6.7 kcal/mol, suggesting that campesterol may be a more potent modulator of FSH receptor activity. Similarly, vitamin E demonstrated strong affinity for the testosterone receptor (-8.6 kcal/mol), while stigmasterol from the ethyl acetate extract showed the highest binding to the androgen receptor (-8.6 kcal/mol).

Table 6. Binding energy calculations for target protein-phytochemical complexes from *M. citrifolia* leaf extracts

No.	Compounds	Binding Affinity (Kkal.mol)		
		FSH	Testosterone	Androgen
1.	Clomiphene	-6.7	-7,1	-6,1
	n-hexane Extract			
1.	Phytol	-4,6	-6	-6,5
2.	Squalene	-6,4	-6	**-8,2**
3.	campesterol	**-8,4**	**-8**	**-8,4**
4.	Vit. E	-5,4	**-6,4**	**-8,6**
5.	Sitosterol	**-8,4**	**-7,9**	**-8,1**
	Ethyl Acetate Extract			
1.	stigmasterol	**-8,2**	**-8,6**	**-8,5**
2.	n-Hexadecanoic Acid	-5,2	-4,8	-5
3.	Phytol	-4,6	-6	-6,5
4.	Squalene	-6,4	-6	**-8,2**
5.	Vit. E	-5,4	**-6,4**	**-8,6**
	Methanol Extract			
1.	2-Deoxy-D-Galactose	-5,7	-5,4	-5,9
2.	4H -Pyran-4-one, 2,3-dihydro- 3, 5- dihydroxy-6 -methyl-	-5,5	-5,4	-5,5
3.	5-Hydroxymethylfurfural	-5,1	-5,2	-5
4.	Eugenol	-5,7	**-6,5**	-6,2
5.	Phytol	-4,6	-6	**-6,5**

The molecular basis for these high-affinity interactions is detailed in Table 7. For the FSH receptor, campesterol formed extensive Van der Waals interactions with residues GLN C:123 and TYR A:89, along with alkyl bonds at HIS A:83. These multiple interaction types contribute to the compound's superior binding affinity and stability. The testosterone receptor, on the other hand, showed a preference for vitamin E, which engaged in diverse intermolecular interactions encompassing both hydrophobic Van der Waals contacts and polar hydrogen bonding with critical residues such as TRP H:108 and PRO L:64.

Table 7. Amino acid residues revolved in molecular interactions between ligands and hormone receptors

Ligand	Amino acid residues
	FSH
Clomiphene	*Van der Waals:* ASP E:41, TYR E:39, GLN D:27, ASN D:15, VAL E:38, ILE E:47, PHE D:17 *Conventional Hydrogen Bond: ARG E:35* *Carbon Hydrogen Bond:* ILE E:47 *Pi-Pi T-Shaped:* PHE D:18 *Alkyl:* ARG E:44, LEU E:37, LYS E:46
Campesterol	*Van der Waals:* GLN C:123, LYS C:146, TYR A:89, THR A:86, GLN A:48, VAL A:61, ALA A:62, LYS A:63, GLU C:73, ILE C:49, LYS C:74, SER A:85, HIS C:98, TYR A:88, TYR A:65, TYR C:124, LEU C:148 *Alkyl:* HIS A:83
Sitosterol	*Van der Waals:* ILE F:49, GLU F:73, HIS F:98, GLN F:123, ALA D:62, TYR D:65, LYS E:40, LYS E:46, THR D:86, SER D:85, LYS F:74, *Conventional Hydrogen Bond:* ASP E:36, TYR D:89, *Alkyl:* HIS D:83, LYS D:63, TYR D:88, VAL D:61
Stigmasterol	*Van der Waals:* ILE F:49, GLU F:73, HIS F:98, GLN F:123, ALA D:62, TYR D:65, LYS E:40, LYS E:46, THR D:86, SER D:85, LYS F:74, *Conventional Hydrogen Bond:* ASP E:36, TYR D:89, *Alkyl:* HIS D:83, LYS D:63, TYR D:88, VAL D:61

Testosterone	
Clomiphene	*Van der Waals:* SER L:48, **TRP H:108**, **PRO L:49**, **ALA H:106**, **TYR H:107**, LEU L:51, **VAL L:63**, **LEU L:52**, **PHE L:60**, **LEU L:42**, **LYS L:44** *Alkyl:* **PRO L:64**, PHE L:67
Stigmastrol	*Van der Waals:* ASN H:56, GLY L:96, PHE L:94, LEU H:105, SER H:50, VAL H:52 *Pi-Sigma:* TYR H:58 *Alkyl:* TRP H:47, PRO L:101, TYR H:102, VAL L:99,
Campesterol	*Van der Waals:* **LEU L:52**, **LEU L:42**, **VAL L:63**, PHE L:67, ASP L:87, GLU L:86, **LYS L:44**, GLY L:62, **LEU L:51**, **PHE L:60**, **ALA H:106**, **TRP H:108**, **TYR H:107**, **PRO L:49** *Conventional Hydrogen Bond:* ARG L:66, GLU L;84 *Alkyl:* LYS L:50, **PRO L:64**
Sitosterol	*Van der Waals:* ASN H:56, GLY L:96, LEU H:105, PHE L:94, SER H:50, TRP H:47, VAL H:52 *Pi-Sigma:* TYR H:58 *Alkyl:* VAL L:99, TYR H:102, PRO L:101
Androgen	
Clomiphene	*Van der Waals:* **PROA:682**, ASN A:756, THR A:755, **GLN A:802**, **PHE A:804**, **TRP A:751**, **GLU A:678**, **PRO A:801** *Carbon Hydrogen Bond:* GLU A:678 *Pi-Sigma:* A:752, LEU A:805 *Pi-Anion:* GLU A:681
Vit. E	*Van der Waals:* PRO A:766, TYR A:763, ALA A:765, LEU A:707, ALA A:748, GLY A:683, **PRO A:682**, GLU A:681, **GLU A:678**, **PRO A:801**, **PHE A:804** *Conventional Hydrogen Bond:* GLN A:711, VAL A:685, *Pi-Cation:* ARG A:752 *Alkyl:* VAL A:684, TRP A:751, LEU A:805
Stigmasterol	*Van der Waals:* ALA A:748, VAL A:684, **PRO A:682**, **ASN A:756**, **TRP A:751**, **PHE A:804**, **GLU A:678**, **GLN A:802**, GLU A:681, GLY A:683, GLN A:711 *Alkyl:* LEU A:805, PRO A:801, ARG A:752, VAL A:685
Campesterol	*Van der Waals:* GLU A:681, VAL A:684, **PRO A:682**, ALA A:748, VAL A:685, GLY A:683, **TRP A:751**, **PHE :804**, **GLU A:678** *Conventional Hydrogen Bond:* GLN A:802 *Alkyl:* ARG A:752, PRO A:801, LEU A:805,

The androgen receptor displayed particularly strong binding with stigmasterol, which interacted with key residues PRO A:682 and GLU A:678. These interactions were mediated through both Van der Waals forces and alkyl bonds, demonstrating the compound's ability to form stable complexes with the receptor. The consistency of these high-affinity interactions across different receptors suggests that *M. citrifolia* compounds may have broad applicability in modulating reproductive hormone signaling.

Figure 2 provides a detailed visualization of their three-dimensional structures, highlighting the distinct binding pockets that facilitate ligand interactions. The structural characteristics of these receptors are particularly noteworthy. The FSH receptor (Figure 2-a) comprises 202 amino acid residues, while the testosterone receptor (Figure 2-b) contains 212 residues, and the androgen receptor (Figure 2-c) consists of 207 residues. These structural differences contribute to their unique binding properties and ligand specificities. The visualization of these receptors underscores the complexity of their binding sites and the importance of molecular complementarity for effective ligand-receptor interactions.

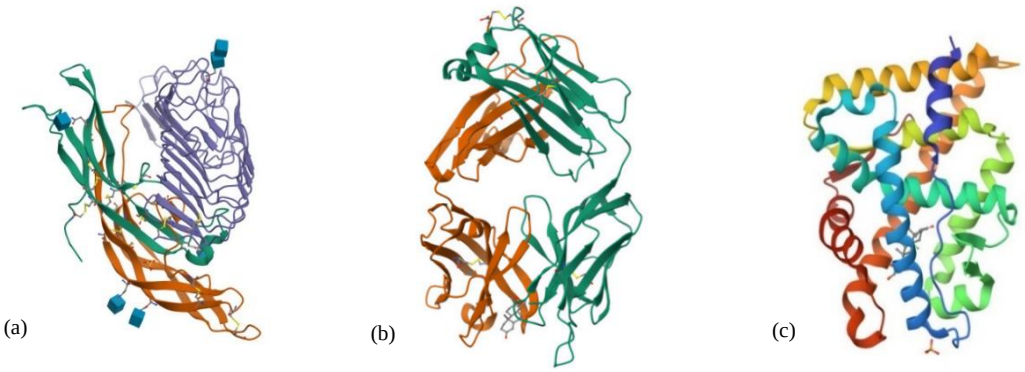
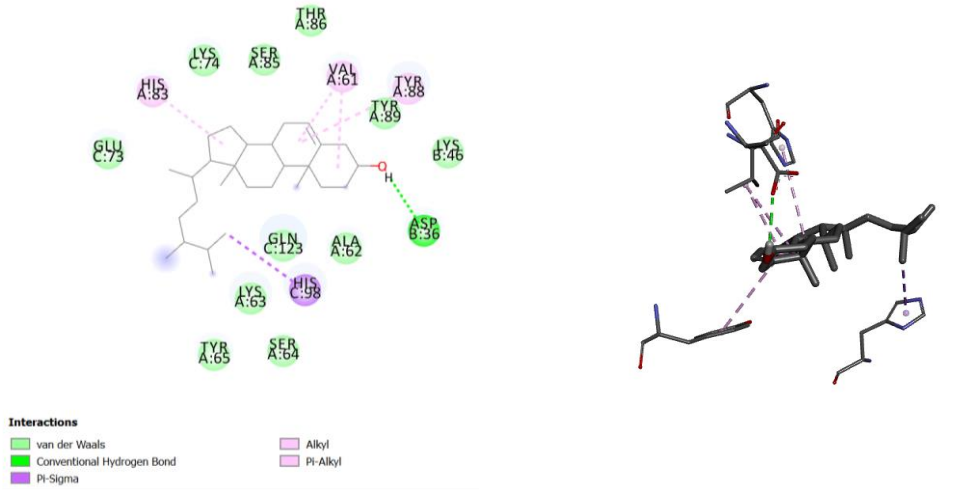
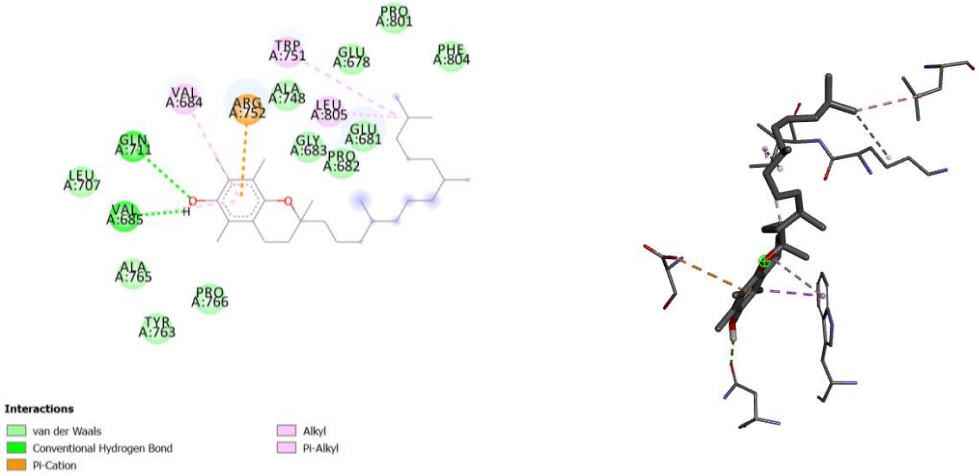


Figure 2. Three-dimensional structures of hormone-binding receptors: (a) FSH (1XWD), (b) Testosterone (1I9J), and (c) Androgen (1E3G)

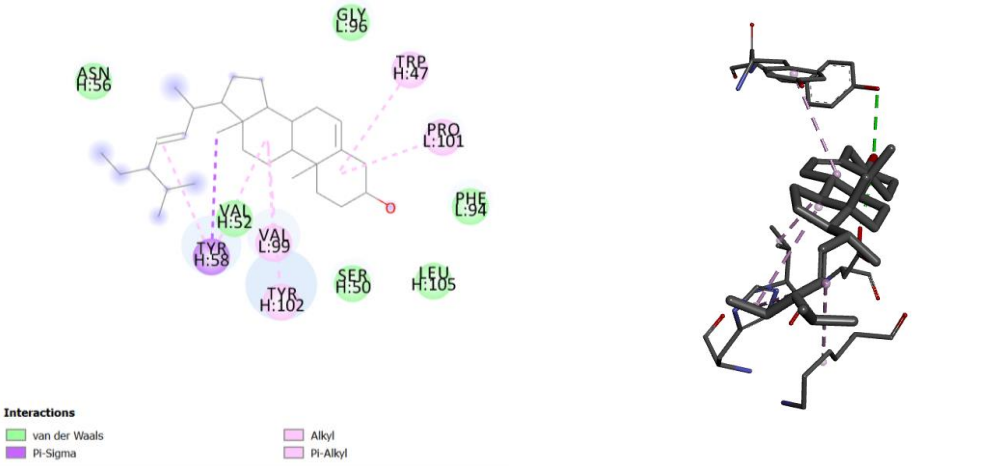
Figure 3 provides visual confirmation of these findings, showing the spatial orientation of high-affinity ligands within the receptor binding pockets. The predominance of Van der Waals interactions in these complexes indicates that shape complementarity and surface contacts play a crucial role in binding stability. This observation is consistent with the notion that non-covalent interactions are often sufficient for high-affinity ligand-receptor binding in biological systems. The molecular docking analysis revealed that the high-affinity binding of campesterol, tocopherol, and stigmasterol to their respective receptors was primarily stabilized by Van der Waals interactions, underscoring the critical role of shape complementarity and extensive surface contact in these receptor-ligand complexes.



Campesterol



Tocopherol (Vit. E)



Stigmasterol

Figure 3. Structural characterization of high-potency ligand-protein binding poses

Campesterol and stigmasterol, as phytosterols, demonstrated particularly strong interactions with the hydrophobic pockets of the receptors, facilitated by alkyl and Pi-Sigma bonds, while tocopherol (Vitamin E) exhibited enhanced binding affinity through additional hydrogen bonding with polar residues. The androgen receptor (1E3G) featured a well-defined hydrophobic binding site that perfectly accommodated sterol-based ligands like stigmasterol, whereas the FSH receptor (1XWD) and testosterone receptor (1I9J) similarly favored non-polar interactions, consistent with the steroid-like nature of the compounds. Figure 3 clearly illustrates the optimal spatial orientation of these ligands within their binding sites, showcasing both the alignment of hydrophobic moieties (e.g., sterol rings) with receptor subpackets and the minor yet critical polar interactions, such as those between tocopherol's hydroxyl group and hydrogen-bond acceptors, which collectively contribute to the stability and specificity of these molecular complexes.

4. Discussion

The phytochemical composition of *M. citrifolia* leaf extract shows significant variability depending on the extraction solvent used. A comparative analysis of three different solvent extracts from *M. citrifolia* leaves revealed distinct phytochemical patterns. The n-hexane extract, as a non-polar solvent, primarily yielded lipophilic compounds including steroids and terpenoids, which reflects n-hexane's ability to dissolve non-polar constituents [33]. The ethyl acetate extract (semi polar solvent) contained a broader spectrum of polyphenolic compounds including flavonoids, phenolics, tannins, terpenoids, and steroids. Alkaloids were conspicuously absent in both the n-hexane and ethyl acetate extracts, a finding that supports earlier research reporting negative alkaloid results in these solvent fractions [26, 34].

The methanol extract exhibited the most comprehensive metabolite profile among the three solvents tested. Methanol's dual polar and non-polar characteristics, owing to its hydroxyl group and methyl moiety, enable it to extract a wider range of phytochemicals compared to more selective solvents like n-hexane or ethyl acetate [20, 35, 36]. These results are consistent with previous studies highlighting the role of solvent polarity in the selectivity of bioactive compound extraction, where polar solvents like methanol tend to extract a broader spectrum of metabolites, including polar compounds like phenolic acids and glycosides [26, 37, 38]. The observed variations in phytochemical screening results extend beyond solvent effects alone. Contemporary studies emphasize that geographic location, climatic conditions, and genetic variation among *M. citrifolia* populations significantly influence secondary metabolite production [38].

This research also indicated that *M. citrifolia* leaf extracts exhibit significant DPPH radical scavenging activity, demonstrating their potential as natural antioxidants. The antioxidant capacity is quantitatively assessed using IC50 values, with lower values representing greater efficacy [39, 40]. Comparative analysis reveals that methanol-extracted *M. citrifolia* leaf samples display superior antioxidant performance compared to other solvent extracts. These results are consistent with the previous finding [34], confirming that polar solvent extracts generally exhibit stronger antioxidant effects than semi-polar or non-polar extracts. This phenomenon can be attributed to the chemical nature of the extracted compounds such as polar molecules, particularly those with hydroxyl groups, exhibit stronger electron or hydrogen-donating capabilities, enabling more efficient neutralization of free radicals compared to non-polar constituents [41].

GC-MS analysis of *M. citrifolia* leaf extracts has revealed a diverse array of bioactive compounds with established antioxidant properties, confirming previous phytochemical reports [15, 42-45]. The study identified three predominant antioxidant compounds: squalene (54.03%), phytol (28.86%), and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP, 15.95%), all of which have been previously documented for their free radical-scavenging capabilities [46-48]. Squalene ($C_{30}H_{50}$), detected at a retention time of 46.69 minutes, demonstrated significant antioxidant activity through its ability to reduce reactive oxygen species (ROS) levels. This hydrocarbon compound has been shown to act synergistically with α-tocopherol and β-sitosterol to effectively inhibit oxidative processes, as reported by Sogandi and Rabima [48]. The diterpenoid alcohol phytol (3,7,11,15-tetramethylhexadec-2-en-1-ol), eluting at 35.39 minutes, contributes to antioxidant defence through its hydroxyl group's hydrogen-donating capacity, which neutralizes damaging free radicals [49]. The flavonoid derivative DDMP emerged as particularly noteworthy for its potent antioxidant characteristics. Previous studies have established DDMP's exceptional free radical-scavenging capacity and served as a primary antioxidant agent [50, 51]. These findings collectively reinforce the substantial antioxidant potential of *M. citrifolia* leaf extracts, aligning with and expanding upon existing phytochemical research.

Molecular docking studies were conducted to evaluate the binding interactions between five major bioactive compounds from *M. citrifolia* leaf extracts and three key reproductive hormone receptors: follicle-stimulating hormone (FSH) receptor (PDB ID: 1XWD), testosterone receptor (PDB ID: 1I9J), and androgen receptor (PDB ID: 1E3G). The high docking scores obtained suggest strong binding affinity of these phytochemicals toward the target proteins. Clomiphene, a selective estrogen receptor modulator (SERM) used in fertility treatments, served as the control ligand. As a SERM, clomiphene acts on estrogen receptors in the hypothalamus and pituitary, stimulating the hypothalamic-pituitary-gonadal (HPG) axis to enhance gonadotropin release, boost testosterone production, and support reproductive function [52, 53]. FSH further contributes to this process by regulating testosterone synthesis and promoting androgen-binding protein secretion from Sertoli cells [54].

Notably, several *M. citrifolia* compounds including campesterol, sitosterol, stigmasterol, and vitamin E exhibited binding affinities comparable to or exceeding that of clomiphene. While none of the phytochemicals shared clomiphene's interaction pattern with the FSH receptor, suggesting distinct mechanisms of action, significant similarities emerged for the testosterone and androgen receptors. Campesterol displayed a clomiphene-like binding mode with the testosterone receptor, forming Van der Waals interactions with residues LEU L:42, LEU L:52, VAL L:63, LYS L:44, LEU L:51, PHE L:60, ALA H:106, TRP H:108, TYR H:107, and PRO L:49, along with an alkyl interaction at PRO L:64. Similarly, tocopherol and stigmasterol mirrored clomiphene's interactions with the androgen receptor, engaging residues PRO A:682, GLU A:678, PHE A:804, TRP A:751, and others via Van der Waals forces.

The differential binding patterns observed between n-hexane and ethyl acetate extracts are particularly intriguing. While both extract types yielded high-affinity compounds, their specific interactions varied, reflecting the diverse chemical profiles of the extracts. For instance, n-hexane extracts were rich in campesterol and squalene, whereas ethyl acetate extracts contained higher levels of stigmasterol and vitamin E. These differences highlight how solvent selection can influence the recovery of bioactive compounds with distinct receptor affinities. The superior performance of *M. citrifolia* compounds compared to clomiphene across all three receptors is a key finding of this study. Clomiphene, a synthetic selective estrogen receptor modulator, is commonly used in fertility treatments. The fact that natural compounds from *M. citrifolia* can match or exceed its binding affinity suggests their potential as natural alternatives for reproductive health applications. This is particularly relevant given the growing interest in plant-based therapeutics with fewer side effects.

5. Conclusion

The current study provides a systematic analysis of the phytochemical composition and bioactivity of *M. citrifolia* leaf extracts obtained using different solvents. Phytochemical screening revealed distinct variations in secondary metabolites, with n-hexane extract containing steroids and terpenoids, while ethyl acetate fraction exhibited high concentrations of polyphenolic constituents, notably flavonoid derivatives, phenolic acids, hydrolyzable tannins, terpenoid compounds, and steroidal structures. Notably, the methanol extract revealed the most diverse metabolite profile, demonstrating the highest antioxidant activity, as reflected by its significantly lower IC50 value (13.10 ppm) in the DPPH radical scavenging assay compared to n-hexane (32.13 ppm) and ethyl acetate (57.20 ppm) extracts. GC-MS characterization revealed multiple pharmacologically significant constituents, including squalene (54.03%), phytol (28.86%), and 4H- pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy- 6-methyl (DDMP, 15.95%), all of which contribute to the observed antioxidant effects. Squalene and phytol were particularly notable owing to their efficacy in ROS attenuation, while DDMP, a flavonoid derivative, exhibited strong free radical scavenging properties. These compounds possess significant antioxidant, anti-inflammatory, and potential therapeutic benefits, supporting the traditional use of *M. citrifolia* in herbal medicine. Molecular docking studies revealed that certain compounds in ethyl acetate extract and n-hexane extracts, such as campesterol, sitosterol, stigmasterol, and Vitamin E, exhibited strong binding affinities to FSH, testosterone, and androgen receptors, comparable or even superior to clomiphene, a known selective estrogen receptor modulator (SERM). Overall, this study highlights two important finding *M. citrifolia* leaves: methanol extracts offer optimal antioxidant potential, while ethyl acetate and n-hexane extracts contain compounds with hormonal activity. This dual functionality positions *M. citrifolia* as a valuable source for both antioxidant and endocrine-targeted therapies warranting further investigation

6. Declarations

6.1. Author Contributions

Conceptualization, R.D., T.N.S., and A.S.; methodology, R.D. and A.S.; software, T.N.S.; validation, A.S. and T.N.S.; formal analysis, A.S. and T.N.S; investigation, R.D. and A.S.; resources, R.D.; data curation, R.D., A.S., and S.W.; writing—original draft preparation, R.D., T.N.S., S.M., and A.S.; writing—review and editing, A.S. and T.N.S.; visualization, S.W.; supervision, T.N.S., S.W., and A.S.; project administration, R.D., and A.S.; funding acquisition, R.D. All authors have read and agreed to the published version of the manuscript.

6.2. Data Availability Statement

The data presented in this study are available in the article.

6.3. Funding

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6.4. Acknowledgments

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6.5. Institutional Review Board Statement

Not applicable.

6.6. Informed Consent Statement

Not applicable.

6.7. Declaration of Competing Interest

The authors declare that there are no conflicts of interest concerning the publication of this manuscript. Furthermore, all ethical considerations, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancies have been completely observed by the authors.

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