

Metabolomic profiling, *in vitro* antidiabetic and anti-inflammatory activities of methanolic extract of *Leonotis leonurus* leaves

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ABSTRACT

The treatment of diabetes mellitus is not yielding the desired positive effect in some individuals, coupled with the side effects of existing synthetic drugs. As a result, this study aimed to identify and quantify the phenolic compounds, evaluate the antioxidant capacity, and assess the *in vitro* anti-inflammatory and antidiabetic effects of the methanolic extract of *L. leonurus* (MELL) leaves. The bioactive phytoconstituents in MELL were analysed using UPLC-ESI-QTOF-MS. Then, *in vitro* investigations such as (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay, α -glucosidase inhibition, glucose uptake assay, pancreatic β -cell proliferation assay, macrophage activation in L6 myoblasts, and C3A hepatocytes were performed. The polyphenol content was estimated to be 43.03 ± 1.28 mg gallic acid equivalent per gram, and the flavonol content was 40.43 ± 1.75 mg quercetin equivalent per gram. For the antioxidant capacity, ferric reducing antioxidant property was estimated to be 197.42 ± 9.78 μ mol AAE/g, 2,2-diphenyl-1-picrylhydrazyl was 115.45 ± 3.08 μ mol trolox equivalent per gram (TE/g), and trolox equivalent capacity was 105.77 ± 2.30 μ mol (TE/g). MELL exhibited α -glucosidase inhibition in a dose-dependent manner with the maximum activity recorded at 1,000 μ g/ml and an IC_{50} of 500 μ g/ml. It enhanced glucose utilization and uptake in L6 myoblasts and C3A hepatocytes. Interestingly, no significant cytotoxic effect was recorded for the extract in L6 or C3A cells. It also exhibited anti-inflammatory activity from a concentration of 10 μ g/ml when screened against RAW 264.7 cells without a significant cytotoxic effect. The results of the study demonstrated the potential of *L. leonurus* in the management of diabetes, indicating a reservoir for isolating lead compounds with potential applications in the development of novel antidiabetic drugs.

1. INTRODUCTION

Diabetes mellitus is a serious metabolic disorder affecting over 537 million adults worldwide, and its prevalence is expected to rise to 643 million by 2030 [1]. Insulin is essential

for maintaining glucose homeostasis because it initiates the movement of blood glucose to the liver and skeletal muscle [2]. Type 1 diabetes is characterized by a lack of insulin synthesis, while type 2 diabetes (T2D) is characterized by insulin resistance. T2D also involves an increase in endogenous glucose release, which elevates blood glucose levels due to decreased glucose uptake. Substances that enhance glucose uptake may help reduce insulin resistance [3]. Although several commercially available drugs are useful in treating diabetes, their use is limited due to their high cost and adverse side effects [4]. This has encouraged the development of potent natural antidiabetic products and medications with fewer adverse effects.

Herbal remedies are becoming more popular due to their lower costs and improved therapeutic outcomes with fewer adverse effects. Because they contain therapeutically

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significant phytochemicals, medicinal plants offer enormous promise for treating various illnesses, including diabetes [5]. The idea that medicinal plants can help control and prevent metabolic problems has gained acceptance among medical professionals [6]. The use of herbal remedies has grown to be a multibillion-dollar industry with global acceptance that spans all demographic and socioeconomic groups [7].

Leonotis leonurus (L.) R.Br. is a perennial shrub from the Lamiaceae family [8]; its English name is lion's ear or wild dagga. It is indigenous to South Africa and can be found in several regions of the country [9,10]. *Leonotis leonurus* (leaves and stems) has long been used to treat a variety of conditions, including diabetes, constipation, intestinal worms, menstrual delays, dermatitis, epilepsy, coughs, colds, influenza, chest infections, and hypertension. The entire plant is used to make a tea that is said to cure rheumatism, cancer, obesity, bladder and renal disorders, piles, and arthritis [11].

Indeed, a wealth of information and assertions regarding the medicinal value of *L. leonurus* are in the public domain and media. The main phytochemicals in *L. leonurus* are alkaloids, diterpenoids, flavonoids, phenolics, triterpenoids, essential oils, saponins, tannins, and sterols. Nevertheless, there is a dearth of experimental and scientifically sound data regarding this plant's safety and effectiveness in treating diabetes. Metabolic profiling is a valuable tool for identifying and quantifying bioactive compounds, understanding their mechanisms of action, and thus understanding the medicinal properties of a plant. It helps to detect potentially harmful metabolites and maximise the therapeutic potential of a plant. It helps to integrate traditional remedies into evidence-based medicine. Lead molecules have been developed through methodical research on medicinal plants and the exploration of their physiologically active constituents, which have proven beneficial to human health and the economy. Therefore, this study aims to identify and quantify the phenolic compounds in *L. leonurus* leaves and assess their antioxidant, antidiabetic, and anti-inflammatory potential using *in vitro* assays.

2. MATERIALS AND METHODS

2.1. Reagents

Phosphate buffer saline (PBS) and Minimal Essential Medium (MEM) were obtained from Cytiva (Marlborough, MA) with and without Ca^{2+} and Mg^{2+} . Penicillin, streptomycin, nonessential amino acids, and foetal bovine serum (FBS) were products of Biowest (Nuaille, France). The American Type Culture Collection provided the C3A hepatocytes produced from human hepatomas (ATCC, Manassas, VA). MEM/EBSS, PBS, and Roswell Park Memorial Institute (RPMI) 1640 Medium from Cytiva (Marlborough, MA, USA). All other reagents were bought from Sigma-Aldrich in St. Louis, MO.

2.2. Plant collection and identification

The leaves of *L. leonurus* were harvested in the Department of Horticultural Sciences at the Cape Peninsula University of Technology, Bellville Campus in September 2021. A botanist from the same department authenticated the plant, and a voucher specimen (3,014) was deposited at their

herbarium. The leaves were rinsed, air-dried for 2 weeks, and subsequently ground into a powder using a grinder. The powder was kept in an air-tight bag in a 4°C refrigerator until use.

2.3. Plant extraction

100 g of the leaf powder was added to a 2 l flask containing 1.5 l of 70% methanol and stirred on a magnetic stirrer for 48 hours using the method of Okaiyeto *et al.* [12]. The mixture was filtered, and the resulting solution was concentrated, lyophilized, and stored until needed. A yield of 22.5% was obtained.

2.4. Estimation of total polyphenols and flavonols

The total polyphenol content of the extract was determined using a standard protocol of Folin-Ciocalteu technique as described by Okaiyeto *et al.* [12]. Briefly, 1 mg/ml of the methanolic extract of *L. leonurus* (MELL) was treated with 5 ml of 10% Folin-Ciocalteu reagent and 4 ml of 7.5% (w/v) sodium carbonate. The mixture was vortexed for 15 seconds and thereafter incubated at 40°C for 30 minutes in the dark. The absorbance was read at 765 nm. The total phenolics were estimated using a standard of gallic acid (0–250 mg/l) as gallic acid equivalent per gram (GAE/g). The total flavonol content was determined using the method of Yermakov *et al.* [13]. Briefly, 2.0 ml of a 50 g/l sodium acetate solution and 3.0 ml of a 20 g/l aluminum trichloride solution were added to 2 ml of 1 mg/ml of MELL. The resultant solution was incubated at 20°C for 120 minutes; after which the absorbance was read at 765 nm. A standard curve of 20 to 80 mg/l quercetin was used to determine the total flavonol as quercetin equivalent per gram sample (QE/g).

2.5. Estimation of antioxidant capacity

The antioxidant capacity, as measured by assays such as trolox equivalent capacity (TEAC), ferric reducing antioxidant property (FRAP), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were determined for MELL according to established procedures described by Re *et al.* [14], Benzie and Strain [15], and Ngxabi *et al.* [16], respectively. For TEAC determination, ABTS^{*+} was formed by allowing an aqueous solution of 8 mM ABTS and 3 mM potassium persulfate to stand for 16 hours in the dark. ABTS^{*+} was then diluted until an absorbance of 0.7 was obtained at 734 nm. 100 μl of MELL was incubated with 2.4 ml of ABTS^{*+} for 6 minutes, and the absorbance was read at 734 nm. A standard curve of trolox (0–1,000 μM) was prepared, and the TEAC of MELL was determined as trolox equivalent per gram sample (TE/g). For FRAP, 10 μl of MELL was added to 300 ml of the FRAP reagent. The mixture was incubated at room temperature for 30 minutes, after which the absorbance was read at 593 nm. Ascorbic acid was used as the standard. For the DPPH assay, MELL and trolox standards were serially diluted to equivalent concentrations of 0.08, 0.04, 0.02, 0.01, and 0.005 mg/ml, respectively, then mixed in a ratio of 1:1. The resultant mixture was vortexed and subsequently incubated at room temperature for 30 minutes. Therefore, 300 μl of the mixture was dispensed in a 96-well micro plate, and the absorbance was read at 517 nm. Antioxidant activity was expressed as micromole/TE/g dry weight (μmol).

2.6. UPLC-ESI-QTOF-MS analysis of methanol extract of *L. leonurus*

Liquid Chromatography-Mass Spectrometry analysis was used to determine the phenolic content as described by Okaiyeto *et al.* [12]. Briefly, mobile phases A and B consisted of water and acetonitrile, respectively, both with 0.1% formic acid. The seal wash was run for 5 minutes, while the solvent flow rate into the column was 0.4 ml/min at a column temperature of 55°C. 100% solvent A was run for 0.5 minutes, after which the gradient was changed to 100% B from 0.5 to 12.5 minutes. It was then changed back to 100% of A from 12.5 to 15 minutes. Sodium formate was used for calibration. The MassLynx software platform was used to process the chromatogram. Metabolite identities were determined by matching masses to entries in databases such as ReSpect MS/MS, Metlin, mass-Bank, and other libraries. Peaks with an accurate mass error greater than 5 ppm were considered unidentified. Compounds were identified and quantified based on retention time, mass fragmentation, and ionization modes using phenolic compounds standards at varying concentrations (3.9, 7.8, 15.6, 31.3, 62.5, 125.0, and 250.0 µg/l). The percentage abundance of the carbon isotopes was used to reduce the number of incorrect annotations.

2.7. MTT cytotoxicity assay

C3A hepatocytes were incubated in a humidified environment with 5% CO₂ in 10 cm culture dishes filled with complete media. 100 µl of the cells were seeded per well in 96-well plates and left to adhere for 1 day. Thereafter, the cells were treated with either 50, 100, or 200 mg/ml of MELL or 30 µM of melphalan (positive control). The medium and treatment were aspirated, and the cells were then stained. Subsequently, the plates were incubated for 30 minutes, after which 10 µl of propidium iodide solution (100 µg/ml) was added to each well. The numbers of living and dead cells were estimated. Afterward, the data were processed and analysed in an MS Excel spreadsheet.

2.8. Alpha-glucosidase determination

The α-glucosidase inhibitory test was carried out using the previously reported procedure, Okaiyeto *et al.* [12]. Briefly, 10 µl of 62.5, 125, 250, 500, and 1,000 µg/ml of MELL was added to the microplate, followed by 70 µl of α-glucosidase. Epigallocatechin was used as the positive control. The resultant solution was incubated at 37°C for 10 minutes, after which 20 µl of 10 mM p-Nitrophenyl-D glucopyranoside (substrate) was added and then incubated again at 37°C for 20 minutes. The reaction was terminated by the addition of 25 µl of 100 mM Na₂CO₃. The absorbance of the resultant mixture was read at 410 nm. The percentage of the glucosidase inhibition was estimated as follows: %-glucosidase inhibition = $(A_{410} \text{ of control} - A_{410} \text{ of test sample}) / (A_{410} \text{ of control}) \times 100\%$.

2.9. Hepatocyte glucose uptake of the plant extract

The impact of MELL on the absorption and utilization of glucose in C3A hepatocytes was assessed using the method of van de Venter *et al.* [17] with slight modification. 2×10^4 cells were seeded per well in microplates, and the cells were allowed to adhere overnight. The cells were incubated for 24

hours with different doses of MELL (25, 50, and 100 µg/ml) or 1 mg/ml insulin (positive control).

After the medium was aspirated, the cells were rinsed with 100 ml of PBS, the media was withdrawn, and 25 ml of incubation buffer was added. 200 µl of freshly prepared glucose oxidase test reagent was added to the plates following a 4-hour incubation period. The changes in glucose concentration were determined spectrophotometrically by measuring absorbance at 510 nm, after 15 minutes of incubation period. The wells for the standard contained all other components as the treatment groups, except that cells were not added to them. Glucose absorption and consumption were calculated from the difference between the standard and test samples based on the amount of glucose (µM) remaining. The absorbance of the purple formazan formed in the (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay was measured at 540 nm to determine the number of viable cells [18].

2.10. Pancreatic β-cell proliferation assay

The assay for β-cell proliferation was carried out using the method of Pringle *et al.* [19]. Briefly, 100 µl of cells were seeded in 96-well plates and allowed to attach overnight. The cells were treated with 5, 10, and 20 µg/ml of MELL and were subsequently incubated for 24, 48, and 72 hours in complete medium (RPMI1640 with 10% FBS). Thereafter, 50 µl of staining solution was added to each well after the culture/treatment medium was gently aspirated following each incubation period.

The plates were then incubated for 30 minutes at 37°C, after which fluorescent micrographs were taken using a 4',6-diamidino-2-phenylindole filter cube and a 10× Plan Fluor. The images were analysed, and the total number of cells was determined.

2.11. In vitro macrophage activation

The *in vitro* macrophage activation assay was carried out using the method of Rampa *et al.* [20]. Briefly, the RAW 264.7 cells were seeded in 96-well plates and allowed to attach overnight. The cells were incubated with 5, 10, 20, and 40 µg/ml of MELL or 500 ng/ml lipopolysaccharide (positive control) and then cultured for 24 hours. 50 µl of the utilized culture medium was transferred to a new 96-well plate and mixed with 50 µl of the sulfanilamide solution. The resultant mixture was incubated at 25°C for 10 minutes in the dark. 50 µl of N-(1-naphthyl)-ethylenediamine dihydrochloride solution was added to each well, the wells were incubated for a further 5–10 minutes at 25°C and in the absence of light. A standard curve of sodium nitrite was used to quantify the Nitric oxide content in each sample. To ensure that toxicity was not a relevant factor, the MTT assay was performed according to the method of van de Venter *et al.* [17], as described earlier, was performed to examine the cell viability with minimal adjustments.

2.12. Data analysis

Data were presented as mean ± standard deviation from three replicates. A one-way analysis of variance was used to analyse the data; *p*-value <0.05 was considered significant. GraphPad Prism was used to determine the IC₅₀ values for cytotoxicity.

3. RESULTS AND DISCUSSION

3.1. Identification of phytochemicals, estimation of polyphenols and flavonols, and antioxidant capacity

Flavonoids and phenols are naturally occurring compounds with significant antioxidative activity. They can scavenge free radicals, suppress, reduce lipid peroxidation, and protect cells from oxidative stress. These properties contribute to the pharmacological effects of medicinal plants [21]. Plants are now the primary source of antioxidants due to the side effects of synthetic antioxidants at higher concentrations [22]. Plants can mitigate ROS-induced oxidative stress without significant side effects.

We obtained 22.5 g of crude extract from 100 g of *L. leonurus* leaf powder soaked in 1 l of methanol. The polyphenol content was estimated to be 43.03 ± 1.28 mg GAE/g, and the

flavonol content was 40.43 ± 1.75 mg QE/g (Table 1). The antioxidant capacity of the plant extract was also investigated, and the following results were obtained: FRAP (197.42 ± 9.78 μ mol AAE/g), DPPH (115.45 ± 3.08 μ mol TE/g), and TEAC (105.77 ± 2.30 μ mol TE/g). Standard solvents for extracting flavonoids include acetone, methanol, ethanol, water, or combinations of these solvents. The extraction efficiency of various soluble flavonoid molecules depends on the compounds' complexity, the chosen solvents, and the system's characteristics, such as its thermal properties, the solvent's capacity to form hydrogen bonds, temperature, pH level, and solvent polarity [23,24]. The antioxidant activity of phenolics is thought to prevent or significantly reduce the risk of many pathological diseases [25]. The identification of the phytochemical compounds was carried out, and the results are depicted in Table 2.

3.1.1. Analysis of phenylpropanoid/hydroxycinnamic acids and diterpenoids

Peaks 1 and 2 were identified as caffeoyl hexoside with MS² fragments at *m/z* 161 [hexoside-H]⁺, 179[caffeoyl-H]⁺. Other phenylpropanoids in peaks 3 and 39 were identified as ferulic acid dimer and hydroxytyrosol β -L-rhamnosyl-(1 $\rightarrow$ 3)- β -D-glucoside, respectively (Fig. 1). Peaks 4, 6, 7, 17, 26, 29, 31, 35, and 36 were identified as peaks of flavonol compounds (Fig. 1). Compounds 18, 30, and 32 are identified as labdane type-diterpenoids, with fragments similar to previous identifications from this plant. A diterpene ester exhibited an MS¹ ion at [M+K]⁺, *m/z* 607, and was identified as 1,2,3-trihydroxy-

Table 1. Polyphenols, flavanols, and antioxidant capacity of MELL leaves.

Extracts	Polyphenols (mg GAE/g)	Flavonols (mg QE/g)	FRAP (μ mol AAE/g)	DPPH (μ mol TE/g)	TEAC (μ mol TE/g)
Methanolic extract	43.03 ± 1.28	40.43 ± 1.75	197.42 ± 9.78	115.45 ± 3.08	105.77 ± 2.30

GAE = Gallic acid equivalents, QE = Quercetin equivalents, Ferric reducing antioxidant power (FRAP), TEAC= Trolox equivalent antioxidant capacity, AAE = Ascorbic acid equivalents, TE = Trolox equivalents.

Table 2. Compounds present in 70% methanolic extract of *L. leonurus* leaves in both positive and negative modes.

No	t _r (min)	UV λ _{max} (nm)	<i>m/z</i> (M-H) ⁻ / (M+H) ⁺	MS/MS	Tentative compound name	Identification
1	1.2		341.1088	179	Caffeoyl hexoside	New
2	7.80	249, 334	341.0899	161, 179	Caffeoyl hexoside	New
3	11.23		387.1629	unfragmented	Ferulic acid dimer	New
4	11.60	270, 331	593.1531	431, 421	Kaempferol-3-O- β -glucopyranosyl-7-O- α -rhamnopyranoside	New
5	15.04	255, 347	447.0925	285	Luteolin 7-O-glucoside	[26]
6	15.20	250, 331	755.2345	447	Quercetin-3-O- α -L-rhamnopyranosyl(1->2)- β -D-glucopyranoside-7-O- α -L-rhamnopyranoside	New
7	15.50	250, 331	623.2007	571	Quercetin 3'-O-diglucoside	New
8	17.10	246, 320	535.1473	187	Uridine diphosphate xylopyranose	New
9	17.44	251, 347	461.1074	255, 285, 447, 299, 151	Luteolin 7-glucuronide/scutellarin	New
10	19.03	250, 318	573.1473	527, 565	Unknown	New
11	20.34	256, 349	285.0401	133, 217	Luteolin	New
12	21.10	284, 334	299.0545	284	Chrysoeriol (3'-O-methyl-luteolin)	New
13	22.10	245, 284, 341	329.0660	285	Tricin	New
14	22.56	281, 331	393.1922	314, 299, 271	Narrulibanoside	[27]
15	22.61	281, 331	393.1925	347, 383	Narrulibanoside	[27]
16	22.90	270, 318	577.1393	269	Galangin 3-(3''-coumaroyl)-Galactopyranoside	[28]

Continued

No	t _R (min)	UV λ _{max} (nm)	m/z (M-H) ⁻ / (M+H) ⁺	MS/MS	Tentative compound name	Identification
17	23.50	243, 268, 334	299.0556	269, 284, 179	3-O-methyl kaempferol	New
18	23.92	241, 283, 335	363.1812	347, 227, 183, 343	Leoleorin D	[29]
19	24.15	246, 321	347.1864	311, 163, 127	Unknown	New
20	24.23	242	313.0706	309	Unknown	New
21	24.32	242	377.1961	309, 227	Unknown	New
22	0.73	264	160.0980	144	L-2-Aminoadipic Acid	New
23	4.28	354, 348	775.3391	197, 307, 433, 535, 333, 271	4'-O-Methyl-2,3-dihydromyricetin derivative	New
24	4.94	243, 330	339.2538**	185, 321	4-hydroxy- dihydromyricetin	New
25	5.36	234, 286, 346	331.0818	319, 301	Tricin	New
26	5.69	269, 331	301.0710	285	Hydroxykaempferol	New
27	6.10	234, 284, 346	345.0983	329, 333, 315, 287	4-O-methyl triclin.	New
28	6.15	243	701.4253	333, 287, 315, 381	4'-O-Methyl-2,3-dihydromyricetin-3'-O-(6''-O-acetyl glucosyl) glucoside	New
29	6.80	245, 311	349.2028	303, 315, 287	Isorhamnetin derivative	New
30	7.00	243, 287, 323	379.2490**	231, 143, 83, 365	Leoleorin H (15-acetoxy-9,13-epoxy-16-hydroxylabd-5-en-7-one)	[29]
31	7.10	251, 335	315.0878	287, 269, 221, 175	Isorhamnetin	New
32	7.21	248	379.2491**	231, 143, 83, 365	Leoleorin H (15-acetoxy-9,13-epoxy-16-hydroxylabd-5-en-7-one)	[29]
33	7.72	247	315.1961	301	3',4'-dimethyluteolin	New
34	8.13	276	315.212	287, 269, 221	3',4'-dimethyluteolin	New
35	8.20	245	317.2118	299, 271, 289, 287	Isorhamnetin	New
36	8.49	248	317.2117	299, 205	Isorhamnetin	New
37	9.32	250	299.2013	229, 217, 273	Naringenin derivative	New
38	11.60	248, 273, 327, 410	593.2761	533, 461, 431	Apigenin-6-C-glucosyl-7-O-glucoside	New
39	12.00	256, 399	623.2870	Unfragmented	Acteoside/ hydroxytyrosol β-L-rhamnosyl-(1->3)-β-D-glucoside	New
40	12.20	243, 272, 326, 407		Unfragmented	1,2,3-trihydroxy-3,7,11,15-tetramethylhexadecan-1-yl-palmitate (dihydroxyphytyl palmitate).	[26]

K=[M+K]⁺ and **=[M+NH₄]⁺ in the positive ion mode. ^{New} means it has been reported from *L. leonurus* for the first time, although it has been previously identified from other plant sources. Compounds 1–21 were detected in the negative ion mode, while compounds 22–40 were detected in the positive ion mode.

3,7,11,15-tetramethylhexadecan-1-yl-palmitate (Fig. 1). This identification underscores the structural diversity of diterpenoids present in the sample.

3.1.2. Analysis of flavonols flavone and flavononol

Flavonols were also identified by UV maximum absorption of about 331 nm or slightly above. Compound 7 was identified as quercetin 31-O-diglucoside. This is due to conjugation with one or several sugar molecules; fragmentation would produce an aglycone ion of quercetin, m/z 301 in negative ion mode. Identification was also supported by high-resolution spectrometry with accurate mass determination. Most flavonoid

O-glycosides fragmented in negative ion mode by removing glycosyl residues and acquiring aglycone ions, such as m/z 285 (kaempferol) and m/z 301 (quercetin/6-hydroxykaempferol, as in peak 26). Neutral loss of the sugar molecules (glucuronide 176 Da), glucose (162 Da), and rhamnose (146 Da) could yield aglycone ions. A compound in peak 6 with m/z 755.2345 was thus identified as quercetin after conjugation with glucose and rhamnose sugars, consistent with a database search (ReSpect MS/MS; <http://spectra.psc.riken.jp/>). Likewise, the compound in peak 4 was identified as kaempferol. The compounds in peaks 31, 35, and 36 were identified as isorhamnetin or its derivative at peak 29. Isorhamnetin exhibited characteristic

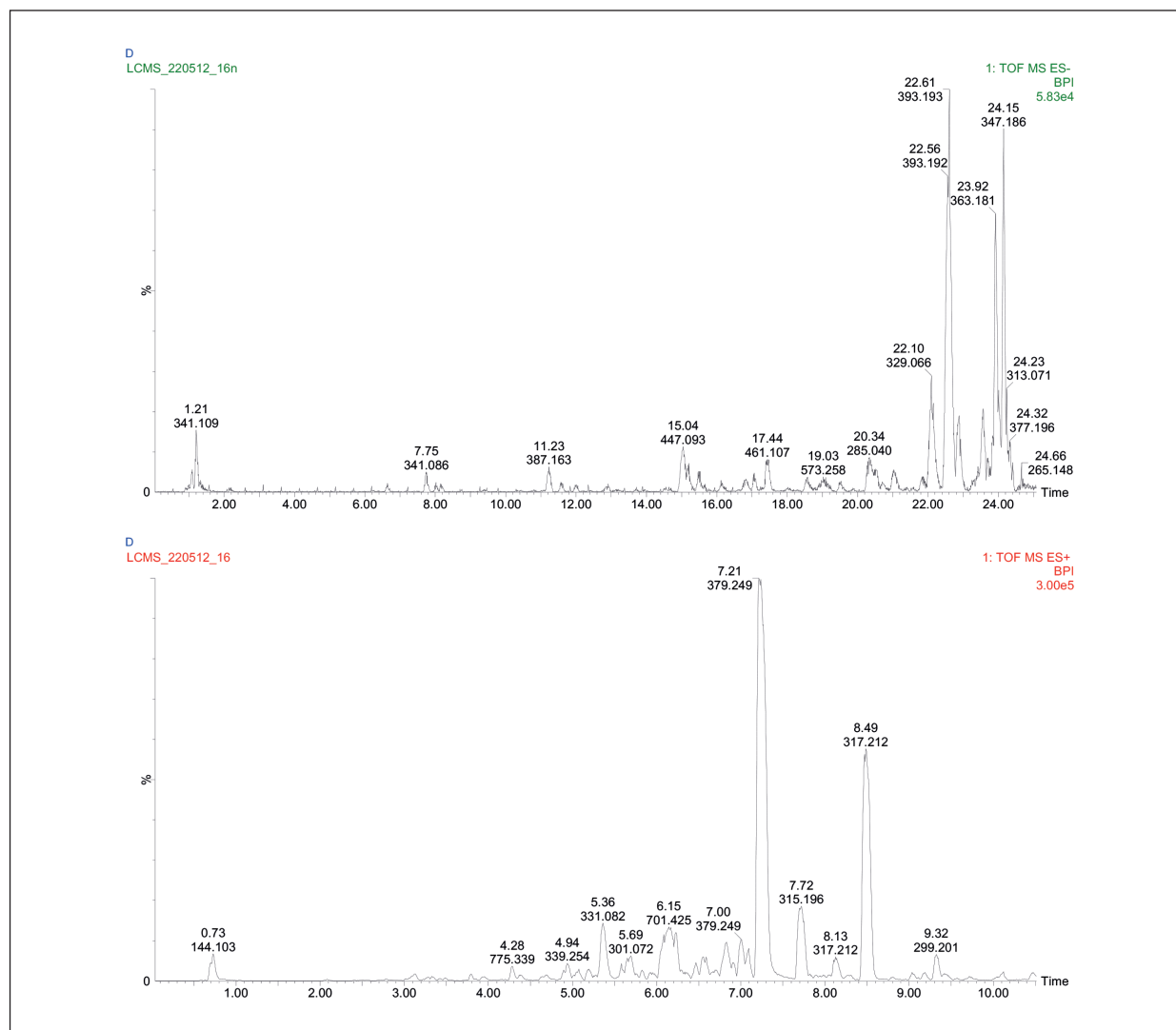


Figure 1. UHPLC-ESI-MS base peak chromatogram for methanol extract of *L. leonurus* leaves analysed in the negative and positive ion modes. Peaks represent count versus acquisition time in minute.

parent and fragment ions; m/z 317 [isorhamnetin + H]⁺, m/z 287 [isorhamnetin + H - 2CH₃]⁺, and RDA cleavage ion m/z 269 [M - H - CH₃ - CO]⁺. The flavonol, Galangin 3-(3111-coumaroyl)-galactopyranoside was identified at peak 16 after simultaneous acylation with coumaroyl and conjugation with galactose.

Flavones exhibit UV $\lambda_{\max}$ of about 348 and 410 nm. Flavone compounds were identified in the following peaks: 5, 9, 11, 12, 13, 16, 25, 33, 34, and 38. Compound 9 (C₂₁H₁₈O₁₂) was identified as scutellarin or Luteolin 7-glucuronide, with the ion [M - H]⁻ at m/z 461.1074 and its fragments [M - H - C₆H₈O₆]⁻ at m/z 285 resulting from the loss of a gluconide residue Agnihotri *et al.* [26]. Peaks 11, 33, and 34 showed precursor ions at m/z 285.0401, 315.1961, and 315.212 [M - H]⁻ identified as luteolin and 31,41-dimethyluteolin, respectively. The precursor ion for Apigenin-6-C-glucosyl-7-O-glucoside was m/z 593.2761 with major MS² fragments at m/z 533 [M + H - 60]⁺, and 431 [M + H - glucose]⁺. This fragmentation was consistent with database searches. Peaks 25 and 27 were identified as tricetin and 4-O-methyl

tricetin, respectively. The UV $\lambda_{\max}$ absorption of 348 nm indicated the presence of flavone flavonoids. The aglycon tricetin exhibited an ion with [M + H]⁺ at m/z 331.0818 with a fragment m/z 301 [M + H - 2 CH₃]⁺, allowing identification of its methyl derivative by the addition of 15 Da to 331. Peaks 23 and 28, attributed to flavononol/flavonone subclass of flavonoids, with many showing the characteristic precursor ion m/z 333 of the 4'-O-methyl-2,3-dihydromyricetin. Naringenin was identified at peak 37 with the main fragment ion 273 [naringenin + H]⁺.

3.1.3. Quantification of phenolic compounds

Phenolics are among the most varied types of bioactive secondary metabolites found in medicinal plants [27]. Secondary metabolites constitute most of the medicinally useful bioactive compounds (phytochemicals) derived from plants. Bioactive plant extracts typically contain complex combinations of components that, when combined, may have an enhanced effect. Figure 2 shows the concentration of different classes of

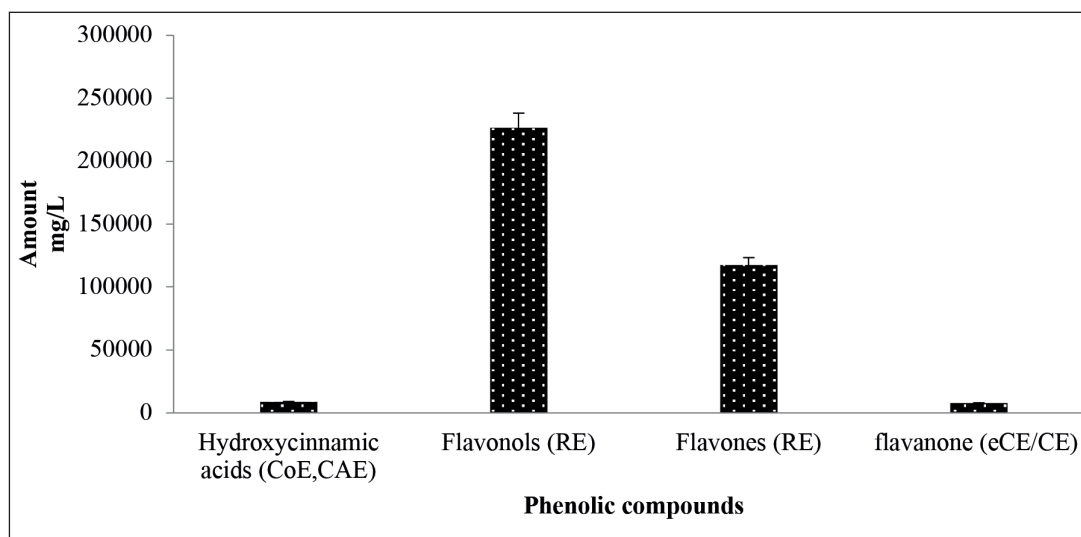


Figure 2. The concentration of phenolic compounds obtained from the methanol extract of *L. leonurus* leaves. CoE = coumaric acid equivalents, CE = Catechin equivalents, CAE = caffeic acid equivalent, RE = rutin equivalent, and eCE = epicatechin equivalent. Results represent mean $\pm$ standard deviation of three replicates; values are significantly different at p -value < 0.05 .

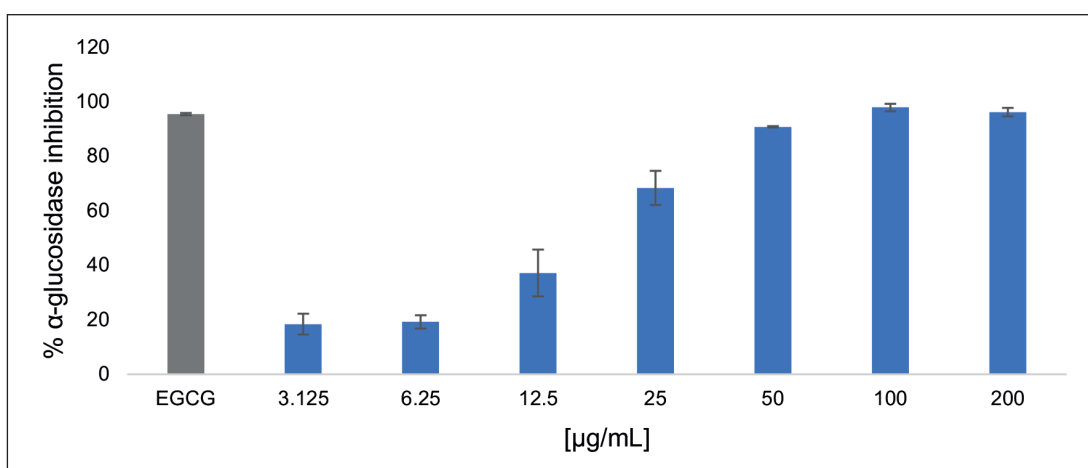


Figure 3. α -glucosidase inhibition by methanol extract of *L. leonurus* leaves. ECGC = positive control. Results represent mean $\pm$ standard deviation of three replicates; values are significantly different at p -value < 0.05 .

compounds quantitated in the 70% MELL. Flavonoids were the most represented phytochemicals. Hydroxycinnamic acids were also present. Diterpenoids subclasses were not quantitated due to the absence of a standard and were not among the most represented compounds. Many antidiabetic flavonoids have been isolated and characterized.

3.2. Alpha-glucosidase inhibition

Complex polysaccharides and disaccharides are hydrolyzed into monosaccharides by digestive enzymes in the small intestine. Inhibiting these enzymes prevents monosaccharides from being released into the bloodstream, limiting their availability to adipose, muscle, and liver tissues [31,32]. This study investigated the hypoglycaemic effect of the MELL on α -glucosidase activity, which is commonly inhibited by T2D medications. The rapid absorption was investigated on α -glucosidase activity, which is often

inhibited by α -glucosidase in the small intestine, causes high blood sugar levels in diabetic individuals. By inhibiting these enzymes, blood sugar levels can be regulated [33]. Glucose is typically derived from the hydrolysis of maltose in the small intestine [34]; thus, delaying maltose absorption can also delay glucose absorption.

The extract exhibited dose-dependent α -glucosidase inhibitory activity, with significant inhibition ($>50\%$) observed starting at 25 $\mu\text{g/ml}$ (Fig. 3). Over 80% inhibitory activity was recorded from 50 $\mu\text{g/ml}$ and above, while the highest α -glucosidase inhibitory activity (approximately 98%) was 100 $\mu\text{g/ml}$. α -glucosidase inhibitors delay glucose uptake [35]. There was no significant difference ($p > 0.05$) between MELL at 100–200 $\mu\text{g/ml}$ and the positive control (ECGC); notably, the extract of *L. leonurus* demonstrated these properties, suggesting its potential therapeutic application in delaying postprandial hyperglycemia.

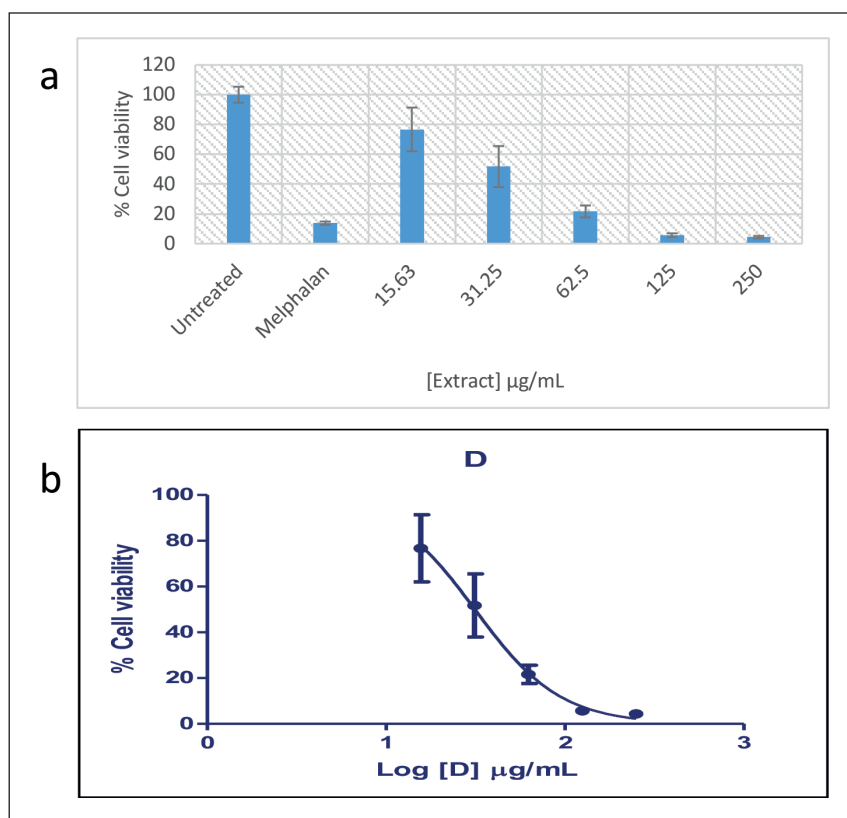


Figure 4. Cytotoxic activity of crude extract of *L. leonurus* leaves against C3A hepatocytes. Results were normalised to cell viability as determined using the MTT assay. Data represent mean $\pm$ standard deviation of three replicates; values are significantly different at p -value <0.05 .

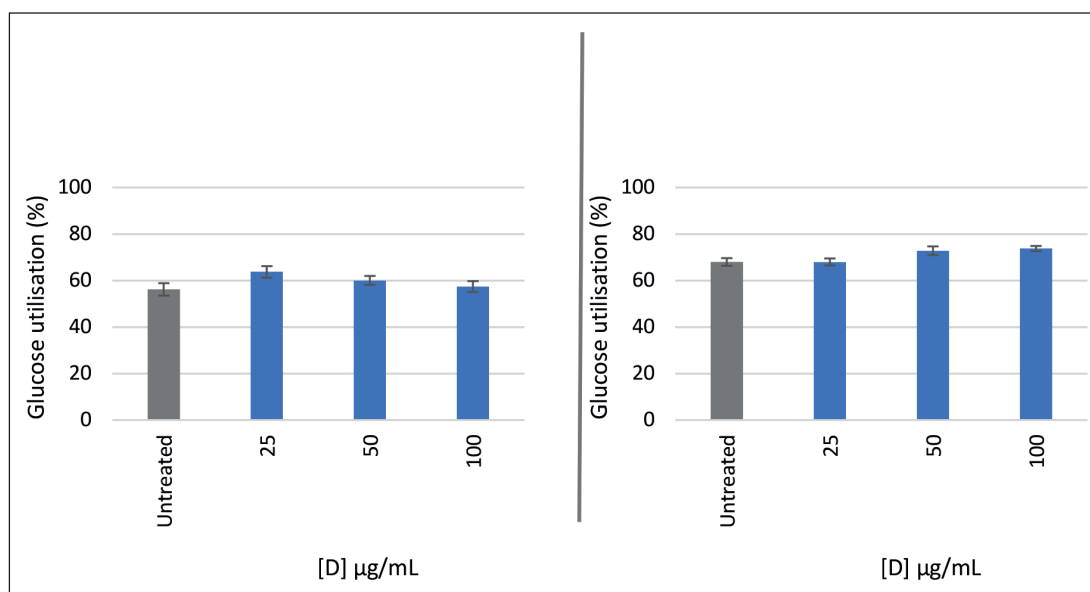


Figure 5. Glucose utilisation (%) after 24 hours of treatment with methanol extract of *L. leonurus* leaves in L6 myoblasts (a) and C3A hepatocytes (b). Data represent mean $\pm$ standard deviation of three replicates; values are significantly different at p -value <0.05 .

Polyphenols have also been reported to enhance insulin's effects on glucose utilization. Additionally, inhibitors of key enzymes, including α -amylase and α -glucosidase, are known to help prevent T2D [36]. Various natural substances, such as

terpenoids, steroids, and flavonoids, serve as α -glucosidase inhibitors.

This study reveals that *L. leonurus* could be used as a lead in the development of drugs aimed at reducing

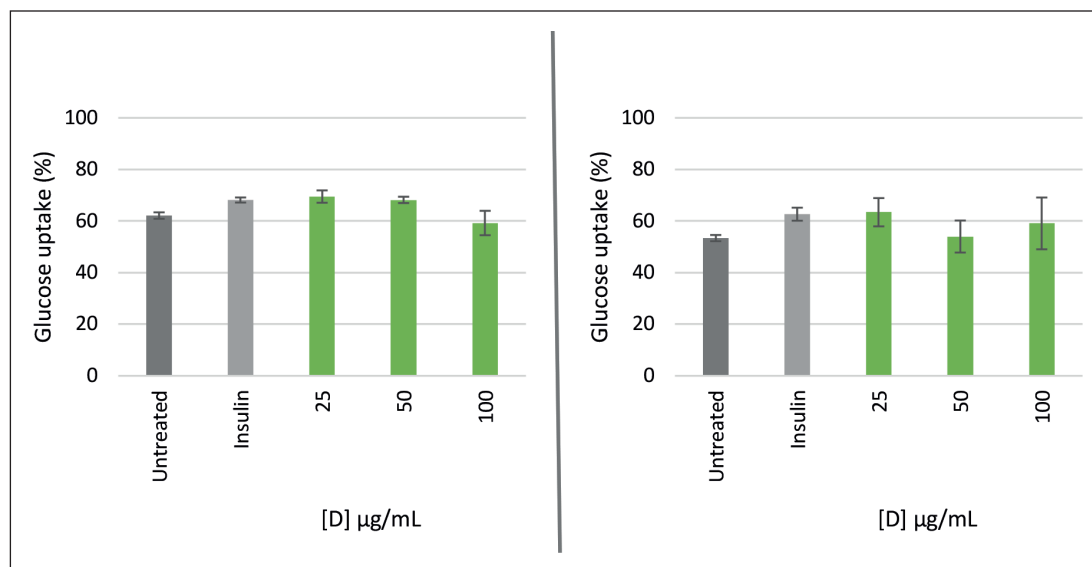


Figure 6. Glucose utilisation (%) after 4 hours of treatment with MELL leaves in L6 myoblasts (a) and C3A hepatocytes (b). Data represent mean $\pm$ standard deviation of three replicates; values are significantly different at p -value <0.05 .

blood sugar through the inhibition of carbohydrate digestion and enhancement of insulin sensitivity. Based on the high concentration of secondary metabolites in *L. leonurus* leaf extract and its α -glucosidase inhibitory activity, it could be further researched for creating nutraceutical formulations to combat T2D. Identified compounds such as quercetin and its conjugates, luteolin, and its derivatives, isorhamnetin, apigenin, naringenin, and kaempferol derivatives have been shown to inhibit α -glucosidase activity initially [37–39]. The mechanisms of action of various diabetes drugs include enabling glucose transporter type 4 to serve as a vehicle for allowing insulin-induced blood glucose uptake into skeletal muscle, stimulating glucose uptake, and activating AMPK phosphorylation. Quercetin has been reported to prevent intestinal glucose absorption and possesses insulin-secretory and insulin-sensitizing properties. It enhances glucose utilization in peripheral tissues, boosts insulin secretion, allows pancreatic β -cells to regenerate, and inhibits sucrose's enzymatic activity [40]. By upregulating adenosine monophosphate-activated protein kinase, naringenin has reduced gluconeogenesis and has shown results similar to those of metformin [39]. According to a study by Alkhalidy *et al.* [37], kaempferol inhibits gluconeogenesis in the liver and increases glucose metabolism in skeletal muscle. Luteolin has been shown to halt sorbitol accumulation in erythrocytes [38]. Caffeoyl glucoside was found to be an active ingredient of the antidiabetic plant, *Equisetum myriochaetum* [41]. The antioxidant properties of phenolics have been thoroughly studied, as evidenced by several existing studies. Flavonoids, phenolic acids, coumarins, and stilbenes have all demonstrated antioxidant potential. For instance, ferulic acid effectively scavenges free radicals and inhibits lipid peroxidation by scavenging superoxide anion radical [39]. Quercetin has been reported to have better antioxidants than kaempferols [42].

In the last stage of digestion, the enzyme α -glucosidase is essential because it breaks down complex carbohydrates like starch and glycogen into their monomers. The influx of glucose from the digestive tract to blood vessels can thus be efficiently suppressed by α -glucosidase inhibitors, lowering postprandial glucose levels [31,43,44]. Therefore, in T2D, inhibiting α -glucosidase may be a useful strategy for regulating blood glucose levels [45,46].

3.3. Cytotoxicity study

There has been a significant increase in the use of herbal remedies worldwide [47]. Due to their wide variety of phytochemical ingredients and lower risk of side effects, plants have garnered more interest in recent years for the prevention and treatment of cancer and other diseases. However, concerns regarding their safety have also increased. This has led to a state of ambivalence in discussions about their applications. Certain therapeutic plants can be inherently toxic due to their ingredients and may have negative side effects if administered improperly. Bioactive compounds found in medicinal plants typically vary in type and content within and across species. Some plants used in traditional medicine (TM) are inherently hazardous because of the potential toxicity of their chemical constituents [48].

The presence of mixtures of various physiologically active plant compounds, or phytochemicals, forms the basis for the medical use of plants. These constituents may act individually, in combination, or synergistically to produce an effect that may be beneficial or detrimental to health [30]. Due to their negative impacts on the biological processes of other organisms, some plants have been classified as poisonous [47].

As a result, these plants can be detrimental to a person's ability to survive or function normally. Plants with known harmful components are avoided or used sparingly

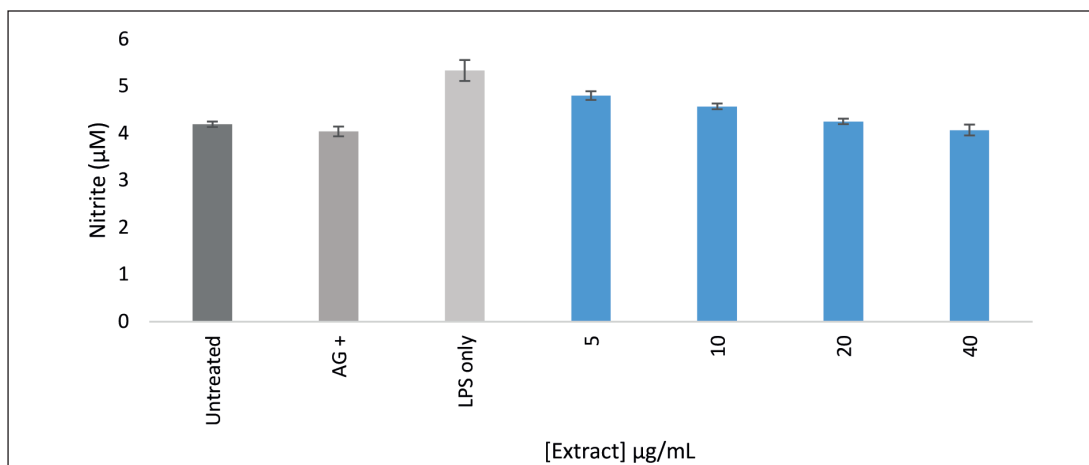


Figure 7. Nitric oxide production in LPS-activated macrophages in treated and untreated groups. Data represent mean $\pm$ standard deviation of three replicates; values are significantly different at p -value <0.05 .

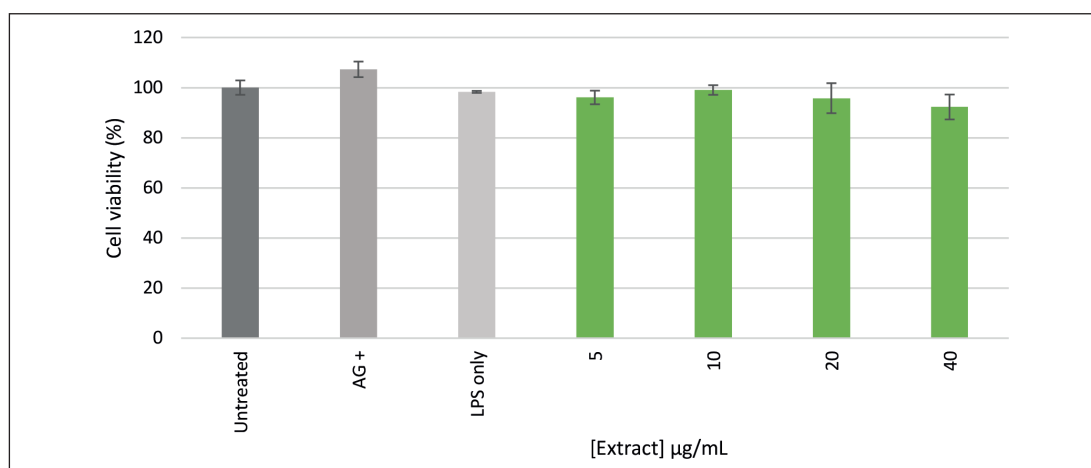


Figure 8. Percentage cell viability (%) of LPS-activated macrophages after 24 hours of treatment exposure with MELL leaves. Data represent mean $\pm$ standard deviation of three replicates; values are significantly different at p -value <0.05 .

in herbal product formulations in TM. Even when these plants are used in pharmaceuticals, they are administered at doses below hazardous levels. Thus, when taken by qualified professionals or experienced individuals, they rarely cause death. In this study, the crude extract was not cytotoxic at concentrations above 15.63 $\mu\text{g}/\text{ml}$, with cell viability remaining below 50% (Fig. 4). There was no significant difference ($p > 0.05$) in percentage cell viability between MELL at 125–250 $\mu\text{g}/\text{ml}$ and the positive control (melphalan). This indicates that the plant should be used with caution in the preparation of herbal products.

3.4. Myocyte and hepatocyte glucose uptake

Most of the body's tissues utilize glucose as their primary energy source. Accordingly, a complex regulatory mechanism involving multiple tissues maintains glucose homeostasis throughout the body [49]. There is currently extensive research being conducted to find new treatments for T2D. Figure 5 shows the effect of MELL on glucose utilization and uptake in C3A and L6 cells. In C3A hepatocytes, there

was a significant difference ($p < 0.05$) in the glucose uptake at treatment concentrations of 50–100 $\mu\text{g}/\text{ml}$ compared to the untreated group. Glucose utilization increased in L6 myoblasts at a treatment concentration of 25 $\mu\text{g}/\text{ml}$. In C3A hepatocytes, an increase in glucose utilization was observed for sample D at concentrations of 50–100 $\mu\text{g}/\text{ml}$ (Fig. 6). Conversely, glucose uptake in L6 myoblasts increased at concentrations of 25–50 $\mu\text{g}/\text{ml}$. Zhao *et al.* [50] reported that the hypoglycemic potential of the ethanolic extract of *Folium sennae* is related to its effect on glucose uptake effect. All treatment groups, including insulin, significantly increased glucose absorption by cells to twice that of the untreated group.

Interestingly, no significant cytotoxic effect was recorded for the extract in either L6 cells or C3A cells. Most of the body's tissues rely on glucose as their primary energy source, and a complex regulatory mechanism involving multiple tissues maintains glucose homeostasis throughout the body [49]. Inter-organ interactions, facilitated by various circulating variables such as hormones and neuropeptides, ensure the distribution of dietary components according to the individual needs of

each organ [51]. After glucose is absorbed from the intestine, consuming carbohydrates causes an immediate rise in blood glucose levels. Pancreatic beta cells respond to rising blood glucose levels by secreting more insulin through a GLUT2-dependent mechanism [49].

3.5. *In vitro* anti-inflammatory

Figures 7 and 8 shows the effect of the extract against RAW 264.7 cells. To precisely establish possible anti-inflammatory activity, the cytotoxic effect of the extracts on Ralph, Raschke, and Watson was assessed. The reduction in nitrite content due to Lipopolysaccharide (LPS) activation of RAW macrophages without affecting cell viability is indicative of anti-inflammatory action [12]. MELL leaves showed anti-inflammatory properties starting at 10 µg/ml. Caffeoyl hexosides have been reported to reduce inflammation by inhibiting the expression of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β . It also mediates mitogen-activated protein kinase pathways, which are activated during inflammatory responses. The compounds might also inhibit the activation of the Nuclear factor kappa-light-chain-enhancer of activated B cells pathway, consequently reducing the expression of various inflammatory mediators [41].

4. CONCLUSION

The study demonstrated the antidiabetic potential MELL leaves in L6 myoblasts and C3A hepatocytes cells. The extract increased glucose utilisation and uptake in both cell types without any significant cytotoxic effect. Interestingly, the extract also exhibited anti-inflammatory activity without significant cytotoxicity. The LCMS analysis revealed the presence of polyphenols and flavonols, which might be responsible for the bioactivity. Its glucosidase inhibitory activity at concentrations of 50–200 µg/ml compares favourably with the positive control, epigallocatechin gallate. Moreover, its effectiveness in glucose utilization is similar to that of insulin in L6 myoblasts and C3A hepatocytes. The compounds present in MELL leaves exhibited antidiabetic activity by enhancing glucose uptake, transport, and glycogen storage. However, further work is needed to fully elucidate the mechanisms underlying its antidiabetic and anti-inflammatory activities.

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6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the

International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

10. PUBLISHER'S NOTE

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11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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