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Novel Blood Pressure-Lowering Effect of Spathulenol in Rats

(Kesan Penurunan Tekanan Darah Novel Spathulenol pada Tikus)

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ABSTRACT

In recent years, natural compounds from plant sources are sought after as alternative anti-hypertensive treatments. Spathulenol (Spa), a terpenoid plant metabolite obtained mainly from the *Origanum* species has shown anecdotal evidence of blood pressure (BP)-lowering properties probably through the effects on vasoreactivity. This study aimed to investigate the BP-lowering activities of Spa *in vivo* as well as to ascertain the mechanisms of action *in vitro*. Male Sprague-Dawley (SD) rats (n=16) and spontaneously hypertensive rats (SHR) (n=16) with their normotensive Wistar-Kyoto (WKY) rats (n=16) as controls were subjected to anaesthesia and administered intravenous boluses of Spa at 0.0, 0.045, 0.90, 0.18, 0.36, and 0.7 mg/kg or positive control losartan at 0.0, 0.6, 1.2, 1.8, 2.4 and 3.0 mg/kg. The BP and heart rate (HR) of the rats were recorded by a computerised physiographical system (Power Lab) through carotid arterial cannulation that was connected to a pressure transducer. The data were analysed by using the Lab Chart 6 software. Spathulenol was also tested for angiotensin-converting enzyme (ACE) inhibitory activity by using the ACE1 inhibitor screening kit (PromoKine). Spathulenol was able to decrease the BP of rats in a dose-dependent manner with the BP-lowering effect being significantly ($p < 0.05$) higher in the SHRs than in the WKY controls. Furthermore, Spa was able to inhibit the ACE activity suggesting the possibility that this could be one of the mechanisms underlying the observed BP-lowering effect.

Keywords: Angiotensin-converting enzyme inhibition; blood pressure; Spathulenol; spontaneously hypertensive rats; Wistar-Kyoto rats

ABSTRAK

Dalam beberapa tahun kebelakangan ini, sebatian semula jadi daripada sumber tumbuhan telah didekati sebagai salah satu bentuk untuk rawatan anti-hipertensif. Spathulenol (Spa), metabolit tumbuhan terpenoid yang diperoleh terutamanya daripada spesies *Origanum* telah menunjukkan bukti anekdot tentang sifat menurunkan tekanan darah mungkin melalui kesan ke atas vasoreaktiviti. Oleh itu, penyelidikan ini dilakukan untuk mengkaji aktiviti penurunan tekanan darah Spa secara *in vivo* serta memastikan mekanisme tindakan secara *in vitro*. Tikus jantan Sprague-Dawley (SD) (n=16), tikus hipertensi spontan (SHR) (n=16) dan tikus Wistar-Kyoto (WKY) (n=16) sebagai kawalan normotensif telah diletakkan di bawah anestesia dan diberikan bolus intravena Spa sebanyak 0.0, 0.045, 0.90, 0.18, 0.36 dan 0.7 mg/kg atau kontrol positif losartan sebanyak 0.0, 0.6, 1.2, 1.8, 2.4 dan 3.0 mg/kg. Tekanan darah dan kadar denyutan jantung tikus direkodkan oleh sistem fisiografi berkomputer (PowerLab) melalui kanulasi arteri karotid yang disambungkan kepada transduser tekanan. Data dianalisis menggunakan perisian LabChart 6. Spathulenol juga telah diuji untuk aktiviti perencatan enzim penukar angiotensin (ACE) menggunakan kit pemeriksaan perencat ACE1 (PromoKine). Spathulenol didapati dapat mengurangkan tekanan darah tikus dalam cara yang bergantung kepada dos dengan kesan penurunan tekanan darah secara signifikan ($p < 0.05$) lebih tinggi dalam SHR berbanding kawalan WKY. Tambahan pula, Spa dapat merencat aktiviti ACE yang menunjukkan kemungkinan bahawa ini boleh menjadi salah satu mekanisme yang mendasari kesan penurunan tekanan darah yang diperhatikan.

Kata kunci: Perencatan enzim penukar angiotensin; Spathulenol; tekanan darah; tikus hipertensi spontan; tikus Wistar-Kyoto

INTRODUCTION

Hypertension (HTN) is considered one of the most prevalent cardiovascular risk factors worldwide and can be defined into a few known stages i.e., (i) stage 1: systolic blood pressure (SBP) between 130 mmHg and 139 mmHg or diastolic (DBP) between 80 mmHg and 89 mmHg, (ii) stage 2: SBP at least 140 mmHg or DBP at least 90 mmHg and (iii) hypertensive crisis: SBP above 180 mmHg and/or DBP over 120 mmHg (American College of Cardiology High and American Heart Association 2017). Hypertension poses a serious threat to the heart leading to continuous elevated pressure with reduced blood flow to the heart can lead to several complications, such as heart attack, heart failure and irregular heartbeat which can lead to sudden death (WHO 2023). Managing hypertension through timely interventions and lifestyle changes is crucial to prevent these severe cardiovascular complications.

Common first-line antihypertensive treatments for high blood pressure include the use of thiazide-type diuretics, calcium-channel blockers (CCB), angiotensin-converting enzyme inhibitors (ACEI) and angiotensin II receptor blockers (ARBS) (Khalil & Zeltser 2023). Antihypertensives can be divided into two major groups; the first group being those which directly or indirectly block the renin–angiotensin system (RAS), such as the ACEIs and ARBS groups. Although acting through multiple mechanisms of action, the predominant effect of these groups is to cause vasodilatation. The second group of drugs works by promoting sodium and water excretion to reduce the intravascular volume or to cause vasodilatation through the non-RAS pathway. These include the diuretics and CCBs (Jackson & Bellamy 2015).

In recent years, there has been an increasing demand for using natural compounds from plant sources as an alternative for treatment and prevention of various diseases including cardiovascular diseases (Al-Akwaa et al. 2020). Demands for new antihypertensives are primarily due to safety issues as synthetic substances record new side effects every year. Indeed in 2015, the Netherlands Pharmacovigilance Centre Lareb reportedly received 15 cases of hallucinations that were associated with the use of ACE inhibitors, such as captopril and lisinopril, undesired effects that were not documented previously (Lareb 2015).

Spathulenol (Spa) is a tricyclic sesquiterpenoid alcohol extracted from plants purported to have medicinal properties, particularly those with antihypertensive effects. Spathulenol has been found to exhibit vasorelaxation of the smooth muscle (Perez-Hernandez et al. 2008) and contains anti-inflammatory, antimicrobial, antiproliferative and antioxidant activity (do Nascimento et al. 2018). Preliminary information by Dib et al. (2017) on *Artemisia campestris* suggests that Spa may significantly affect vasoreactivity and BP. However, the mechanisms of action are scarcely studied. This was then further elaborated by Suručić et al. (2017) on the combination of vasorelaxant and ACE inhibitory effects of the analysed *Seseli pallasii* essential oil containing Spa which might have the potential therapeutic significance in hypertension. Suručić et al. (2017) explained that part of antihypertensive activity of Spa is achieved through ACE inhibition wherein Spa had exhibited the best binding affinity.

The prospects of Spa as an alternative form of treatment for hypertension are evident. Although empirical research surrounding Spa has grown over the past decade, there remains a lack of extensive studies regarding the effectiveness of Spa on its blood pressure (BP)-lowering properties and the mechanism of actions involved. This study was therefore conducted to systematically elucidate the eventual antihypertensive effect of Spa in an *in vivo* intra-arterial direct blood pressure measurement set-up and an *in vitro* ACE1 inhibitor screening assay kit to ascertain the mechanisms of action.

MATERIALS AND METHODS

All studies were undertaken in accordance with the criteria approved by the University of Malaya Faculty of Medicine Institutional Animal Care and Use Committee (FOM IACUC) (approval code 2022-240210/PHYSIO/R/HSZ).

ANIMALS USED

A total number of forty-eight adult male rats of the strains Sprague-Dawley (SD) (n = 16), spontaneouly hypertensive (SH) (n = 16) and Wistar-Kyoto (WKY) (n = 16), between the ages of 10 weeks to 12 weeks and weighing approximately 250 g to 300 g were randomly selected from the specific-pathogen free animal house, Animal Experimental Unit (AEU), University of Malaya (UM). The rats were subsequently housed in a conventionally maintained animal facility at the Satellite Animal Facility, Department of Physiology, UM. The rats were anaesthetised with 5% pentobarbital sodium (50 mg/kg) intraperitoneally (ip) prior to the invasive procedure as suggested by the Institutional Animal Care and Committee (IACUC) for procedures that would potentially cause pain or distress (Silverman, Macy & Preisig 2017). The rats were placed under general anaesthesia throughout the whole procedure and were euthanised via an intravenous (iv) overdose of injectable anaesthesia following the end of the procedures to prevent the suffering of the rats from the invasive nature of the surgical procedures. All procedures were conducted at the Physiology Laboratory, Faculty of Medicine, UM.

CHEMICALS USED

The compound spathulenol (Spa) used was purchased from ChemFaces (China) in the form of an oil at a concentration of 10 mM in 1 mL dimethyl sulfoxide (DMSO) and has a purity of >= 98%. The molecular weight of Spa is 220.4 g/mol according to the safety data sheet provided. The source of the Spa was obtained from the herbs of *Salvia sclarea*, commonly known as clary sage. Spathulenol is insoluble in water and the solvents recommended to be used as a vehicle are chloroform, dichloromethane, ethyl acetate, DMSO, or acetone. A diluted concentration of 0.5% DMSO was chosen as the vehicle as recommended by Jamalzadeh et al. (2016) as it shows minimal toxicity towards living organisms. Losartan (Los) was obtained from Sigma-Aldrich (USA) in the form of white powder with a purity of > 98%. The molecular weight of Los is 461.0 g/mol according to the safety data sheet provided. Losartan is highly soluble in water and thus distilled water (dH_2O) was used as the vehicle for dilution purposes.

In vivo STUDY: DIRECT MEASUREMENT OF BP

All surgical techniques were performed as previously shown by Lin et al. (2019) who described the technique of intra-arterial cannulation as an alternative way to obtain intra-arterial pressure waveform recordings and systemic BP measurements. The rats were subjected under general anaesthesia of 5% pentobarbital sodium via ip injection prior to the cannulation of the trachea with a silicone endotracheal tube to facilitate respiration, cannulation of the right jugular vein with a polyethylene tube to facilitate iv administration of the compound and cannulation of the left carotid artery with a heparinised (with heparinised saline, 10 U/mL) polyethene tube for continuous BP monitoring. The cannulated carotid artery was connected to a pressure transducer and subsequently to a computerised physiographic system (Power lab, AD Instruments, USA) for continuous BP monitoring and recording. Small animal electrographic leads were attached to the limbs of the rat to record the heart rate (HR) and to obtain the electrocardiogram (ECG). The set-up was equilibrated before injecting the test solutions and the rats were kept warm (35 ± 2 °C) throughout the experiments.

After achieving equilibrium, Spa (0.0, 0.045, 0.09, 0.18, 0.36 and 0.70 mg/kg) or Los (positive control) (0.0, 0.6, 1.2, 1.8, 2.4 and 3.0 mg/kg) were administered to each rat as a bolus. Each injection was standardised to a fixed volume of 0.1 mL per 300 g body weight. After recording the changes in BP, the rats were allowed to return to the resting level before each subsequent dose administration. The mean arterial pressure (MAP) and the decrease in BP (%) were calculated from the formula:

$$\text{MAP (mmHg)} = \text{DBP} + (\text{SBP} - \text{DBP})$$

$$\text{Decrease in BP (\%)} = \frac{\text{MAP before administration of compound} - \text{MAP after administration of compound}}{\text{MAP before administration of compound}} \times 100$$

In vitro STUDY: ESTIMATION OF ACE1 INHIBITION

Spathulenol was also tested for angiotensin-converting enzyme (ACE) inhibitory activity by using the ACE1 inhibitor fluorometric screening kit (PromoKine, Germany). The assay kit was used to screen for potent inhibitors of ACE1 activity to regulate hypertension. The positive controls used were captopril and lisinopril, two of the most common ACE inhibitors used for the treatment of hypertension. Spathulenol was dissolved in absolute ethanol and diluted with the ACE1 assay buffer. A volume of 10 **µ**L containing 0, 0.02204, 0.2204, 2.204 and 22.04 mg/ml of Spa was added to each of the 96-well microplate. This was then followed by 40 **µ**L of assay substrate mix (assay buffer with substrate) from the ACE1 kit. The reaction mixture was incubated at 37 **°C for 60** min to 120 min. Fluorescence was measured at Ex/Em = 330/340 nm using an absorbance microplate reader. The inhibitory activity of samples was calculated by choosing two-time points (T1 and T2) and obtaining corresponding values for the fluorescence (RFU1 and RFU2). The slope for all samples is calculated by ΔRFU/ΔT. The relative activity was measured by the following formula:

$$Relative\ activity = \frac{\Delta\text{RFU of S}}{\Delta\text{RFU of EC}} \times 100$$

STATISTICAL ANALYSIS

Statistical analyses were carried out via a combination of IBM Statistical Packages for the Social Sciences (SPSS) ver. 26 and GraphPad Prism ver. 8. For the SD rats, the effect of different dosages of Spa on the BP of the SD rats was analysed by using one-way ANOVA followed by a *post hoc* multiple comparisons Tukey test. For the comparison between SHR, WKY and SD rats, the effect of different dosages of Spa towards the BP of the three strains of rats was analysed by using two-way ANOVA followed by a *post hoc* multiple comparison Tukey test. A p-value of < 0.05 was considered as significant.

RESULTS

In vivo STUDY: DIRECT MEASUREMENT OF BP

In this study, the direct measurement of BP using a pressure transducer was conducted to observe and monitor the effects of Spa on the BP of the rats.

Effects of Spa on blood pressure of Sprague-Dawley (SD) rats

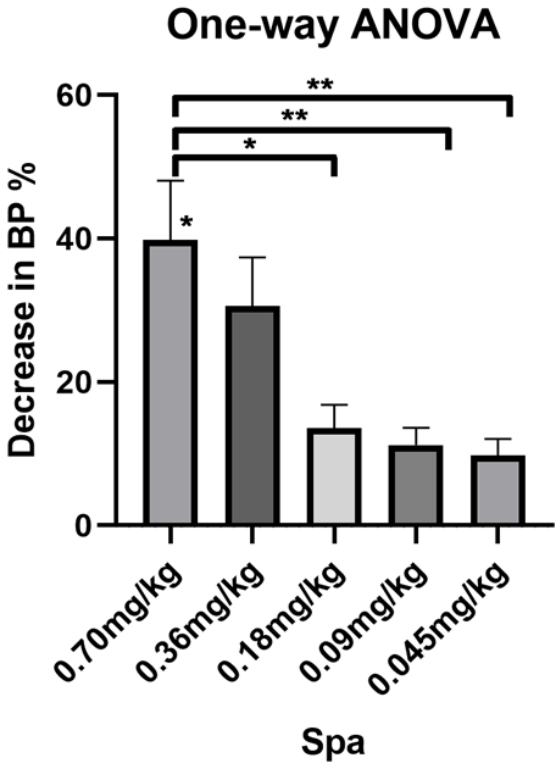
Direct BP measurement of the SD rats showed a dose-dependent response towards Spa as seen in Figure 1. This study showed that the BP of the SD rats reacted to a decrease in BP (%) upon administration of the Spa. The decrease in BP was higher when the concentration of Spa administered was highest at 0.07 mg/kg as compared to the other concentrations ($p < 0.05$). The decrease in BP (%) subsequently lowered with a decrease in the concentration of the Spa from 0.7, 0.36, 0.18 and 0.09 mg/kg and the lowest reduction in BP when administered at 0.045 mg/kg Spa. A typical response that can be expected is shown in Figure 2.

In comparison, the positive control Los was expected to show similar results of a decrease in BP (%). However, the decrease in BP was highest when the concentration of Los administered at the dosage of 3.0 mg/kg. Unexpectedly, the dosages between 0.6 and 2.4 mg/kg showed a reduction in BP as opposed to these two dosage points. However, no significant difference in BP decrease of SD rats was detected between the different dosages of Los amongst all groups (Figure 3).

Effects of Spa on blood pressure of SD, SHR and WKY rats

Comparison between the effects of the different doses of Spa towards the 3 strains of SD, SHR and WKY rats that could also be seen in a dose-dependent manner as seen in Figure 4 (visualisation by line chart) and Figure 5 (bar chart). This showed that all three strains of rats react similarly towards Spa with the highest decrease in BP when the Spa concentration was at 0.07 mg/kg and subsequently reduced with each decreasing dose.

In contrast, a comparison between the effects of different dosages of the positive control Los on the three strains of SD, SHR and WKY rats showed that the highest reduction in BP (%) was at the administration of the lowest concentration of Los (0.6 mg/kg) and subsequently, decrease in BP reduced with the higher concentration as seen in Figure 6. Two-way ANOVA showed that there is a significant difference ($p < 0.01$) between different dosages (3.0 mg/kg) of Los and (0.6 mg/kg) of Los towards the three strains of rats, as seen in Figure 7.



Data are presented as mean ± SEM; n = 8 rats. *p<0.05, **p<0.01 compared between different dosages using one-way ANOVA followed by post hoc Tukey multiple-comparison test

FIGURE 1. Dose-dependent response of Sprague-Dawley rats towards Spa (mg/kg)

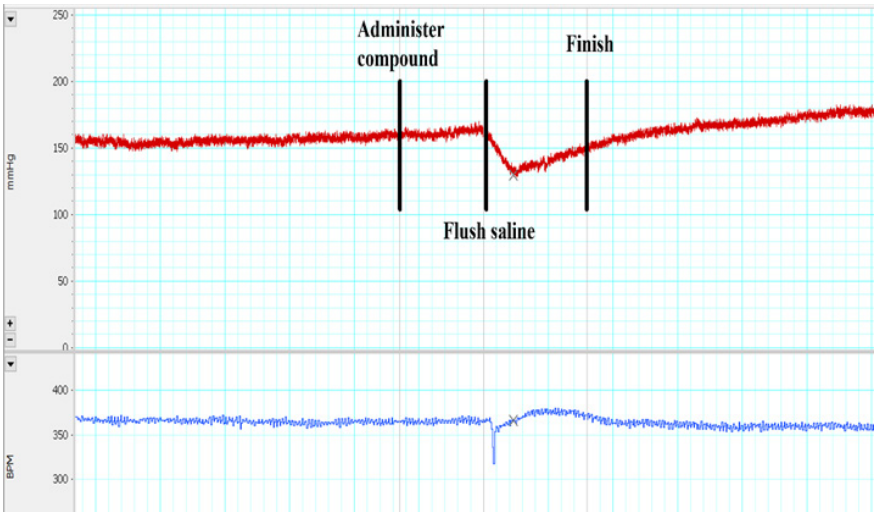
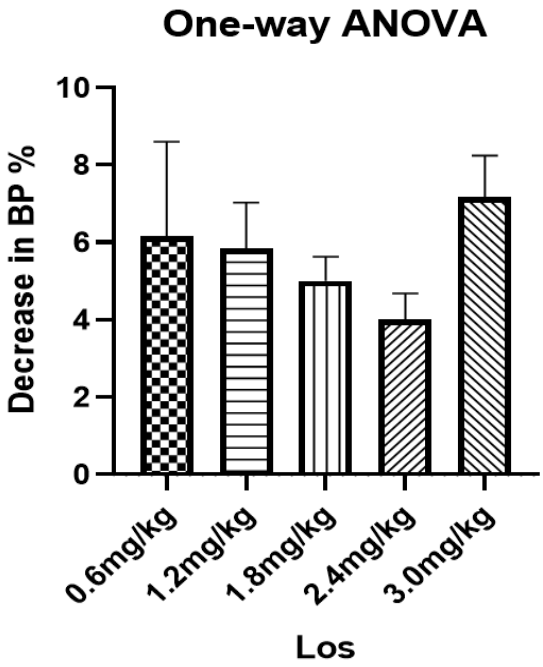


FIGURE 2. Typical recording of blood pressure (red) and heart rate (blue) in response to Spa injection. The black vertical lines indicate the injection time



One-way ANOVA indicates no significant difference between different dosages towards SD rats

FIGURE 3. Blood pressure response of Sprague-Dawley rats towards Los (mg/kg)

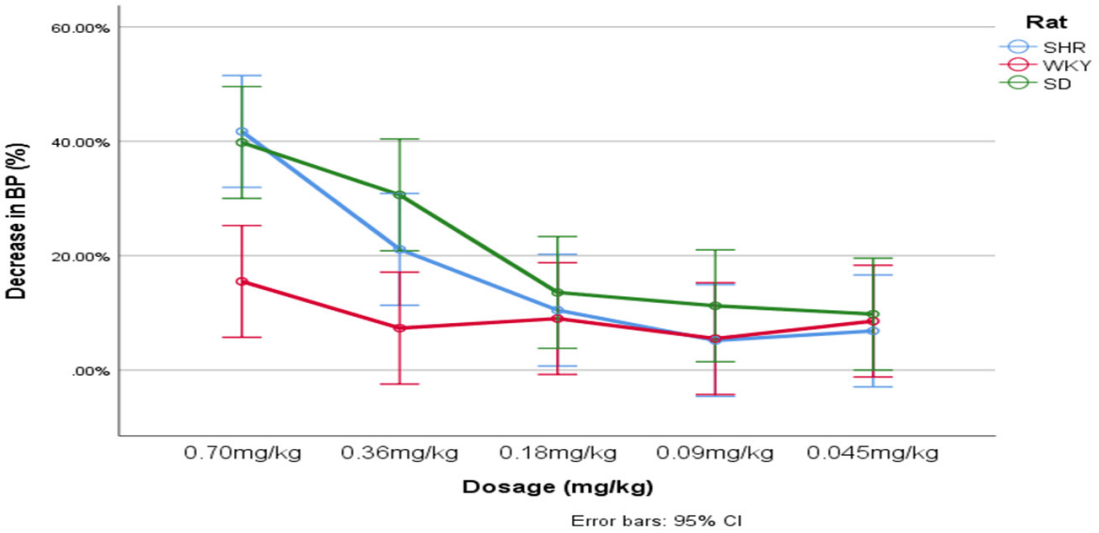
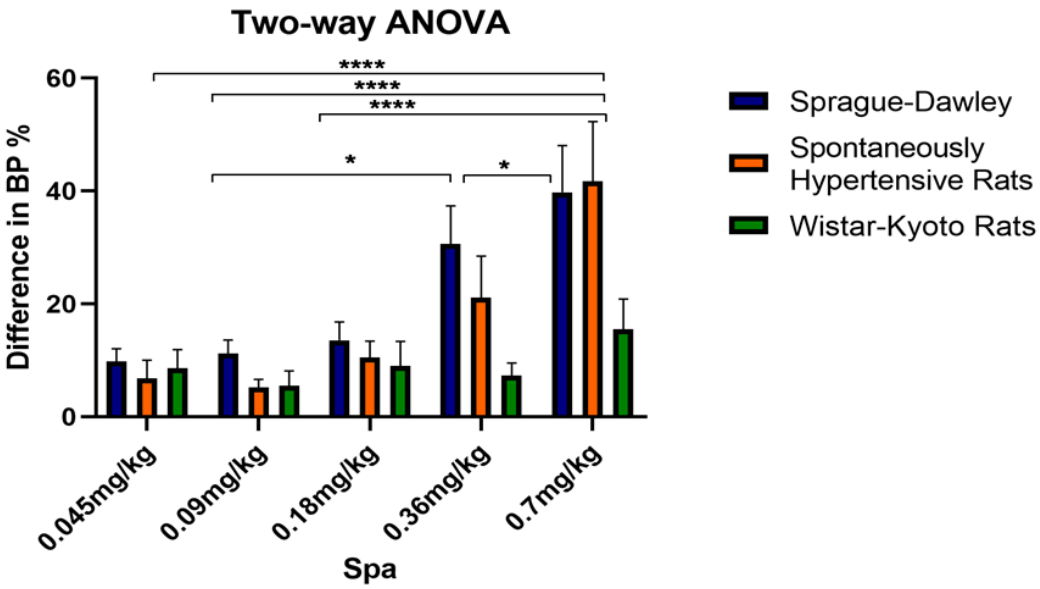


FIGURE 4. Dose-dependent response of Sprague Dawley, Spontaneously Hypertensive Rats and Wistar-Kyoto rats towards Spa (mg/kg)



Data are presented as mean ± sem; n = 8 rats. *p<0.05, ***p<0.001 compared between different dosages using two-way ANOVA followed by post hoc Tukey multiple-comparison test

FIGURE 5. Dose-dependent response of SD, SHR and WKY rats towards Spa (mg/kg)

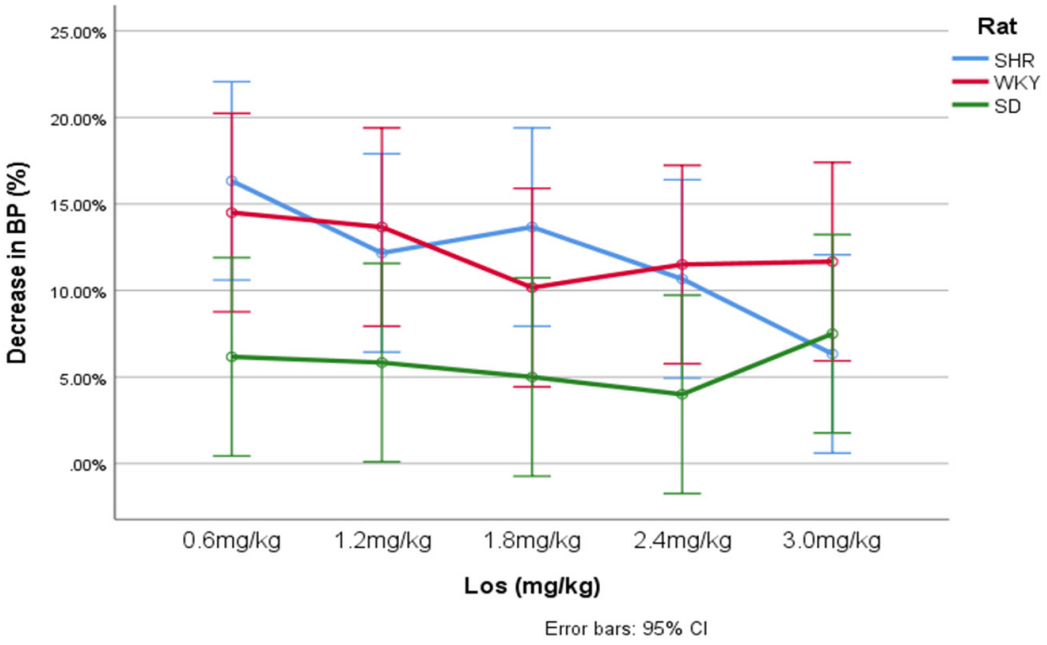
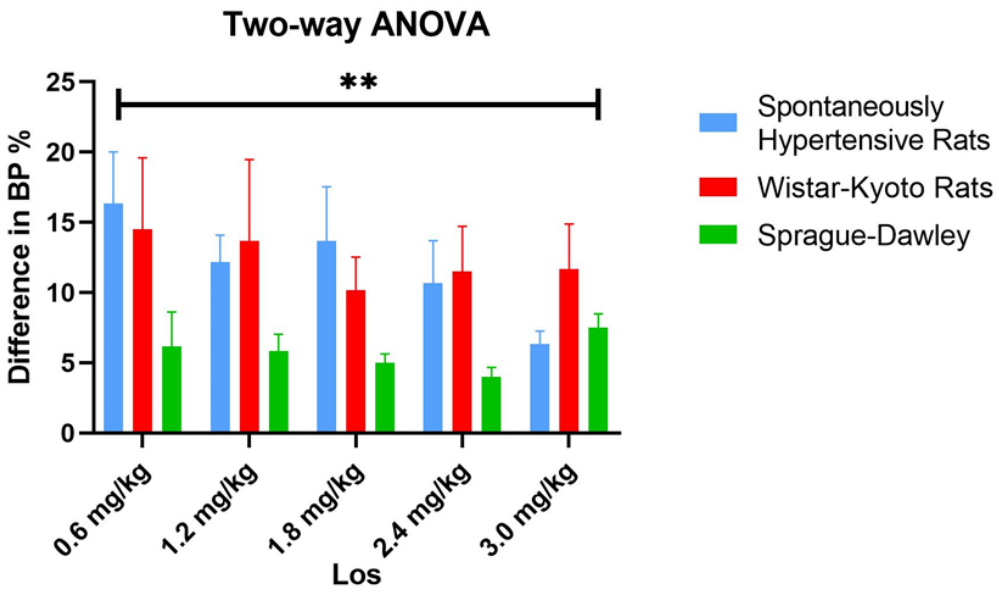


FIGURE 6. Blood pressure response of Sprague Dawley, Spontaneously Hypertensive Rats and Wistar-Kyoto rats towards Los (mg/kg)



Data are presented as mean ± SEM; n = 8 rats. **p<0.01 compared between different dosages using two-way ANOVA followed by post hoc Tukey multiple-comparison test

FIGURE 7. Blood pressure response of Sprague Dawley, Spontaneously Hypertensive Rats and Wistar-Kyoto rats towards Los (mg/kg)

In vitro STUDY: ESTIMATION OF ACE1 INHIBITION

To investigate the mechanism of ACE1 inhibition, Spa was compared with the positive controls Captopril (Cap) and Lisinopril (Lis), two of the most common ACE inhibitors used in the treatment of HTN. The principle of the assay screening kit is that the presence of an ACE1-specific inhibitor will cause the enzyme to lose its peptidase activity and subsequently lead to a decrease in fluorescence intensity or relative fluorescence unit (RFU). As shown in Figure 8, the RFU of all three compounds Cap, Lis and Spa showed a similar decrease in RFU except for Spa which exhibited a slight increase at the 8th cycle before RFU decreased again at the 9th and 10th cycle over 45 min for all concentrations.

Spathulenol demonstrated the ability to inhibit the ACE activity, suggesting the possibility that this could be one of the mechanisms underlying the observed BP-lowering effect. However, the response was not shown to be dose-dependent as expected (Figure 9) as compared to the positive controls (Cap and Lis). This may indicate that there are other predominant mechanisms of action involved in the RAS for Spa. However, more study is warranted to further investigate this.

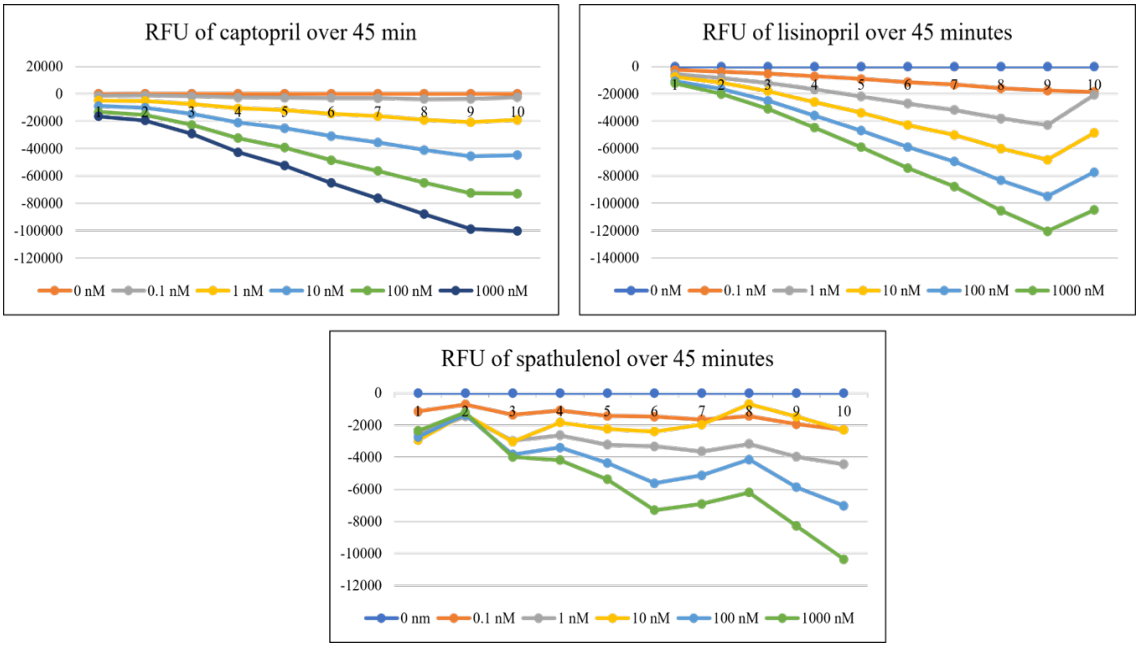


FIGURE 8. ACEi activity (RFU) of captopril, lisinopril and spathulenol over 45 min

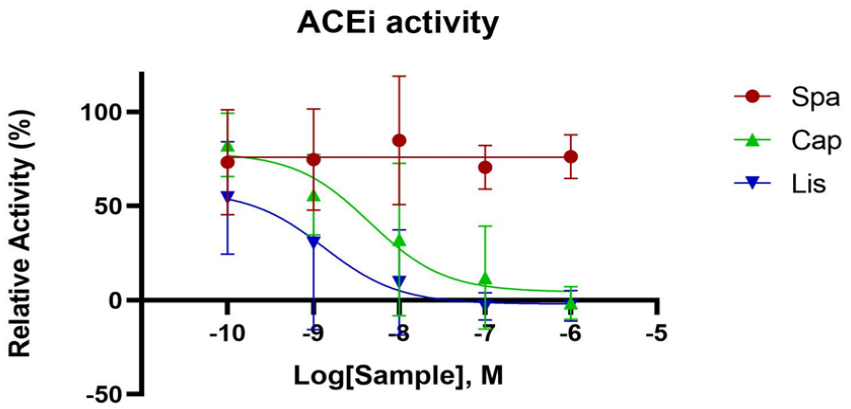


FIGURE 9. Relative activity (%)

DISCUSSION

The present study is the first *in vivo* study of antihypertensive activities focusing solely on Spa by using SD, SHR, and WKY rats. In this particular study, the injection of the controls of 0.5% DMSO and dH_2O with the same volume as Spa and Los had minimal effects on the BP of the control groups. The control 0.5% DMSO used as a vehicle for Spa caused a reduction in blood pressure by 10% which is expected as multiple studies have shown that DMSO itself can cause hypotension (Kollerup Madsen et al. 2018). Thus, this was noted when considering the antihypertensive effect of DMSO-dissolved Spa. This study also showed that Spa acted in a dose-dependent manner to lower the blood pressure of laboratory rats. Spathulenol was observed to reduce blood pressure by up to 40% when administered at 0.7 mg/kg iv in SD and SHR rats and up to 18% when administered in WKY rats. Overall, the rats showed a similar response towards Spa with a higher decrease in BP with a higher dose of Spa administered from 0.7 mg/kg to 0.045 mg/kg. This is different from the positive control of losartan administered from a higher concentration of 3.0 mg/kg to a lower concentration of 0.6 mg/kg as observed from previous readings. Losartan was shown to be able to decrease the BP even at a lower dosage of 0.6 mg/kg. It is hypothesised that at a dose higher than 0.6 mg/kg, no further decrease in BP can be observed. This is probably due to a blockade of corresponding receptors (Antlanger et al. 2017).

Losartan was used as a key component of the study as a positive control to compare the BP-lowering effects of Spa as this drug represents a common antihypertensive medication in human use. Losartan acts as a selective angiotensin II receptor blocker at the AT1 receptor site resulting in the elevation of the renin and angiotensin I levels. Losartan also inhibits angiotensin II-induced vasoconstriction and the action of aldosterone, which in turn lowers BP. Compared to ACE inhibitors, angiotensin II-receptor blockers effectively inhibit the renin-angiotensin system, however, they do not affect the kallikrein-bradykinin pathways (Mulla & Siddiqui 2022).

As explained by Suručić et al. (2017) who examined the *Saselli paselli* essential oil containing Spa, part of the antihypertensive activity is achieved through ACE inhibition and best binding affinity. However, through the findings of this present study, it can be shown that this may not be the predominant mechanism of action. Although it is one of the possible mechanisms of action for Spa, the authors believe that it may not be primarily affected by this as Spa did not show a dose-dependent response towards ACE inhibition. Despite showing a similar reduction in RFU as its positive controls Cap and Lis, Spa did not show a similar curve in relative activity (%) as to its positive control as expected the reduction in relative activity to be reduced with a decreasing concentration of the compounds screened in the assay kit. Unexpectedly, Spa showed a stagnant relative activity throughout the decreasing concentration. The IC_{50} of Spa was thus not able to be calculated due to the ambiguous state of the curve as shown in comparison in Figure 9.

The ACE inhibitor group is typically used for the treatment of hypertension by causing vasodilatation and hypovolemia which results in decreasing blood pressure. Their major mechanism of action is to inhibit the ACE of the RAS. It is proposed that ACE inhibitor through blockade of angiotensin I conversion, limits the generation of angiotensin II, and causes the reduction of vasoconstriction and water and sodium retention (Malik, Siddiqui & Zafar 2016).

The authors plan to further investigate the possible predominant mechanism of action of Spa specifically targeting the effects of Spa on the following axes: (i) renin-Ang II-Ang II Type 2 receptor (renin-Ang II-AT2R), (ii) angiotensin-converting enzyme 2-angiotensin (1-7)-mitochondrial assembly receptor (ACE2-Ang (1-7)-MasR) and (iii) kallikrein-bradykinin (BK)-bradykinin Type 2 receptor (K-BK-BK2R) expecting to elucidate the whole mechanism of action, and thus classify Spa into its appropriate class under the antihypertensive treatment.

Due to the paucity of information on Spa, there is a need to elaborate on the characteristics of this compound. Although Spa is found to be soluble in chloroform, dichloromethane, ethyl acetate, DMSO or acetone, it is highly insoluble in water. This poses a problem for *in vivo* animal and potential human use as the vehicles used were mainly toxic to cells at high concentrations (Joshi & Adhikari 2019). An appropriate amount of 0.5% DMSO was used as suggested according to Jamalzadeh et al. (2016) as levels higher than the suggested amount may be toxic to the cells. Due to this issue, less than 3 mg/mL was obtained in the dilution of Spa when paired with the solvent of choice. A higher concentration was difficult to obtain as the concentration of the vehicle would be too high and be toxic for *in vivo* use. Spathulenol was also found to be recommended to be stored at 2 °C to 8 °C according to the safety data sheet provided by ChemFaces (China). Several findings in the literature mentioned freezing the compound at -20 °C for long-term storage of up to 1 month (MedChemExpress, 2023). However, the authors observed a decline in hypertensive activity when stored at -20 **°C** and recommended storage at room temperature instead. This may suggest the unstable nature of Spa at certain temperatures possibly due to physiological stress caused by the fluctuations which may affect the active compounds of Spa and possibly its antihypertensive activity. Nevertheless, more samples would be required for further study to investigate and prove this assumption. Principally, this information may be noteworthy in the future usage of Spa in research.

CONCLUSION

In conclusion, Spa was found to be able to decrease the BP of rats in a dose-dependent manner. This shows the potential of applying these findings to clinical trials for humans after considering no observed adverse effect level (NOAEL) in the animals before converting to the human equivalent dose (HED). The results also showed that the percentage of decrease in BP caused by Spa in SHR is more significant as compared to the WKY. This information may be applied to the difference in the effects of Spa on hypertensive vs normal patients in the future. Furthermore, Spa showed the ability to inhibit the ACE activity suggesting the possibility that this could be one of the mechanisms underlying the observed BP-lowering effect. However, more studies should be conducted in a widened spectrum of the concentration of Spa and also, on the possible mechanism of action in terms of the involvement of Spa with the Ang II Type 2, Ang 1-7-Mas and BK receptors which have been planned for the near future.

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