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Research article

LC-TOFMS analysis, *in vitro* and *in silico* antioxidant activity on NADPH oxidase, and toxicity assessment of an extract mixture based on *Marrubium vulgare* L. and *Dittrichia viscosa* L.

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Abstract

In this study we explored the chemical composition of the extracts of *Marrubium vulgare* L. and *Dittrichia viscosa* L. and their mixture. The antioxidant activity of these extracts was evaluated using the ABTS assay. Next, we examined the acute toxicity and sub-acute toxicity of the mixture. The molecular docking was conducted to discover the potential of compounds detected on NADPH oxidase. In the extract of *D. viscosa* the results revealed the presence of 4 compounds *p*-coumaric acid, caffeic acid, palmitic acid and stearic acid. The mixture of the two plants showed the presence of 2 fatty acids (palmitic acid and stearic acid), caffeic acid and daidzein. Concerning the antioxidant activity, the mixture (IC_{50}= 0.13 ± 0.04 mg/mL) has the highest effect in the inhibition of ABTS. During 28 days of the test, no sign of toxicity was observed in the rats treated with the two doses (500 and 800 mg/kg). No significant difference was observed between the control group and the groups treated with the two doses in ALP, UREA, and CREA. However, a slight elevation of AST was observed in the group treated with the 500 mg/kg dose. Daidzein, myricetin, and vanillic acid were most active against NADPH oxidase with a glide gscore of -7.13, -6.597, and -6.12 kcal/mol, respectively. In conclusion, both plants are effective as antioxidant agents and their mixture shows no sign of toxicity as an herbal preparation.

Keywords: *M. vulgare*; *D. viscosa*; Mixture; Toxicity; Antioxidant activity; Molecular docking

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1. Introduction

Oxidative stress is recognized as an imbalance case between antioxidants and reactive oxygen species (ROS). This situation is occurred when ROS exceed the normal balance, and these molecules are the principal origin of various human diseases, including cancer, atherosclerosis, chronic obstructive pulmonary disease, and Alzheimer's disease (Forman and Zhang, 2021; Ngenge Tamfu et al., 2022). Thus, plant-derived compounds (PDCs) constitute an important source of natural antioxidants to treat diseases associated with oxidative stress. Accordingly, plant-based antioxidants are of great importance to fight against excess of ROS. Consequently, researchers are focusing on the potential of these substances to reveal their strength against ROS (Atay Balkan et al., 2018). Moreover, PDCs may be involved in the management of many diseases according to several studies due to their antioxidant power (Bhavyasree and Xavier, 2022; Sinda et al., 2022). Therefore, the study of novel PDCs could lead to the discovery and development of new drug destinated to treat oxidative stress (AlMousa et al., 2022).

Currently, plants and PDCs are attracting attention as potential drugs and a novel therapeutic approach for the management of several diseases, constituting approximatively 25% of conventional medicines (Vilkickyte

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et al., 2022). However, plants produce a wide range of compounds, some of which may be beneficial or potentially toxic to mankind (Kale et al., 2019). Furthermore, it is known that the usage of pharmaceutical drugs may be therapeutic at one dose and toxic at another. Thus, it is necessary to ensure the safety of new PDCs-based drugs by studying and investigating the toxicity profiles of herbs claimed to be beneficial to humans and animals before deciding to use them (da Silva Moreira et al., 2019; Zhao et al., 2020). Accordingly, to study the safety of plant extracts and compounds, usually, the main tests used for this purpose are acute and sub-acute toxicity. For this reason, studies mainly start with an acute toxicity test, which is the first step to evaluate the adverse effects of PDCs or plant extracts within 14 days after administration of single or multiple doses (Alelign et al., 2020; Rhiouani et al., 2008). Regarding subacute toxicity, some studies administered extracts at doses of 2000 and 5000 mg/kg for 28 days (Alelign et al., 2020).

Marrubium vulgare L. (Lamiaceae), and *Dittrichia viscosa* L. (Asteraceae), are two Mediterranean medicinal plants known to possess high concentrations of phytochemical compounds such as phenolic acids, flavonoids, tannins, monoterpenoids and sesquiterpenoids, and are reported as antioxidant, antimicrobial, anti-inflammatory, analgesic, anti-diabetic and for wound healing (Amessis-Ouchemoukh et al., 2014; Amri et al., 2017; Chahmi et al., 2015; Danino et al., 2009; Fathiazad et al., 2016; Gharred et al., 2019; Grauso et al., 2020; Haoui et al., 2015; Mahmoudi et al., 2016; Rhimi et al., 2017; S et al., 2002). In our previous studies, we evaluated the antioxidant, antimicrobial, insecticidal, analgesic, anti-inflammatory and healing activity of these two plants in Morocco (Mssillou et al., 2022b, 2022a, 2021a, 2021b). Although individual medicinal plants are effective against several diseases, more emphasis is placed on promoting polyherbal formulations because of their efficacy and minimal occurrence of side effects (Dev et al., 2019; Talekar et al., 2017).

Here in this study, we try to explore the individual effectiveness of *M. vulgare*, and *D. viscosa* against oxidative stress using ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) assay and the potential application of these plants in mixture to treat oxidative stress. Also, this study aims to identify the chemical components of these plants and their combination using LC-TOFMS. Next, we investigated the acute and subacute toxicity of their mixture for 14 and 28 days, respectively. Finally, we performed an *in silico* study to predict the importance of our compounds against NADPH oxidase.

2. Materials and Methods

2.1. Plants material and extraction

The plants used in this study were collected around the city of Fez in Morocco (34°03′41.3″ N 5°03′45.5″ W) in December 2020. *D. viscosa* and *M. vulgare* were brought to the laboratory and identification was carried out by Pr. Bari Amina, a professor at Sidi Mohamed Ben Abdellah university. A voucher sample was specified for each plant: *D. viscosa* (DV20201214) and *M. vulgare* (RM001617). Leaves were cut from fresh plants and left to dry for a week in the laboratory; then the leaves were ground into fine particles using a Waring® blender. To prepare the hydro-ethanolic extracts, 30 g of each plant was macerated using 210 mL of pure ethanol (99%) and 90 mL of distilled water at a rate of 70% (v/v) and 30% (v/v), respectively. Maceration was performed for 72 h at laboratory ambient temperature. The macerates were filtered using a Whatman paper No°1 and evaporated by a rotary evaporator at 37 °C. The mixture was prepared by mixing the hydro-ethanolic extract of *D. viscosa* and *M. vulgare* at a rate of 50% (*w/w*).

2.2. LC-TOFMS analysis

LC–TOFMS analysis was performed as previously described by Ojanperä et al., (2006) with some modifications. Samples were dissolved in 1 mL of ACN and vortexed for 10 s. Thereafter, they were let stand for 30 min and then vortexed again for 10 s. 200 µL of extract was taken and diluted with 2 mL of ACN. The resulting solutions were filtered (0.45 um CLARIFY-PTFE 13 Syringe filter) and added to the HPLC-vials. A blank from pure ACN was treated in the similar way as the samples. The acetonitrile (ACN) used in all these experiments was Honeywell/Riedel-de Haën Chromasolv HPLC Gradient grade. The instruments were Agilent 1200 HPLC equipped with diode array detector online coupled to Bruker Esquire 3000+ ion trap mass spectrometer with electrospray ionization.

Eluent A for HPLC was Milli-Q Water with 0.1 % (v/v) Formic acid. Eluent B was Acetonitrile with 0.1 % (v/v) Formic acid (AnalaR NORMAPUR). The gradient was as follows: 1 min 1% B, 30 min linear gradient form 1% B to 99% B, hold 99% B until 40. The injection volume was 20 µL. Eluent flow rate was 0.7 mL/min. Column temperature was set at 30 °C. Th column used was Agilent C18, 4.6 mm ID, 250 mm length.

The mass analyzer was a Bruker Daltonics MicroTOF MS with an electrospray ionization (ESI) source and a six-port divert valve (Bremen, Germany). The instrument's controls were performed with HyStar 3.1 and micrOTOF Control 1.1 (Bruker Daltonics) software. The nominal resolution of the instrument was 10,000. The instrument was operated in negative ion mode at m/z 50–1000. The capillary voltage was 5000 V, and the capillary exit was -90 V. The nebulizer gas pressure was 0.7 bar, and the drying gas flow was 6.0 L/min. The drying temperature was 250 °C. The transfer time was 8.0 µs, and the hexapole RF was 120 Vpp.

2.3. ABTS (2,2'-Azinobis (3-Ethylbenzothiazoline-6-Sulphonic Acid)) assay

This test was performed using the method described by Shah and Modi, (2020). In purpose to prepare the solution of

$ABTS^{\bullet+}$, the same volume of aqueous solution of ABTS (7 mM) was mixed with 2.45 mM aqueous solution of potassium persulfate. The solution was kept in the dark for 12-16 hrs at room temperature. Next, the solution was diluted to obtain an absorbance of 0.70 ± 0.02 at 734 nm. Then, 2.0 mL of the solution was mixed with 1 mL of the extract of our plants and their mixture at different concentrations. The incubation was conducted at room temperature for 10 min in the dark. The absorbance was measured against a blank at 734 nm using a spectrophotometer. BHT was used as a standard. The percentage of scavenging activity of each extract on $ABTS^{\bullet+}$ was calculated as percentage of inhibition (I%) using the following equation (1):

$$I\% = [(A_0-A_s)/ A_0] \times 100 \quad (1)$$

Where A_0 is the absorption of control and A_s is the absorption of the tested extract solution.

2.4. Toxicity assessment

2.4.1. Animal

Swiss albino mice weighing between 120 and 140 g were used to perform the study. Animals were obtained from the pharmacology laboratory of the Faculty of Sciences Dhar El-Mehraz, department of biology, Fez-Morocco. The animals were divided into groups with 5 in each and kept in cages for acclimatization under laboratory conditions for 7 days (temperature 20-22°C, photoperiod of 12 hours of light) (Bourhia et al., 2019). The procedures used in the current research are in accordance with the internationally accepted guidelines for care and use laboratory animals. The Animal Ethics Review Committee of the Faculty of Sciences of Fez, Morocco, reviewed and approved this study.

2.4.2. Preparation of test Solutions

The extract was dissolved in distilled water, then, the mixture was homogenized by stirring (3-5 min) with a magnetic stirrer. The solution obtained was kept in a closed plastic jar and placed in a refrigerator after each use. The volume of solution chosen to be administered to animals was determined by the following formula (2):

$$V = \frac{D \times P}{C} \quad (2)$$

where V= volume of solution chosen to be administered (mL); D=dose (mg/kg); P= weight of animal (kg); C= concentration of solution chosen to be administered (mg/mL).

2.4.3. Acute oral toxicity

This experimental study was conceptualized according to the guideline 423 (OECD, 2001). Animals were divided into 4 groups with 5 mice in each. Doses of 1000 and 2000 mg/kg were chosen to be orally administered to treated mice, meanwhile the control group received physiological solution (vehicle) after fasting for 18 h under acute toxicity conditions according to the earlier protocol. Afterward, animals were placed under monitoring for recording immediate clinical symptoms for 14 days.

2.4.4. Subacute oral toxicity

Subacute toxicity study was conducted according to OECD, 2008, Guideline No. 407 (OECD, 2008). After dividing the animals into 3 groups of 5 mice each, treated groups received the mixture daily (500 mg/kg (group II) and 800 mg/kg (group III)), meanwhile by the control group (group I) received physiological solution (vehicle) for 28 days. Signs and symptoms of toxicity were observed for 28 days, and body weight was measured weekly. On day 28, the animals were sacrificed after anesthesia for the collection of blood and organ.

2.4.5. Analysis of serum biochemistry

Biochemical analysis of serum was performed at the end of the experiment. The blood was transferred into heparin tubes then centrifuged at 3500 rpm for 10 minutes. The serum was recovered and stored into tubes for analysis. Creatinine (CREA), urea (UREA), alkaline phosphatase (ALP), aspartate aminotransferase (ASAT), and alanine aminotransferase (ALAT) were evaluated using an automated analyzer.

2.5. Molecular docking

2.5.1. Ligand preparation

To evaluate the antioxidant activity of the compounds identified in extracts and mixture of *M. vulgare* and *D. viscosa*, all compounds were downloaded from the PUBCHEM platform in SDF format. Then, these molecules were prepared using the LigPrep subsystem in Maestro 11.5 from Schrödinger suite (version 2018, Schrödinger). The minimization process employed the OPLS3 force field, and the generation of all possible ionic states was achieved at a target pH of 7.2 ± 2 using Epik. Additionally, for each ligand, plausible stereo isomers and lower-energy ring conformations were generated (Ouahabi et al., 2023).

2.5.2. Receptor preparation

The structure of human NADPH oxidase (PDB ID: 2CDU) (Herrera-Calderon et al., 2021) was downloaded from PDB data Bank (RCSB) and prepared using protein preparation wizard of the Schrödinger suite, involving tasks such as assigning bond orders, adding hydrogen, filling empty side chains and loops with PRIME, and ultimately removing all water from the crystal structures. Following the optimization of these crystal structures, a restrained minimization with a root mean square deviation (RMSD) of 0.3 Å was performed using the OPLS3 force field (Abdali et al., 2023).

2.5.3. Grid generation and molecular docking

The minimized protein structure was then utilized to generate grids through the "Receptor Grid Generation" panel. A grid was established with default parameters, including a Van Der Waals scaling factor of 1.00 and a charging cut-off value of 0.25, in accordance with the

OPLS3 force field. A cubic receptor grid box with dimensions of 20 Å × 20 Å × 20 Å was centered on the selected co-crystallized ligand. The molecular docking assay utilized the Standard Precision (SP) scoring method of Glide, integrated into the Schrödinger suite-Maestro version 12.5 (Amrati et al., 2023).

2.6. Statistical analysis

All tests were conducted in triplicates, and values were expressed as mean ± standard deviation. The statistical analysis of the results was performed using GraphPad Prism software (version 8), by one-way analysis of variance (ANOVA), followed by the Tuckey test, and differences at $p<0.05$ were considered significant.

3. Results and discussion

3.1. LC-TOFMS analysis

The extract of each plant and the mixture were analyzed by CL-TOFSM and the results are shown in the chromatograms (figure 1, 2 and 3) and in tables 1, 2 and 3.

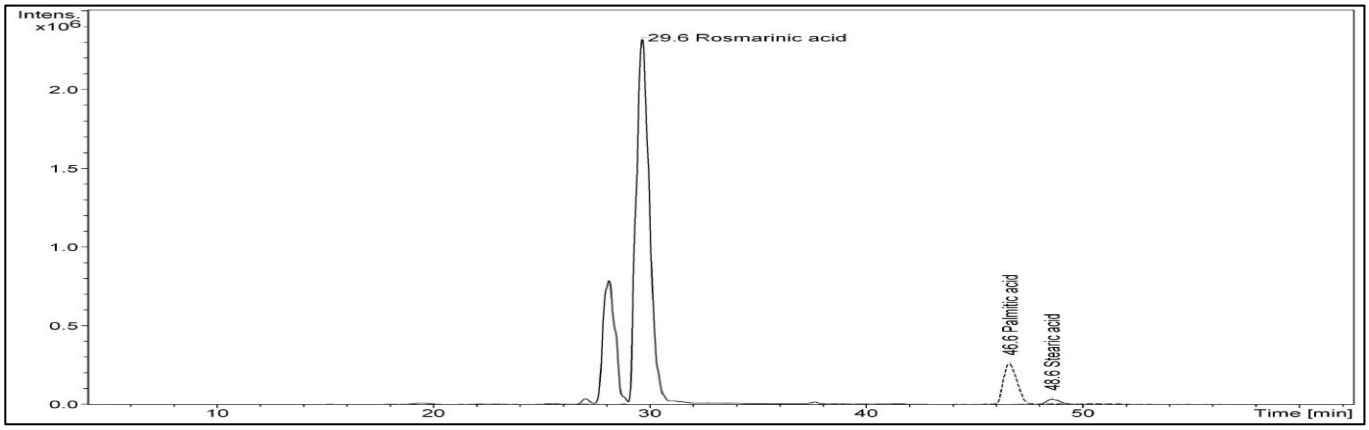

Figure 1: Chromatogram of the hydro-ethanolic extract of *D. viscosa*.

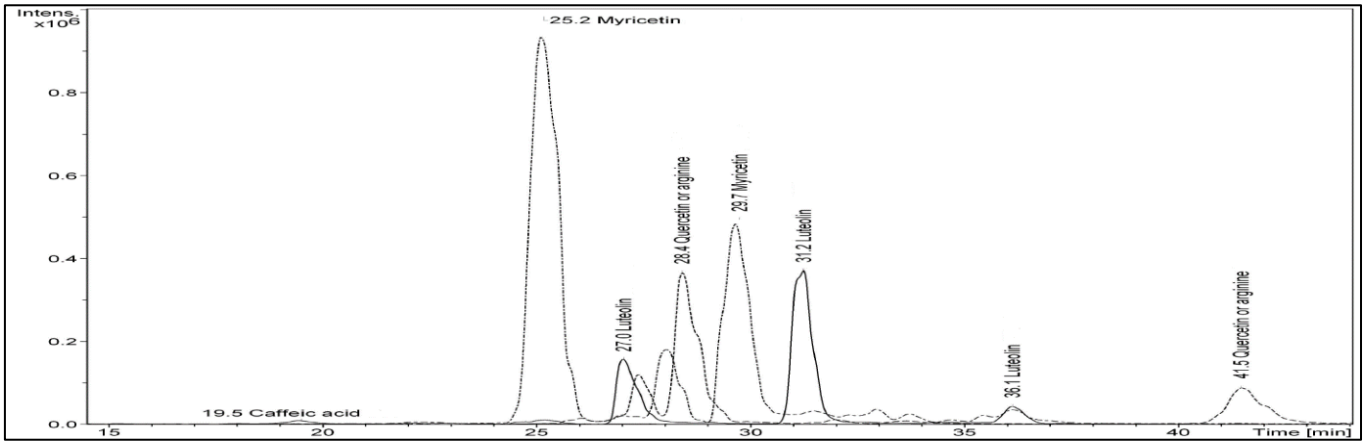

Figure 1: continued.

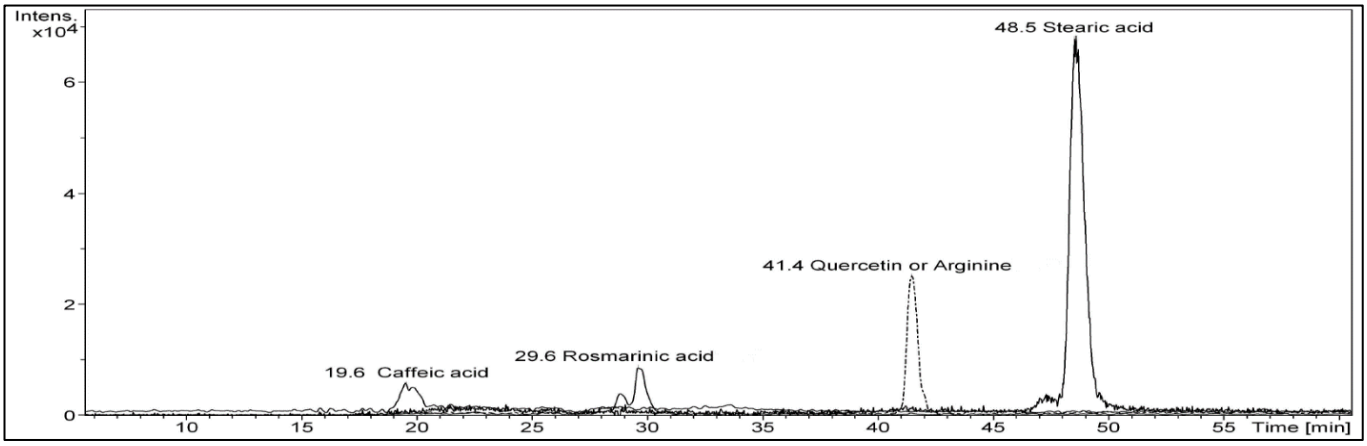

Figure 2: Chromatogram of the hydro-ethanolic extract of *M. vulgare.*

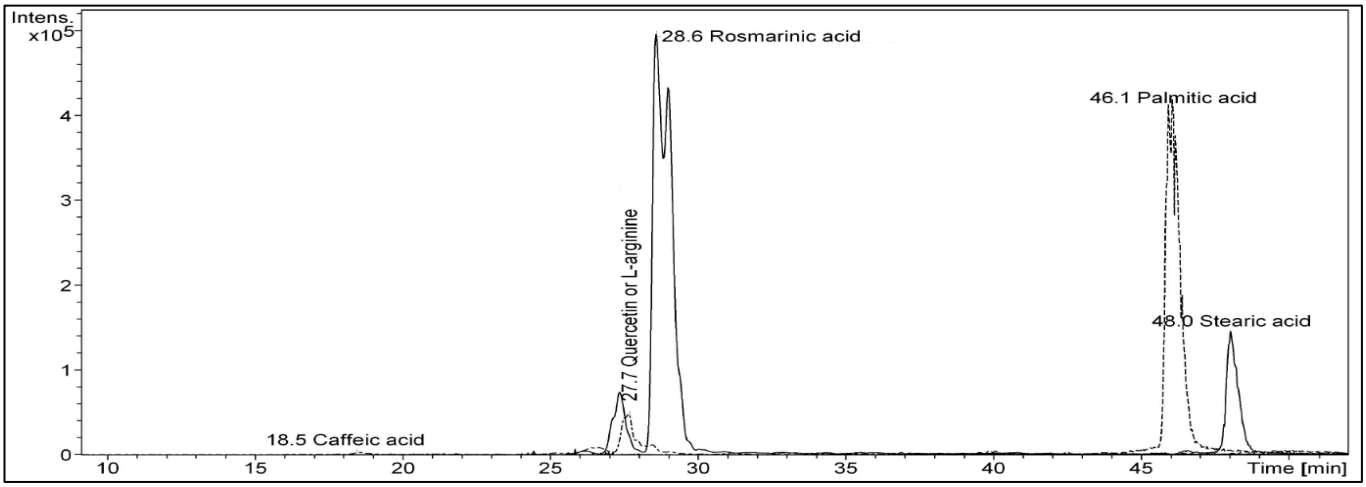

Figure 3: Chromatogram of the mixture of *M. vulgare* and *D. viscosa*.

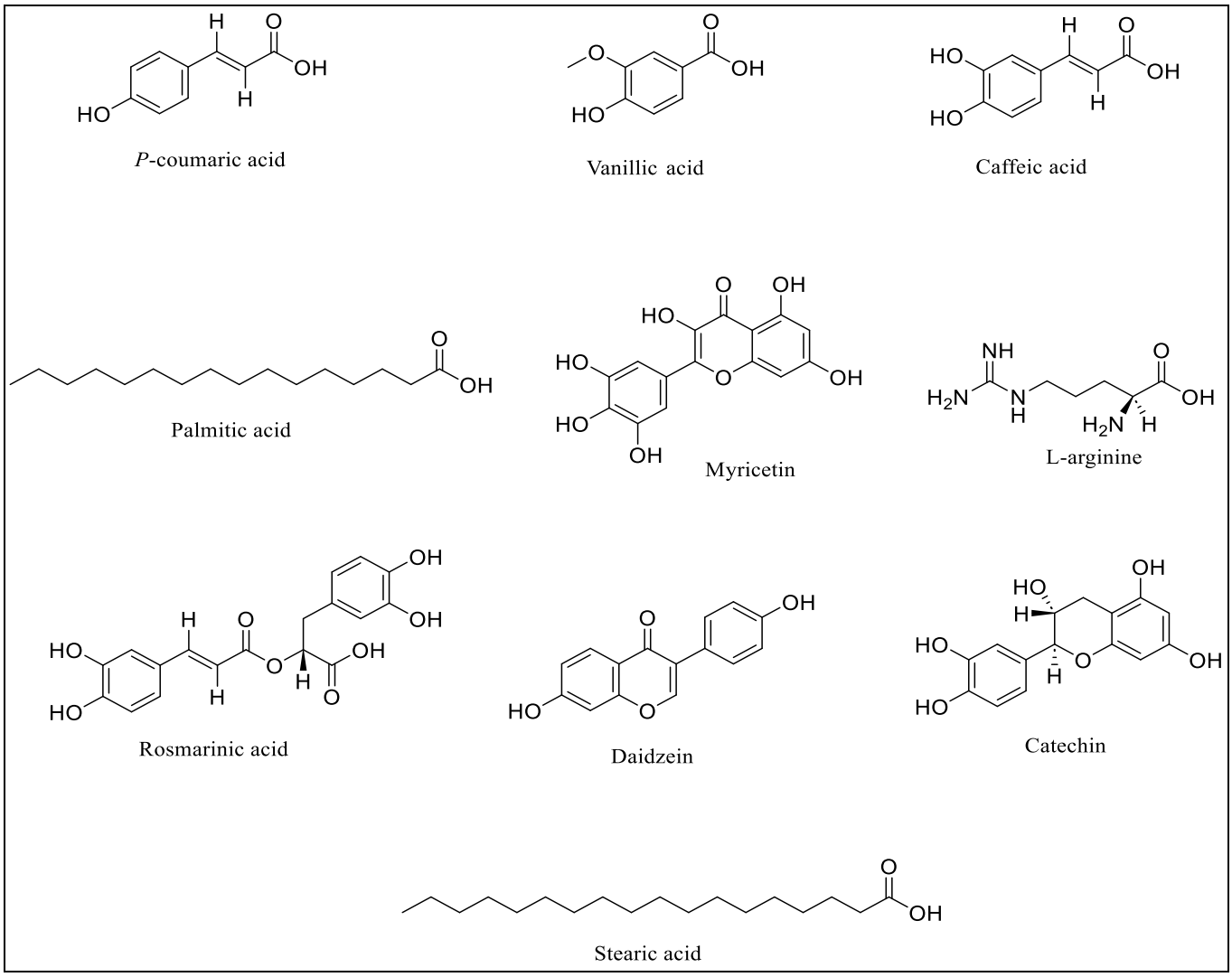

Figure 4: Chemical structure of compounds identified in both plants and their mixture.

In the extract of *D. viscosa*, the results revealed the presence of 4 compounds by comparison of mass and retention time with those of the standards. These compounds are *p*-coumaric acid, caffeic acid, palmitic acid and stearic acid (Table 1). The lowest ppm errors were recorded for palmitic acid (8.79) and stearic acid (8.66). Other molecules have been identified in *D. viscosa* by the molecular mass in the absence of standards, which are vanillic acid, L-arginine, myricetin and rosmarinic acid.

Regarding *M. vulgare*, only 3 compounds were identified by mass and retention time with standards, these are caffeic acid, stearic acid and palmitic acid which gives the lowest ppm error (-5.67). Additionally, rosmarinic acid was revealed by molecular weight (Figure 2 and Table 2).

The mixture showed the presence of 2 fatty acids (palmitic acid and stearic acid), caffeic acid and daidzein. Two other compounds were identified by their molecular mass, namely catechin and rosmarinic acid. Unfortunately, due to the lack of standards we have not identified all the compounds contained in these extracts.

The use of time-of-flight spectrometry allows the identification of interesting compounds in the extract of these two plants and their mixture. *M. vulgare* and *D. viscosa* are well known for their richness in phytochemicals, such as monoterpenes (Grauso et al., 2020; Khanavi et al., 2005), sesquiterpenes (Hamdaoui et al., 2013; Santos et al., 2016), phenolic acids (Boulila et al., 2015; Rhimi et al., 2019), flavonoids (Grauso et al., 2020; Hayat et al., 2020), and fatty acids (Ohtera et al., 2013; Rhimi et al., 2018).

P-coumaric acid and caffeic acid have already been identified in *D. viscosa* (López-Orenes et al., 2018; Mrid et al., 2022; Mssillou et al., 2022b). Several previous studies have proven the antioxidant and antimicrobial effects of these two compounds (Gülçin, 2006; Kiliç and Yeşiloğlu, 2013; Sato et al., 2011; Stojković et al., 2013). Pragasam et al., (2013) reported the immunomodulatory and anti-inflammatory effect of *p*-coumaric acid. Also, caffeic acid has been proved for its anti-inflammatory, analgesic and ulcerogenic activity (Al-Ostoot et al., 2021). Caffeic acid has already been identified in *M. vulgare* (Pukalskas et al., 2012).

Palmitic acid has already been identified in *M. vulgare* (Ohtera et al., 2013), and *D. viscosa* (Mahmoudi et al., 2016). Palmitic acid is a saturated fatty acid naturally present in vegetable oil (van Rooijen and Mensink, 2020). This compound is known to possess antioxidant and antimicrobial properties (Davoodbasha et al., 2018; Ngamakeue and Chitprasert, 2016; Prasath et al., 2021). Palmitic acid is known for its inhibitory effects on prostate cancer cell proliferation and metastasis (Zhu et al., 2021). Some studies reported that this compound can acts as antiviral, anti-inflammatory, analgesic and lipid metabolism regulatory activities (Librán-Pérez et al., 2019; Mayneris-Perxachs et al., 2014; Sawada et al., 2012). In the same context, stearic acid is a fatty acid known for its bioactive interest. The study conducted by Wang et al., (2007) revealed that stearic acid has a protective role of cortical neurons against glutamate or H_2O_2 damage. In a study conducted on stearic acid, it has been revealed that this compound contributes in the elevation of interleukin-10 in hepatocytes (Nishitani et al., 2007). Moreover, it has been confirmed that saturated fatty acids have lower melting points and have clearly a good oxidative stability (Jubie et al., 2012).

Daidzein is the only compound identified in the mixture and not detected in the extracts of the two plants. This compound has already proven its antioxidant, antimicrobial, and anti-inflammatory power (Buchmann et al., 2022; Foti et al., 2005; Liu et al., 2009).

Table 1: Phytochemical compounds identified in *D. viscosa*.

Compounds	Formula	Calculated mass [M]	Calculated mass [M-H]-	Observed mass [M-H]-	Error ppm	RT	RTs
P-coumaric acid	$C_9H_8O_3$	164.047	163.040	163.083	-265.16	20.6-21.6	20.6-21.7
Vanillic acid	$C_8H_8O_4$	168.042	167.034	167.035	-1.96	21.1-21.4	N.A
Caffeic acid	$C_9H_8O_4$	180.042	179.035	179.032	14.70	18.8-20.1	19.0-20.4
Palmitic acid	$C_{16}H_{32}O_2$	256.240	255.233	255.230	8.79	46.1-47.2	46.2-47.3
Stearic acid	$C_{18}H_{36}O_2$	284.271	283.264	283.261	8.66	48.1-49.2	48.1-49.4
L-arginine	$C_{16}H_{14}O_6$	302.079	301.071	301.066	18.44	28.1-28.9	N.A
Myricetin	$C_{15}H_{10}O_8$	318.037	317.030	317.060	-93.74	-	N.A
Rosmarinic acid	$C_{18}H_{16}O_8$	360.084	359.077	359.077	-1.81	29.2-30.2	N.A

RT: retention time of the compound; RTs: retention time of the standard; N.A: not available

Table 2: Phytochemical compounds identified in *M. vulgare*.

Compounds	Formula	Calculated mass [M]	Calculated mass [M-H]-	Observed mass [M-H]-	Error ppm	RT	RTs
Caffeic acid	$C_9H_8O_4$	180.042	179.034	179.033	7.89	19.1-20.3	19.0-20.4
Palmitic acid	$C_{16}H_{32}O_2$	256.240	255.233	255.234	-5.67	46.3-47.1	46.2-47.3
Stearic acid	$C_{18}H_{36}O_2$	284.271	283.264	283.262	6.76	48.1-49.2	48.1-49.4
Rosmarinic acid	$C_{18}H_{16}O_8$	360.084	359.077	359.076	3.12	28.7-30.2	N.A

RT: retention time of the compound; RTs: retention time of the standard; N.A: not available

Table 3: Phytochemical compounds identified in the mixture.

Compounds	Formula	Calculated mass [M]	Calculated mass [M-H]-	Observed mass [M-H]-	Error ppm	RT	RTs
Caffeic acid	C9H8O4	180.042	179.034	179.033	9,12	18.1-19.1	19.0-204
Daidzein	C15H10O4	254.057	253.0506	253.145	-373.59	24.7-25.5	24.8-25.6
Palmitic acid	C16H32O2	256.240	255.233	255.234	-6.92	45.7-466	46.2-47.3
Stearic acid	C18H36O2	284.271	283.264	283.265	-5.81	47.7-48.5	48.1-49.4
Catechin	C15H14O6	290.079	289.071	289.074	-10.82	-	N.A
Rosmarinic acid	C18H16O8	360.084	359.077	359.083	-16.46	28.4-29.2	N.A

RT: retention time of the compound; RTs: retention time of the standard; N.A: not available

3.2. ABTS assay

The antioxidant activity of each plant and their mixture was performed using ABTS test. The results are presented in Figure 5. As shown in this figure, the mixture (IC_{50}=0.13±0.04 mg/mL) has the highest effect in the inhibition of ABTS followed by *M. vulgare* (IC_{50}=0.143±0.03 mg/mL) and *D. viscosa* (IC_{50}=0.162±0.05 mg/mL). Both plants and the mixture have not exceeded the potential of BHT (IC_{50}=0.09±0.05 mg/mL) which showed the highest antioxidant activity.

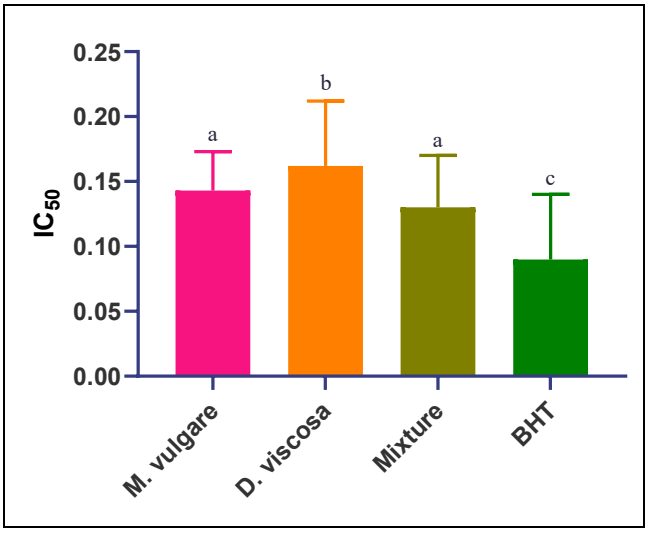

Figure 5: IC_{50} of the hydro-ethanolic extract of *M. vulgare*, *D. viscosa* and their mixture compared to the standard BHT. Different letters above bars indicate significant difference at $p<0.05$.

Several studies have evaluated the potential of these plants, using many tests as DPPH and FRAP. In our previous research we have evaluated the effect of these plants and we have proved their effects as good antioxidant using DPPH, FRAP and total antioxidant capacity (Mssillou et al., 2021a, 2021b). In the present study the test of ABTS is a very interesting way to prove that both plants have a high antioxidant activity and their mixture stimulate their effect.

3.3. Acute toxicity

In acute toxicity, behavioral and body weight changes are the major signs involved in the assessment of toxicity (Ngaffo et al., 2022). For 14 days and even a few hours after the administration of the mixture the rats did not show any sign of toxicity for the 2 doses administered (1000 and 2000 mg/kg) and no change in behavior and mortality was observed. Figure 6 shows that there is no significant change in the body weight of the rats treated with the two doses compared to the control (NaCl (0.9%)). In addition, it has been observed that the administration of the mixture at 1000 mg/kg and 2000 mg/kg does not produce diarrhea, immobility, excitation, writhing and does not affect food and water consumption.

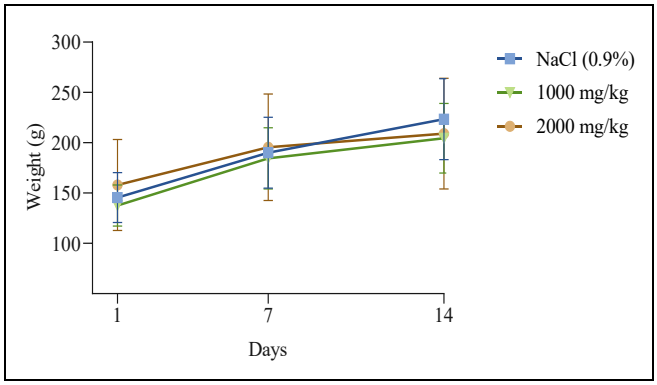

Figure 6: Effects of the administration of the mixture at 1000 and 2000 mg/Kg on the body weight of rats for 14 days compared to the control (NaCl (0.9%)).

In the study conducted by Paula de Oliveira et al., (2011), they suggested that the acute oral toxicity of *M. vulgare* is greater than 2000 mg/kg. concerning *D. viscosa*, the study conducted by Martin et al., (1988) showed that when determining the LD_{50} of the methanolic extract of this plant, it showed the low toxicity obtained in metabolic cage experiments. The repeated administration of the methanolic extract of *D. viscosa* at 500 mg/kg did not cause abnormal symptoms concerning animal weight, on the contrary, the animals treated gained body weight as well as weight of faeces compared to controls (Martin et al., 1988).

In another study conducted on the extract of the leaves of *D. viscosa*, the results showed that the 400 and 800 mg/kg doses have no acute toxicity (Ouahchia et al., 2017). Another study found the petroleum ether extract was the most toxic (626 mg/kg) and the aqueous extract has been free of acute toxic effects up to 8000 mg/kg body weight (Al-Dissi et al., 2001).

3.4. Subacute toxicity

3.4.1. Clinical observations and body weight

During 28 days of the test, no sign of toxicity was observed either for the rats treated with the two doses (500 and 800 mg/kg) or the control group (NaCl (0.9%)). In addition, the administration of the doses did not affect the feeding behavior of the rats, and no mortality was recorded during the test period.

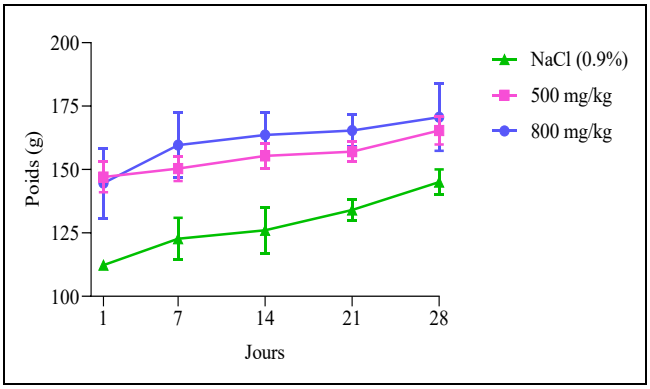

Figure 7: Progression of the weight of the rats receiving two doses (500 and 800 mg/kg) of the mixture for 28 days compared to the control (NaCl (0.9%)).

The change in the weight of the rats is presented in figure 7. In particular, we recorded a progression of the weight in the two groups treated and the control, and no significant difference was obtained for the doses and the control groups because the evolution of the weight was in parallel for the three groups. Weight data and observation of behavior of rats indicate that the administration of the mixture for 28 days has no toxic effect on rats.

3.4.2. Effects of the mixture on Biochemical Parameters of serum

Figure 8 presents the results of the biochemical parameters of the serum of the groups treated with the mixture (500 and 800 mg/Kg) and the control (NaCl (0.9%)). According to this figure, a slight elevation of ASAT was observed in the group treated with the dose of 500 mg/kg. After the analysis, a slight decrease was recorded in the group treated with the dose of 800 mg/kg for ALAT compared to the control. No significant difference was observed between the control group and the groups treated with the two doses in ALP, UREA, and CREA. These results could confirm the harmlessness of the mixture at these two doses.

ALAT and ASAT, are enzymes located in the cytoplasm of hepatocytes, an elevated levels in the bloodstream, signaling the existence of liver injury (Awe and Banjoko, 2013; Slighoua et al., 2021). Moreover, elevated levels of these enzymes are associated with hepatitis, liver necrosis, and liver toxicity, rendering them valuable in diagnosing liver disorders (Mia et al., 2022). A decrease in ALTA levels could imply that the combination exhibits hepatoprotective characteristics (Iheagwam et al., 2021). ASAT is found in various tissues such as myocardium, skeletal muscle, brain, kidney, and liver. Whereas, ALT is primarily concentrated in the liver (Yi-Chen et al., 2018). Elevated levels of ALP have been documented in connection with liver damage or myocardial infarction (Witthawaskul et al., 2003). In our study, no significant difference was observed in ALP between the treated groups and the control. The levels of CREA and UREA constitute important indicators of kidney function. An increase of these indicators often correlate with evident damage to functioning nephrons (Lameire et al., 2005; Yi-Chen et al., 2018). Apart from the slight increase in ASAT in this study, other serum parameters indicate that oral administration of the mixture has no toxic effect.

3.5. Molecular docking

The inhibition of NADPH, a crucial cofactor in various enzymatic reactions contributing to cellular antioxidant defenses, plays a vital role in regulating cellular redox balance and antioxidant defense mechanisms.

Daidzein, myricetin, and vanillic acid were the molecules most active against NADPH oxidase with a glide gscore of -7.13, -6.597, and -6.12 kcal/mol,a glide emodel of -60.845, -67.752, and -45.821 kcal/mol, and a glide energy -40.807, -47.249, and -27.679 kcal/mol. While Stearic acid and Palmitic acid showed the weakest inhibitory effect against NADPH oxidase (Table 4).

Table 4: Docking results in ligands in active site of NADPH oxidase.

Compounds	Glide gscore (Kcal/mol)	Glide emodel (Kcal/mol)	Glide energy (Kcal/mol)
	NADPH Oxidase (PDB: 2CDU)		
Myricetin	-6.597	-67.752	-47.249
Vanillic acid	-6.12	-45.821	-27.679
Catechin	-5.55	-54.058	-40.233
Caffeic acid	-5.484	-47.45	-33.437
Rosmarinic acid	-5.215	-63.574	-45.588
P-coumaric acid	-5.017	-44.68	-31.018
Daidzein	-7.13	-60.845	-40.807
L-arginine	-2.054	-17.892	-16.176
Stearic acid	-0.552	-33.55	-36.675
Palmitic acid	-0.006	-32.986	-35.35

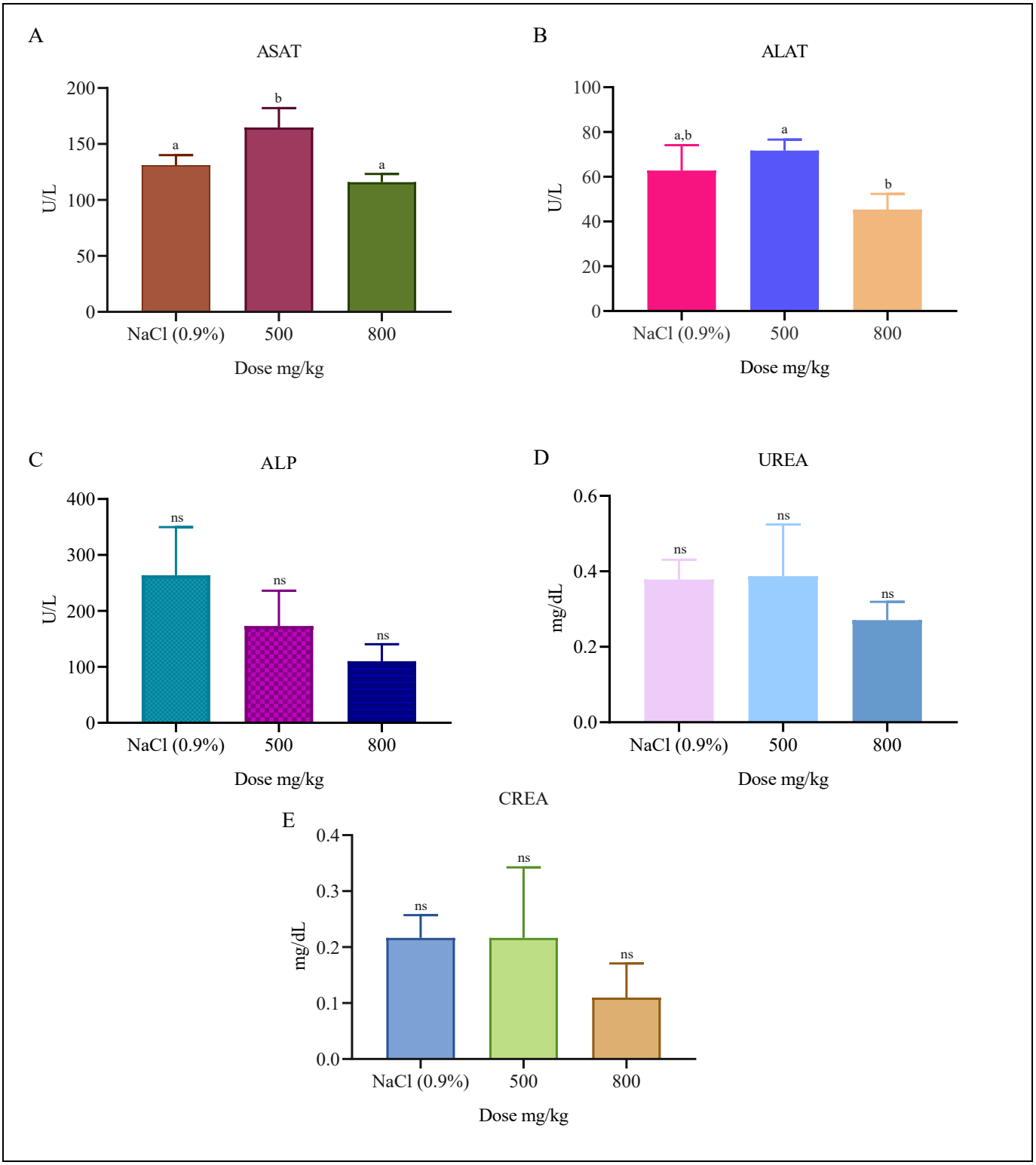

Figure 8: Effect of administration of the mixture on serum biochemical parameters: (A) AST, (B) ALT, (C) ALP, (D) UREA, (E) CREA.

In the active site of NADPH oxidase, Daidzein established a single hydrogen bond with residue PRO 298 and a salt bridge with residue LYS 187 (Figure 9A and 10A). While myricetin established two hydrogen bonds with residues ASP 179 and VAL 214 and one PI-cation bond with residue LYS 187 (Figure 9B and 10B).

In this same active site of NADPH oxidase, vanillic acid established a single bond with residue VAL 214 and salt bridge with residue LYS 213 (Figure 9C and 10C).

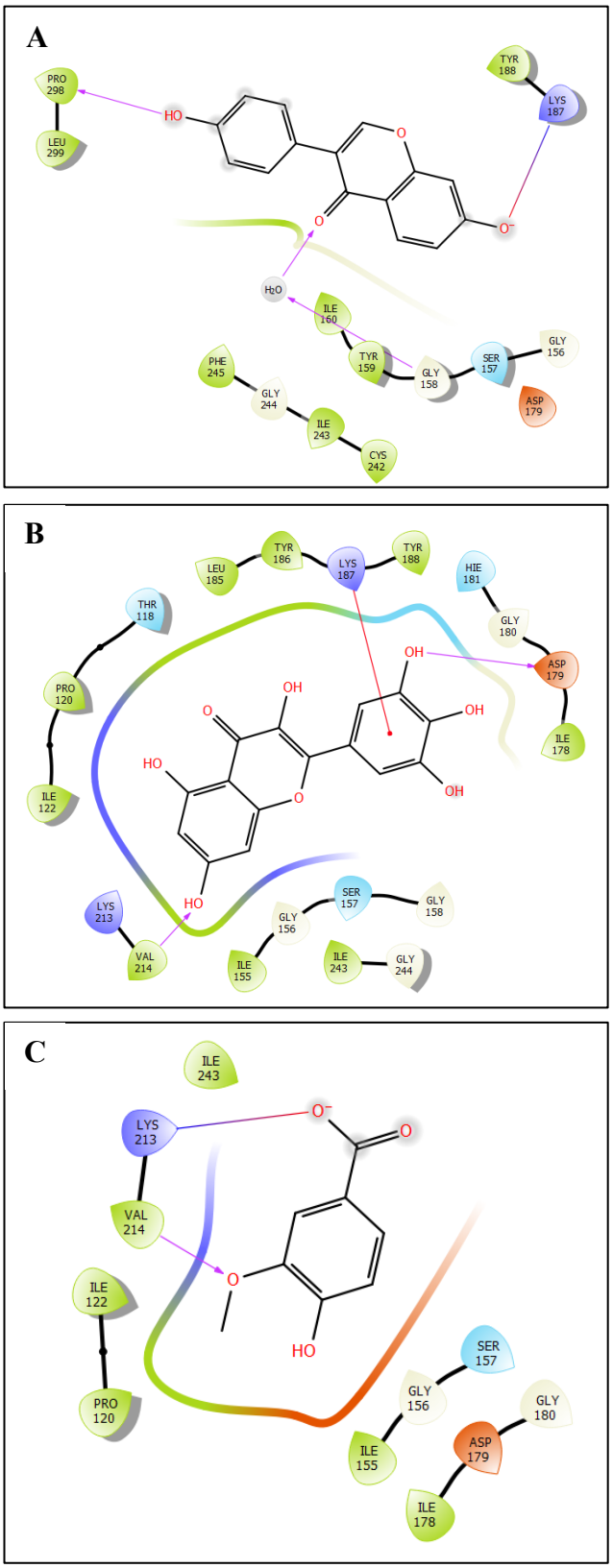

Figure 9: The 2D viewer of ligands interactions with the active site of NADPH oxidase. **A:** Daidzein; **B:** myricetin; **C:** vanillic acid

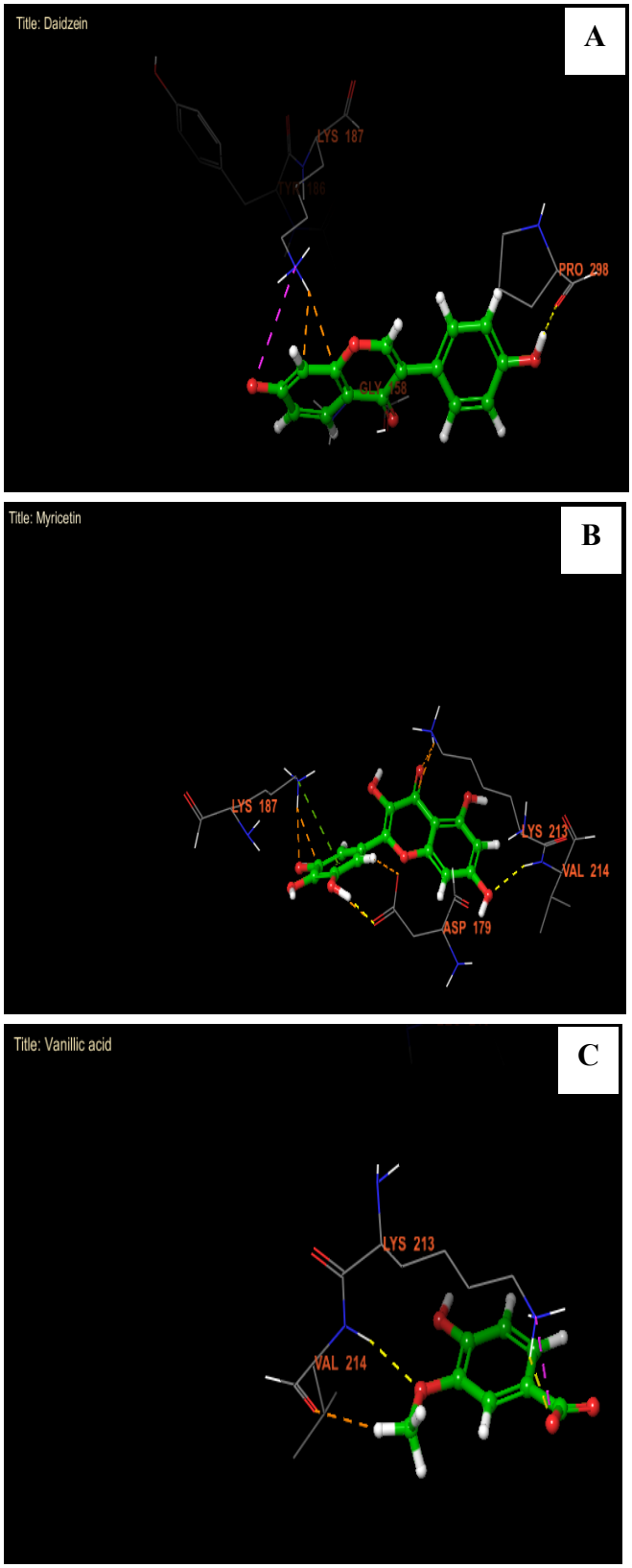

Figure 10: The 3D viewer of ligands interactions with the active site of NADPH oxidase. **A:** Daidzein; **B:** myricetin; **C:** vanillic acid.

4. Conclusion

In this study we investigated the chemical composition of *M. vulgare* and *D. viscosa* using LC-TOFMS and the extracts contain some interesting compounds which required more deep studies to explore their biological and pharmacological potential. Also, these plants and their mixture revealed a good antioxidant activity. Furthermore, the toxicity tests revealed that the mixture of these plants has no undesirable effects. In conclusion. The extract of these plants could be used as good antioxidant, and the mixture required some clinical studies to prove its safeness.

Finding

This research received no external funding

Conflicts of Interest

The authors declare no conflicts of interest.

Data availability statement

The data was not deposited in public repositories

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