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Research Article

Effect of pyrrole and xanthine derivatives on the contractility of *A. basilaris*

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Abstract

Oxidative stress plays a key role in the progression of neurodegenerative processes. Based on the etymology of these diseases, another common factor is impaired blood supply, which leads to cerebral ischemia. In the current study, the effects of pyrrole and xanthine derivatives on the contractility of the rat cerebellum *A. basilaris* were evaluated. To aim this purpose, the *A. basilaris* was isolated from 10 white rats, and the contractility was elucidated through wire myography. The obtained results demonstrated a good vasodilating dose-dependent effect of compound ethyl 5-(4-bromophenyl)-1-(3-hydrazinyl-3-oxopropyl)-2-methyl-1H-pyrrole-3-carboxylate (**12**), lowering arterial contractility, while the caffeine derivatives N-(1-methyl-2-phenylethyl)-2-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl-sulfanyl)-acetamide (**JTA3**) and N-(tetrahydrofuran-2-ylmethyl)-2-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylsulfanyl)-acetamide (**JTA5**) increases vascular tone, with **JTA5** having a transient effect.

Keywords

pyrrole, xanthine, contractility, *A. basilaris*

Introduction

Caffeine is the most commonly studied methylxanthine and is known as a psychostimulant. The compound increases cognitive function with a decrease of blood flow in brain vessels in animals and humans (Cappelletti et al. 2015). The reduction in cerebral blood flow is induced at doses that boost cerebral consumption of glucose. This action profile confirms the lack of use of caffeine as a drug for the treatment of dementia or other neurodegenerative symptoms (Rômulo et al. 2020).

Numerous studies have been conducted to determine the effects of caffeine on the cardiovascular system as well as on different types of blood vessels. The obtained results revealed that certain substances found in caffeinated beverages (theobromine and theophylline – used to treat respiratory diseases) may have effects on changing blood pressure, heart rate, vascular contractility, and vascular endothelial activity. In higher doses, caffeine demonstrated a significant improvement of endothelial function in patients with and without coronary artery disease, which is also associated with low plasma markers of inflammation (Echeverri et al. 2010).

Additional *in vitro* studies displayed a significant vasodilatory effect (approximately 75%) at micromolar (μM) concentrations of caffeine, comparable to those normally consumed by humans (Lopez 2001). The relative system-

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atic response to caffeine, along with the various factors that influence the metabolism and its consequences, has not been assessed in any *in vitro* experiments. Thus, the mechanisms of action predominating *in vivo* are still unclear (Lopez 2001).

It is worth mentioning the direct effect of caffeine on endothelial cells by stimulating the production of NO, which by itself is synthesized by nitric oxide synthase (eNOS) from L-arginine and oxygen (Umemura et al. 2006).

In addition, the vascular effects of caffeine are also related to its stimulation of the release of Ca^{2+} from the endoplasmic reticulum and the increase of its concentration in the cytoplasm (iCa^{2+}), which further favors the activation of eNOS. In VSMC, the Ca^{2+}-influx mechanisms responsible for sustained cell activation are generally mediated by both voltage-gated Ca^{2+} channels and a specific receptor (Leung et al. 2008).

Overall, the effect of caffeine on the vascular endothelium results in greater expression of NO (autocrine effect), acting on the same endothelial cell to increase Ca^{2+} and its rapid diffusion outward (paracrine effect) (Higashi 2019).

Furthermore, caffeine competitively inhibits 3'-5'cGMP phosphodiesterases and stimulates even greater accumulation of cGMP (Echeverri et al. 2010).

Some *in vitro* studies suggest caffeine induction of strong arterial vasodilation in the presence or absence of preserved endothelial function, using rabbit arteries and human arteries (Echeverri et al. 2008).

As a summary, it may be defined that in general the caffeine intake is related to the appearance of a number of vascular effects with various mechanisms.

Some recent studies suggest the appearance of cerebral blood flow by cyclooxygenase (COX) with relatively unknown effects during hypoxia. The studies imply that COX contributes to both basal and hypoxic cerebral vasodilation, with COX-mediated vasodilation in the posterior being greater than in the anterior cerebral circulation. These results indicated the large, uniform contribution of COX to cerebrovascular tone during normoxia and its privation for hypoxic vasodilation in the regions supplied by large extracranial or intracranial arteries (Kellawan et al. 2020).

Additional studies pointed towards the evaluation of the potential role of COX isoforms on brain disorders that has been conducted (Kaufmann et al. 1997; Minghetti and Levi 1998; O'Banion 1999), suggesting the existence of a relationship between the COX-2 overexpression and appearance of acute neurotoxicity, in means of hypoxia/ischemia and seizures. However, currently the exact role of COX-2 is still controversial in inflammatory and neurodegenerative brain pathologies (Minghetti 2004).

Expression of COX-2 leads to increased production of prostanoids (Hempel et al. 1994, Akarasereenont et al. 1995). In the brain, prostanoids are potent vasoactive (Ellis et al. 1979) and inflammatory substances (Schaad et al. 1991). In addition, certain pathophysiological conditions, including ischemia and hypoxia, are associated with increased expression of COX-2 in the brain (Brian et al. 1998).

Another COX-related molecule, causing controversial vasoactive effects, is prostaglandin E2 (PGE2). A bimodal vasomotor response is observed by PGE2 due to some astrocytic release of PGE2, contributing to neurovascular coupling responses and some cerebral vasospasm associated with subarachnoid hemorrhage with overproduction of PGE2. Some studies have identified that at low concentrations, PGE2 dilates the human cerebral parenchymal arterioles, while at higher concentrations, it constricts them (Czigler et al. 2020).

These data suggest that a new possible indirect path for modification of vascular effects is through moderation of COX-2 activity. This points our attention towards a group of N-pyrrolylcarboxylic acids, reported to be potent COX-2 inhibitors (Bocheva et al. 2006, Lessigiarska et al. 2005).

A thorough understanding of the mechanisms that regulate cerebrovascular tone is essential for further improving our knowledge of various brain injuries' pathophysiology.

By definition, the vascular tone is the contractile state of the vascular smooth muscle cells lining the blood vessels.

A key role in the proper function of the cerebral musculature is played by the appropriate sustenance of the cerebrovascular tone necessary for preventing conditions such as ischemia or hemorrhage, arising due to either decreased or increased cerebral blood flow and/or imbalance in the needs of the brain's metabolic demands. Often, the appearance of disturbances in the regulation of the cerebrovascular tone can cause various cerebrovascular diseases, including insults, stroke, or brain injury (Hu et al. 2017).

The following main mechanisms are involved in the regulation of cerebrovascular tone: neural, endothelial, and myogenic (Cipolla 2009). The neural control is mediated primarily through the autonomic nervous system, with various pathways responsible for modulation in the vessel diameter. The myogenic mechanisms are more related to a phenomenon known as autoregulation, explained by the responsive changes in the intravascular pressure, aimed at maintaining a constant cerebral blood flow independent of the changes in the blood pressure. The production of vasodilators such as nitric oxide (NO) and prostacyclin, and vasoconstrictors like endothelin 1, is the endothelium's contribution to cerebrovascular tone regulation (Salvagno et al. 2024).

Thus, the aim of the current study was pointed towards evaluation of the effects of one pyrrole-containing compound (**12**) and two xanthine derivatives (**JTA3** and **JTA5**) on the contractility of *A. basilaris* as a potential target for prevention of neurodegenerative appearance.

Materials and methods

The studies were conducted on 10 male white rats, breed Wistar. The animals were obtained from the National Breeding Center of the Bulgarian Academy of Sciences, Slivnitsa, Bulgaria, and were reared under standard conditions in Plexiglas cages with free access to water and food and a 12 h/12 h light/dark regime at a temperature of 20 °C–25 °C. Twelve hours before each particular study, the animals were deprived of their food. The experiments

were carried out in accordance with Regulation No. 15 on the minimum requirements for the protection and humane treatment of experimental animals (SG № 17, 2006) and the European Regulation on working with experimental animals and were approved by the Bulgarian Food Safety Agency with permit № 323, valid until 22.12.2026.

Synthesis of the target compounds

The pyrrole-based representative ethyl 5-(4-bromophenyl)-1-(3-hydrazinyl-3-oxopropyl)-2-methyl-1H-pyrrole-3-carboxylate (**12**) (Fig. 1) was synthesized through a classical Paal-Knorr cyclization as explained in detail in our previous study (Kondeva-Burdina et al. 2022).
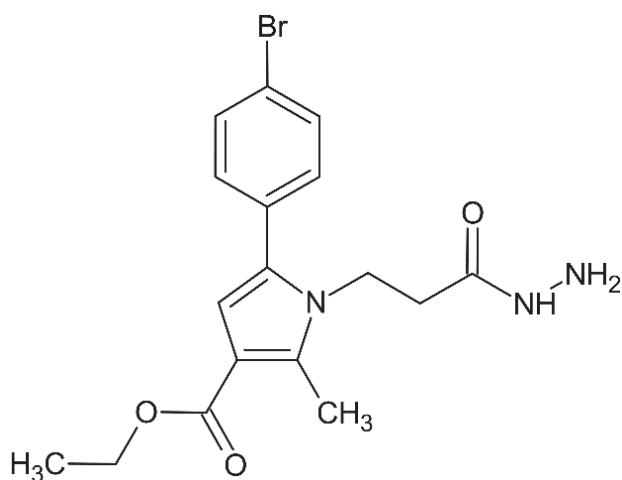

Figure 1. The structure of selected compound **12**.

The xanthine-based derivatives N-(1-methyl-2-phenylethyl)-2-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl-sulfanyl)-acetamide (**JTA3**) and N-(tetrahydrofuran-2-ylmethyl)-2-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylsulfanyl)-acetamide (**JTA5**) was obtained in our laboratory as per the procedure defined at Kasabova-Angelova et al. 2018 and Mitkov et al. 2007, respectively.

The experiments were conducted with native endothelium. The defined procedures were applied as follows:

Solvents:
To evaluate the contractile activity of vascular preparations, three varieties of modified Krebs solution with different compositions (in mM) were prepared:

For isolation and assertion of vascular segment: NaCl 112.5, KCl 4.75, NaH_2CO_3 25.00, KH_2PO_4 1.19, $MgSO_4.7H_2O$ 1.8, $CaCl_2$ 0.00 or 0.16, glucose 11.5. The absence of $CaCl_2$ in the solution is necessary to ensure a relaxed (dilated) state of the vascular musculature. Thus, mechanical trauma to the arterial wall during the preparatory procedures is minimized (Hessellund et al. 2003).

For conservation of vascular segment: NaCl 118.0, KCl 5.0, $CaCl_2$ 2.5, glucose 11.5, taurine 10.0, pyruvic acid 5.0, HEPES 25.0 (pH 7.4–7.5).

PSS for contraction experiments — the compositions of the solutions depended on the blood vessels that were assessed, as follows:

For recontracting of vascular segments: KCl 125.00, $MgSO_4.7H_2O$ 1.80, NaH_2CO_3 25.00, KH_2PO_4 1.19, EDTA 0.03, $CaCl_2$ 2.50, glucose 5.50.
For *A. basilaris*: NaCl 112.50, KCl 3.50, $MgSO_4.7H_2O$ 1.20, NaH_2CO_3 25.00, KH_2PO_4 1.19, EDTA 0.03, $CaCl_2$ 1.50, glucose 5.50.

Method for isolation of *A. basilaris*

The brain is extirpated from the skull of the laboratory animal 10 minutes after its death. For this purpose, after removing the parietal bones, the brain is carefully lifted with a spatula in its frontal part in order to cut the cranial nerves and *n. opticus*. The aim is to protect the meninges at the base of the brain from damage, thereby also protecting the *A. basilaris*. Under a binocular magnifier, the corresponding meninges are removed, and *A. basilaris* is carefully dissected in ice-cold solution from the base of the brain using fine scissors and forceps. Then the prepared *A. basilaris* was stored in an ice-cold PSS storage solution for 20 min. After this period, segments of the artery are mounted on the wire myograph.

Method for registration of vascular segments' contractile activity

Arterial vascular segments, with a length of 1.8–2 mm, after being dissected free from the surrounding tissues (fatty and connective), are fixed on two stainless steel wires with a thickness of 40 μm in a solution with the same composition as the one in which they were isolated. The fixed preparations were attached with steel jaw screws to a volume-controlled organ tub of a wire myograph (model 410A, JP Trading, Denmark) containing modified Krebs solution (PSS for contraction studies) of pH 7.4, which was continuously aerated with a gas mixture (95% O_2, 5% CO_2). One jaw is connected to a micrometer that adjusts the distance between the two wires, and the second wire is fixed on a second jaw connected to a piezoelectric strain gauge. Finally, connective tissue and fat residues around the vessel are carefully removed using fine scissors and tweezers. The procedure for dissecting the arterial preparations and fixing the segments of the apparatus was conducted for an average of 1 hour at the temperature of the working solutions of 2–4 °C.

Normalization

Fixed, the vessel was equilibrated in the bath for 30 min in the medium of working PSS solution and 2.5 mM $CaCl_2$ at 37 °C and continuous aeration with carbogen (95% O_2 and 5% CO_2). After a 30 min equilibration period, the vessel preparation was normalized to a passive tension equivalent to 90% of their diameter (diameter D100) when the vessel was exposed to a transmural pressure of 100 mmHg. The normalization procedure is as follows: initially in a com-

pletely unloaded state, the preparations were stretched incrementally by moving apart the two wires on which the arterial vessel was fixed. Each stretch step increased in tension, and in this state the vessels remain for about 100–120 sec, during which time the vessels, after a rapid and brief contraction, reach a stable level of tension. This process is repeated as many times as necessary to reach such a stretching of the vessels as to generate a tension value equivalent to 90% of the passive diameter, and at this diameter, the effective pressure is 13.3 kPa (100 mmHg) (Corcoran et al. 2014). The normalization procedure examines the differences in the length and diameter of the segments, thus allowing the examination of the different vessels under identical conditions of intravascular pressures, which are similar to those in intact biological objects. After normalization, the vascular segments were incubated for about 30 min before the actual experimental procedure.

In each experiment, the viability of the preparation and the presence of intact endothelium were tested. The first is accomplished by sequential applications of PSS to recontract vascular segments at the beginning of the experiment after the normalization procedure. The presence of functionally intact endothelium was verified by acetylcholine-evoked relaxation (ACh) (10^{-5} M), applying at 125 mM K^+-induced contraction.

Preparation of the applied solutions of 12, JTA3, and JTA5

The stock solutions of the target compounds **12, JTA3,** and **JTA5** are prepared at 1 mM concentration in DMSO. The desired working solutions are prepared by dilution of the corresponding stock solutions with distilled water.

Statistical processing of the results

The obtained experimental data on contractility were processed by the method of variational analysis ANOVA. Results were presented as arithmetic mean values obtained from preparations that were isolated from individual experimental animals. The standard error of the arithmetic mean (±S.E.M.) was also estimated. Statistical significance when comparing means was determined by Student's t-test and Tukey-Kramer multiple comparison test for paired and group data for statistical significance $P < 0.05$. Computer programs were used for the statistical processing of the data, the construction of the graphs, and the overall layout of the figures: "GraphPad Instat" v2.04 and v3.02, "Origin Pro 7.5 PRO," "Corel Draw," and "MyoData" v2.02.

Results and discussion

In order to assess the neurological properties of the selected molecules, an evaluation of the effect of the target molecules on the vascular tone was performed.

Two are the main controlling mechanisms for vascular tone regulation in cerebral vessels: muscular and endothelial (Cipolla 2009). The former is regulated by myogenic

mechanisms originating from vascular smooth muscle. The latter is regulated by endothelial factors, such as nitric oxide and endothelin, which can decrease or increase the vascular tone, respectively (Salvagno et al. 2024).

The available data suggested that the regulation of cerebrovascular tone involves a complex interaction of multiple factors and is vital for preserving cerebral homeostasis, as its disruption can result in neurological deficits. To investigate the influence of the target compounds on the cerebral vasculature, the corresponding effect on the vessel contraction is measured for the pyrrole derivative **12** and the caffeine-based **JTA3** and **JTA5**.

The pyrrole-based representative ethyl 5-(4-bromophenyl)-1-(3-hydrazinyl-3-oxopropyl)-2-methyl-1H-pyrrole-3-carboxylate (**12**) (Fig. 1) was selected due to its lowest neurotoxicity and highest neuroprotection on all evaluated cellular and subcellular parameters, including synaptosomal viability and GSH and MDA levels, as revealed in our previous study (Kondeva-Burdina et al. 2022). In addition, the determined *h*MAOB inhibitory effect suggested the molecule could perform effects related to the decrease of the formation of oxidative stress. In this relation, any tentative information on its vascular (*A. basilaris*) tone effects will be essential for the full assessment of this compound.

Effect of compound 12 on vascular wall contractility of *A. basilaris*

For the performance of the experiments, a series of concentration-differing solutions of compound **12** were prepared, aiming for concentrations of 10^{-10}, 10^{-9}, 3×10^{-9}, 10^{-8}, 3×10^{-8}, 10^{-7}, 3×10^{-7}, and 10^{-6} mol/l. Each solution was added to the isolated vessel and kept in contact for 30 minutes. The corresponding change in the tension was measured in mN/mm. The obtained results established that compound **12** exhibits a good concentration-dependent, vasodilating effect on the brain *A. basilaris* (Figs 2–4).

Preclinical measurement of vasoactivity is considered an effective method for evaluation of the efficacy of certain target molecules during the drug development process. Altered vasoactivity is related to some cardiovascular, oncological, and currently neurological disorders. In this means, the appearance of vasodilators, which are regionally selective, would be advantageous for maximizing treatment efficacy while avoiding side effects, such as systemic hypotension. Thus, the obtained results present for the first time the vasodilating effect of pyrrole-based hydrazide on *A. basilaris*, pointing to this derivative as promising for obtaining selective dose-dependent vascular tone effects.

Effect of xanthine derivate JTA3 on vascular wall contractility of *A. basilaris*

Results from human clinical trials suggest that moderate doses of coffee consumption may have a protective effect against the risk of Parkinson's disease due to the caffeine in it, but this activity is not entirely clear. Many studies have
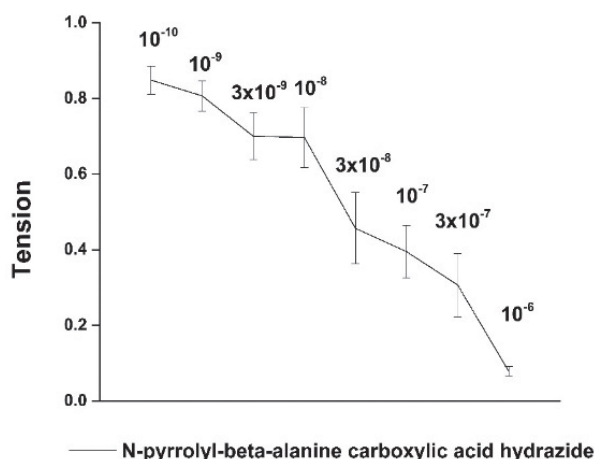
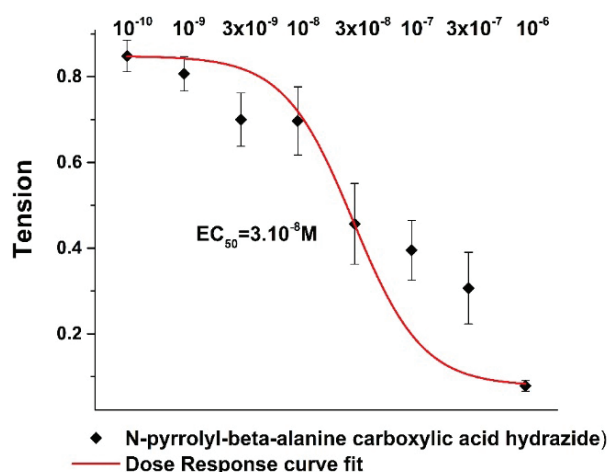**Figure 2.** Effect of compound **12** on contractility of cerebral *A. basilaris*.

Figure 3. EC_{50} of compound **12** on contractility of cerebral *A. basilaris*.
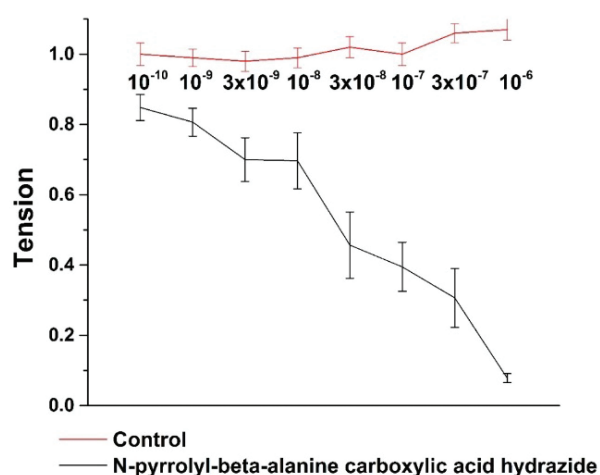

Figure 4. Effect of compound **12** (grey line) and a control (red line).

been conducted to support this hypothesis that caffeine has a protective effect against Parkinson's disease. These results support the hypothesis of a possible protective ef-

fect of moderate doses of caffeine on the risk of Parkinson's disease (Ascherio et al. 2001).

In addition, caffeine is a commonly used psychostimulant that also produces cerebral vasoconstriction by antagonizing adenosine receptors. Chronic caffeine use results in an adaptation of the vascular adenosine receptor system, presumably to compensate for the vasoconstrictive effects of caffeine. Evidence from receptor binding, physiology, and behavioral studies suggests an adaptation to the effects of caffeine following chronic intake. This adaptation presumably accounts for the development of tolerance to the vasoconstrictive and psychostimulant effects of caffeine. To what extent adenosine receptors can upregulate to compensate for the effects of caffeine is a pertinent health question (Addicott et al. 2009).

These neurological effects of caffeine and the suggestion that it affects the vasodilation and vasoconstriction in the brain, together with the promising neuroprotective, antihypoxic, and vasodilating effects of two caffeine-8-thioglycolic acid derivatives: N-(1-methyl-2-phenylethyl)-2-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl-sulfanyl)-acetamide (**JTA3**) and N-(tetrahydrofuran-2-ylmethyl)-2-(1,3,7-trimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-ylsulfanyl)-acetamide (**JTA5**) (Fig. 5) led our study to investigate their effects on *A. basilaris* contractility (Kasabova-Angelova et al. 2018; Mitkov et al. 2007).

The application of **JTA3** with a concentration of 10 μM revealed a significant increase in the vascular tone of *A. basilaris* in comparison with the non-selective adenosine receptor blocker caffeine administered at the same concentration (Fig. 6).

The results demonstrated the absence of effect on vascular tone with the application of **JTA3** in combination with tetrodotoxin (Fig. 7).

Effect of xanthine derivative JTA5 on vascular wall contractility of *A. basilaris*

Administered alone in a concentration of 10 μM, the compound **JTA5** transiently increased the smooth muscle tone of the vascular segments of *A. basilaris* (Fig. 8). However, the obtained result was statistically significant.

The combination of **JTA5** with tetrodotoxin (100 μM) demonstrated the absence of effect on vascular tone (Fig. 9).

In the presence of the non-selective adenosine receptor blocker caffeine (10 μM), **JTA5** does not affect the vascular tone (Fig. 10).

Caffeine is a xanthine derivative with various effects and mechanisms of action in vascular tissue. In endothelial cells, it increases intracellular calcium, stimulating the production of nitric oxide through the expression of the endothelial nitric oxide synthase enzyme. Nitric oxide is diffused to the vascular smooth muscle cell to produce vasodilation. In vascular smooth muscle cells, its effect is predominantly a competitive inhibition of phosphodiesterase, producing an accumulation of cAMP and vasodilation. In addition, it blocks the adenosine receptors present in the vascular tissue to produce vasoconstriction (Echeverri et
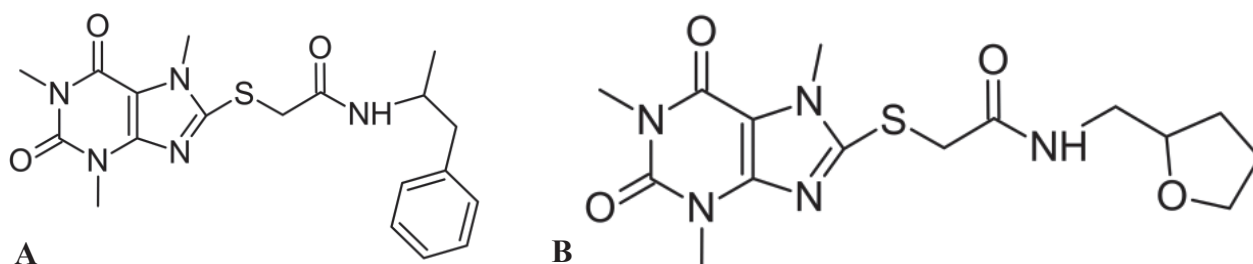
Figure 5. Molecular structures of compound **JTA3** (**A**) and **JTA5** (**B**).
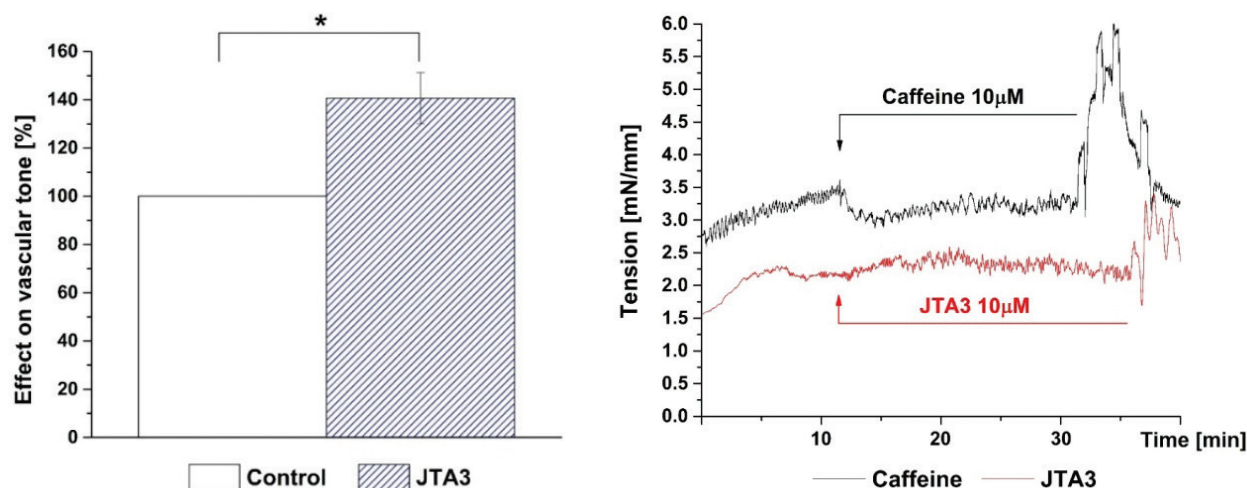

Figure 6. Effect of xanthine derivative **JTA3** on vascular tone of *A. basilaris* in comparison with the effect of caffeine.
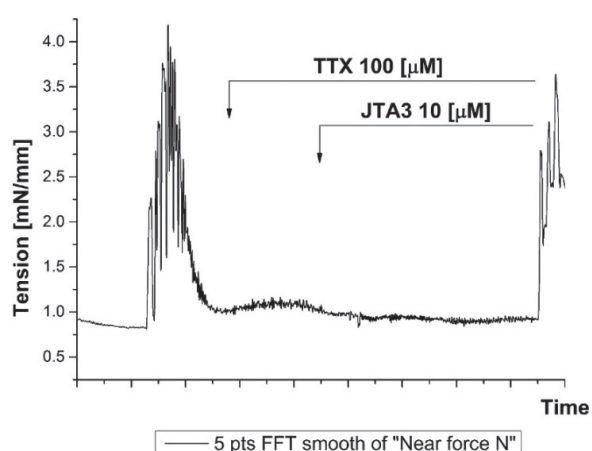

Figure 7. Effect of compound **JTA3** on vascular wall contractility (*A. basilaris*) in combination with tetrodotoxin (TTX 100 μM).

Figure 8. Effect of xanthine derivative **JTA5** on vascular tone of *A. basilaris*.

al. 2010). The effect of caffeine on the vascular endothelium is a greater expression of the enzyme NO synthase or greater production of nitric oxide (Rosenberg et al. 1988), which has an autocrine effect, acting on the same endothelial cell to increase Ca^{2+}, potentiating the response, and coming out of the endothelial cell to diffuse rapidly to the VSMC in a paracrine fashion (Shin et al. 1996).

The observed results indicated that the introduction of a substituent on the 8th position of the caffeine structure does not change the vascular effects of the initial molecule. In combination with tetrodotoxin, the evaluated **JTA3** and
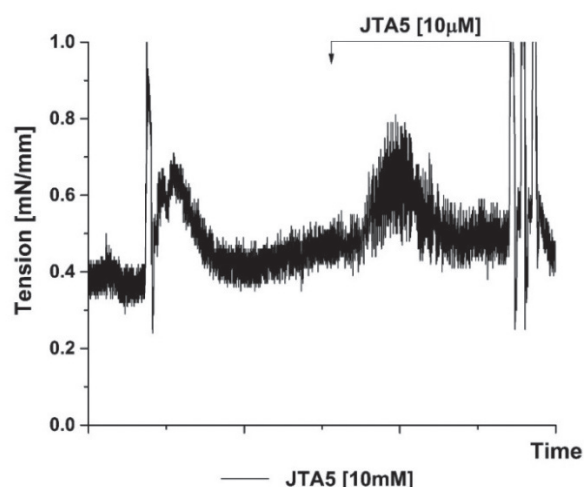
JTA5 did not cause any change in the *A. basilaris* behavior. These results may be considered indicative of the vasodilative mechanism.

The simultaneous incubation of *A. basilaris* in a mixture of tetrodotoxin and each of the corresponding caffeine derivatives **JTA3** and **JTA5** results in blockage of the sodium ionic channels on one hand (characteristic for tetrodotoxin) and, at the same time, blockage of the calcium ionic channels (related to the effect of the derivatives **JTA3** and **JTA5**). Thus, the lack of contracting effect of these combinations
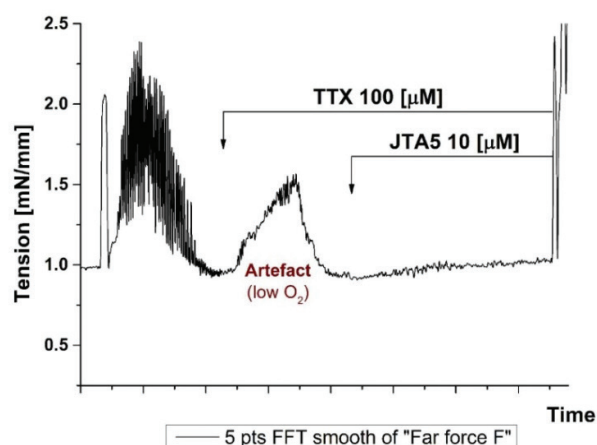
Figure 9. Effect of compound **JTA5** on vascular wall contractility (*A. basilaris*) in combination with tetrodotoxin (TTX 100 μM).
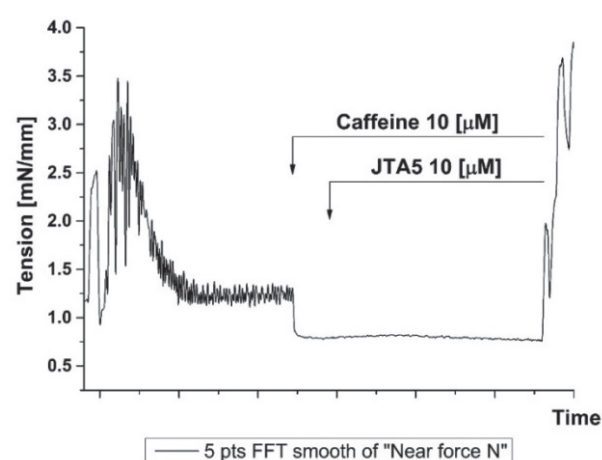

Figure 10. Effect of compound **JTA5** on vascular wall contractility (*A. basilaris*) in the presence of caffeine (10 μM).

on *A. basilaris* may be considered as affecting the contractility through another possible mechanism of vasodilation, most likely related to the involvement of iNOS and nitric oxide (NO) production.

Conclusion

Two caffeine-8-thioglycolic acid derivatives and one pyrrole molecule were selected for investigation of their effects on vascular wall contractility of *A. basilaris* alone or in the presence of caffeine and tetrodotoxin. The obtained results revealed a good vasodilating effect of compound **12**, lowering arterial contractility in a dose-dependent manner, while the caffeine derivatives **JTA3** and **JTA5** increase the vascular tone.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statements

The authors declared that no clinical trials were used in the present study.

The authors declared that no experiments on humans or human tissues were performed for the present study.

The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.

Experiments on animals: The experiments were carried out in accordance with Regulation No. 15 on the minimum requirements for the protection and humane treatment of experimental animals (SG №. 17, 2006) and the European Regulation on working with experimental animals, and were approved by the Bulgarian Food Safety Agency with permit № 323, valid until 22.12.2026.

The authors declared that no commercially available immortalised human and animal cell lines were used in the present study.

Author contributions

Conceptualization: M.K-B., B.K.; methodology: B.K.; formal analysis: Y.M., M.G.; data curation: all co-authors.; writing – A.M., M.K-B., M.G.; visualization: A.Z.

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Data availability

All of the data that support the findings of this study are available in the main text.

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