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Phytochemical Characterisation and LC-MS Profiling of *Ziziphus mauritiana* Leaf Extracts for Anti-inflammatory Potential

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Abstract

Background: Inflammation is the immune system's first reaction to an injury or infection. Corticosteroids, immunosuppressants, and Non-Steroidal Anti-Inflammatory Drugs (NSAID) often used to treat inflammatory illnesses have serious adverse effects, such as cardiovascular, renal, and gastrointestinal damage. To lessen the negative effects of currently available medications, there is an urgent need to create innovative anti-inflammatory regimens derived from natural sources. **Aim:** The current study evaluated *Ziziphus mauritiana* for its anti-inflammatory potential, as it contains numerous bioactive constituents like phenolics, flavonoids, and saponins, which can contribute to the development of safer and cost-effective plant-based therapeutics for the treatment of inflammation. **Methods:** Methanol, hexane and ethyl acetate leaf extracts were evaluated for anti-inflammatory potential using LPS-stimulated RAW 264.7 macrophages at concentrations of 3-100 µg/mL for 24 hours. Nitric oxide production was evaluated by the Griess assay. Liquid Chromatography- Mass Spectrometry (LC-MS) analysis for characterisation of various phytoconstituents was determined on a Waters Alliance e2695 High Performance Liquid Chromatography (HPLC) system using a gradient of water, acetonitrile, methanol, and acetic acid. **Results:** Qualitative estimation shows the presence of phenols, triterpenoids, flavonoids and cardiac glycosides in methanol and ethyl acetate extract. *In-vitro* cytotoxicity evaluation demonstrated that the methanol and ethyl acetate extracts maintained more than 50% cell viability at 3 to 20 µg/mL, whereas the hexane extract showed reduced viability, indicating toxicity. Nitric oxide production in RAW 264.7 macrophages was inhibited significantly by methanol and ethyl acetate extract, with maximum inhibition at a concentration of 20µg/mL. LC-MS profiling of ethyl acetate fraction has shown the presence of various bioactive compounds such as L-carnitine, Delphinidin, caffeine, ononin, saccharopine, sinapine, Kaempferol-7-O-neohesperidoside, fortunellin, diosmin, marein etc. **Conclusion:** Results indicate that leaves of *Z. mauritiana* are rich in bioactive compounds and exhibit significant anti-inflammatory potential, mediated by quenching nitric oxide production, highlighting their potential for the development of plant-derived anti-inflammatory medications that are safe to use and have minimal side effects.

Major Findings: *Ziziphus mauritiana* leaf extract has shown significant anti-inflammatory potential by the inhibition of nitric oxide production in the RAW 264.7 cell line. The anti-inflammatory potential of this plant may be attributed to the presence of various bioactive compounds such as L-carnitine, *Delphinidin,* caffeine, sinapine, Kaempferol-7-O-neohesperidoside, fortunellin, diosmin, marein, etc., analysed by LC-MS.

Keywords: Inflammation, LC-MS Analysis, Nitric Oxide, RAW 264.7 Macrophages, *Ziziphus mauritiana*

Abbreviations: LC-MS (Liquid chromatography-mass spectrometry), HPLC (High performance liquid chromatography), ZM (MeOH) (*Ziziphus mauritiana* methanol extract), ZM (EA) (*Ziziphus mauritiana* ethyl acetate extract), ZM (Hex) *Ziziphus mauritiana* hexane extract, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), DMEM (Dulbecco's Modified Eagle Medium).

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1. Introduction

Inflammation is the vital biological defence system of the body, shielding tissues from harm and infection[1]. Fundamentally, inflammation is caused by immune cell activation, the release of signalling molecules such as cytokines and chemokines, and modifications in blood flow to the afflicted area[2]. Inflammation is now seen in contemporary research as a key component connecting several immune, cardiovascular, and metabolic disorders rather than just as a symptom. In this regard, the goal of both conventional and natural therapies is to maintain the protective qualities of the immune response while lowering harmful inflammation[3]. So, natural products continue to be used as models and sources for drug development and discovery, from conventional herbal remedies to contemporary pharmaceuticals[4]. In traditional medicine, plant-derived phytoconstituents have been used for the treatment of various ailments, including inflammation. Among the vast array of medicinal plants, genus *Ziziphus* is considered to be the powerhouse of the phytochemicals in Ayurveda as it is the richest source of bioactive metabolites like flavonoids, triterpenoid saponins, and cyclopeptide alkaloids, which can contribute to the biological potential of the plant. Various species under the genus *Ziziphus* have been widely employed in folk medicine and studied for their pharmacological actions. Among them, *Z. mauritiana* has attracted researchers for its ethnomedicinal uses and extensive phytochemical content, which contributes to the anti-inflammatory potential[5]. Perhaps the most abundant source of therapeutic compounds is found in plants, which generate a diverse range of secondary metabolites, known for their defensive roles in nature[6,7].

Ziziphus mauritiana, also known as Indian Jujube, is a tropical fruit tree species belonging to the family *Rhamnaceae*. Traditionally, it is used for typhoid, indigestion, diarrhoea, wound healing and abdominal cramps[8]. According to the recent research, inflammatory processes may be the primary cause of numerous clinical ailments, including diabetes, atherosclerosis, cancer, and certain neurological diseases[9,10]. This fact emphasises how crucial it is to comprehend inflammatory mechanisms precisely and how urgent it is to find effective anti-inflammatory treatments. Plant-based drug moieties play an important role in the treatment of inflammation with fewer side effects and better effectiveness[11].

Our research is focused on evaluating the anti-inflammatory potential of various extracts of *Z. mauritiana* using RAW 264.7 macrophages as an *in-vitro* model. The leaf extracts of *Z. mauritiana* were determined for the presence of phytoconstituents by various standard qualitative phytochemical tests. Among all the extracts, the ethyl acetate fraction showed the presence of flavonoids, triterpenoids, phenols and tannins, etc. LC-MS analysis of the ethyl acetate fraction for compound identification and isolation was followed by an anti-inflammatory assessment, shedding light on the possible ways that *Z. mauritiana* might have therapeutic effects. This study supports the investigation of *Z. mauritiana* as a natural source of anti-inflammatory agents by combining functional bioassays with phytochemical characterisation.

2. Materials and Methods

2.1 Chemicals and Reagents

Analytical grade solvents (Methanol, Hexane, Dichloromethane, and Ethyl acetate) were procured from Rankem Laboratories, India. For LC-MS, HPLC-grade acetonitrile, methanol, acetic acid, and ammonium formate were also procured from Rankem Laboratories, India. LPS *(Escherichia coli)* was purchased from Sigma-Aldrich. Dulbecco's Modified Eagle Medium (DMEM) High glucose (Himedia AL007S), fetal bovine serum (Gibco) and trypsin (Cytiva SV30037) were used for the cell culture.

2.2 Collection and Extraction

The leaves of *Z. mauritiana* collected from the Ramban district of Jammu and Kashmir were identified and authenticated by a taxonomist, Dr Akhtar Malik, at the University of Kashmir, bearing specimen number 4301-KASH, dated October 10, 2021. Accurately weighed 2kg of plant material was extracted using methanol by the cold maceration process. The extraction was carried out in methanol, as it is a highly polar solvent (polarity index 5.1) and capable of trapping diverse phytoconstituents contained in the plant material. The methanol extract was further subjected to fractionation using solvents of varying polarity, i.e., hexane, dichloromethane and ethyl acetate[12].

3. Phytochemical Analysis

All the extracts (methanol, hexane, dichloromethane and ethyl acetate) were screened by various qualitative tests for the presence of active phytochemical constituents[13,14].

3.1 *In-vitro* Anti-inflammatory Activity

Anti-inflammatory activity was assessed in Lipopolysaccharide (LPS)- stimulated RAW 264.7 cells by measuring inhibition of key inflammatory response mediators. Cells were maintained at passages ranging from 5 to 20. The composition of the complete culture media includes DMEM, High glucose (AL007S) with 10% heat-inactivated Fetal Bovine Serum (FBS) (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin (Pen Strep 15140122). Cells were incubated in complete media in a 5% CO_2 humidified atmosphere at 37°C. In order to prevent mycoplasma contamination, cells were pre-treated with EZkill™ (CCK006) according to the manufacturer's recommendations.

3.1.1 MTT Assay

Following an overnight culture in a 96-well plate (2 x 10^4 cells/well, 100µL medium/well), the cells were exposed to various extracts at concentrations ranging from 3 µg/mL to 100 µg /mL for 24 hours, as well as lipopolysaccharide (LPS). After that, each well received a 0.5 mg/mL (MTT) solution before being incubated at 37 °C for 4 hours. Finally, using a microplate reader (PerkinElmer), the absorbance of each well was measured at 570nm[15].

3.1.2 Inhibition of Nitric Oxide Production

When RAW 264.7 cells are stimulated by various stimuli such as LPS, inflammatory cells like macrophages express iNOS, which results in the overproduction of NO. As NO has a very short half-life and quickly forms stable products, including nitrite and nitrate, nitrite formation through the Griess reaction is a useful indicator of inflammation. Following an overnight culture in a 24-well plate (1 x 10^5 cells/well, 500µL medium/well), the cells were pre-treated with plant extracts for 1 h and lipopolysaccharide for another 24 h[16,17]. LPS-stimulated RAW 264.7 macrophages were designated as the positive control, and untreated RAW 264.7 macrophages were designated as the negative control. The culture supernatant from each well was collected at the conclusion of the planned experiments and used to calculate the amount of NO produced. 50 µL of cell culture medium and 100µL of Griess reagents I and II were combined in a 96-well plate for a 10-minute reaction at Room Temperature (RT), and the absorbance was then measured at 540 nm using a microplate reader (Perkin Elmer)[18]. Based on the Griess reaction, the NO production was calculated.

3.1.3 LC-MS Analysis

LC-MS on a Waters Alliance e2695 HPLC system connected to a Triple Quadrupole (TQD) mass spectrometer was used to analyse the *Z. mauritiana* leaf extract. In order to eliminate particles, samples were first prepared by solvent extraction and then filtered through a 0.22µm membrane. Using a gradient of water, acetonitrile, methanol, and acetic acid, separation was carried out on a SUNFIREC18, 250X2.1, 2.6µm, at a flow rate of 1 mL/min while the column temperature was maintained at 35°C. With optimised source parameters for both positive and negative ion detection based on the analytes, the mass spectrometer functioned in Electrospray Ionisation (ESI) mode between m/z150 and 2000[19]. The compound identification was based on MS/MS fragmentation patterns and retention time, enabling accurate qualitative and quantitative analysis of the phytochemicals contained in the extract.

3.2 Statistical Analysis

All the experimental results were represented as mean ± SD of three replicates (n=3). One-way ANOVA was performed to determine significant differences among treatment groups, with a p-value of < 0.05 considered statistically significant. All statistics were carried out on GraphPad Prism version 9.0 (GraphPad software, San Diego, CA, USA).

4. Results

4.1 Plant Extraction and Yield

The coarsely powdered drug (2kg) was extracted with methanol, yielding a % yield of 10.83w/w. This was followed by fractionation using hexane, dichloromethane, and ethyl acetate, generating a % yield of 14w/w, 3w/w and 23w/w, respectively.

4.2 Phytochemical Analysis

Table 1. Qualitative phytochemical screening of *Ziziphus mauritiana* extracts

Phytochemical Tests	Extracts				
	Methanolic	Hexane	DCM	Ethyl acetate	Aqueous
Tests for Alkaloids					
Mayer's test	+	-	+	+	-
Wagner's test	+	-	-	+	-
Dragendroff's test	+	-	+	+	-
Tests for Carbohydrates					
Molisch's test	+	+	-	-	-
Benedict's test	-	-	-	-	-
Fehling's test	+	-	-	-	-
Test for Cardiac Glycosides					
Kellar Killani test	+	-	-	+	-
Legal's test	+	+	-	++	-
Test for Phytosterols					
Libermann Buchard's test	+	+	+	+	+
Test for Triterpenoids					
Salkowski's test	++	+	-	++	-
Test for Phenols					
Ferric chloride test	+	+	+	+	-
Tests for Tannins					
Gelatin test	+	-	-	+	+
Ferric chloride test	+	-	-	+	+
Tests for Flavonoids					
Shinoda test	++	+	-	++	-

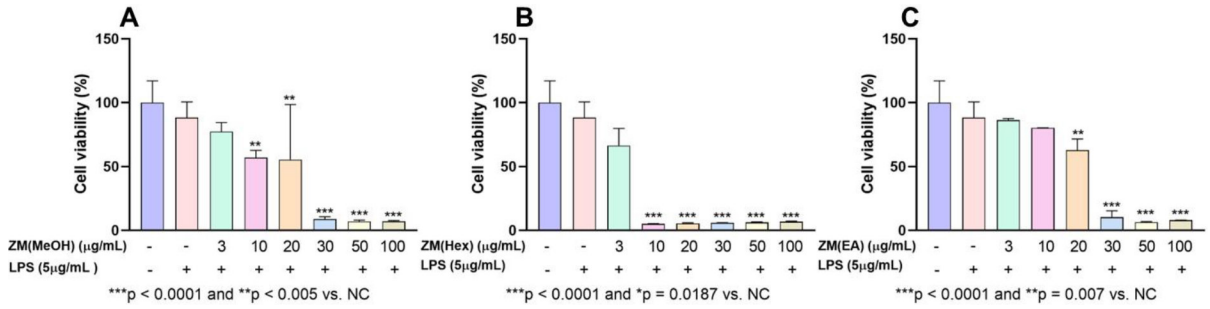


Figure 1. Effect of *Ziziphus mauritiana* extracts on RAW264.7 macrophage viability. RAW264.7 macrophages were cultured with 24-hour *Z. mauritiana;* **(A).** methanol [ZM(MeOH)], **(B).** hexane [ZM(Hex)], and **(C).** ethyl acetate [ZM(EA)] extracts in concentrations of 3-100µg/mL. LPS (5µg/mL) was employed as a positive control. Cell viability was ascertained by MTT assay and expressed as a percentage of untreated controls (NC). Data are mean ± SEM (n = 3).

4.3 Effect of *Ziziphus mauritiana* Extracts on Cell Viability by MTT Assay

The cell viability was determined by MTT assay, a colourimetric method to determine cytotoxic or cytoprotective effect of test samples on cultured cells. It is used to establish non-cytotoxic doses, which would allow safe use in future studies without compromising the viability of the cells under study (RAW 264.7). *Ziziphus mauritiana* leaf extracts were screened for cytotoxicity against RAW 264.7 macrophages at 3 to 100µg/mL concentrations. ZM (MeOH) and ZM (EA) have shown more than 50% cell viability at 20 µg/ml

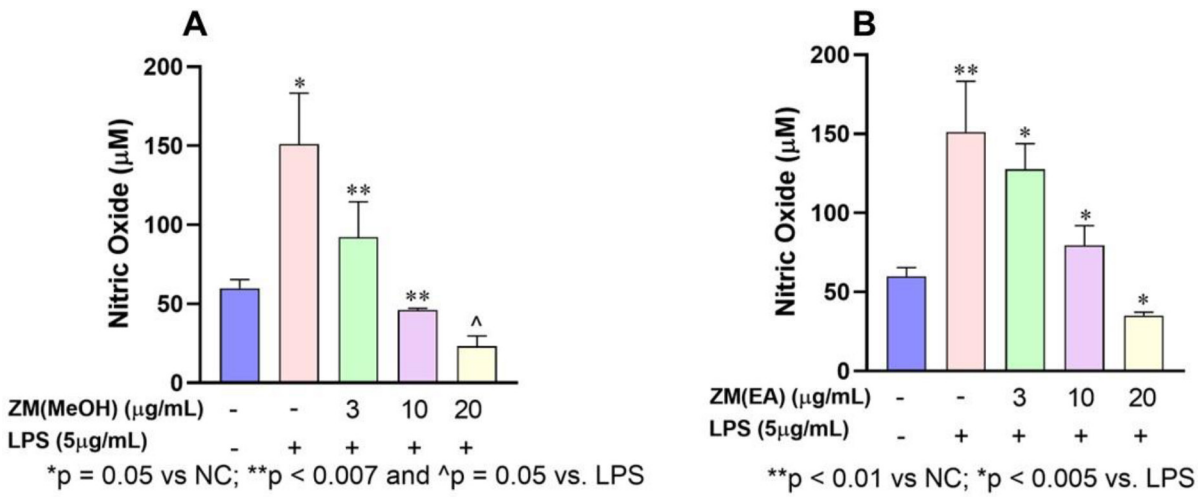


Figure 2. Effect of *Ziziphus mauritiana* extracts on nitric oxide (NO) production in LPS-stimulated RAW264.7 macrophages. RAW264.7 macrophages were pretreated with *Z. mauritiana* **(A).** methanol [ZM(MeOH)] and **(B).** ethyl acetate [ZM(EA)] extracts at indicated concentrations, then stimulated with LPS (5 µg/mL) for 24 hours. Nitric oxide production was measured in culture supernatants using the Griess assay. Data are mean ± SEM (n = 3).

($p<0.001$) (Figures 1A and C). On the other hand, ZM (Hex) (Figure 1B) shows 50% cell viability at 3 µg/ml and very little cell viability at all the concentrations ranging from 10 to 100 µg/ml. So, ZM (Hex) fraction may be toxic to be used for further evaluation of other parameters.

4.4 Effect of *Ziziphus mauritiana* Extracts on the Production of Nitric Oxide (NO)

The Griess assay was used to estimate the production of nitric oxide. The effect of Ziziphus *mauritiana* extracts (Methanol and ethyl acetate) was examined on nitric oxide production in LPS-activated RAW264.7 cells to determine their anti-inflammatory activity. Both the extracts (ZM (MeOH) and ZM (EA)) (Figures 2A and B) have shown a decrease in concentration of nitric oxide with increasing the extract concentration. Hence, both these extracts displayed a dose-dependent decrease.

4.5 LC-MS Analysis

LC-MS analysis of the ethyl acetate extract of *Z. mauritiana* revealed the presence of 21 compounds in positive mode (Figure 3A) and 8 compounds in negative mode (Figure 3B) as depicted by the LC-MS chromatogram. The identified compounds (Figure 4), along with their respective retention times and molecular masses, are shown in Table 2.

5. Discussion

The current study sought to identify the phytochemical components of leaf extracts from *Z. mauritiana* and assess anti-inflammatory properties. Depending on our study design, the results displayed that the leaf extracts are abundant in bioactive substances, which could be a major factor in the pharmacological effects that have been observed. Numerous secondary metabolites, such as flavonoids, alkaloids, tannins, phenolic saponins, and terpenoids, were found by phytochemical screening (Table 1). *Ziziphus mauritiana* extracts were further screened for cytotoxicity against RAW 264.7 macrophages at 3 to 100µg/mL concentrations. ZM (MeOH) and ZM(EA) have shown more than 50% cell viability at 20 µg/ml (Figures 1A and C), but ZM(Hex) extract (Figure 1B) has shown very little cell viability, indicating the extract to be toxic. ZM (MeOH) and ZM (EA) have shown a dose-dependent decrease in inhibition of nitric oxide production with increasing the concentration of extracts (Figures 2A and B). Since ethyl acetate extract has shown promising results in the quenching of nitric oxide production, it was subjected to LC-MS analysis for the identification of various compounds. The compounds such as L-carnitine, Delphinidin, caffeine, ononin, saccharopine, sinapine, Kaempferol-7-O-neohesperidoside, fortunellin, diosmin, marein etc., (Figure 4) were identified by the

Table 2. Identification of compounds of leaves of *Ziziphus mauritiana* by LC-MS analysis

Retention Time	Score	Compound Name	Ion	Formula	Exact Mass	Observed Mass	Mass Difference
1.16	0.996	D-Carnitine Hydrochloride salt	$[M+H]^+$	$C_7H_{15}NO_3$	161.2	160.2059	0.9941
1.36	0.979	L-Carnitine	Positive	$C_7H_{16}NO_3$	162.113	160.1712	1.9418
3.79	0.757	Harmaline hydrochloride dihydrate	$[M+H]^+$	$C_{13}H_{14}N2_O$	214.28	214.2180	0.062
6.59	0.869	L-Tryptophane	$[M+H]^+$	$C_{11}H_{12}N_2O_2$	204.28	203.2214	1.0086
6.86	0.771	Chalcone	Positive	$C_{15}H_{12}O$	208.088	207.4188	1.0086
7.57	0.82	Delphinidin	Positive	$C_{15}H_{11}O_7$	303.05	303.1756	0.1256
8.23	0.97	3,4-Dihydroxy-L-Phenylalanine	[M+H]+	$C_9H_{11}NO_4$	197.19	197.2200	0.03
8.40	0.881	Caffeine Anhydrous	Positive	$C_8H_{10}N_4O_2$	194.08	197.289	3.209
8.81	0.763	D-Erythro-Dihydrosphingosine	Positive	$C_{18}H_{39}NO_2$	301.298	303.0231	1.7251
10.15	0.766	Ononin	Positive	$C_{22}H_{22}O_9$	430.126	430.9217	0.7957
14.75	0.813	L-saccharopine	Positive	$C_{11}H_{20}N_2O_6$	276.132	277.3530	1.221
15.38	0.588	Sinapine	Positive	$C_{16}H_{24}NO_5$	310.165	309.1284	1.0366
17.16	0.776	Cytidine5′-Diphosphocholine	Positive	$C_{14}H_{26}N_4O_{11}P_2$	488.107	490.1702	2.0632
18.31	0.59	N-Palmitoyl-D-Erythro-Sphingosine	Positive	$C_{34}H_{67}NO_3$	537.512	535.4391	2.0729
21.33	0.712	Kaempferol-7-O-Neohesperidoside	Positive	$C_{27}H_{30}O_{15}$	594.158	593.2306	0.9274
29.35	0.694	MalonylCoenzymeA Lithium Salt	Positive	$C_{24}H_{38}N_7O_{19}P_3S$	853.115	852.2179	0.8971
29.93	0.689	Fortunellin	Positive	$C_{28}H_{32}O_{14}$	592.179	591.2534	0.9256
33.66	0.742	Neodiosmin	Positive	$C_{28}H_{32}O_{15}$	608.174	607.4877	0.6863
34.01	0.844	Diosmin	Positive	$C_{28}H_{32}O_{15}$	608.174	607.3837	0.7903
39.11	0.848	HarmalineHydrochlorideDihydrate	Positive	$C_{13}H_{14}N_2O$	214.11	214.1833	0.733
7.59	0.911	Spiraeoside	Negative	$C_{21}H_{20}O_{12}$	464.095	463.2864	0.8086
13.56	0.755	D-(+)-Melezitose Monohydrate	Negative	$C_{18}H_{32}O_{16}$	504.169	501.5482	2.6208
15.62	0.878	Cytidine-5′-Triphosphate Disodium Salt	Negative	$C_9H_{16}N_3O_{14}P_3$	482.984	485.4872	2.5032
16.22	0.726	Cytidine-5′-Diphosphocholine Sodium Salt Dihydrate, From Yeast, Solid	Negative	$C_{14}H_{26}N_4O_{11}P_2$	488.107	485.4872	2.6198
17.53	0.914	2′-Deoxyuridine-5′- Triphosphate Sodium Salt	Negative	$C_9H_{15}N_2O_{14}P_3$	467.973	471.5077	3.5347
24.90	0.879	Daidzein	[M-H]-	$C_{15}H_{10}O_4$	254.25	255.4293	1.1793
27.20	0.92	Marein	Negative	$C_{21}H_{22}O_{11}$	450.116	451.4575	1.3415
27.45	0.96	Flavanomarein	Negative	$C_{21}H_{22}O_{11}$	450.116	451.4575	1.3415

LC-MS chromatograms in both positive and negative mode (Table 2), demonstrating the diverse presence of phytoconstituents in *Z. mauritiana*. It is worth noting that most of these compounds (diosmin, neodiosmin, Kaempferol-7-O-neohesperidoside spiraeoside, daidzein) belong to the flavonoid class of secondary metabolites that have been found to exhibit significant antioxidant activity and anti-inflammatory activity by suppressing nitric oxide generation, and causing downregulation of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6. It is noteworthy to mention that diosmin and neodiosmin happen to be the most widely studied molecules in relation to their potential to improve vascular inflammation and oxidative stress

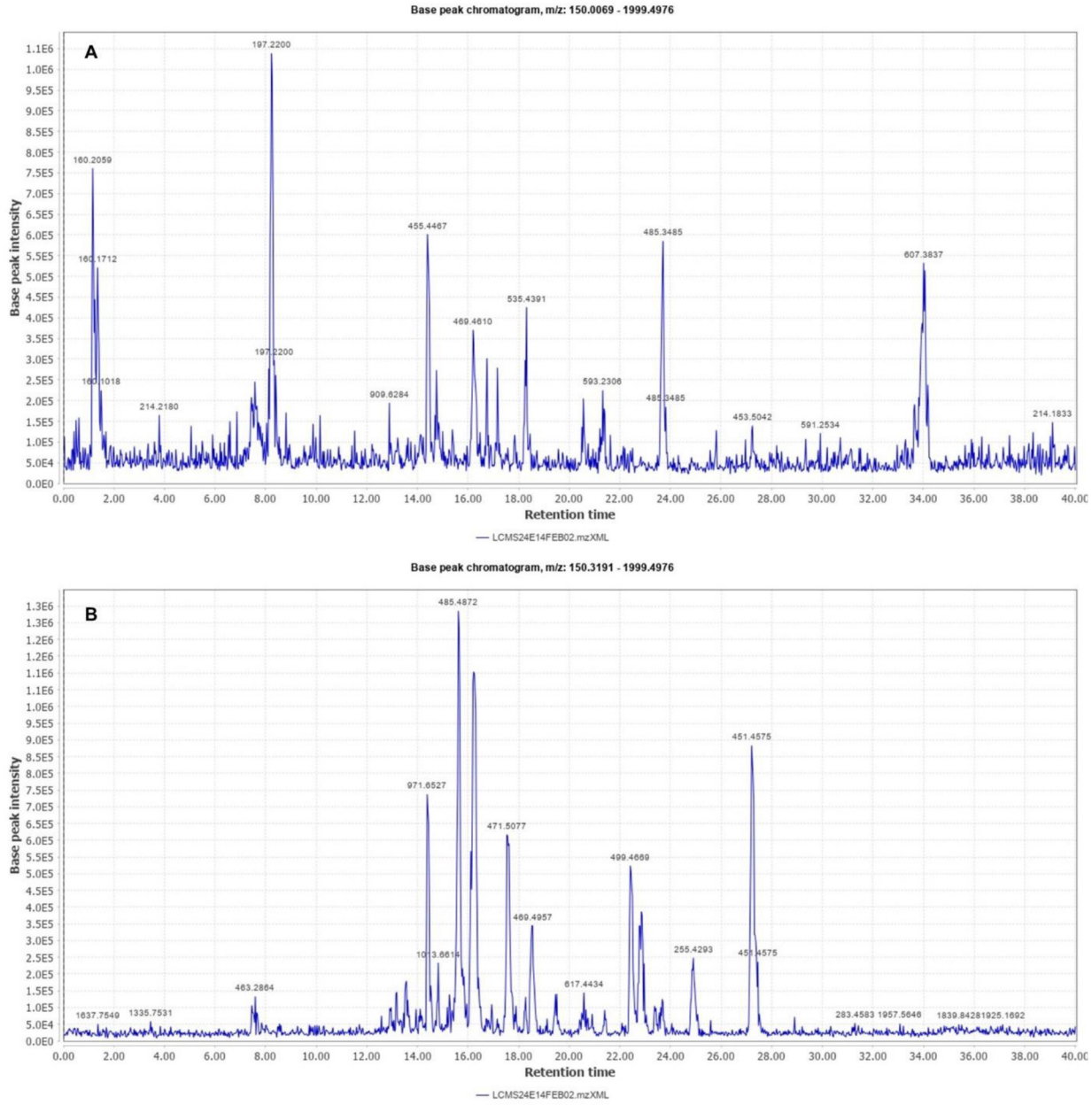


Figure 3. LC-MS base peak chromatograms of ZMEA. Representative base peak chromatograms showing the chemical profile of *Z. Mauritiana* ethyl acetate extract analysed by liquid chromatography-mass spectrometry. **(A).** Positive ion mode (m/z range 150.0069-1999.4976) and **(B).** negative ion mode (m/z range 150.3191-1999.4976). Major peaks are labelled with their corresponding m/z values and retention times. The chromatograms demonstrate the complex phytochemical composition of the extract, with multiple compounds detected across the 40-minute analytical run.

by inhibiting the inflammatory mediators[20]. Thus, inhibition of nitric oxide by ZM (EA) as well as ZM (MeOH) can be ascribed to the combined effects of the identified phytoconstituents, which most likely mediate via a variety of pathways.

Natural remedies have always been the source of therapeutic drugs for the treatment of inflammatory disease due to the presence of secondary metabolites, and it holds because *Z. mauritiana* has also shown the presence of various phytoconstituents[21]. The plant-derived bioactive agents with anti-inflammatory potential have fewer side effects and a better therapeutic effect[22]. Previous studies have shown that nitric oxide inhibition can control inflammation, as nitric

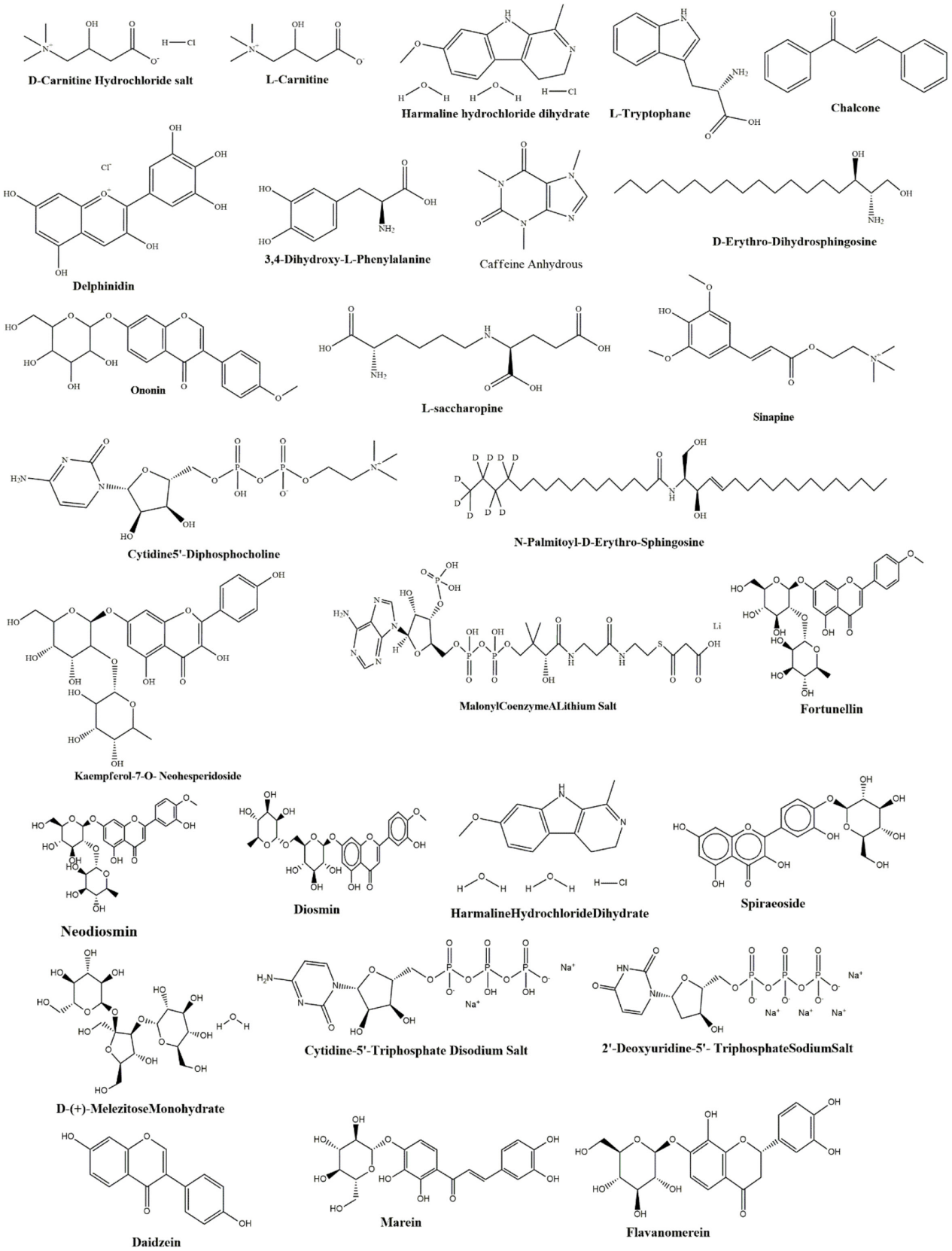


Figure 4. Structural representation of bioactive compounds identified in *Z. mauritiana* ethyl acetate extract.

oxide is the key mediator of inflammation[23]. It is well known that flavonoids and phenolic compounds, in particular, inhibit important mediators and enzymes involved in the inflammatory process, including pro-inflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6), Cyclooxygenase (COX), and Lipoxygenase (LOX)[24]. *Ziziphus mauritiana's* traditional use in the treatment of inflammatory disorders is supported by the abundance of bioactive compounds contained in the plant[9]. Prostaglandins, pro-inflammatory cytokines, nitric oxide, and other important inflammatory mediators can all be modulated by *Z. mauritiana*. According to earlier research, *Z. mauritiana* extracts inhibit the cyclooxygenase and lipoxygenase pathway, which lowers the production of inflammatory eicosanoids[25].

5. Conclusion

In conclusion, phytochemical diversity and LC-MS profile of *Z. mauritiana* leaf extract demonstrate its potential as a natural source of anti-inflammatory compounds. The quenching of nitric oxide production demonstrates the anti-inflammatory activity of *Z. mauritiana* leaf extract. Its use in traditional medicine is supported by the presence of important flavonoids and phenolic compounds, which also encourage further pharmacological research. It is imperative to note that this study has several limitations, which include the lack of understanding of the molecular mechanism of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β), pro-inflammatory enzymes (COX-2, iNOS), and pro-inflammatory signal transduction pathways (NF-κB, MAPK). Findings were limited to *in-vitro* evaluation. Also, the identification of various active phytoconstituents through LC-MS was not further substantiated by their isolation or structural elucidation.

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Author Contribution

Insha Qadir: Conceptualisation, Methodology, Investigation, Data Curation, Formal Analysis, Visualization, Writing original draft. Mubashir Hussain Masoodi: Conceptualisation, Supervision, Resources, Writing review and editing. Both authors contributed to the interpretation of the results, critically reviewed the manuscript, and approved the final version for publication.