

Full Length Research Paper

The effects of an aqueous leaf extract of *Clausena anisata* (Willd.) Hook.f.ex Benth. on blood pressure, urine output, angiotensin II levels and cardiac parameters in spontaneously hypertensive rats

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Received 26 April, 2016; Accepted 11 July, 2016

***Clausena anisata* (Willd.) Hook.f. ex Benth (Rutaceae) is a medicinal plant indigenous to Southern Africa and scientific studies report on its biomedical activities and possible antihypertensive property by demonstrating *in vitro* angiotensin converting enzyme inhibition. This study investigated the antihypertensive effects of an aqueous leaf extract of *Clausena anisata* in a spontaneously hypertensive rat model, and determined whether these blood pressure lowering effects could be attributed to diuresis, the inhibition of the renin-angiotensin-aldosterone blood pressure control system and/or possible negative inotropic or chronotropic cardiac effects. Aqueous extracts were prepared from ground leaves of *Clausena anisata*. Four groups of ten rats each received 50, 100, 200 or 400 mg/kg.bw of aqueous extracts intra-arterially respectively to obtain a dose response relationship. Another two groups of fifteen rats each received either plain water (control group) or the plant extract added to their drinking water (experimental group) for 40 days. Urine output was measured at 10 day intervals. All cardiovascular data was recorded with a Powerlab 200 recording system in anaesthetised rats that were catheterised via the carotid artery. Plasma angiotensin II levels were determined using a commercially available enzyme immunosorbent assay kit. The results showed that *Clausena anisata* was effective in reducing aortic blood pressure at the highest dose tested (400 mg/kg.bw). Furthermore, *Clausena anisata* significantly reduced the blood pressure over 40 days. This appears to have occurred via a mechanism that causes a reduction in plasma angiotensin II levels and not via diuresis or negative chronotropic effects.**

Key words: angiotensin-converting-enzyme inhibitor (ACE inhibitor), negative inotropic effect, diuresis, antihypertensive agents, traditional medicinal plants, lethal dose 50 cardiac contractility, South Africa.

INTRODUCTION

In 2008, almost one billion individuals globally had hypertension, with the vast majority residing in developing countries. The highest number of reported cases came

from the Sub-Saharan African region (WHO, 2011). Because of the high cost involved in treating and managing hypertension with conventional pharmaceutical

drugs and the inadequate supply of these drugs to many rural areas with limited resources (Patwardhan, 2005), these populations have to rely on treatment by traditional healers who use plant remedies that have not been adequately tested for their effectiveness. . Approximately 80% of the world's population, primarily those in developing countries, relies on plants and plant-derived medicines for primary health care requirements (Gurib-Fakim, 2006; Talha et al., 2011). South Africa has a very large biodiversity that contains more than 30,000 species of higher plants (Van Wyk et al., 2002). However, only a limited number of plant species have been investigated for their possible antihypertensive effects (Duncan et al., 1999; Mackraj and Ramesar, 2007).

Clausena anisata (*C. anisata*) belongs to the plant family Rutaceae and is indigenous to Southern Africa where it is commonly known as 'isifudu' in Zulu. It usually grows in areas with high rainfall, such as evergreen forests and woodlands, and along streams and rivers (Coates Palgrave et al., 2000). In traditional medicine, *C. anisata* is used as a remedy against worm infections, respiratory ailments, heart disorders, and hypertension (Hutchings et al., 1996). In addition, traditional healers claim that the steam arising from boiling leaves of *C. anisata* strengthens the heart, cures rheumatism and decreases fever (Coates Palgrave et al., 2000). A number of studies have also shown the therapeutic potential of the plant as an antimalarial (Clarkson et al., 2004), hypoglycaemic (Ojewole, 2002) and antiparasitic (Makanju, 1983) remedy. Phytochemical studies reported that the plant contains large amounts of coumarins, such as chalepin, osthole, coumarins, scopoletin, xanthoxyletin, and heliottin (Duncan et al., 1999). Also, the presence of carbozole alkaloids, such as clausenol and clausamine, has shown to exhibit antimicrobial properties (Chakraborty et al., 1995). Furthermore, many terpenoid hydrocarbons, sesquiterpenoids and fatty acids have been isolated from the pericarps, roots and leaves of this plant (Reisch et al., 1985). However, very little is known about the antihypertensive properties of the plant as claimed by traditional healers. One study, however, suggested that the plant may possess a possible antihypertensive property by showing angiotensin converting enzyme (ACE) inhibition when an aqueous leaf extract was tested *in vitro* (Duncan et al., 1999). The current study was therefore conducted to investigate whether an aqueous leaf extract of *C. anisata* has blood pressure (BP) lowering effects in the spontaneously hypertensive rat (SHR) and, to further investigate whether the antihypertensive effects could be attributed to diuresis, inhibition of the renin-angiotensin-aldosterone

BP control system, possible negative inotropic or chronotropic cardiac effects or a combination of these effects.

MATERIALS AND METHODS

Preparation of the plant extract

Fresh leaves of *C. anisata* were collected from the South African National Biodiversity Institute in Pretoria, South Africa. The plant was authenticated by the botanist, Mrs J. Maphuta as *C. anisata* (Willd.) Hook.f. ex Benth (the plant name has been checked on www.theplantlist.org) and a voucher specimen (Genspec 3346-1) was deposited at the institute's herbarium. Directly after collection, the fresh leaves were washed with water and air-dried at room temperature for 48 h. The dried leaves were then ground into a fine powder using a coffee grinder, vacuum packed at -0.8 bar (La.va V300 vacuum packing machine, La.va, South Africa) and stored at 4°C.

The leaf powder was used to prepare a crude aqueous extract using a procedure similar to the method described by Duncan et al. (1999). Briefly, 15 g of the powdered leaves were added to 150 mL of distilled water in a beaker that was surrounded by ice and subjected to ultra-sonication for 60 min (Soniprep 150 ultrasonic disintegrator, MSE Scientific Instruments, England). After sonication, 150 mL of warm distilled water (40°C) was added to the mixture, stirred for 1 h on a magnetic stirrer and then left to stand overnight to allow the plant residues to settle to the bottom. The supernatant was then filtered (Whatman no 1 filter paper), and the plant residue was then repeatedly (2 times) extracted with 50 mL of distilled water (40°C). The solution was then stirred for 5 min and left standing for 2 min; then, the supernatant was filtered, after which the filtrate was centrifuged for 10 min at 3000 x g (17RS, Heraeus Sepatech centrifuge, USA). The supernatant was then decanted into an empty pre-weighed Petri dish and was oven dried at 40°C for 24 h. The dried yield was weighed, sealed and then stored at 4°C in a refrigerator until testing.

Toxicity testing

Toxicity testing of the plant extract was performed by using the brine shrimp toxicity assay as described by Krishnaraju et al. (2006). Briefly, artificial sea water was prepared by adding 38 g of artificial sea salt (Tropic Marin®) to 1 L of distilled water (3.8% artificial sea water), and the pH was adjusted to 8.5 using 1 M NaOH. *Artemia salina* eggs were then incubated in an Erlenmeyer flask containing artificial sea water at room temperature. An aerator was placed into the flask that was exposed to an artificial light source for 48 h. Once the eggs hatched, the completely hatched nauplii were used as the test organism. Varying concentrations of plant extract (0.5 mL) were added into sample vials containing 10 nauplii in 4.5 mL artificial sea water solution to achieve a final concentration of 0 to 10 g plant extract/L. Determinations were done in quadruplicate. After the 24-h incubation period, at room temperature and exposed to a light source, the number of dead nauplii was counted and recorded. The following equation was used to correct for the control:

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$$\% \text{ Mortality} = \frac{x-y}{z} \times 100$$

Where x = the number of dead nauplii in the treated group after 24 h, y = the number of dead nauplii in the control group after 24 h and z = the number of nauplii at 0 h. From these data, the median lethal dose (LD₅₀) value was extrapolated from a best fit line of log concentration vs % mortality.

Animals and experimental design

The animal design, administration of anaesthesia, recording of aortic and cardiac parameters was performed using a procedure as described by Hay et al. (2008). Seventy six-month-old SHR, weighing between 300 and 350 g, were obtained from the animal unit of the University of Cape Town, South Africa. The rats were housed in individual standard rat cages and were kept in an environmentally controlled room (comfortable room temperature approximately 24°C, relative humidity between 45 and 55%, proper ventilation, 12 h light/dark cycles and a quiet environment). The animals had access to a sufficient amount of tap water (40 mL/day) and a nutritionally balanced rat diet (supplied by Epol Pty Ltd, RSA). The investigation conforms to the South African Guide for the care and use of laboratory animals (South African National Standard, 2008) in accordance with the internationally accepted principles for laboratory animal use and care (Directive 86/606/EEC, 1986). Ethical approval was obtained from the Medical Research and Ethics Committee of the University of Limpopo (Medunsa Campus).

Methodology for dose response experiments

Forty SHR were randomly divided into four groups of 10 each to obtain dose-dependent effects. Each animal was anaesthetised with an intra-peritoneal injection of a 3:1 mixture of Anaket-V (ketamine 100 mg/mL, Kyron Laboratories Pty Ltd. Johannesburg, SA) and Chanazine (2%) (xylazine 20 mg/mL, Centaur labs, SA) at a concentration of 1.3 mL/kg of bodyweight (*bw*). Directly before the experiments started, the Hewlett-Packard pre-conditioner (8805D) and quartz pressure transducer (1290A) were calibrated with a mercury manometer according to the manufacturer's instructions.

The right carotid artery was surgically exposed and carefully separated from the surrounding structures in a sterile environment using standard surgical techniques. A catheter (Arrow 20 G x 12 cm) was inserted into the exposed carotid artery, advanced into the aorta and the proximal end connected to a fluid-filled Hewlett-Packard quartz pressure transducer (1290AH-E01) that was interfaced with a Hewlett-Packard multi-channel (3968A) and Powerlab 200 (AD Instruments) recording system. The following parameters were obtained from the aortic blood pressure (AOP) curve: Systolic blood pressure (SBP); diastolic blood pressure (DBP); mean arterial pressure (MAP), calculated as $[SBP+DBP]/2$; and pulse pressure (PP). The data obtained were captured and analysed with a Powerlab 200 (AD Instruments) application program (Chart 4.2.3 for Windows).

Four different concentrations of *C. anisata* (50, 100, 200, and 400 mg/kg of *bw*) were prepared from the dried yield and brought to 0.5 mL with saline. The four different concentrations were intra-arterially administered over one minute as 0.5 mL bolus injections. Baseline aortic BP values were recorded followed by a bolus injection and recorded for 20 min. Anaesthesia was maintained throughout the procedure by further administration of the anaesthetic as needed. A constant anaesthetic plane was continually assessed by means of the tarsal pinch reflex.

Methodology for chronic experiments over 40 days

Water consumption and urine output measurements

During the 40-day feeding period, urine output and water consumption was measured at 10-day intervals from the two groups of rats as follows: six rats were randomly selected from each group and placed in individual metabolic cages situated in the same animal room where the other rats were kept. These rats were placed in the metabolic cages a day before the actual measurements were taken. Both water consumption and urine output was measured over a 48-hour period.

Recording of BP and cardiac parameters

For these experiments, 30 SHR were randomly divided into two groups. The control group received a known quantity (40 mL/day) of tap water and the standard rat diet *ad libitum* for 40 days, and the experimental group was treated as above, except that the group received the leaf extract of *C. anisata* at 400 mg/kg of *bw* dissolved in 40 mL of drinking water. At the end of the 40-day feeding period, each animal was anaesthetised and catheterised as previously described under the methodology for dose response experiments. However, for chronic experiments the catheter was also advanced into the left ventricle for recording the left ventricular pressure (LVP) to obtain the left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), heart rate (HR) and maximum rate of the rise of LV pressure (dP/dt_{max}), indicating LV contractility. The pressure recordings were continuously monitored to confirm the position of the catheter either in the aorta for AOP recordings or in the left ventricle for LVP recordings.

Determination of plasma angiotensin II levels

At the end of the AOP and LVP recordings, 5 mL of arterial blood was withdrawn via the arterial catheter and collected in ethylenediaminetetraacetic acid (EDTA) coated tubes (Transpharm, Pretoria), mixed thoroughly by gentle tilting and placed immediately on ice. After enough blood was collected, the rats were sacrificed by further exsanguination. The blood samples were then centrifuged at 3000 x g for 20 min at 4°C. Plasma was removed by aspiration, placed into Eppendorf tubes and immediately stored at -21°C. Once all of the samples were accumulated, an angiotensin II (All) enriched fraction was prepared using hydrophobic interaction chromatography with phenyl cartridges (SPI-Bio/Bertin, Part Number PH-S-00/3) according to the manufacturer's instructions. Briefly, this entailed loading the plasma samples on prewashed columns. Unbound material was washed off with water. The All enriched fraction was eluted with methanol that was dried under a stream of nitrogen at 37°C. The All levels were thereafter determined with a sandwich enzyme linked immunosorbant assay kit (SPI bio/Bertin, Catalogue Number A05880) according to the manufacturer's instructions. All determinations were performed in triplicate.

Statistical analysis

Toxicity data were evaluated using Excel Microsoft 2010. All other data were analysed using an interactive statistical program and were expressed as the mean ± standard error of the mean (SEM) (SigmaStat version 8.0, SPSS Science, USA). Data were also analysed for statistical significance and comparison among groups using Student's t-test and Paired t-test, One Way ANOVA (bonferoni test) as applicable. A p-value of <0.05 was considered to

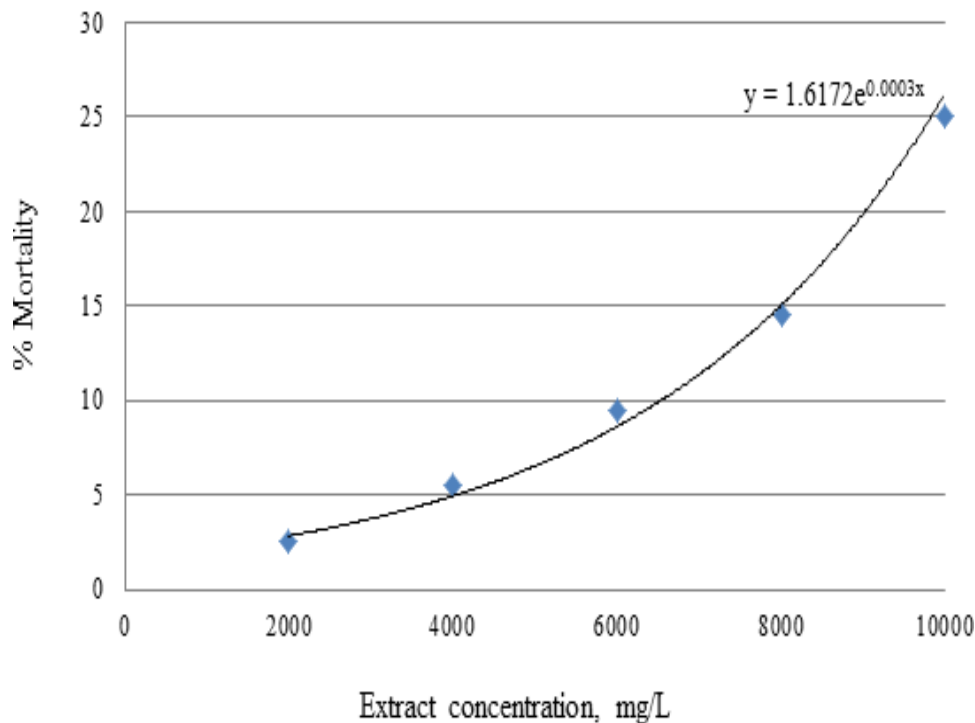


Figure 1. The brine shrimp toxicity result showing that a crude extract of *C. anisata* (10 g/L) had a very low toxicity (LD_{50} of 27172.5 mg/L).

be statistically significant, and all graphical presentations were performed using either Excel Microsoft 2010 or SigmaPlot (version 8.0, SPSS Science, USA).

RESULTS

Toxicity results

An exponential relationship of increasing doses of aqueous leaf extracts of *C. anisata*, ranging from 0 to 10 000 mg/L, and toxicity was established (Figure 1). The results indicated that the leaf extract had a very low toxicity; at a concentration of 10 g/L, only 30% of nauplii were killed. The LD_{50} was calculated using the formulae of the fitted line to be 27172.5 mg/L. A dosage of 400 mg/kg of *bw/day* should therefore have no substantial toxic effect on rats.

Dose response relationship of different concentrations of *C. anisata* on aortic BP over 20 min

As indicated in Figure 2; at 50 mg/kg of *bw*, SBP was significantly decreased by 33 mmHg; DBP was decreased by 34 mmHg and MAP was decreased by 25 mmHg. Furthermore, a 100 mg/kg of *bw* dose significantly reduced SBP by 41 mmHg, reduced DBP by 37 mmHg, and reduced MAP by 35 mmHg. In addition, 200 mg/kg of

bw significantly decreased SBP by 47 mmHg, decreased DBP by 39 mmHg, and decreased MAP by 37 mmHg. However, the 400 mg/kg of *bw* produced the largest significant decrease in SBP by 50 mmHg, decreased DBP by 40 mmHg, and decreased MAP by 40 mmHg. Compared to other concentrations, there is only a slight difference between the 200 mg/kg of *bw* and the 400 mg/kg of *bw*. PP did not show any significant change for all concentrations.

Chronic effects of *C. anisata* (400 mg/kg of *bw/day*) on water consumption, urine output, BP, cardiac parameters and plasma All levels

Effects on water consumption and urine output

Figures 3 and 4 show that administration of *C. anisata* (400 mg/kg of *bw/day*) over a 40-day period did not significantly influence the urine output or water consumption of SHR compared to day 0; control rats receiving plain tap water had significantly larger urine output and water consumption over the same period.

Effects on aortic blood pressure

Figure 5 shows that administration of *C. anisata* (400

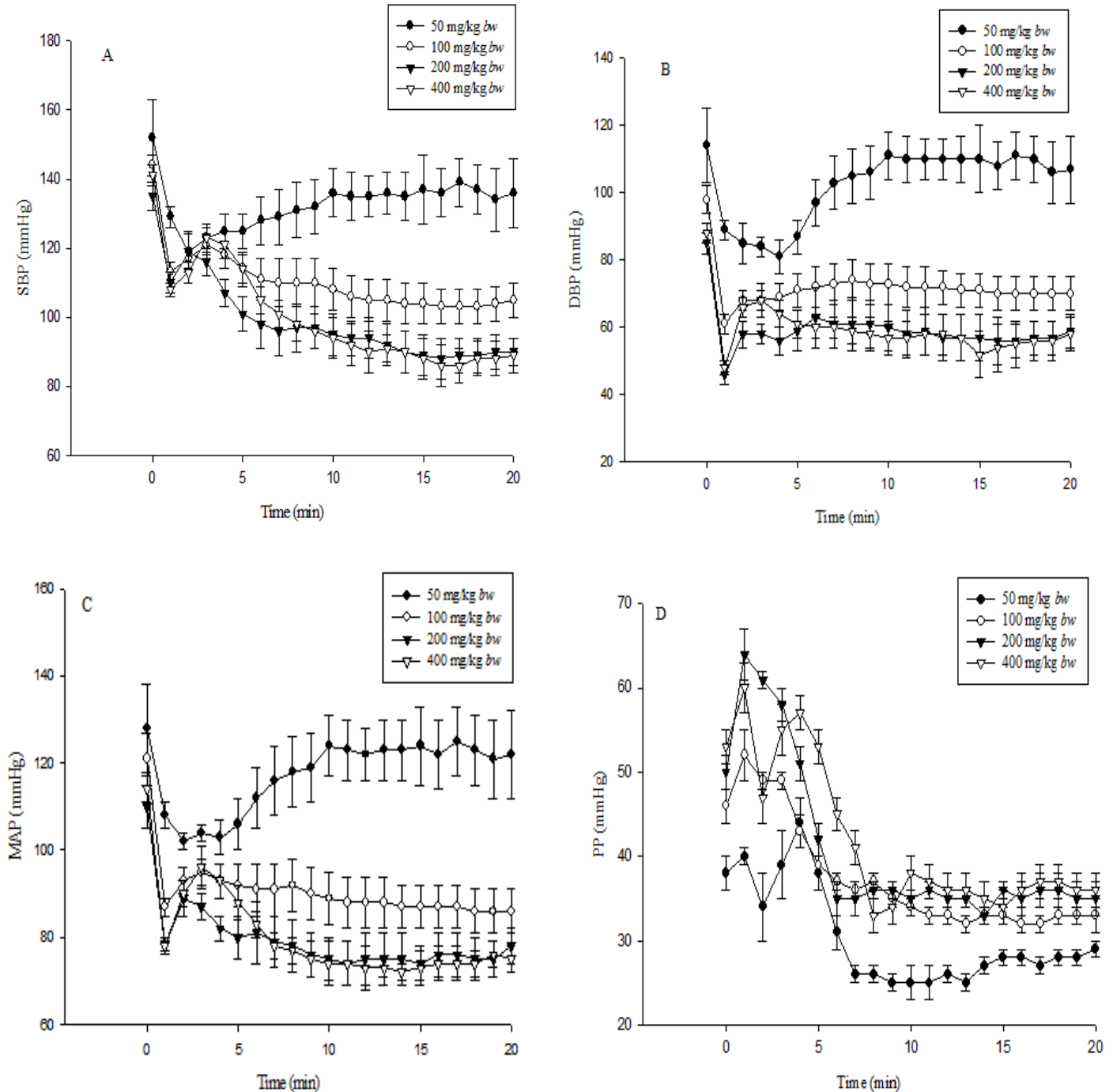


Figure 2. Effects of different concentrations of *C. anisata* (50, 100, 200 and 400 mg/kg of *bw*) on aortic BP over 20 minutes. (A) Changes in systolic BP; (B) Changes in diastolic BP; (C) Changes in mean arterial pressure, and (D) Changes in pulse pressure. The 400 mg/kg of *bw* showed the largest decrease in BP. Data was expressed as mean \pm SEM. Paired t-test used to compare changes over 20 min with time 0. One Way ANOVA (Bonferoni test) did not show significant changes between the different groups (data not shown) ($n = 40$).

mg/kg of *bw*/day) significantly ($p < 0.05$) decreased SBP by 17 mmHg (6%), decreased DBP by 13 mmHg (7%) and decreased MAP by 13 mmHg (5%), while PP did not change significantly.

Effects on the left ventricular pressure

Figure 6 indicates that *C. anisata* significantly decreased LVEDP by 3.6 mmHg (9%), while LVSP was not

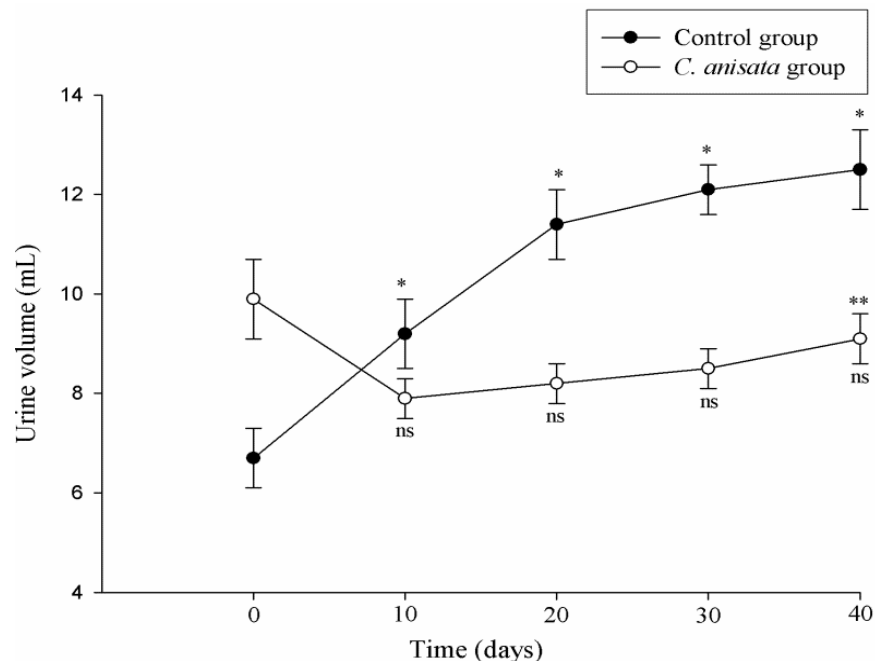


Figure 3. Effects of *C. anisata* (400 mg/kg of *bw/day*) on urine output over 40 days. Control rats receiving plain water had a significantly larger urine output compared to day 0, while rats treated with a leaf extract of *C. anisata* did not show significant changes in urine output when compared to day 0, * $p < 0.05$ and ns (non-significant) (Paired t-test). ** $p < 0.05$ compared to control group (Student's t-test). Data was expressed as mean \pm SEM (n = 12).

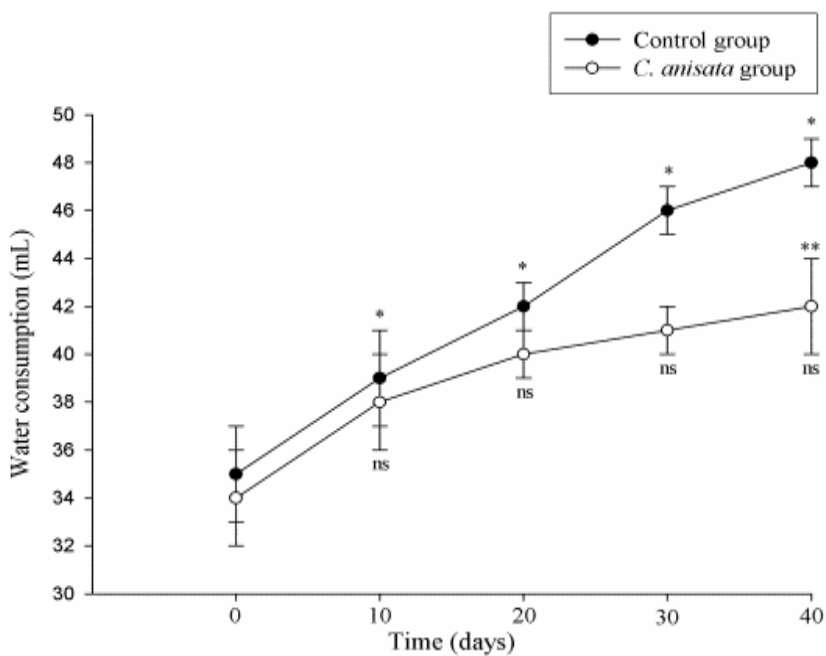


Figure 4. Effects of *C. anisata* (400 mg/kg of *bw/day*) on water consumption over 40 days. Control rats receiving plain water had significantly larger water consumption, while rats treated with *C. anisata* did not significantly influence water consumption when compared to day 0, * $p < 0.05$ and ns (non-significant) (Paired t-test). ** $p < 0.05$ compared to control group (Student's t-test). Data was expressed as mean \pm SEM (n = 12).

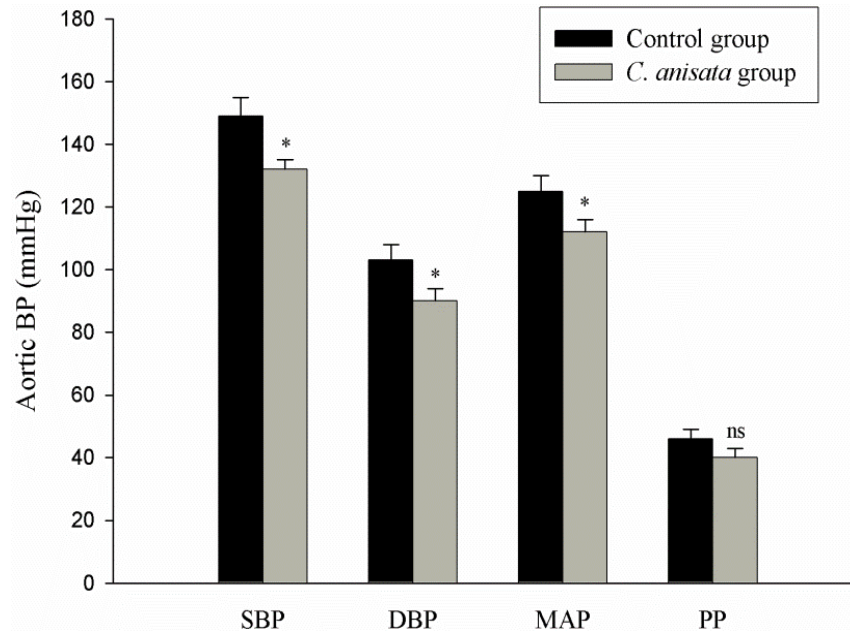


Figure 5. Effects of *C. anisata* (400 mg/kg of *bw/day*) on systolic BP, diastolic BP, mean arterial BP and pulse pressure. Administration of *C. anisata* (400 mg/kg of *bw/day*) significantly decreased all the BP parameters after 40 days. * $p < 0.05$ and ns (non-significant) compared to control group (Student's t-test). Data was expressed as mean \pm SEM ($n = 30$).

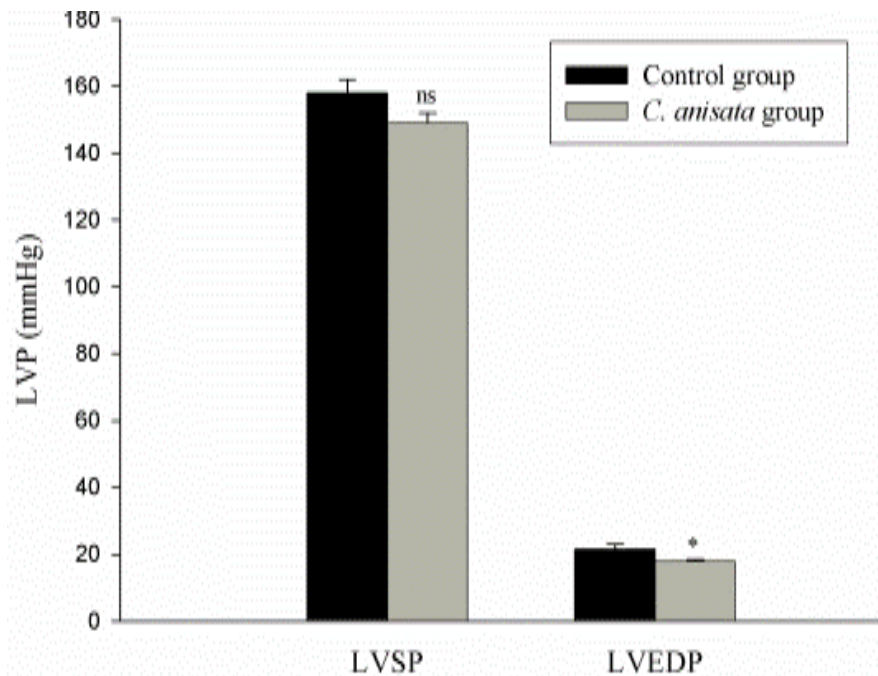


Figure 6. Effects of *C. anisata* (400 mg/kg of *bw/day*) on left ventricular systolic and end-diastolic pressures after 40 days. *C. anisata* significantly decreased LVEDP by 9%, while LVSP was not significantly affected. * $p < 0.05$ and ns (non-significant) compared to control group (Student's t-test). Data was expressed as mean \pm SEM ($n = 30$).

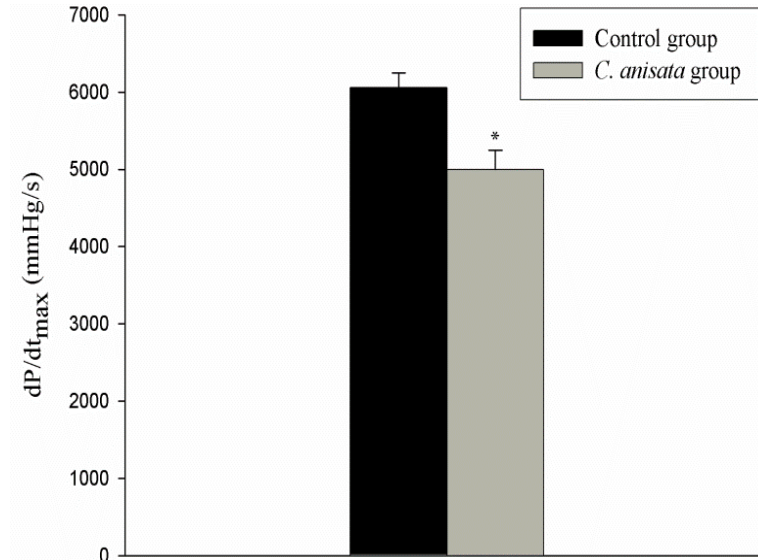


Figure 7. Effects of *C. anisata* (400 mg/kg of *bw/day*) on dP/dt_{max} after 40 days suggested that the left ventricular contractility was significantly reduced. * *p* < 0.05 compared to control group (Student’s t-test). Data was expressed as mean ± SEM (n = 30).

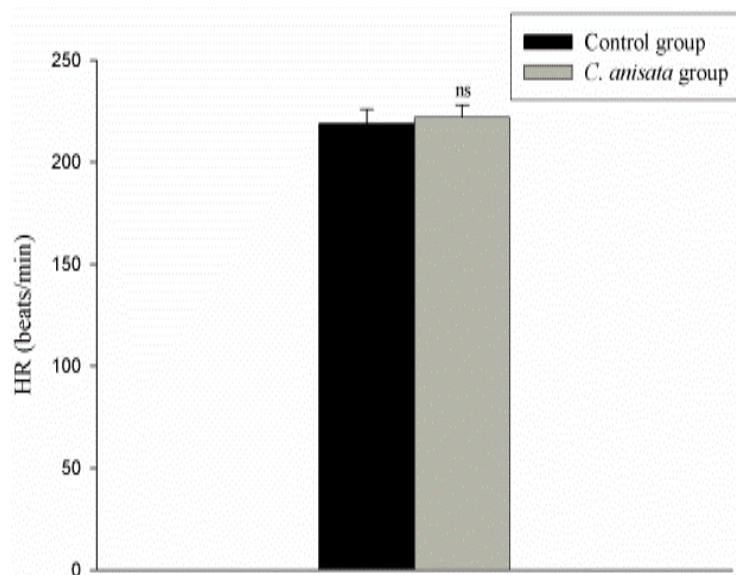


Figure 8. *C. anisata* (400 mg/kg of *bw/day*) did not significantly affect heart rate after 40 days. ns (non-significant) compared to control group (Student’s t-test). Data was expressed as mean ± SEM (n = 30).

significantly affected.

significantly affected (Figure 8).

Effects on the left ventricular dP/dt_{max} and heart rate

Figure 7 shows that *C. anisata* significantly reduced dP/dt_{max} by 1062 mmHg (10%), while HR was not

Effects of *C. anisata* on plasma All levels

Figure 9 show that *C. anisata* significantly decreased plasma All levels by 92.8 ± 25.3 pg/mL (31%) after 40

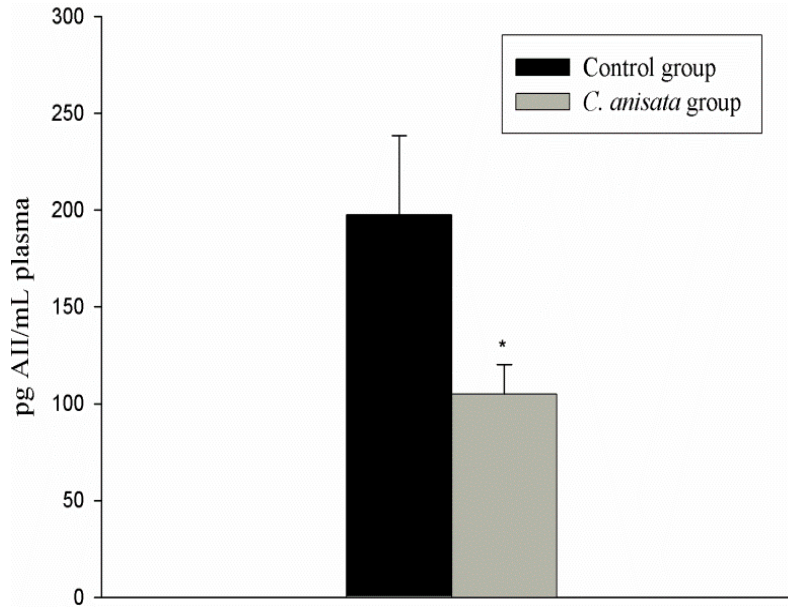


Figure 9. Effects of *C. anisata* (400 mg/kg of *bw/day*) on plasma angiotensin II after 40 days. Rats treated with *C. anisata* significantly decreased plasma angiotensin II levels by 31% after 40 days. * $p < 0.05$ compared with control group (Student's t-test). Data was expressed as mean \pm SEM ($n = 30$).

days.

DISCUSSION

Medicinal plants have been used to treat a wide variety of human ailments (Duncan et al., 1999; Hutchings et al., 1996; Osamaor and Owumi, 2010). In South Africa, Duncan et al. (1999) identified twenty plant species showing some ACE inhibitory potential *in vitro*. *C. anisata* is one of those plants and is used by traditional healers to treat hypertension. However, its antihypertensive potential has not been fully investigated to substantiate the antihypertensive claims that are made by the traditional healers. The current study showed that bolus injections of *C. anisata* (50, 100, 200, and 400 mg/kg of *bw*) to hypertensive rats significantly decreased aortic blood pressures, with 400 mg/kg of *bw* producing the largest effect. The study further showed that a leaf extract of *C. anisata* (400 mg/kg of *bw*) added daily to the drinking water of SHR significantly reduced SBP, DBP and MAP after 40 days. A possible diuretic effect, which is a common occurrence for many plant species to reduce BP (Bhadoriya et al., 2010; Reddy et al., 2011), was, however, ruled out because the results did not show any significant increases in urine output. Similarly, the non-significant changes in heart rate could not be offered as an explanation for the decrease in BP. Although phytochemical screening was not carried out in this

study, it has been shown that *C. anisata* is biologically active due to compounds such as the carbozole alkaloids and coumarins (Chakraborty et al., 1995; Duncan et al., 1999). The biological activities reported for coumarins have been shown to possess antihypertensive properties (Gilani et al., 2000). It is therefore possible that the decrease in BP observed in this study could be attributed to these compounds.

In contrast to the parameters discussed above, LV dP/dt_{max} , which is an index for LV contractility, decreased significantly. However, its overall contribution to the decrease in BP is weakened by the lack of a matched change in LVSP. A possible explanation for this lies in the role of AII in contractility. It is known that AII has a direct positive inotropic effect by affecting myocardial calcium channels (Mori and Hashimoto, 2006), increasing intracellular calcium (Petroff et al., 2000); because the plant extract used in our study significantly reduced AII levels, this most likely results in the negative inotropic effect of the plant extract. In addition, because AII is also known to be a very powerful vasoconstrictor (Rosenthal, 1992), the significant decrease in the AII levels could therefore explain the significant decreases in BP parameters, especially the lower DBP reported in this study. AII is a vasoconstrictive hormone that increases the systemic blood pressure, renal perfusion pressure and the glomerular filtration rate. Therefore, the decrease in the AII levels could also provide impetus to the *in vitro* observation that *C. anisata* possesses ACE inhibitory

activity, as suggested by Duncan et al. (1999).

Conclusion

The results suggest that *C. anisata* extracts significantly reduced the BP of hypertensive rats. The mechanism most likely involves a reduction in the AII levels, which might act through an ACE inhibitory mechanism as suggested by Duncan et al. (1999). This study provides an important basis for further investigations into the isolation and characterization of the active compounds that might be responsible for lowering the BP. Further investigations should also be conducted on isolated hearts to investigate a possible direct negative inotropic effect and possible active compounds of the plant extract on ventricular muscle. The findings of this study should then provide traditional healers with a scientific basis for the antihypertensive effects of *C. anisata*. We also acknowledge the fact that more research is needed on human subjects to determine the effectiveness and safety of this plant as an antihypertensive remedy in humans.

Conflict of Interests

The authors declare that there is no conflict of interests including any financial, personal, or other relationships with people or organizations

ACKNOWLEDGEMENTS

The authors thank Prof. A Mogale and Ms. S du Plooy for assistance with extract preparations, Ms. A de Freitas for assisting with animal care and treatment as well as anaesthetic and surgical procedures, Ms. SM Nkadimeng and Ms. LE Moagi for their technical assistance and the South African National Biodiversity Institute for supplying the plant material for the study. The Department of Human Physiology funded the project.

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