Vasorelaxant effects of *Teucrium takoumitense* via receptor-activated calcium channels (ROCC) and voltage-gated calcium channels (VDCC) and its subacute toxicity in rats

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Abstract:

Introduction: Teucrium takoumitense is used traditionally for cardiovascular disorders, but its vascular effects and safety profile remain poorly characterized.

Objective: This study evaluated the vasorelaxant activity of Teucrium takoumitense aqueous extract (TTAE) and its subacute toxicity.

Material and Methods: Isolated thoracic aortic rings from Wistar rats were precontracted with epinephrine (EP, 10 μM) or KCl (80 mM). Cumulative concentrations of TTAE (0.5–2 mg/mL) were applied to intact and endothelium-denuded rings to assess vasorelaxation. Mechanistic experiments evaluated the extract's effect on extracellular Ca2+-induced contractions to probe inhibition of receptor-operated (ROCCs) and voltage-dependent calcium channels (VDCCs). For subacute toxicity, rats received TTAE by gavage daily for 28 days (200 mg/kg) with a control group given vehicle. Body weight, food intake, relative organ weights, hematological parameters (LYM, MCV, MCHC, PLT) and biochemical markers (urea, creatinine, triglycerides, total cholesterol, LDL, AST) were measured.

Results: TTAE induced concentration-dependent relaxation of EP- and KCl-precontracted aortic rings (0.5–2 mg/mL) in both endothelium-intact and -denuded preparations. Mechanistic data indicate vasorelaxation is mediated, at least partly, by inhibition of extracellular Ca2+ entry via ROCCs and VDCCs. Subacute administration (200 mg/kg) significantly increased relative weights of liver, kidneys, spleen, and lungs. Hematological changes included increased MCV and MCHC in females, and increased LYM, MCV, MCHC and PLT in males. Female rats showed elevated urea and creatinine and decreased triglycerides, total cholesterol, LDL and AST compared with controls.

Conclusion: TTAE demonstrates potent vasorelaxant activity via Ca2+ blockade, but 28-day administration at 200 mg/kg indicating potential toxicity and dose-finding needed.

Keywords: *Teucrium takoumitense*; Hypertension; Vasorelaxant; receptor-activated calcium channels (ROCCs); Subacute toxicity.

1- Introduction

In recent years, HBP (high blood pressure) has become a real communal health problem, affecting more than a billion people worldwide [1]. This abnormality leads to mortality from coronary heart disease, myocardial infarction, Both peripheral vascular disease and cardiac failure [2]. In developed nations, it is the primary cause of death [3]. And the second for underdeveloped countries such as the African continent after parasitic diseases such as malaria. Cardiovascular diseases, especially HBP, are linked to several risk factors, including dyslipidemia. Antihypertensive medications are the mainstay of treatment for high blood pressure; however, their many side effects, including irregular heartbeat, dry cough, fatigue, and kidney failure, pose a significant challenge. Using natural items has emerged as a substitute for investigating other antihypertensive medications. In fact, Hundreds of plants are utilized in traditional medicine to alleviate hypertension, according to several ethnobotanical reports [4].

Teucrium takoumitense is an aromatic and medicinal plant endemic to Morocco belonging to the family Lamiaceae and the genus *Teucrium*, which is the second largest genus of the subfamily Abugidas. The discovery (determination) of this small *Teucrium* was in 1995 by German botanists Förther and Podlech, at the foot of the cliffs overlooking the source of Takoumit, in Tazougart, north of Boudenib. This plant forms small tufts rooted in the cracks of the rock. The leaves are serrated, oval. The flowers have a calyx extended by two hairy brownish teeth from which emerge [5]. Aqueous extracts of *Teucrium* plants have historically been the most widely utilized treatment for a number of illnesses, such as oxidative stress-related conditions and cardiovascular ailments. The phytochemical diversity especially valuable secondary metabolites, antioxidant potential and biological properties of this genus explain their use in traditional medicine [6]. Consequently, a significant antioxidant activity characterized this plant as well as a powerful anti-inflammatory effect were caused by the volatility of oil. In addition, Escherichia coli was susceptible to the antimicrobial effects of T. takoumitense's volatile oil, Staphylococcus aureus and Pseudomonas aeruginosa [5]. This study aimed to evaluate the vasorelaxant properties and subacute toxicological effects of TTAE. In fact, some non-toxic plants can have negative effects on some human or animal organs if consumed in excess or absorbed over a prolonged period [7]. Despite the fact that a variety of biological actions have been demonstrated for *T. takoumitense* and its derivatives, but the vasorelaxant action and subacute toxicity have not been discussed.

2- Material and methods

2.1 Plant material and preparation of the aqueous extract

In the village of Tazouguerte north of Boudenib (Errachidia region, Morocco) in the Daraa-Tafilalet region (semi-arid zone), the aerial parts of *T. takoumitense* were picked fresh (the plant having already been recognized) and air-dried at 40 °C in April 2023. The Faculty of Sciences and Technology of Errachidia received a reference specimen (TT1125). The most traditional Moroccan method, decoction, was used to create the aqueous extract of the dried and crushed plant material [8].

2.2. Experimental animals

After being acquired from Missour, Morocco (experimental center), Wistar strain adult male albino rats in good health, weighing 130–260 g, were kept in individual polyethylene cages under regular conditions. They were also given at least three weeks to acclimatize to the stress of transport. The experiments were conducted in compliance with the standards of the FSTE/2015 local committee. In the subacute toxicity study, 12 rats of both sexes were utilized, with six rats per group, consisting of three females and three males [9].

2.3. Chemical reagents and drugs

We purchased indomethacin, 4-aminopyridine (4-AP), norepinephrine-nitro-l-arginine methyl ester (L-NAME), and acetylcholine chloride from Sigma Chemical Co. (St. Louis, USA). Every other reagent was obtained locally and were of analytical grade. Distilled water was used to dissolve all drugs mentioned.

2.4. Measurement of vascular relaxation and evaluation of mechanisms involved

Male rats belong to the Wistar strain weighing between 262 and 305 grams were produced and kept in cages of six each in rooms with a regulated light/dark cycle at 25 °C and unlimited access to food and water. The vasorelaxant activity of TTAE was studied in accordance with the approach of Ajebli and Eddouks [8].

2.5. Subacute oral toxicity test

Subacute oral toxicity studies were conducted over a period of 28 days using the repeated dose toxicity technique according to the OECD standard [10]. Whereas twelve rats in all were split into two groups (I and II) at random, with three female and three male rats in each group. As a control, distilled water was administered to group I. For 28 days, group II received oral gavage

once daily at a dose of 200 mg/kg body weight of TTAE. the lethal dose (LD50) was greater than 500 mg/kg [11]. Weekly weight measurements were taken for four weeks during the 28-day clinical observation period.

Pentobarbital was used to anesthetize the animals following an overnight fast on day 29 during which they were allowed unlimited access to water. Blood was extracted from the retro-orbital sinus of each animal and placed in tubes covered with non-EDTA for biochemical analysis and anticoagulant for hematological analysis. After the blood samples were collected, the rats were killed by cervical dislocation. After the rats were dissected, the liver, kidneys, lungs, spleen, ovaries, and testes were carefully removed, cleaned with distilled water, weighed, and examined under a microscope for any obvious anomalies.

The organs were weighed and their body weight index was calculated using the following formula [12]: Relative organ weight (ROW) (g) = weight of organ/body weight of rats on the day of sacrifice × 100%.

2.6. Assessment of hematological and biochemical parameters

To evaluate different hematological parameters, The Automated Hematology Analyzer (Symex-RX, 21, Japan) was used to analyze samples of blood that had been collected in test tubes containing EDTA.White blood cell (WBC) and leukocyte count (LYM), red blood cell (RBC) count, lymphocyte count (RCDW), hematocrit (HCT), hemoglobin (HGB) level, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet count (PLT) were among them. Blood samples obtained in anticoagulant-free tubes were allowed to coagulate at room temperature in order to facilitate biochemical analyses. After that, the samples were centrifuged for 10 minutes at 3000 rpm to separate the serum for biochemical analysis to separate the serum for biochemical analysis, the samples were centrifuged for ten minutes at 3000 rpm. in an electrical centrifuge (HUMAX-K, HUMAN-Germany). A German-made Humastar 200 chemical analyzer was used to measure parameters like total cholesterol (TC), total triglycerides (TGs), HDL, LDL, aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, creatinine, total protein (TP), albumin (Alb), and bilirubin (BT) [13].

2.7. Preliminary phytochemical screening of *T. takoumitense*

A preliminary screening of TTAE was conducted to identify the presence of bioactive compounds. These analyses were carried out as previously described [8].

2.8. Determination of total polyphenol content

The total phenolic contents of TTAE were measured according to the method described by Raposo F et al [14]. Briefly, 100 µL of aqueous extract and 500 µL of Folin-Ciocalteu reagent (diluted tenfold with water) were combined, followed by the addition of 400 µL of aqueous sodium carbonate solution (7.5% w/v). After incubation for 60 minutes at room temperature, the absorbance of the mixture at 765 nm was measured. Gallic acid was used to generate the calibration curve. The gallic acid equivalent, measured in milligrams per gram of extract, was used to express the total phenolic components.

2.9. Determination of flavonoid content

The total flavonoid content of TTAE was measured according to the method of Kim et al [15]. In short, 1 mL of distilled water was combined with 250 µL of diluted filtrate. After that, 75 µL of a 5% w/v aqueous sodium nitrite solution and 75 µL of a 10% w/v aqueous aluminum chloride solution were added. One milliliter of sodium hydroxide (1 M) was added to the mixture after it had been incubated for five minutes at room temperature. The final volume was then adjusted to five milliliters using distilled water. At 510 nm, the absorbance was measured. Rutin was used to prepare the calibration. Mg rutin equivalent (RE)/1 g of extract was the unit of measurement used to express the results.

2.10. Determination of tannin content

The tannin content was measured employing a slightly altered version of the technique described by Broadhurst et al., with catechin serving as the standard reference compound [16]. 3 mL of vanillin solution (4% in methanol) and1.5 mL of strong hydrochloric acid were combined with 400 µL of extract. The absorbance was measured at 500 nm after an incubation time of 15 minutes. Condensed tannins are represented as mg of E. Catechin/1 g of extract.

2.11. Statistical Analysis

The mean ± SEM is used to express all values. Two-way ANOVA was used to assess for statistical significance between more than two groups, and the Bonferroni multiple comparisons test for vasorelaxant activity was then performed, and by T-test for Ca^{2+}-induced extracellular contractions in calcium-free Krebs solution. One-way ANOVA followed by Bonferroni multiple comparisons test was used for study the effect of TTAE on body weight in rats. While Student's unpaired two-tailed t-test was used to examine organ weights, hematological and

biochemical data independently for each sex. Statistical analyses were carried out using GraphPad Prism version 8, with a p-value of less than 0.05 regarded as statistically significant.

3-Results

3.1. Vasorelaxant activity

3.1.1. Effect of TTAE on relaxation of isolated rat aortic ring

Regarding the evaluation of vasodilatation mechanisms involved in the aqueous extract of the aerial part of *T. takoumitense* (TTAE) on intact rat aortic rings with endothelium, After the relaxing of four concentrations (0.5, 1, 1.5, and 2 mg/ml), a number of tests were conducted. Using rat aortic rings pre-contracted by EP (10 µM) and KCl (80 mM), a concentration-dependent technique was employed. Figure 1 provided an illustration of the findings. From the third dose (1.5 mg/ml, $p < 0.0001$) to the final chosen concentrations (2 mg/ml, $p < 0.0001$), TTAE considerably dilated the EP precontracted aortic rings, as shown in Figure (1A). Whereas, in the KCl (80 mM) pre-contracted aortic rings (Fig.1B), TTAE caused significant relaxation from the first concentration to the last concentration (0.5, 1, 1.5 and 2 mg/ml, $p < 0.0001$). The rat aortic ring did not significantly relax when the maximum proportion of vehicle volume (distilled water) was applied. Several medications were employed in this study prior to pre-contraction by EP (10 µM) in order to assess the mechanism of action behind the vasorelaxant activity of TTAE.

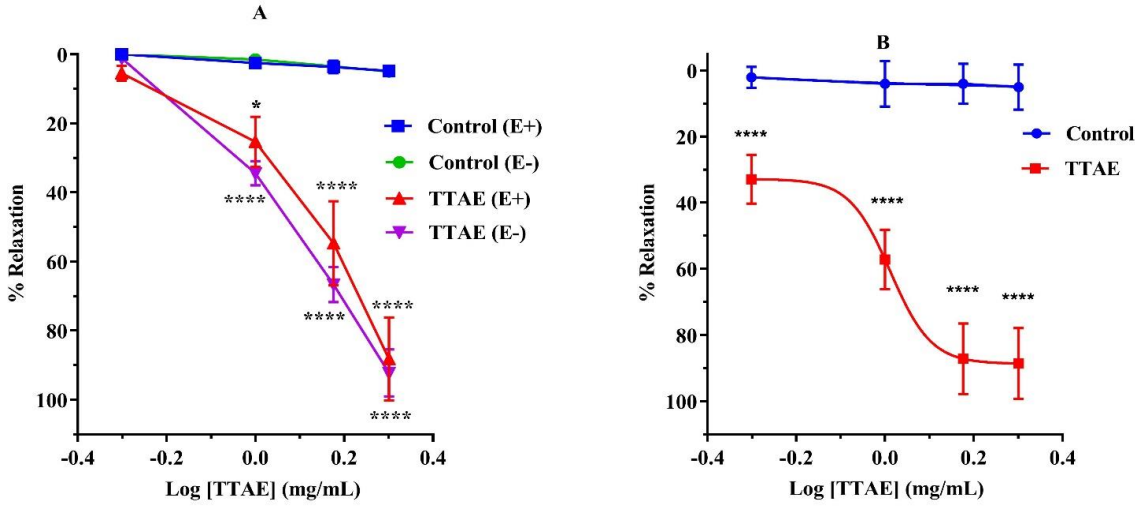

Figure 1: Effect of TTAE on vasorelaxation of aortic rings. Concentration-dependent cumulative curves for TTAE- and vehicle-induced (distilled water) relaxation in rat aortic rings**.**

3.1.2 Effect of NO synthase inhibitor and prostaglandin synthesis Inhibitor on Aortic Responses to TTAE.

To determine the involvement of NO (nitric oxide) in the relaxation effect induced by TTAE, aortic rings were pre-treated with L-NAME (10^{-4} M) for 20 minutes prior to EP-induced pre-contraction (10 µM). The results showed that L-NAME (10^{-4} M) did not significantly reduce the vasorelaxant response to TTAE (Figure 2A). Similarly, the vasorelaxant effect of TTAE on EP-pre-contracted aortic rings without indomethacin (10^{-5} M) (a prostaglandin syntesis inhibitor) pre-incubation resulted in significant dilation from the third concentration (1.5 mg/ml, $p < 0.0001$) to the last selected concentrations (2 mg/ml, $p < 0.0001$). The findings indicated that pre-incubation of aortic rings with indomethacin (10^{-5} M) for 20 minutes prior to EP-induced pre-contraction (10 µM) did not significantly inhibit the vasodilatory responses elicited by TTAE (Figure 2B). These findings (Figure 2) imply that TTAE's vasorelaxant action is unrelated to the vascular cyclooxygenase and direct nitric oxide (NO) pathways.

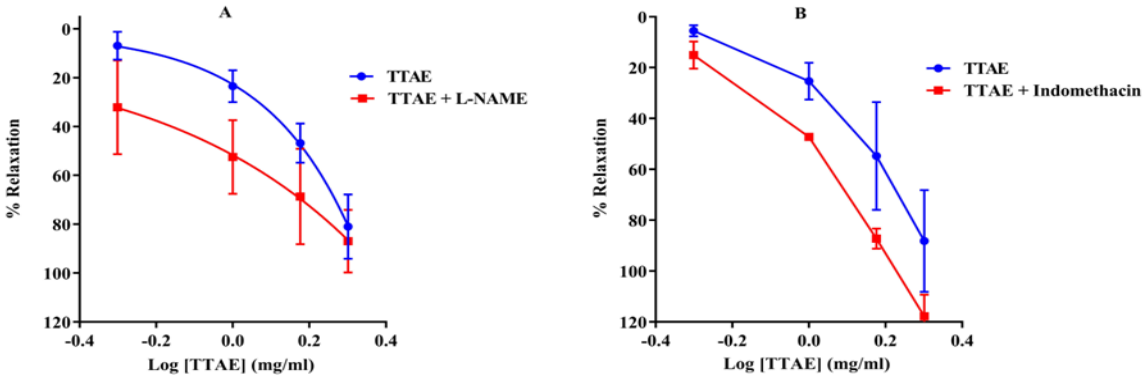

Figure 2: Effect of pre-incubated drugs on TTAE-induced relaxation in EP-pre-contracted aortic rings, comparing the presence and absence of L-NAM and Indomethacin

3.1.3. Aortic Responses to TTAE in the Absence and Presence of Methylene Blue or Nifedipine

In this study, rat aortic rings were pre-treated for 20 minutes with nifedipine (10^{-5} M), an L-type calcium channel blocker, and methylene blue (10^{-5} M), an inhibitor of the NO-cGMP signaling pathway. before pre-contraction of the EP (10 µM), The findings were examined and depicted in Figure 3. These findings demonstrated that in response to varying dosages of TTAE, methylene blue and nifedipine did not lessen the relaxation of rat aortic rings.in fact, TTAE-treated aortic rings precontracted by EP without preincubation with methylene blue or nifedipine were significantly dilated by the third concentration of TTAE (1.5 mg/ml, $p <$

0.0001) to the last selected concentrations (2 mg/ml, $p < 0.0001$). These findings imply that the NO-cyclic guanosine monophosphate (NOcGMP) pathway and L-type calcium (Ca^{2+}) channels have no bearing on the vasorelaxant action of TTAE.

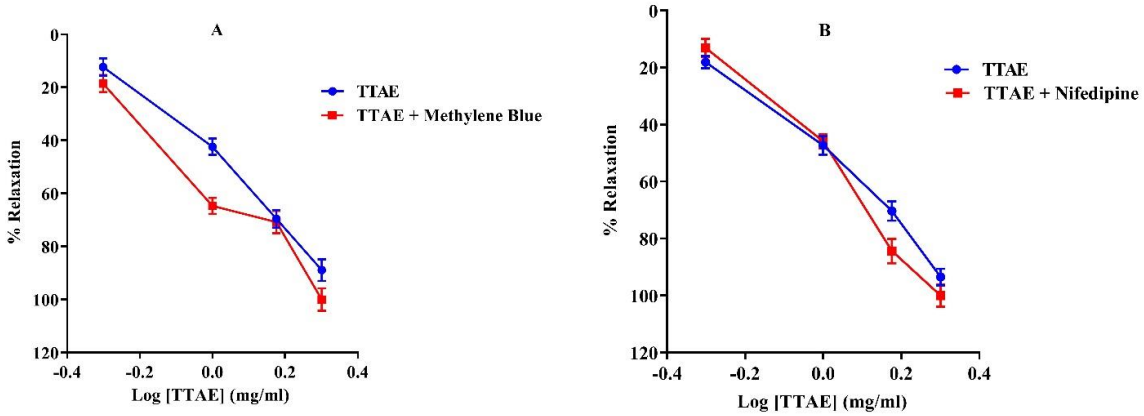

Figure 3: Effect of pre-incubated drugs on TTAE-induced relaxation in EP-pre-contracted aortic rings, comparing the presence and absence of Methylene blue and Nifedipine

3.1.4. Aortic Responses to TTAE in the Absence and Presence of Propranolol or Glibenclamide

The findings of this experiment were examined and shown in Figure 4. Rat aortic rings were pre-incubated with Glibenclamide (10^{-5} M), an ATP-sensitive K^+ channel blocker, and Propranolol (10^{-5} M), a β-adrenergic receptor blocker, for 20 minutes prior to EP pre-contraction (10 µM). Compared with TTAE-treated aortic rings pre-contracted by EP without Glibenclamide (GC) or Propranolol preincubation, the results showed that Glibenclamide (GC). Moreover, Rat aortic rings' relaxation in response to varying TTAE dosages was not decreased by propranolol. These findings demonstrate that the vasodilatation effect of TTAE was not mediated by the vascular ATP-sensitive K^+ channel pathway or by the adrenergic receptor pathway.

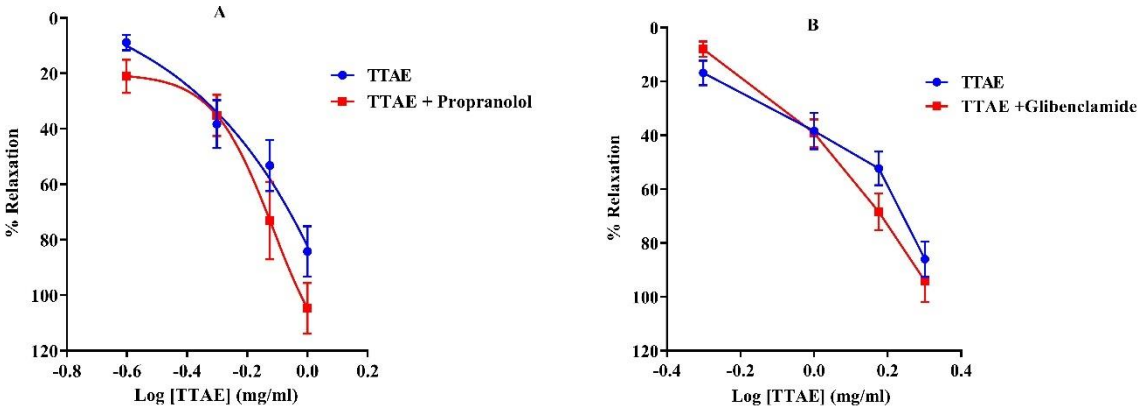

Figure 4: Effect of pre-incubated drugs on TTAE-induced relaxation in EP-pre-contracted aortic rings, comparing the presence and absence of Propranolol and Glibenclamide.

3.1.5. Aortic Responses to TTAE in the Absence and Presence of $Bacl_2$ and 4-Aminopyridine

In this experiment, pre-incubation of rat aortic rings with 4-aminopyridine (4-AP) (10^{-4} M), a voltage-gated K^+ channel blocker (KV blocker), and 10^{-4} M barium chloride ($Bacl_2$), an inwardly rectifying potassium channel blocker (KIR blocker). After release for 20 min before EP pre-contraction (10 µM), the results were analyzed and illustrated in (Fig.5). Compared with TTAE-treated aortic rings pre-contracted by EP without 4-aminopyridine or barium chloride preincubation, the findings demonstrated that in response to cumulative doses of TTAE (0.5, 1, 1.5, and 2), 4-AP and $Bacl_2$ did not lessen the relaxation of rat aortic rings. These findings imply that TTAE's vasorelaxant action occurs independently of inward rectifying potassium channels (KIR) and voltage-gated K^+ channels (KV).

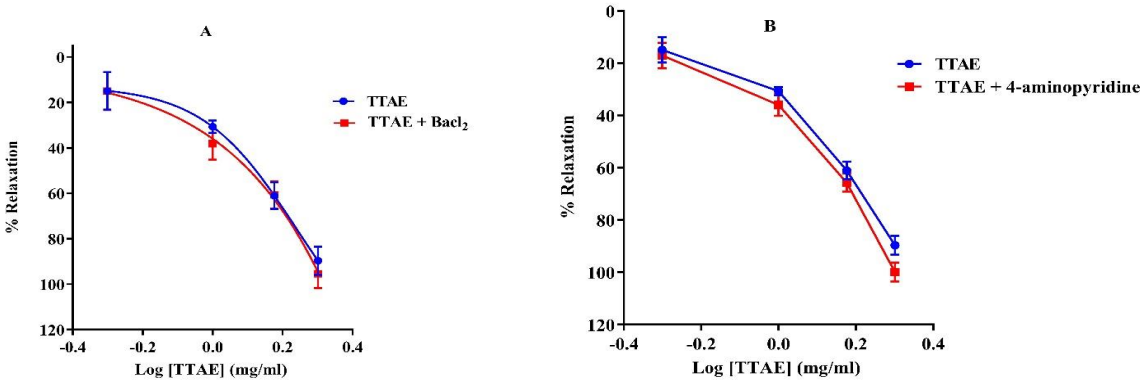

Figure 5: The vasorelaxant effect of varying concentrations of TTAE in EP-precontracted aortic rings was observed, with and without pre-incubation with $Bacl_2$ and 4-aminopyridine (4AP).

3.1.6. Effect of TTAE on extracellular Ca^{2+}-induced contraction

Through precontraction with EP (10 µM) or KCl (80 mM) in Ca^{2+} free KH buffer, with and without TTAE preincubation (control) for 20 minutes, we investigated the contraction response generated by a single concentration of calcium chloride ($CaCl_2$, 0.3 mM) in aortic rings. $CaCl_2$-induced contraction responses were measured in the presence and absence (control) of low-concentration TTAE pretreatment. In Ca^{2+}-free KH buffer, addition of a single dose of $CaCl_2$ (0.3 mM) induced a progressive increase in tension in rat thoracic aortic rings. As shown in Figure 6, TTAE preincubation (0.5 mg/ml) significantly ($p<0.0001$) prevented the extended contractions brought on by the inclusion of extracellular calcium chloride ($CaCl_2$, 0.3 mM) in PE (Figure 6A) and KCl (Figure 6B) precontracted aortic rings compared with the control. These results suggest that the vasorelaxant effect of TTAE is dependent on receptor-gated calcium channels (ROCCs) and voltage-gated calcium channels (VDCCs) located on cell membranes.

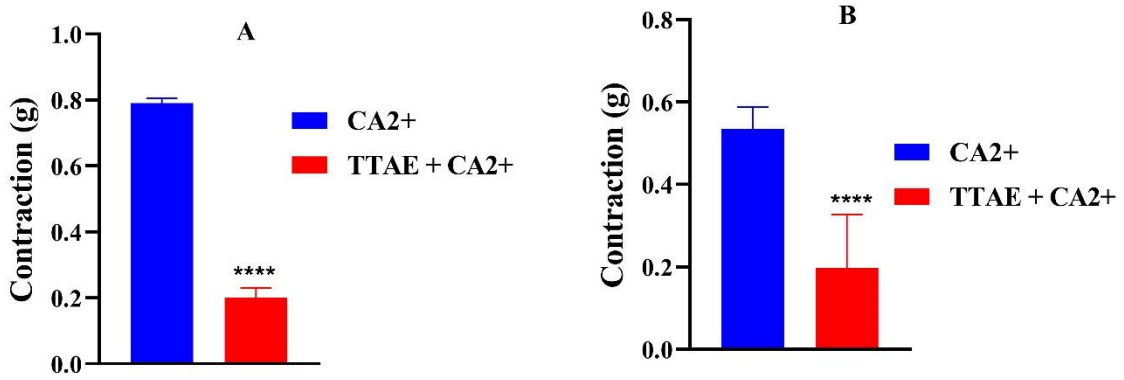

Figure 6: Inhibitory effect of low concentration TTAE (0.5 mg/ml) on contraction induced by the addition of a single concentration of extracellular Ca^{2+} in rat thoracic aortic rings pre-contracted with EP, and pre-contracted with KCl in the presence and absence of TTAE.

3.2. Subacute Toxicity

During the 28-day treatment period, every male and female rat given a dose of 200 mg/kg body weight lived. Compared with the control group, rats treated with the extract showed obvious

signs of toxicity such as diarrhea and alteration of the rats' fur. Additionally, significant behavioral alterations were also observed in treated rats, including reduced activity and decreased desire to eat.

3.2.1. Effect of TTAE on the body weight in rats

During the subacute experimental period, male rats given the 200 mg dose of TTAE and untreated (control) rats showed a consistent and expected increase in mean body weight. However, no significant change in mean body weight was observed compared to the control group (Figure 7). Whereas, female rats treated with TTAE showed a significant decrease in mean body weight ($p<0.001$) during day 28, compared to the control group (Figure 7).

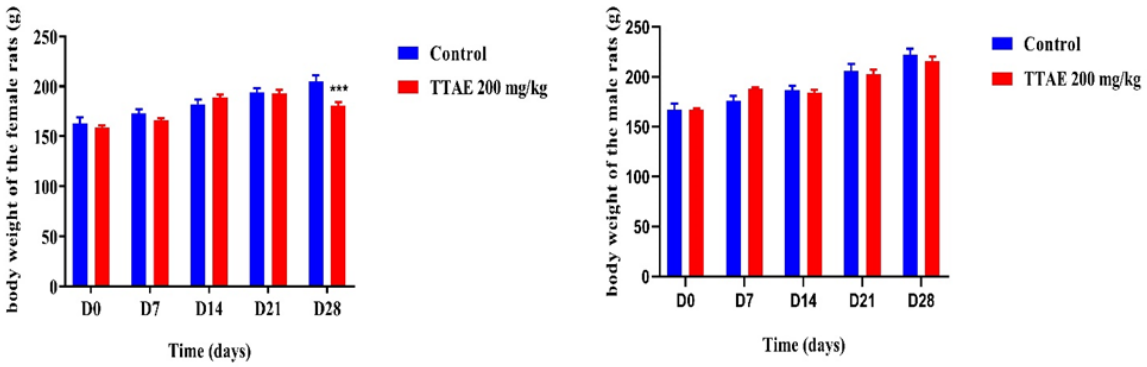

Figure 7: Body weight of female and male rats, control group and treated with aqueous extract of *T. takoumitense* at doses of 200 mg/kg bw in the subacute toxicity study; Values expressed as mean ± SEM, n=3

3.2.2. Effect of TTAE on relative organ weight

In female rats, significant increases were observed in the relative weights of heart ($p<0.01$), lungs ($p<0.05$), liver ($p<0.0001$), kidneys ($p<0.01$) and spleen ($p<0.05$) compared to the control group. However, in male rats, significant increases in the relative weights of kidneys ($p < 0.01$), spleen ($p < 0.01$), liver ($p < 0.01$), testes ($p < 0.0001$) and heart compared to control rats were also observed (Table 1).

316 **Table 1**: Effect of *T. takoumitense* aqueous extract on relative organ weights in female and
317 male Wistar rats treated at 200 mg/kg bw for 28 consecutive days. Data are expressed as mean
318 ± standard deviation, n = 3 rats for each sex within each group. *p < 0.05, ** p<0.01, ***
319 p<0.001, **** p<0.0001; compared with control.

Rats	**Organ**	**Relative organ weight (%)**	
		2000 mg/kg b.w	**Control group**
Females	Heart	0 .79±0.18**	0.41 ± 0.02
	Lungs	1.41± 0 .31*	0.81 ± 0.10
	Liver	10.83 ± 0.51***	4.41 ± 0.62
	Kidneys	1.79 ± 0.26**	0.73 ± 0.02
	Ovary	0.51 ± 0.24	0.45 ± 0.08
	Spleen	0.60 ± 0.25*	0.24 ± 0.02
Males	Heart	1.02 ± 0.17***	0.31 ± 0.01
	Lungs	1.75 ± 0.43	0.90 ± 0.7
	Liver	11.87 ± 0.97**	3.74 ± 0.72
	Kidneys	2.13 ± 0.32**	0.60 ± 0.03
	Testicles	4.07 ± 0.16****	1.81 ± 0.09
	Spleen	0.61 ± 0.17**	0.17 ± 0.003

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3.2.3. Effect of TTAE on hematological parameters

MCV ($p<0.001$) and MCHC ($p<0.05$) significantly increased in female rats' hematological parameters. while the other parameters LYM, RBC, HGB, MCV, HCT, HGB, and PLT were not significantly different compared to the control. Similarly, the hematological parameters of male rats showed a significant increase in the parameter LYM ($p<0.05$), MCV ($p<0.05$), MCHC ($p<0.001$) and PLT ($p<0.0001$) compared to the control rats (Table 2).

Table 2: Hematological parameters of rats treated with TTAE for 28 days in female and male rats. Data are expressed as means ± SD, n=3 rats for each sex within each group. *p<0.05; **p<0.01; ****p<0.0001 when compared to the control.

Rats	Hematological parameters	200mg/kg b.w	Control group
Females	WBC ($\times 10^9$/L)	4.06± 0.61	6.69 ± 2.43
	LYM ($\times 10^9$/L)	2.28± 0.61	4.37 ± 2.30
	RBC ($\times 10^{12}$/L)	5.48±0.76	7.33 ± 0.12
	HGB (g/dL)	11.2±1.10	14.49 ± 0.37
	HCT (%)	30.68±1.84	37.82 ± 0.75
	MCV (fL)	55.33± 0.62**	50.67 ± 0.57
	MCH (pg)	20.4± 0.55	19.70 ± 0.3
	MCHC (g/dL)	37.3± 0.54*	38.70 ± 0.6
	PLT ($\times 10^9$/L)	510.6± 8.39	502.3 ± 11.59
Males	WBC ($\times 10^9$/L)	5.70± 0.63	8.26 ± 1.16
	LYM ($\times 10^9$/L)	2.77± 0.58*	5.02 ± 0.24
	RBC ($\times 10^{12}$/L)	6.99± 0.41	7.18 ± 0.69
	HGB (g/dL)	13.16± 0.56	13.80 ± 0.75
	HCT (%)	37.15± 0.97	34.75 ± 4.23
	MCV (fL)	53± 0.57*	48.00 ± 1.73
	MCH (pg)	18.9± 0.18	19.2 ± 01.21
	MCHC (g/dL)	35.5±0.4**	39.73 ± 1.13
	PLT ($\times 10^9$/L)	788 .66±8.86****	464.00 ± 12.50

3.2.4. Effect of TTAE on biochemical parameters

The biochemical characteristics of female rats throughout subacute toxicity showed a significant increase in Urea ($p<0.001$) and Creatinine ($p<0.0001$) and a significant decrease in TG ($p<0.01$), TC ($p<0.01$), LDL ($p<0.01$) and AST ($p<0.01$) while other parameters HDL,

ALAT, BT and Alb were not significantly different compared to the control. Biochemical parameters of male rats showed a significant increase in Urea ($p<0.001$) and a significant decrease in TG ($p<0.01$), TC ($p<0.001$), LDL($p<0.001$), ALAT ($p<0.01$) and AST($p<0.001$). Other parameters Creatinine, HDL, BT and Alb were not significantly different compared to the control (Table 3).

Table 3: Biochemical parameters of rats treated with *T. Takoumitense* aqueous extract for 28 days in female and male rats. Data are expressed as means ± SD, n=3 rats for each sex within each group. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 when compared to the control.

Rats	Biochemical parameters	200mg/kg b.w	Control group
Females	Urea mg/dl	62.67 ± 4.13***	24.00 ± 4.58
	Creat mg/dl	0.54 ± 0.04****	0.34 ± 0.02
	TC mg/dl	70.21± 2.81**	82.67 ± 2.52
	TG mg/dl	20 .00 ± 3.35**	34.00 ± 3.60
	HDL mg/dl	52.33 ± 2.89	53.00 ± 3.00
	LDL mg/dl	11.77 ± 2.54**	22.67 ± 2.51
	AST U/L	132.2±12.65****	298.30 ±13.78
	ALAT U/L	68.7± 8.36	76.47 ± 8.85
	BT mg/dl	0.09 ± 0.02	0.08 ± 0.008
	Alb g/dl	3.25 ± 0.5	3 .85 ± 0.14
Males	Urea mg/dl	44.00 ± 1.74***	28.00 ± 1.73
	Creat mg/dl	0.45± 0.082	0.46 ± 0.04
	TC mg/dl	53.53 ± 6.00***	74.00 ± 1.73
	TG mg/dl	21.34 ± 3.21**	32.67 ± 2.88
	HDL mg/dl	39.33 ± 0.67**	40.00 ± 1.73
	LDL mg/dl	13.00 ± 2.01***	26.00 ± 1.00
	AST U/L	237.6±12.47***	304.03 ± 12.05
	ALAT U/L	80.8±4.15**	98.57 ± 0.28
	BT mg/dl	0.098 ± 0.005	0.091 ± 0.002
	Alb g/dl	3.31 ± 1.20	3.39 ± 3.04

3.3. Phytochemical analysis

3.3.1. Preliminary phytochemical screening of TTAE

Preliminary phytochemical analysis of TTAE revealed the presence of polyphenols, flavonoids, tannins, saponins, sterols, terpenoids, reducing sugars, quinones, anthraquinones and glycosides. On the other hand, the absence of alkaloids was noted (Table 4).

Table 4: Phytochemical screening of aqueous extract of TTAE.

Detected phytochemical group	Test	Results of the test
Polyphenols	Ferris chlorid test	(+)
Flavonoids	Shinoda test	(+)
Tannins	Ferricchlorid test	(+)
Saponins	Frothing test	(+)
Quinones		(+)
Anthraquinones		(+)
Sterols and terpenoids	Libermann_Buchard test	(+)
Glycosides	Borntrager's test	(+)
Carbohydrates		(+)
Reducing sugars	Fehling's test	(+)
	Wagner’s test	(-)
Alkaloids	Mayer’s test	(-)
	Borntrager's test	(-)

3.3.2. Measurement of total phenolic, flavonoid and tannin contents

Total phenolic content in TTAE was found to be 39.39±12 mg gallic acid equivalent per 1g of extract (39±12 mg GAE/1g TTAE), using gallic acid as a standard to establish the calibration curve (Figure 8). Total flavonoid content in the same extract was estimated to be 674.65±10.8 mg Rutin equivalent per 1g of extract (674.65±10.8 mg RE/1g TTAE), Rutin, a common flavonoid, was used to generate the calibration curve (standard flavonoid). (Figure 9). While the tannin content was found to be 36.92±0.082 mg catechin equivalent per 1g of extract (36.92±0.082 mg CE/1g TTAE), with catechin being utilized as the calibration curve's standard (Figure 10).

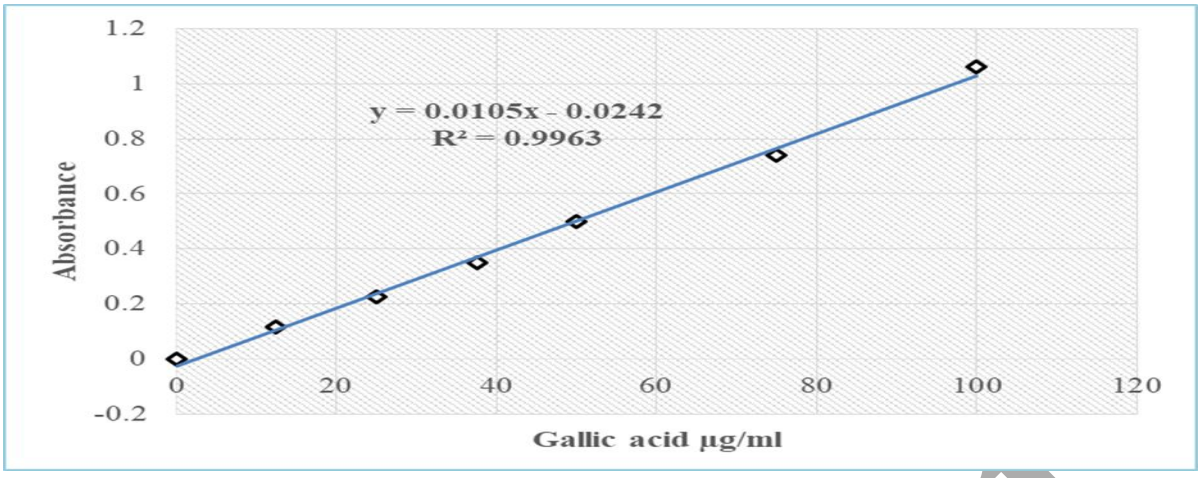

Figure 8: Calibration curve of polyphenol contents established with gallic acid (µg/ml).

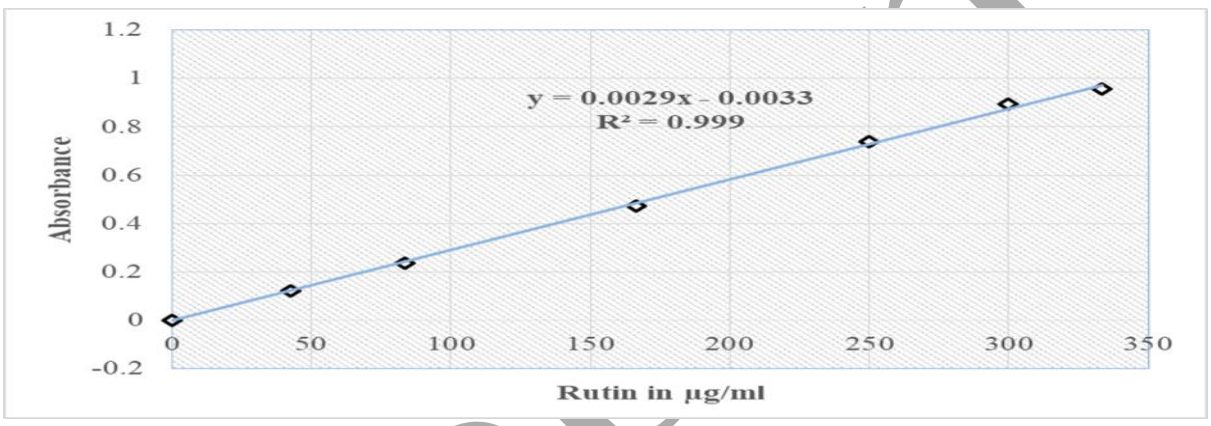

Figure 9: Calibration curve of flavonoid contents established with Rutin (µg/ml).

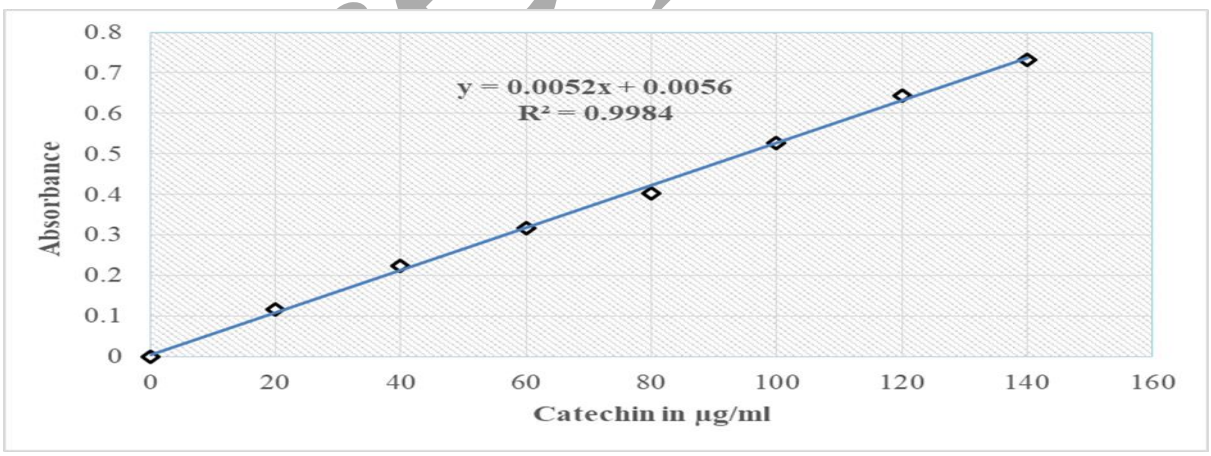

Figure 10: Calibration curve of tannin contents established with Catechin (µg/ml).

4- Discussion

An in vivo investigation was conducted using isolated aortic rings from Wistar rats to explore a possible mechanism through which this medicinal plant exerts its antihypertensive effects via vasorelaxation. Therefore, our findings demonstrated that *T. takoumitense* aqueous extract (TTAE) induced pre-contracted aortic rings to completely relax when exposed to EP (10 μM) and KCl (80 mM). This investigation examined the route underlying the vasorelaxant activity of TTAE using eight reference medications: L-Name, Indomethacin, Methylene Blue, nifedipine, Propranolol, Glibenclamide, $Bacl_2$ and 4-Aminopyridine.

The current investigation demonstrated that the vasorelaxant effect of TTAE was unaffected by the eight reference medications used. This suggests that the direct NO pathway, NO-cGMP pathway, vascular cyclooxygenase pathway, β-adrenergic receptor pathway, ATP-sensitive K^+ channel pathway, and L-type calcium channel pathway did not mediate the vasorelaxant effect of TTAE. According to the study's findings, this medication had no influence on TTAE vasorelaxant effects.

After being pre-contracted by EP (10 μM) or KCl (80 mM), the vasorelaxant effect of low concentration TTAE (0.5 mg/mL) on Ca^{2+}-induced extracellular contractions was examined to ascertain if calcium channels integrated on vascular smooth muscle cells are involved in this vasodilation activity. When the receptor-operated calcium channels (ROCCs) and voltage-dependent calcium channels (VDCCs) on cell membranes open, the free cytoplasmic Ca^{2+} level rises, which causes vascular smooth muscle contraction. PE for ROCC, high extracellular K^+ for VDCC, or the release of Ca^{2+} from the sarcoplasmic reticulum (SR) can all activate these channels. while, smooth muscle cell relaxation and vasodilation occur when these calcium channels are inhibited or suppressed, as this prevents calcium from entering or exiting the cytoplasm of vascular cells. After precontraction with EP or KCl in Ca^{2+}-free KH buffer with and without TTAE preincubation, the $CaCl_2$-generated contraction response was assessed in aortic rings. The sarcoplasmic reticulum releases $Ca^{2+,}$ which causes the initial phasic contraction and the transient Ca^{2+} increase with norepinephrine [17].

The results demonstrated that, when precontracted by EP (10 μM) or KCl (80 mM), a low concentration of TTAE (0.5 mg/mL) pre-incubated for 20 minutes significantly inhibited the prolonged contraction of aortic rings induced by the addition of a single concentration of extracellular calcium chloride ($CaCl_2$ 0.3 mM) in a calcium-free Krebs solution. These findings show that the vasorelaxant effect of TTAE is related to the inhibition of extracellular Ca^{2+} influx

via receptor-activated calcium channels (ROCC) and voltage-dependent calcium channels (VDCC). Interestingly, several studies have documented the vasorelaxant potential of medicinal plants and their derivatives via Ca^{2+} channel blockade [17]. To assess the safety of TTAE, a comprehensive toxicological study is necessary because the scientific information on the toxicological activities of TTAE is still insufficient. In the present subacute toxicity study is used to assess the deleterious consequences resulting from continuous or recurrent exposure to the TTAE for a duration of 28 days. The accumulation of chemical compounds and their progressive effects on specific tissues and organs are provided by these toxicity profile studies. Thus, TTAE was evaluated at a dose of 200 mg/kg bw in rats of both sexes for this subacute toxicity study. A thorough assessment of hematological and biochemical parameters, as well as body weight changes, and calculation of relative organ weights were all included in this assessment. During the 28-day duration of subacute oral administration of the extract. All male and female rats treated with the dose of 200 mg/kg body weight survived throughout the treatment period. Compared to the control group, rats treated with the extract showed obvious signs of toxicity such as diarrhea and alteration of the rats' coat. In addition, notable behavioral changes were observed in animals treated with TTAE and a significant decrease in the average body weight of female rats during the 28th day, compared to the control group. Absolutely the chemical composition of this plant influences their potential toxicity [18].

On the other hand, in female rats, significant increases were observed in the relative weights of some organs of rats such as the heart, lungs, liver, kidneys and spleen. However, in male rats, significant increases in the relative weights of the kidneys, spleen, liver, testes and heart compared to control rats were also observed. Significant changes in the relative weights of organs are considered indicative of possible adverse effects of the substance [19]. Where The increase in relative liver weight of rats observed in the present study could be associated with hepatic edema, as edema can induce increased organ weights and a slight increase in serum liver enzyme levels, suggesting organ hypertrophy. Alterations in hematological and biochemical parameters are frequently used as important indicators of toxicity in acute and long-term toxicological research [20]. The harmful effects of chemicals from *T. takoumitense* extracts on blood components may be demonstrated by evaluating hematological parameters. Comparing the treated groups to the control group, the majority of the values in the obtained hematological results were normal. However, some data, including MCV and MCHC, leukocyte count (LYM), and platelet count (PLT), differed considerably from the control group. In general, MCV reflects the size of red blood cells and MCHC reflects the mean corpuscular

hemoglobin concentration. The observed elevation in these parameters could be attributed to enhanced production of hematopoietic regulatory factors, such as erythropoietin (EPO), within the bone marrow. This suggests that TTAE may possess anti-anemic properties [21]. Furthermore, the increase in MCV levels might indicate alterations in the size of red blood cells in the treated rats.

The process by which ammonia in some animals including humans is transformed into urea and then excreted is known as the urea cycle. Through a series of metabolic processes, the kidneys normally remove this cycle from the body in the urine after it is created in the liver. Repeated administration of the extract (200 mg/kg) throughout the subacute toxicity period resulted in a significant increase in serum creatinine levels in female rats ($p < 0.0001$) while a statistically significant increase in urea levels was observed in both male and female rats ($p < 0.001$). to assess the concentrating and diluting capacity of renal tubular functions in addition to the glomerular filtration rate. A commonly used marker is serum urea. Elevated serum urea levels may be a sign of reduced renal function. Furthermore, in clinical settings, elevated creatinine levels indicate chronic renal failure, while elevated urea levels indicate acute renal dysfunction [22].

The risk of cardiovascular disease is estimated by measuring the serum lipid profile nearly every day. Triglycerides, total cholesterol, HDL cholesterol, and LDL cholesterol are the four crucial components of this thorough examination. The results show that repeated oral administration of the extract at a dose of 200 mg/kg to male and female rats resulted in a significant decrease in triglycerides ($p < 0.01$ and 0.01 respectively), LDL cholesterol ($p < 0.01$ and 0.01 respectively) and TC ($p < 0.001$ and 0.01 respectively), while HDL cholesterol did not differ significantly from the control. However, these results suggested that *T. takoumitense* has antihyperlipidemic properties. It is important to remember that the kidneys also play a role in the lipid metabolism process, with the liver being a key organ in this regard. In addition, hyperlipidemia, the degree of which varies depending on the severity of the disease, is observed in people with chronic liver disease [23].

The cytoplasm of liver cells is where most AST and ALT production takes place. When liver cells are damaged by hepatotoxic substances, these enzymes are released into the bloodstream. These enzymes are essential markers for predicting potential chemical toxicity and their increase signifies liver damage. It is noteworthy that, in contrast to AST, ALT is also extensively distributed throughout the kidneys, testes, heart, and skeletal muscles, making it the

most sensitive sign of liver dysfunction or toxicity. It is therefore possible that some phytochemicals causing liver inflammation or damage are responsible for the significant decrease in ALT enzyme levels observed in male rats, after administration of an aqueous extract of *T. takoumitense* at a dose of 200 mg/kg body weight for 28 days. In contrast, AST levels in both male and female rats were significantly decreased, which may indicate that the enzyme is inhibited or that its activity has decreased [24]. Additional particular toxicity tests that reveal more about the extract's harmful effects on the liver and kidneys include histological sections.

Therefore, this vasorelaxant and toxicological effect is linked to the chemical composition of TTAE. Preliminary phytochemical analysis of TTAE revealed the presence of polyphenols, flavonoids, tannins, saponins, sterols, terpenoids, reducing sugars, quinones, anthraquinones, and glycosides. In contrast, no alkaloids were detected. Anthraquinones, however, have been identified as secondary metabolites potentially linked to toxic effects such as muscle weakness and weight loss [25]. These results suggest that TTAE (200 mg/kg) induces adverse effects with prolonged use. Furthermore, additional studies are needed to investigate the genotoxicity, nephrotoxicity, and neurotoxicity of TTAE, as well as the toxicity of its bioactive compounds. Furthermore, our future research should aim to determine the maximum tolerated dose with minimal adverse effects and the minimum effective dose for in vivo studies. Moreover, the study shows that the aqueous extract of *T. takoumitense* induces significant vasorelaxation at the level of the aortic rings pre-contracted by phenylephrine (PE, 10 μM) and potassium chloride (KCl, 80 mM). This vasorelaxant activity is probably due to the blockade of receptor-activated calcium channels (ROCCs) and voltage-gated calcium channels (VDCCs). These characteristics are attributed to the chemical composition of the extract, and in particular to its secondary metabolites. It is therefore highly likely that these metabolites contain compounds that, once purified, could exhibit pharmacological activity. Further phytochemical and preclinical research is needed to identify, isolate, and purify the active constituents involved in this activity. Finally, the role of *T. takoumitense* in the management of hypertension could be clarified by appropriate additional studies.

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2- Author's Contribution

El aydy S conducted the vasorelaxation, phytochemical, and toxicological studies and wrote the manuscript. Bouadid I, Amssayef A, and Qabouche A participated in the subacute toxicity study. Hebi M and El-Haidani A participated in the study design and performed the statistical analysis. Azzaoui B and Khalloukiont F participated in the phytochemical study. Eddouks M designed the study and participated in its design and coordination.

3-Conflict of interests

The authors declare no conflict of interest, financial or otherwise.

4-Ethics approval

No human was involved in the study. All animal procedures were performed in accordance to the local ethical committee guidelines, Faculty of Sciences & Techniques Errachidia, Morocco, N° FSTE/205

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6- Data Availability

Not applicable.

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