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Original research article

In vitro biological activities of *Calamintha nepeta* L. aqueous extracts

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Abstract
Aim: This study aimed to investigate the phenolic composition, antioxidant capacity, and toxicity of aqueous extracts of *Calamintha nepeta* L. leaves and their potential vasorelaxant effects.
Methods: Aqueous extracts of *Calamintha nepeta* L. were prepared by three extraction methods: decoction, infusion, and maceration. The total phenolic contents of the extracts and their antioxidant properties were investigated. The toxicity was evaluated by *Artemia salina* lethality bioassay. The decoction extract was analyzed by HPLC for its chemical profile and was also used to evaluate the vasorelaxant effect on thoracic aortic rings isolated from healthy Sprague Dawley rats. Pre-contraction was induced by phenylephrine, followed by cumulative doses of the extract (0.001 up to 250 μg/ml).
Results: Aqueous extracts of *Calamintha nepeta* L. showed noticeable radical scavenging and chelating activities. However, the decoction extract exhibited the most powerful antioxidant capacity. No toxicity was recorded for the extracts obtained by decoction and infusion. Caffeic acid, quercetin, and rosmarinic acid were the main identified compounds. Notably, the aqueous extract obtained by decoction induced significant relaxation in endothelium-intact aortic rings at lower concentrations, and at higher concentrations in denuded aortic rings.
Conclusion: This study reveals that *Calamintha nepeta* L. extracted with a decoction method possesses potent antioxidant capacity and has an endothelium-dependent vasorelaxant effect.

Keywords: Antioxidant activity; Aqueous extracts; *Calamintha nepeta* L.; Polyphenols; Vasorelaxation

Highlights:
- *Calamintha nepeta* L. leaf extracts show high total phenolic content.
- Aqueous plant extract obtained by decoction has strong antioxidant activity.
- *Calamintha nepeta* L. extract has an endothelium-dependent vasorelaxant effect.
- *Calamintha nepeta* L. extract has no toxic effect on *Artemia salina* larvae.

Abbreviations:
ACh: Acetylcholine; ABTS: 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate radical scavenging assay; cGMP: Cyclic guanosine monophosphate; DPPH: 1,1-diphenyl-2- picrylhydrazyl free radical scavenging assay; dw: Dry weight; FRAP: Ferric reducing power assay; IC_{50}: Concentration providing 50% inhibition of radicals; LC_{50}: Lethal concentration inducing 50% lethality; LD_{50}: Lethal dose inducing 50% lethality; NO: Nitric oxide; PE: Phenylephrine; ROS: Reactive oxygen species; sGC: Soluble guanylate cyclase

Introduction

Nowadays, the importance of aromatic and medicinal plants is recognized worldwide due to their effective pharmacological activities, economic viability, and low toxicity (Ayoub and Mehta, 2018). The biological properties of medicinal plants used in traditional medicine are mainly associated with the phenolic compounds they contain. These constitute a large group of compounds that result from the secondary metabolism of plants and are known to display strong antioxidant activities (Boy et al., 2021). The main groups of phenolic compounds are phenolic acids, flavonoids, and non-flavonoids (Li et al., 2014). The redox properties and chemical structure of these compounds, which neutralize free radicals, chelate metals, and decompose peroxides, are responsible for their antioxidant properties (Moon and Shibamoto, 2009). The latter is known to delay the development of diseases related to increased oxidative stress, such as hypertension (Rodrigo et al., 2011).

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Calamintha nepeta L. is known by its local vernacular name "manta", "t'minţa" or "nebta" (El-Hilaly et al., 2003; Fakchich and Elachouri, 2014). It is widely used in traditional medicine to treat various health issues such as muscular pains, fever, menstruation induction, arthritis, and as an expectorant (Neves et al., 2009). It is also used for the treatment of hypertension, respiratory diseases, gastroenteric and infectious diseases (Jouad et al., 2001; Slimani et al., 2016).

Several studies have reported that *Calamintha nepeta* L., (mainly its essential oil), has many biological properties, including antioxidant, antibacterial, and antiproliferative (Arantes et al., 2019; Gormez et al., 2015). Analgesic and anti-inflammatory properties were also reported in Wistar rats (Pacifico et al., 2015). Notably, the majority of studies focus on its essential oils, while aqueous extracts, which are commonly used in traditional medicine, remain under-explored.

The composition and properties of aqueous extracts of *Calamintha nepeta* L. have not been extensively studied, in particular their potential to improve vascular function. Therefore, the aim of the present study is to evaluate the phenolic contents, antioxidant capacity, and vasorelaxant potential of the leaf aqueous extract of *Calamintha nepeta* L.

Materials and methods

Plant material

Calamintha nepeta L. was collected in March 2021 in the Ksar El Kebir region (North of Morocco) at an altitude of 134 m (34°06'03.11''N, 03°56'35.94''W). After the taxonomic identification, a specimen of *Calamintha nepeta* L. was carefully prepared, and deposited in the Scientific Institute Herbarium of Rabat (Morocco) under the reference number RAB114271.

The plant was dried in the shade at ambient temperature. Thereafter, an electric grinder was used to grind the plant leaves into a fine powder.

Preparation of the extracts and plant yield

Extraction was performed using three traditional methods: decoction, maceration, and infusion. The decoction extract was obtained by boiling 30 g of the plant leaf powder in 300 ml of distilled water for 15 min. The infusion extract was prepared by mixing 30 g of plant leaf powder with 300 ml of boiling distilled water, and the mixture was left to infuse for 15 min at room temperature. The maceration extract was obtained by macerating 30 g of the plant leaf powder in 300 ml of cold distilled water under low agitation for 24 hours (Mohti et al., 2019). Whatman paper (No. 4) was used to filtrate all obtained extracts, which were then concentrated in a rotary evaporator (BüchiRotavapor R-200) at 45 °C. All residues obtained were kept at 4 °C until further investigation.

The yield of the dry residue obtained by each extraction technique was calculated by the following equation: (weight of extract and recipient – weight of empty recipient)/initial weight of dried plant (Cao et al., 2022).

Total phenolic content (TPC)

As previously described (Bouhlali et al., 2016), the amount of TPC was measured using the Folin–Ciocalteu reagent. In brief, the samples prepared with the different extraction methods were prepared at a concentration of 0.4 mg/ml. 500 µl of Folin–Ciocalteu reagent (1/10) was added to 100 µl of each sample extract. Then, a sodium carbonate aqueous solution was prepared (7.5% w/v) and a volume of 400 µl was added to the mixture. The total solution was incubated for 60 min at the temperature of the laboratory. The absorbance was measured at 765 nm. Gallic acid is a standard used to elaborate the calibration curve, TPCs were represented as milligrams of gallic acid equivalents per gram of dry residue (mg GAE/g extract). All measurements were performed in triplicate.

Total flavonoid content (TFC)

The TFC of samples extracted by the different extraction methods was assessed according to the $AlCl_3$ method (Mohti et al., 2019). 500 µl of sample extract at a concentration of 2 mg/ml was mixed with 1.5 ml of methanol, 100 µl of aluminum chloride (10%), and 100 µl of potassium acetate (1 M). A total volume of 5 ml was achieved by adding 2.8 ml of distilled water. Test tubes were incubated in the dark for 30 min at the temperature of the laboratory and the absorbance was measured at 415 nm. Quercetin was used to elaborate the standard curve. The results were represented as milligrams of quercetin equivalents per gram of dry residue (mgQE/g extract). The assays were carried out in triplicate.

Antioxidant activity

DPPH radical scavenging activity

The ability of the extracts to scavenge free radicals was evaluated by DPPH free radicals as described by (Mohti et al., 2019). Different concentrations of plant extracts were prepared, 0.5 ml of each sample extract was mixed with 3 ml of a freshly DPPH (0.1 mM) methanolic solution. The obtained mixtures were kept at ambient temperature under darkness for 20 min. Then the absorbance was measured at 517 nm. The DPPH radical scavenging efficiency of each sample was then estimated as a percentage of inhibition using the formula:

$$\% \text{ inhibition} = [(A_{(control)} - A_{(sample)})/A_{(control)}]^*100$$

where $A_{(control)}$ is the absorbance of the control and $A_{(sample)}$ is the absorbance of the extract or standard. Under identical test circumstances, ascorbic acid was employed as a positive control. By plotting inhibition percentages versus extract concentrations, the concentrations providing 50% radical inhibition (IC_{50}) were determined. The assays were carried out in triplicate.

ABTS radical scavenging activity

This assay was performed as previously described (Re et al., 2007). The ABTS radical cations ($ABTS^{\bullet+}$) were produced by reacting 2.45 mM of potassium persulphate aqueous solution with 7 mM of an ABTS aqueous solution. Prior to use, the resulting solution was kept at room temperature for 12 to 16 hours in the dark, an absorbance of 0.700 ± 0.005 at 734 nm was then established after dilution with deionized water. Extracts were prepared at different concentrations, 10 µl of each concentration was added to 990 µl of $ABTS^{\bullet+}$ solution, which was then left to sit for 6 min at ambient temperature. At 734 nm, the absorbance was subsequently determined. The following formula was used to determine the percentage of ABTS•+ radical inhibition:

$$\% \text{ inhibition} = [(A_{(control)} - A_{(sample)})/A_{(control)}]^*100$$

$A_{(control)}$ is the absorbance of the control reaction and $A_{(sample)}$ is the absorbance of the sample or standard (Ascorbic acid).

The results were presented as IC_{50}, which is the concentration that scavenges 50% of $ABTS^{\bullet+}$ radicals. There were three duplicates of each measurement.

Ferric reducing antioxidant power assay

This assay measures the capacity of an antioxidant to reduce ferric tripyridyl-triazine (Fe^{3+} –TPTZ) to ferrous (Fe^{2+} – TPTZ) at pH = 3.6 (Benzie and Strain, 1999). Briefly, A 0.3 M acetate buffer (pH = 3.6), a 10 mM solution of TPTZ (2,4,6-tripyridyl-s-triazine) solubilized in 40 mM hydrochloric acid, and a 20 mM ferric chloride ($FeCl_3$) solution were used to prepare the FRAP reagent with volumes 10:1:1 respectively. Before each experiment, a fresh solution was prepared. 10 µl of each sample obtained from one of the three extraction methods was mixed with 2 ml of FRAP reagent and incubated for 10 min at the temperature of the laboratory. The absorbance was then measured at 593 nm. The standard curve was constructed using Trolox. The results were expressed as mmol Trolox equivalent / 100 g dry weight.

Artemia salina *lethality bioassay*

The brine shrimp lethality assay was used to evaluate the preliminary cytotoxicity of plant extracts and bioactive compounds as previously described (Taviano et al., 2018). Thirty-two grams of sea salt were dissolved in 1 liter of deionized water to prepare artificial seawater, the resulting solution was filtered before being used for sample preparation and shrimp eggs hatching. Shrimp eggs were hatched after 48 h of incubation at 25–28 °C. Samples were prepared at final concentrations of 10, 100, 500, and 1000 µg/ml. Each test tube contained a final volume of 5 ml of artificial seawater with or without the test extract, and ten larvae were introduced into each. After 24 h, the number of mobile larvae was counted. The assay was repeated six times (n = 6) and lethal concentration inducing 50% lethality (LC_{50}) values were determined. LC_{50} value of greater than 1000 µg/ml was considered non-toxic, while an LC_{50} value of less than 1000 µg/ml was considered toxic (Olowa and Nuneza, 2013).

HPLC analysis

High-performance liquid chromatography coupled with UV visible detection (UV-HPLC) (HPLC series 1100, Agilent UV detector) allowed the profiling of the extracts and identification of major polyphenols. The peaks were identified by comparison with authentic standards of retention times and UV spectra.

Chromatographic conditions were as follows: C18 column (PRP-C18 5 µm 4.6 × 250 mm). The apparatus is equipped with a binary pump (G1312A, Agilent 1100) and an automatic injector (G1330B). The mobile phase consisted of two solvent systems (A: 2% acetic acid in water; B: acetonitrile) at a constant flow rate of 1.2 ml/min. The injection volume was 20 µl. The signals were detected at 280 nm by the UV detection system.

Assessment of the vasomotor function of aortic rings

General procedure

Healthy male or female Sprague–Dawley rats (Laboratoires Charles River, France) were used. The European Directive 2010/63/EU relating to animal experimentation was strictly adhered to during animal experimentation and approved by the local ethics committee (Ethical Committee for Animal Experimentation, UHasselt, Belgium, ID 202072K). Rats were housed in standard cages under controlled environmental conditions, fed a standard diet of pellets, and provided with water *ad libitum*. Rats were injected intraperitoneally with 1000 U/kg of heparin prior anesthetized by dolethal (150 mg/kg). The descending thoracic aorta was removed after dissection and immersed in ice-cold Krebs–Henseleit solution prepared from: 118.3 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.026 mM EDTA, and 5.5 mM glucose. After removing connective tissue, the aorta was cut into small rings of 3–4 mm in length (Haesen et al., 2020). For specific experiments regarding the function of the endothelium in the process of vasorelaxation, the endothelium was damaged by gently rubbing the aortic intima.

Vasocontraction and vasorelaxation response

The aortic rings were placed horizontally in a tissue bath chamber filled with Krebs solution (95% O_2; 5% CO_2) and maintained at 37 °C. They were then subjected to a passive tension of 8 g and the tension variation was isometrically detected by a force transducer connected to PowerLab, AD Instruments (Haesen et al., 2020). After 60 min equilibration with washing every 20 min with Krebs solution, 10^{-7} M phenylephrine (PE) was added to induce aortic ring contraction. Then, cumulative doses of acetylcholine (ACh) were added to check endothelial integrity (Basri et al., 2018). Intact endothelium exhibited more than 60% ACh-induced relaxation, whereas damaged endothelium showed less than 10% in PE-precontracted aortic rings (Basri et al., 2018).

Intact and denuded-endothelium aortic rings were used to study the endothelium-dependent and independent vasorelaxation of the plant samples obtained with the decoction method as the preferred extraction method. Aortic ring precontraction was first induced by 10^{-7} M phenylephrine, and after reaching a plateau, the relaxation properties of the extract were assessed by adding a range of cumulative concentrations from 0.001 to 250 µg/ml. Data were calculated as a percentage of relaxation in response to PE-induced contraction.

Data analysis

GraphPad Prism was used for statistical analysis. Data of phenolic content, antioxidant activities, and *Artemia salina* lethality bioassay were statistically evaluated by one-way analysis of variance (ANOVA), or using the non-parametric Kruskal–Wallis test followed by Dunn's multiple comparison tests. Linear correlation analysis was performed using XLstat. Data were represented as mean ± SEM. Relaxant responses were normalized and analyzed using one-way ANOVA followed by Dunnett's multiple comparison tests. Results were expressed as percentage relaxation to maximal PE-induced contraction. $P < 0.05$ was considered statistically significant.

Results and discussion

Yield of extraction and phenolic contents

The highest yield of *Calamintha nepeta* L. aqueous leaf extracts was obtained using the infusion method (13.3%). The yield of the leaf extracts obtained by decoction and maceration was lower at 10% and 7.7% respectively.

The total phenolic compounds obtained from *Calamintha nepeta* L. leaves by the three extraction methods used (decoction, infusion, and maceration) are presented in Table 1.

As shown in Table 1, extraction by the decoction method resulted in a significantly higher phenolic content (163.03 ± 3.18 mg GAE/g extract) compared to infusion and maceration methods (123.24 ± 5.64 and 131.17 ± 2.90 mg GAE/g extract, respectively). Regarding flavonoid content, we found no significant difference between decoction and infusion extract (67.91 ± 0.84 and 70.28 ± 1.30 mgQE/g extract, respectively, $p > 0.05$) while the maceration method resulted in a significantly lower level (57.70 ± 1.46, $p < 0.05$).

Table 1. Total phenolic and flavonoid content in *Calamintha nepeta* L. extracts obtained by the different extraction methods

	Total Phenolic content (mg GAE/g extract)	Flavonoid content (mg QE/g extract)
Decoction	163.03 ± 3.18[a]	67.91 ± 0.84[a]
Infusion	123.24 ± 5.64[b]	70.28 ± 1.30[a]
Maceration	131.17 ± 2.90[b]	57.70 ± 1.46[b]

Note: Data in the same column showing different lower cases indicates a significant difference ($p < 0.05$). Results are ranked in descending order; a > b. mgGAE/g extract: milligrams of gallic acid equivalents per gram of extract; mgQE/g extract: milligrams of quercetin equivalents per gram of extract. Data were statistically evaluated using the non-parametric Kruskal–Wallis test followed by Dunn's multiple comparison tests. Each value is represented as mean ± SEM.

The observed difference in total phenolic and flavonoid contents, namely hot versus cold extraction, is likely due to the method itself. Indeed, as also shown by others, high temperatures weaken cell walls, promoting fluid diffusion through the cells, and improving solute solubility and diffusion coefficients, which in turn enhances extraction (Li et al., 2006). Several similar studies have reported that decoction is the best technique to extract phenolic compounds of either phenolic acids or flavonoids (Hmidani et al., 2019; Martins et al., 2014, 2015), confirming our results.

Decoction extraction method displays better antioxidant activity

To provide a better assessment of antioxidant capacity, the extracts were tested by three different but complementary *in vitro* model systems, including free radical scavenging activity DPPH, ABTS, and ferric reducing power (FRAP). As indicated in Table 2, the decoction extract was the most effective radical scavenger against DPPH (IC_{50} = 28.1 ± 0.5 µg/ml) and ABTS (IC_{50} = 9.96 ± 0.09 µg/ml). However, it is worth mentioning that this anti-oxidant ability remained 10-times weaker than our positive control Ascorbic Acid, which had IC_{50} values of 3.3 ± 0.5 µg/ml for DPPH and 2.15 ± 0.08 µg/ml for ABTS. The extract obtained with the decoction method remains the most powerful for reducing the ferric ions into ferrous ions (108.49 ± 2.42 mmol TE/100 g dw). Extracts obtained by infusion (55.05 ± 0.65 mmol TE/100 g dw) and maceration (55.31 ± 0.43 mmol TE/100 g dw) were not significantly different from each other ($p > 0.05$). Unlike our results, Khodja et al. (2018) did not report a difference between the two extraction methods, namely the decoction and infusion in reducing power, whereas the infusion showed strong anti-radical activity against DPPH radical. It is worth mentioning that the results of DPPH scavenging activity were a lot lower (IC_{50} = 0.248 ± 0.002 µg/ml for infusion, and 112.3 ± 5.4 µg/ml, for decoction extract) compared to the ones obtained in the present study. This difference in results can be attributed to the different collection areas of *Calamintha nepeta* L., which are characterized by different climates, and thus different biotic and abiotic environmental conditions that affect the production of secondary metabolites by plants, resulting in differences in antioxidant activity.

As shown in Table 3, a strong correlation was observed between total phenolics and antioxidant activity. Polyphenol content was negatively correlated with both the DPPH (r = –0.974) and ABTS (r = –0.944) assays, indicating that an increase in phenolic content is associated with lower IC_{50} values for both assays. Since a lower IC_{50} value means higher antioxidant activity, these results indicate that the higher polyphenol content of aqueous extracts was responsible for their high antioxidant activity by scavenging DPPH and ABTS radicals. The significant positive correlation of polyphenols with FRAP (r = 0.957) also indicates that polyphenols have the ability to reduce Fe^{3+} to Fe^{2+} by transferring electrons. In line with our results, Hmidani et al. (2019) have also found a strong correlation between polyphenols and FRAP, whereas a moderate correlation was recorded between polyphenols, flavonoids, and DPPH. In our study, a high correlation between ABTS, DPPH, and FRAP was demonstrated, suggesting a pos-

Table 2. Antioxidant activity of *Calamintha nepeta* L. leaves using different extraction methods

		DPPH IC_{50} (µg/ml)	ABTS IC_{50} (µg/ml)	FRAP mmol TE/100 g dw
	Decoction	28.1 ± 0.5[b]	9.96 ± 0.09[b]	108.49 ± 2.42[b]
	Infusion	39.6 ± 0.2[c]	14.58 ± 0.83[c]	55.05 ± 0.65[a]
	Maceration	36.8 ± 0.3[c]	15.21 ± 0.22[c]	55.31 ± 0.43[a]
Ascorbic acid		3.30 ± 0.46[a]	2.15 ± 0.08[a]	

Note: Data in the same column with different lower cases indicates a significant difference ($p < 0.05$.) Results are ranked in ascending order; a < b < c. IC_{50}: Concentration providing 50% inhibition of radicals. DPPH: 1,1-diphenyl-2-picrylhydrazyl free radical scavenging assay; ABTS: 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate radical scavenging assay; FRAP: Ferric reducing power assay. Data were statistically evaluated using the non-parametric Kruskal–Wallis test followed by Dunn's multiple comparison tests. Data are presented as mean ± SEM.

Table 3. Correlation coefficients between phenolic components and antioxidant activities

Variables	Phenolic	Flavonoids	DPPH	ABTS	FRAP
phenolic	1				
flavonoids	0.1317	1			
DPPH	–0.9742	–0.1092	1		
ABTS	–0.9448	–0.4254	0.9232	1	
FRAP	0.9579	0.3326	–0.9724	–0.9773	1

sible antioxidant activity relationship of the compounds that react in these methods although they have different reaction mechanisms.

The relationship between phenolic compounds and antioxidant activity has been demonstrated in several studies. Indeed, phenolic substances are characterized by the presence of at least one aromatic ring to which one or more hydroxyl groups are associated, their free radical scavenging efficiency seems to depend on the configuration of these free hydroxyl groups (Brewer, 2011). Natural antioxidants have no harmful effects on the human body and can inhibit or prevent cell damage mainly due to their free radical scavenging properties (Lobo et al., 2010).

Calamintha nepeta *L. aqueous decoction and infusion extracts appear to be safe*

A lethality bioassay on *Artemia salina* was carried out to assess the safety profile of the aqueous plant extracts.

The percentage mortality of *Artemia salina* larvae induced by the extracts, after testing the different concentrations is shown in Fig. 1. The increase in mortality was directly proportional to the increase in concentration, particularly for the maceration extract which induced 95% mortality at a concentration of 1000 µg/ml. As previously indicated, all lethal concentrations inducing 50% mortality below 1000 µg/ml are considered toxic, while values above 1000 µg/ml are considered non-toxic (Meyer et al., 1982). Both decoction and infusion extracts exhibited no toxicity against brine shrimp, even at high concentrations (LC_{50} > 1000 µg/ml). However, the leaf maceration extract showed moderate toxicity (LC_{50} = 542.7 ± 47.3 µg/ml), this may be attributed to the maceration method itself, which involves a longer cold extraction period and can extract a wider range of compounds, including potentially toxic substances not effectively extracted by decoction or infusion methods. Additionally, high-temperature extraction techniques, such as decoction and infusion, can degrade or modify the chemical structure of some sensitive compounds, reducing their efficacy or altering their properties (Fig. 1B). To our knowledge, the potential toxicity nature of the aqueous extracts of *Calamintha nepeta* L. has yet to be evaluated. In contrast to our results, other studies indicated a strong cytotoxic effect of the essential oil of *Calamintha nepeta* L., with an LC_{50} value of 128 µg/ml against *Artemia salina* (Arantes et al., 2019). However, this high toxicity level for brine shrimp was not confirmed *in vivo*. Indeed, the acute toxicity of *Calamintha nepeta* essential oil was assessed in Swiss albino mice. The oral lethal dose (LD_{50} ≥ 1500 mg/kg) showed low toxicity associated with no changes in the behavior of the animals (Arantes et al., 2019). Altogether, the data suggest that both the aqueous and essential oil of *Calamintha nepeta* L. are non-toxic in nature.

HPLC analysis

As the extract obtained with the decoction method showed the highest level of phenolic content, strong antioxidant activity, and low toxicity, it was subsequently used to analyze its phytochemical profile, as well as to assess its potential vasorelaxant effect.

The chemical profile of *Calamintha nepeta* L. decoction extract was analyzed using HPLC. The results are presented in Fig. 2. Eleven compounds were identified, among them caffeic acid (8.3%), quercetin (21.1%), catechin (0.7%), cinnamic acid (1.2%), coumaric acid (1.2%), gallic acid (1.4%), rosmarinic acid (4.8%), rutin (2.5%), vanillin (2.5%), and vanillic acid (0.5%).

Numerous studies have shown that caffeic acid, chlorogenic acid, and rosmarinic acid have hypotensive properties, reducing systolic blood pressure and heart rate, as well as the activity of angiotensin-1-converting enzyme, acetylcholinesterase, butrylcholinesterase, and arginase. They also improve nitric oxide bioavailability and increase catalase activity (Agunloye et al., 2019; Prasannarong et al., 2019). Similarly, recent studies have shown that quercetin, a phenolic compound belonging to the flavonoid family, induces a reduction in blood pressure in hypertensive patients. The various mechanisms of its antihypertensive effect include reduction of oxidative stress, inhibition of angiotensin-converting enzyme activity, improvement of endothelial function, direct action on vascular smooth muscle, or modulation of cell signaling and gene expression (Popiolek-Kalisz and Fornal, 2022).

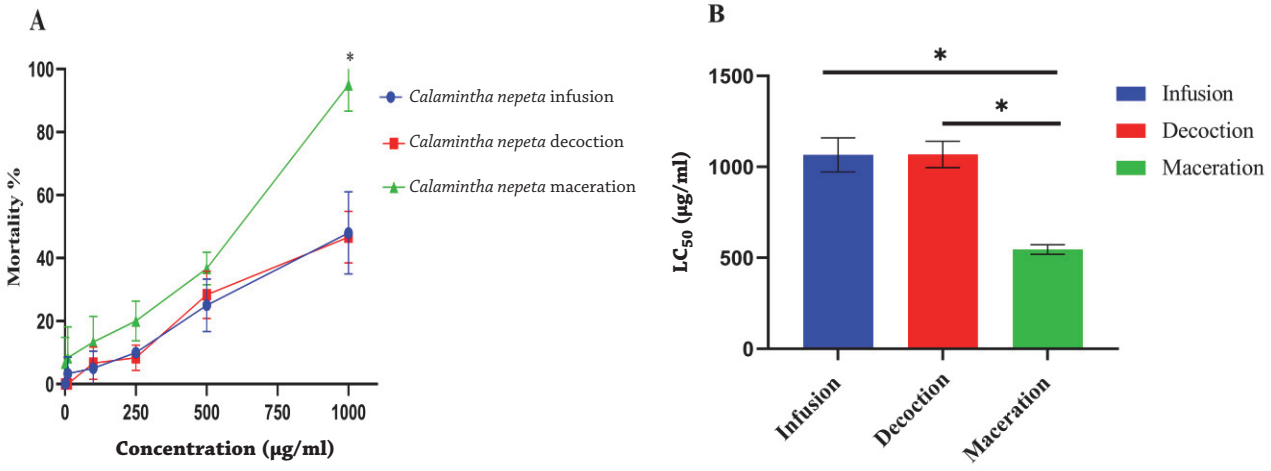

Fig. 1. Determination of the toxicity of *Calamintha nepeta* L. extracts obtained by different methods using brine shrimp lethality bioassay. **(A)** Mortality percentage of different *Calamintha nepeta* L. extracts according to the concentrations. (**B**) LC_{50} of *Calamintha nepeta* L. extracts. LC_{50}: lethal concentration inducing 50% lethality (n = 6). Results were statistically evaluated by one-way analysis of variance (ANOVA). Data are represented as mean ± SEM.* indicated P < 0.05.

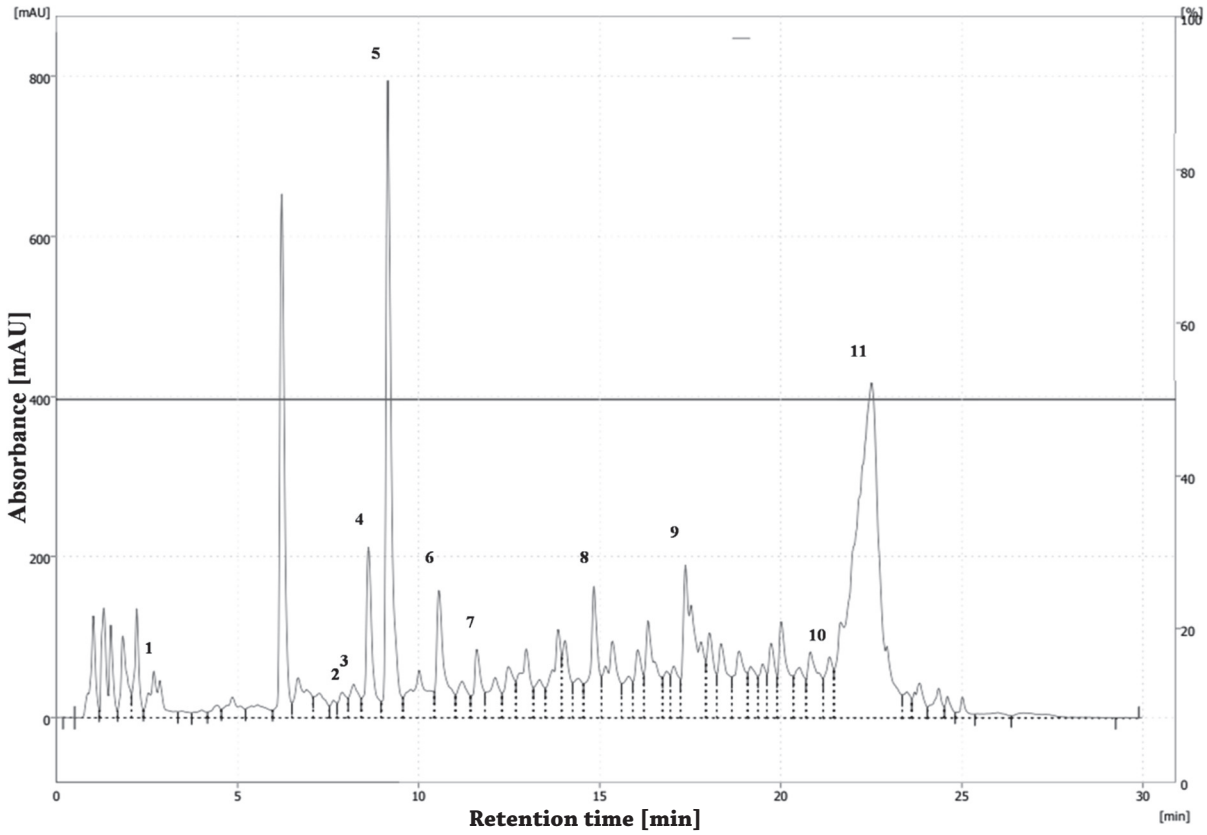

Fig. 2. HPLC profile of phenolic compounds from *Calamintha nepeta* L. aqueous decoction extract. Identified peaks: (1) Gallic acid, (2) Vanillic acid, (3) Catechin, (4) Chlorogenic acid, (5) Caffeic acid, (6) Vanilline, (7) Coumaric acid, (8) Rutin, (9) Rosmarinic acid, (10) Cinnamic acid, (11) Quercetin.

Vasorelaxant endothelium-dependent effect of the decoction extract

The descending thoracic aorta was isolated and cut into aortic rings to assess relaxation responses (Fig. 3). Endothelium-dependent vasorelaxation was assessed in intact rings precontracted with 10^{-7} M of PE. The addition of cumulative doses of *Calamintha nepeta* L. decoction extract to the organ baths elicited a significant concentration-dependent relaxation at all concentrations, as shown in Fig. 4.

The vasorelaxation produced by *Calamintha nepeta* L. aqueous extract was markedly attenuated after denudation of the aorta; low concentrations did not affect the vasorelaxation response. However, the vasorelaxant effect was only observed at the high concentrations of 100 and 250 µg/ml, which induced relaxation in the order of 17 ± 8 and 28 ± 15%, respectively. In fact, cumulative concentrations of *Calamintha nepeta* L. extract provoked a total relaxation of the contracted aorta, which appears to be endothelium-dependent, as this effect disappeared in denuded aortas (Table 4).

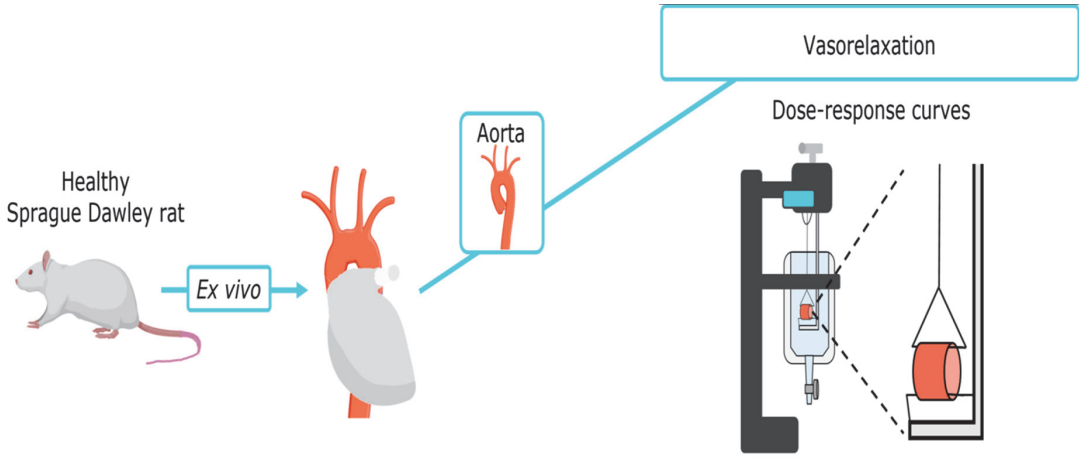

Fig. 3. Visual representation of the experiments performed with the aortic rings

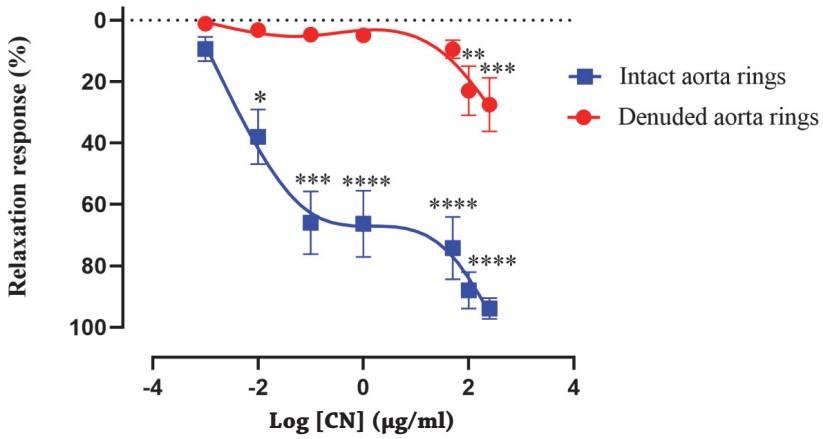

Fig. 4. The effect of *Calamintha nepeta* L. decoction extract on PE-induced contraction in endothelium aortic rings. Relaxation response was measured against PE-induced contraction in endothelium-intact aortic rings (n_{rings} = 16) and in endothelium-denuded aortic rings (n_{rings} = 9). Relaxation was calculated as % pre-contraction induced by 10^{-7} M PE. Relaxant responses were normalized and analyzed using one-way ANOVA followed by Dunnett's multiple comparison tests. Values are expressed as mean ± SEM. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, **** indicates $P < 0.0001$ vs PE-induced contraction.

Table 4. Pharmacological parameters of the relaxant effects induced by *Calamintha nepeta* L.

***Calamintha nepeta* extract**	
Intact aortic rings	
EC_{50} (µg/ml)	16 ± 11
E_{max} relaxation (%)	93 ± 13
Denudedaortic rings	
EC_{50} (µg/ml)	114 ± 26**
E_{max} relaxation (%)	28 ± 15

Note: Maximal relaxation (E_{max} relaxation) is expressed as % of PE-induced precontraction. EC_{50} is the half-maximal effective dose. Data are presented as mean±SEM and analyzed using one-way ANOVA. ** indicates $P < 0.01$ vs EC50 obtained in intact aortic rings.

Vasorelaxation can effectively control high blood pressure by inducing vasodilation which reduces vascular systemic resistance and enhances blood flow, resulting in a reduction in arterial pressure (Brozovich et al., 2016). It occurs when receptors or channels on the vascular endothelium or vascular smooth muscle are activated by endogenous or exogenous vasoactive mediators (Al-Akwaa et al., 2020). Nitric oxide, prostacyclin, and endothelium-derived hyperpolarizing factors, among other vasodilating factors, are liberated by endothelial cells, which exert a major effect on vascular tone regulation (Dib et al., 2017). It was demonstrated that *Calamintha officinalis*, a subspecies of the genus *Calamintha*, has an important antihypertensive effect. In hypertensive rats, *Calamintha officinalis* aqueous extract lowered diastolic, systolic, and mean arterial blood pressure. The vasorelaxant property of this extract was mediated by the cyclooxygenase pathway, the sGC-cGMP signaling pathway, and subsequently the opening of K^+ channels (Azzane et al., 2022). Whether similar mechanisms are used by *Calamintha* nepeta L. needs to be investigated.

It has recently been suggested that oxidative stress is a major factor in the development of high blood pressure. Nitric oxide generated by the endothelium during the vasorelaxation process is rapidly degraded by superoxide anion, an oxygen-derived free radical, limiting its bioavailability and subsequently affecting hypertension regulation (Nasri et al., 2014). Antioxidant therapy can improve vascular function and prevent hypertension. Antioxidants are substances with the ability to neutralize free radicals and capture ROS (Moon and Shibamoto, 2009). The most known natural antioxidant that is widespread in vegetables and fruits is polyphenols. Generally, polyphenols have two major mechanisms in antioxidant reactions, the transfer of the hydrogen atom or electrons to neutralize free radicals, and the chelation of metal ions through their multiple OH groups and the carbonyl part (Li et al., 2014).

As indicated previously, the aqueous extract of *Calamintha nepeta* L. obtained with the decoction method contains polyphenols, notably caffeic acid, chlorogenic acid, quercetin, rosmarinic acid, and coumaric acid. It has been demonstrated that these phenolic molecules play an important protective role against hypertension. They activate the NO-synthase enzyme responsible for nitric oxide production and increase glutathione levels. Moreover, they inhibit ROS-producing enzymes like xanthine oxidase and NADPH. Enhancing vascular functioning and subsequently normalizing vascular tone, they have an overall antihypertensive effect (Nasri et al., 2014). In line with our data, others reported comparable vasorelaxation effects of *Calamintha nepeta* L. from hydro-methanolic extract. Indeed, the main constituents of *Calamintha nepeta* L. hydro-methanolic extract were caffeic acid and its derivatives, acacetin, rosmarinic acid, quinic acid, and salvianolic acid B (Pacifico et al., 2015). Therefore, it is likely that *Calamintha nepeta* L. extract by decoction may contribute to improving vascular tone and preventing hypertension through the same underlying mechanisms as described by others using different extraction methods. However, further studies identifying the exact composition of the extract are yet to be performed.

Calamintha nepeta L. is a medicinal plant used by the Moroccan population for its medicinal virtues using traditional extraction techniques. From our study, it appears that the decoction of the leaves of the plant is rich in polyphenols (163.03 ± 3.18 mg GAE/g extract) and flavonoids (67.91 ± 0.84 mg QE/g extract). These compounds have important antioxidant activity, as revealed by a potential reduction of free radicals of the order of 90% of the DPPH radical at the concentration of 50 µg/ml, and the ABTS radical at the concentration of 20 µg/ml. As a result, *Calamintha nepeta* extracts are able to protect against oxidative stress and its damaging effects,

thanks to their potential antioxidant activity. These results can be associated with the vasorelaxant effect recorded at the dose of 250 μg/ml, the equivalent of 0.5 g/200 ml in the form of decoction of the leaves of *Calamintha nepeta* L., which may improve blood pressure in people suffering from hypertension.

Conclusion

The present study reveals that the aqueous extract of *Calamintha* nepeta L. leaves has a high phenolic content, and possesses potent antioxidant activity associated with a non-toxic profile. This extract also showed an important endothelium-dependent vasorelaxant effect. Though our results suggest promising beneficial effects of *Calamintha nepeta* L. leaves used as a phytoalternative treatment for hypertension, further analysis is required to identify the underlying mechanisms.

Author's contributions

NS conducted the experiments, performed the statistical analysis, and wrote the manuscript. *DD* supervised the laboratory work on animals and revised the draft article. *VB* and *LER* supervised the entire work; they discussed and approved all study protocols and reviewed and corrected the article several times. *AZ* is responsible for project management and funding acquisition. All authors read the manuscript and approved the final version.

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Ethical aspects and conflict of interest

The authors declare that they have no financial interests or personal relationships with other people or organizations that could have influenced the work reported in this paper.

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