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HYPOTENSIVE EFFECTS OF AQUEOUS EXTRACT OF *Eugenia polyantha* LEAVES ARE PARTLY MEDIATED VIA CHOLINERGIC RECEPTOR

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ABSTRACT

Eugenia polyantha leaves, a popular fresh salad ('ulam') used by the Kelantanese has been claimed as a cure for hypertension. In this study, the hypotensive effects of aqueous extract of *Eugenia polyantha* leaves (AEEP) on the anaesthetized male normotensive Wistar-Kyoto rats were described. Increasing intravenous doses of AEEP (0.01, 0.1, 1, 10 and 100 mg/kg) significantly reduced the mean arterial blood pressure (MAP) of twelve animals by 6.06 ± 4.1 %, 8.02 ± 3.9 %, 6.35 ± 0.6 %, 9.34 ± 1.6 % and 30.19 ± 2.0 % (Wilcoxon-Signed Rank test or WSR, $p<0.01$) respectively. However, only reductions in MAP, by the doses of 0.1, 1, 10 and 100 mg/kg of AEEP and by the positive control (5 µg/kg acetylcholine, 37.87 ± 1.7 %) were significantly higher than the reduction of MAP by the negative control (0.9 % normal saline, 5.372 ± 0.5 %) (WSR test, $p<0.05$). From the dose-response curve, the ED_{50} value for hypotensive effects of AEEP was 35.5 mg/kg. Subsequently, a pharmacological antagonistic study was carried out either of pre-treatment with propranolol (2 mg/kg) or by pre-treatment with atropine (2 mg/kg). Pre-treatment with atropine (2 mg/kg) significantly reduced the hypotensive effect of 100 mg/kg of AEEP (WSR test, $p<0.05$) whilst pre-treatment with propranolol (2 mg/kg) does not significantly reduced the hypotensive effect of 100 mg/kg AEEP (WSR test, $p>0.05$). This attenuation suggested that the hypotensive effect of AEEP may be partly mediated by cholinergic but not by β-adrenergic receptor pathways.

Keywords: *Eugenia polyantha, mean arterial blood pressure, dose-response curve, β-adrenergic receptor, cholinergic receptor*

INTRODUCTION

Cardiovascular diseases are one of the leading causes of mortality worldwide. In 2005, thirty percent from 58 millions of deaths worldwide had resulted from various types of cardiovascular diseases [1]. One of the underlying risk factors for cardiovascular diseases is hypertension [2]. Hypertension is highly prevalent in Malaysia where 27.8 % of adults aged ≥ 15 had hypertension [3]. In the state of Kelantan (situated in the East-coast of Malaysia), the disease was considered as common (which is lower than the national average where the overall prevalence of hypertension was 13.9 %) [4].

There is abundance of anti-hypertensive drugs available in the market such as direct renin inhibitors, calcium channel blockers, α- and β-blockers, diuretics, angiotensin converting enzymes (ACE) inhibitors and angiotensin receptor blockers. Nevertheless, these drugs sometimes are not specific, rendering some unwanted side effects that leads to deficiency in the management and control over the disease. In Malaysia, among 32.4 % of the hypertensives who is on anti-hypertensive medication, only 26.8 % of them had their blood pressures under control [3]. In a larger context, about one-third of the hypertensives worldwide with only 8.6 % in Malaysia had controlled over their blood pressures (<140/90 mmHg) [3, 5].

In the meantime, this lack of control over hypertension has urged more research to be done in order to seek for a more precise anti-hypertensive therapy. The sources of drugs discovery are broad, ranging from natural resources such as plants, microorganisms and soils or from chemical synthesis of new or improved version of drugs. Nonetheless, one of the oldest sources of drugs discovery is plant. Ethno-medicinal use of few medicinal plants can certainly guide the screening of a new potential drug from plant. In fact, many researches worldwide have verified hypotensive or anti-hypertensive properties of few ethno-medicinal plants such as *Andrographis paniculata* [6], *Loranthus ferrugineus* Roxb. [7], *Averrhoa carambola* L. [8] and *Eugenia uniflora* L. [9].

Interestingly, one of the ethno-medicinal plants which are claimed by the Kelantanese and Indonesian as a treatment for hypertension is Eugenia polyantha. The decoction from the leaves of *E. polyantha* was boiled in water before being consumed by the hypertensives [10]. Apart from this medicinal use, the plant has been regularly consumed by the Kelantanese community as a fresh salad (‘ulam’) or as a flavour enhancer in local foods such as ‘nasi kerabu’ and ‘laksa’.

In addition, this plant has been scientifically proven to possess several biological activities such as anti-microbial activity against *Staphylococcus aureus* [11], anti-fungal activities against *Alternaria alternata* and *Colletotrichum capsicii* [12], anti-nematodal activity against the pine wood nematode, *Bursaphelenchus xylophilus* [13], anti-tumor promoting activity [14], anti-diabetics [15] and anti-oxidant activity [16].

Due to the arising interest in its biological activities, the chemical compounds in the essential oil from the leaves of *E. polyantha* had been profiled [15, 17]. Few of the major groups include terpenoids, phenolic compounds (e.g. eugenol), tannins and flavonoids. The most important chemical compounds among these are the eugenol and terpenoids, which had been associated with some vasorelaxant properties and the ability to mediate hypotensive effects in both in vivo and in vitro studies [7, 18]. In addition, this plant is taxonomically related to Eugenia uniflora L., an ethno-medicinal plant used as an anti-hypertensive medicine in North-eastern Argentina. E. uniflora L. has been scientifically verified to mediate hypotensive effects majorly via direct action on blood vessels with a minor action as diuretics [9].

Despite these factors, neither the hypotensive effects nor any cardiovascular effects of this plant has been scientifically proven. Therefore, the aims of this study are to determine the hypotensive effects of the aqueous extracts of *E. polyantha* leaves by undertaking an in vivo experimentation. These will further help in elucidating its possible mechanisms of action.

MATERIALS AND METHODS

Collection and extraction of aqueous extracts from *Eugenia polyantha* leaves
Two kilograms of fresh E. polyantha leaves were collected from Gunong, Bachok, Kelantan. The leaves were removed from the stem, weighed using digital weighing balance (AND HV-60KGL), washed under tap-water and then rinsed with distilled water. Excess water was air-dried in an oven (Memmert) at a pre-set temperature of 50 °C for 3 days. The dried leaves were ground into powder in a laboratory blender (WARING Commercial®) and were sieved off from the filtrate by mechanical siever (No. 35). Powdered sample was then re-weighed using weighing balance (Delta Range® Mettler Toledo XP205) before being stored in an air-tight sealed bottle at room temperature until required for extraction.
Powdered samples were immersed in distilled water (with ratio of solute to solvent of 1:10) and were heated on a hot plate (Erla® EMS-HP-700) at 80-90 °C with continuous stirring by magnetic stirrer for 30 mins. Then, the extract was filtered through Whatman No. 1 filter paper (Whatman® Schleicher & Schuell). The filtrate was then evaporated to dryness in an oven (Memmert) at a preset temperature of 50 °C until half of its original volume. The extract was then stored in a deep freezer (Fiocchetti 340) at temperature of -20 °C before being lyophilized in a freeze-dryer (ilShin®, ilShin Lab Co., Ltd.). The lyophilized form of the sample or the AEEP was finally weighed using a weighing balance (Delta Range® Mettler Toledo XP205) before being kept in an air-tight bottle and stored in a freezer (National NR-B53FE) at 4°C until use.

Preparation of samples and drugs

AEEP and drugs were freshly prepared prior to use. AEEP was dissolved in 0.9 % normal saline (sodium chloride was bought from Merck (M) Sdn. Bhd.) to achieve the dose of 100 mg/kg. Then, the dose was diluted by a factor of 10 to achieve the doses of 10, 1, 0.1 and 0.01 mg/kg. AEEP was then homogenized using a homogenizer (Ultra-Turrax® T25 Basic, Ika Laborteknik) at 24,000/min for 1 min.
Isoproterenol hydrochloride (Sigma®), acetylcholine chloride (Sigma-Aldrich®) and atropine sulphate (Sigma-Aldrich®) were dissolved in 0.9 % normal saline whereas propranolol hydrochloride (Sigma®) was dissolved in 0.9 % normal saline plus with 2% dimethylsulfoxide (Sigma®). Sodium pentobarbital (Nembutal®, Ceva-Sante Animale, France) were bought as an injectable solution. Heparin (Heparinol®-5000, Ain Medicare Sdn. Bhd.) was diluted in 0.9 % normal saline to achieve 5 IU/ml.

In vivo preparation

Male adult normotensive Wistar-Kyoto (WKY) rats (280 - 350 g) were supplied by Laboratory Animal Research Unit, Health Campus, Universiti Sains Malaysia. Ethical approval for the use of laboratory animals was obtained from Animal Ethics Committee, Universiti Sains Malaysia. These animals were kept in standard rat cage, allowed to acclimatize for 14 days in the standard environmental condition (25° C with 60-70 % humidity) on a 12-hrs light-dark cycle. Animals were given free access to food (Shipsi Classic Heimtierbett) and tap water.
WKY rats were anaesthetized with 50 mg/kg sodium pentobarbital (Nembutal®, Ceva-Sante Animale, France) via intraperitoneal injection before being placed on a thermally-controlled heating table (37 ± 1°C). Upon tracheostomy, an endo-tracheal tube was inserted into the trachea to prevent from airway obstruction. One poly-ethylene tube filled with heparinized 0.9 % normal saline (5 IU/ml) was cannulated through right common carotid artery for measurement of mean arterial blood pressure (MAP). The response was measured via pressure transducer connected to a computer with BIOPAC® software. Another polyethylene tube was cannulated through the left jugular vein for extracts/ drugs injection.

Effects of increasing intravenous doses of 0.01, 0.1, 1, 10 and 100 mg/kg AEEP on MAP
After a 20-mins equilibration period, vehicle (0.9 % normal saline), increasing intravenous doses of AEEP (0.01, 0.1, 1, 10, 100 mg/kg) and positive control (5 µg/kg acetylcholine chloride) were intravenously administered at a fixed volume of 0.2 ml. In between these doses, an additional 0.2 ml of heparinised 0.9% normal saline (5IU/ml) was flushed intravenously to ensure complete delivery of the extracts/drugs. MAP response was allowed to return to its baseline value (pre-treatment value) before administration of the following dose. The MAP response was expressed as a percentage of reduction in MAP. The experiment was performed twelve times.

Pharmacological antagonistic study: Pre-treatment with 2 mg/kg propranolol
Using similar set up, pharmacological antagonistic study [6] was performed in another set of six animals. Isoproterenol hydrochloride (1.2 µg/kg) was used as an agonist whereas propranolol hydrochloride (2 mg/kg) was used as an antagonist to block β-adrenergic receptors.
The sequence of treatments intravenously administered was as follows; i) 100 mg/kg of AEEP, ii) isoproterenol hydrochloride (1.2 µg/kg), iii) propranolol hydrochloride (2 mg/kg), iv) isoproterenol hydrochloride (1.2 µg/kg) and lastly v) 100 mg/kg AEEP.

Pharmacological antagonistic study: Pre-treatment with 2 mg/kg atropine
Similarly, pharmacological antagonistic study was performed [6] in another set of five animals using acetylcholine chloride (5 µg/kg) as an agonist and atropine sulphate (2 mg/kg) as an antagonist to block the cholinergic receptor.
The sequence of treatments intravenously administered was as follows; i) 100 mg/kg of AEEP, ii) acetylcholine chloride (5 µg/kg), iii) atropine sulphate (2 mg/kg), iv) acetylcholine chloride (5 µg/kg) and finally v) 100 mg/kg AEEP.

Statistical analysis
Results were expressed as mean ± standard error of mean (S.E.M). Data were analyzed using Wilcoxon-Signed Rank test using statistical software, SPSS 16.0 for Windows® (SPSS Inc.). The p-value of < 0.05 was considered to be significant. The ED_{50} value was calculated from the plotted dose-response curve.

RESULTS AND DISCUSSION

Yield of AEEP
At the end of the drying process, the yield of powdered samples was 43.6 % of the fresh leaves. The aqueous extraction of the powders has yielded 2.76 % of AEEP.

Effects of increasing intravenous doses of 0.01, 0.1, 1, 10 and 100 mg/kg AEEP on MAP
Figure 1 illustrates the hypotensive effects of AEEP on twelve rats. Negative control (0.9 % normal saline) shows a small reduction in MAP (5.372 ± 0.5 %, p<0.01, WSR test) which could b due to the presence of residue upon complete delivery of extracts/ drugs. Further administration of the lowest dose, 0.01 mg/kg AEEP has reduced slightly the MAP (6.06 ± 4.1 %, p<0.01, WSR test). However, its reduction in MAP was not significantly different from the reduction of MAP by the negative control (p>0.05, WSR test). Administrations of the higher doses of AEEP (0.1 and 1 & 10 mg/kg) induced a 1-min significant fall of MAP (8.02 ± 3.9 % & 6.35 ± 0.6 % & 9.34 ± 1.6 % respectively, p<0.01, WSR test). The most prominent effect was seen upon administration of 100 mg/kg AEEP when it induced the most significant fall of MAP (30.19 ± 2.0 %, p<0.01, WSR test) that was sustained for about 4-mins before the MAP returns completely to its normal baseline value. Acetylcholine chloride (5µg/kg) which acts as the positive control produced only a 1-min significant reduction of MAP (37.87 ± 1.7 %, p<0.01, WSR test).

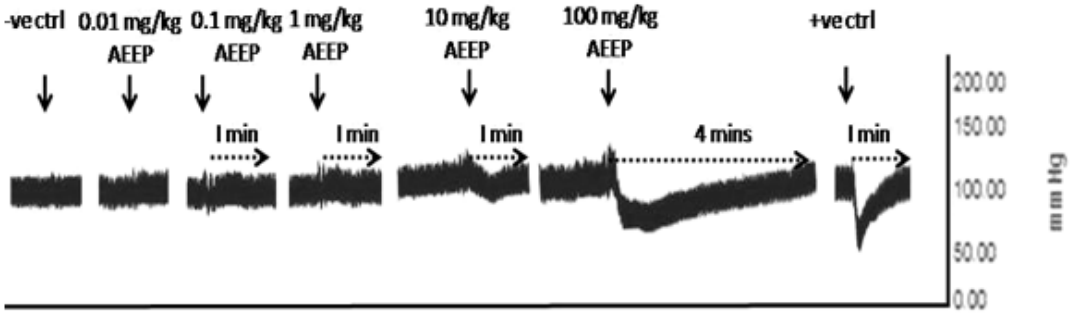

Figure 1: The illustrative figure on the hypotensive effects of AEEP. ▼ = point of dministration; ·····▶ = time; –ve ctrl = 0.9 % normal saline; +ve ctrl = 5 µg/kg acetylcholine.

Dose-response curve for hypotensive effects of AEEP is depicted in Figure 2. The effective dose which produced 50 % of the maximal reductions of MAP, (ED_{50}) was calculated to be 35.5 mg/kg AEEP. The calculated ED_{50} value of AEEP was higher when compared to the ED_{50} value of crude water extracts and its semi-purified fractions (butanolic and aqueous fractions) of fresh aerial part from Andrographis paniculata (11.4, 5, & 8.6 mg/kg respectively) [6]. In order to completely verify the effective dose of AEEP, a dose response curve for the hypotensive effects of AEEP at doses of 10 to 100 mg/kg AEEP needs to be done in future study.

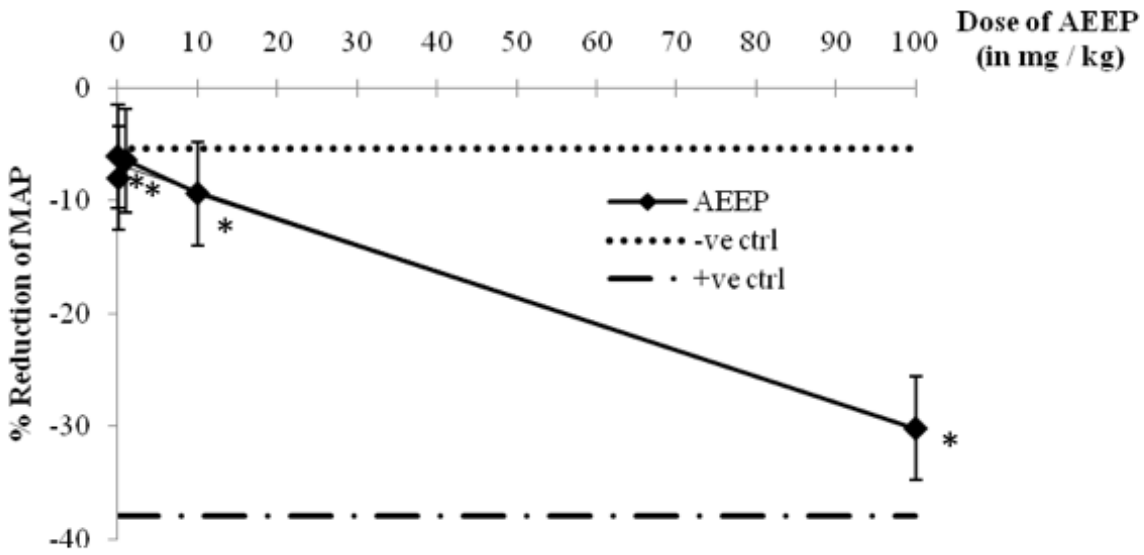

Figure 2: Dose-response curve for hypotensive effects of AEEP. MAP = mean arterial blood pressure; -ve ctrl = 0.9 % normal saline; +ve ctrl = 5 µg/kg acetylcholine chloride. Each points represent the average for percent changes of MAP of twelve animals in each group while bars indicate standard error of mean (S.E.M.) * denotes significant mean percent changes for MAP ($p < 0.01$, WSR test).

Pharmacological antagonist studies: Pre-treatment with 2 mg/kg propranolol

Figure 3 depicts the effect of 2 mg/kg propranolol (β-adrenergic blocker) on the hypotensive effect of 100 mg/kg AEEP. The test dose (100 mg/kg AEEP) produced a 4-mins significant fall of MAP (30.75 ± 3.7 %, p<0.01, WSR-test). Isoproterenol hydrochloride (1.2 µg/kg) which acts as a positive control produced a 2-mins significant fall of MAP (48.64 ± 3.0 %, p<0.01, WSR-test). When the agonist (1.2 µg/kg isoproterenol hydrochloride) was once again administered after blockage with the antagonist (2 mg/kg propranolol hydrochloride), its action on MAP was almost abolished (6.79 ± 2.0 %, p<0.01, WSR-test). Next, the original action of the test dose (100 mg/kg AEEP) on MAP response remained significant even after blockage (31.63 ± 4.2 %, p<0.01, WSR-test).

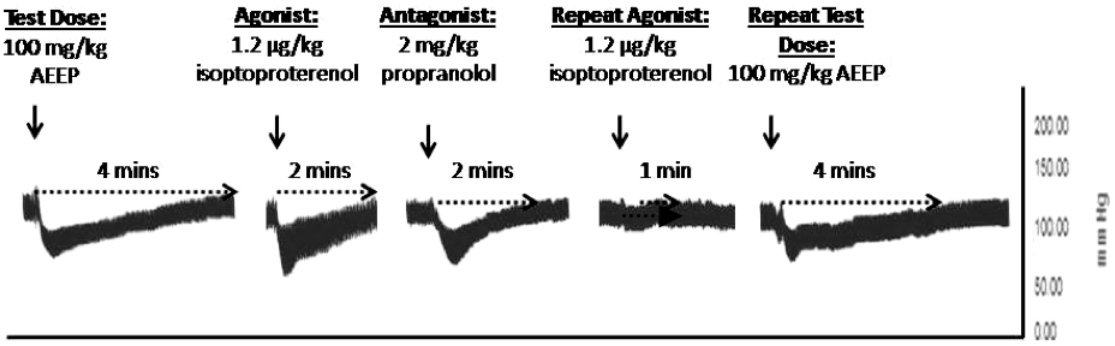

Figure 3: The effect of β-adrenergic receptor blocking agent (propranolol) on the hypotensive effect of AEEP. ▼ : represent point of administration. ·····▶ : represent time

Figure 4 shows tabulation of MAP data (n=6) for the effects of propranolol on the hypotensive effect of AEEP. The hypotensive effect for 100 mg/kg AEEP was not significantly changed after blockage with 2 mg/kg propranolol hydrochloride (WSR test, p>0.05) whereas similar dose of propranolol hydrochloride has blocked the action of the agonist (1.2 µg/kg isoproterenol hydrochloride) by 85.51 ± 2.9 % (p<0.05) (Figure 4). These results taken together suggest that the hypotensive effects of AEEP were not mediated by β-adrenergic receptors.

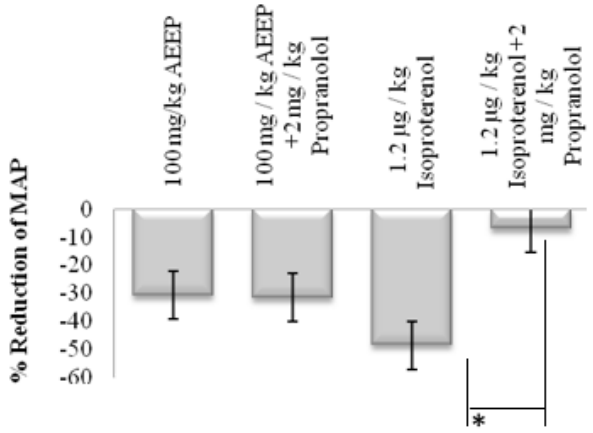

Figure 4: Effects of β-adrenergic receptor blocking agent (propranolol) on the hypotensive effects of AEEP.

Columns represent the average percentage for reduction of mean arterial blood pressure (MAP) of six animals while bars indicate standard error of measurement.

*denotes significant difference (WSR test, p<0.05)

Pharmacological antagonist studies: Pre-treatment with 2 mg/kg atropine

Figure 5 depicts the effect of 2 mg/kg atropine (cholinergic blocker) on the hypotensive effect of 100 mg/kg AEEP. The test dose (100 mg/kg AEEP) produced a 4-mins significant fall of MAP (30.97 ± 1.25 %, p<0.01, WSR-test). Acetylcholine chloride (5 µg/kg) which acts as a positive control produced a 1-min significant fall of MAP (38.39 ± 2.69 %, p<0.01, WSR-test). When the agonist (5 µg/kg acetylcholine chloride) was once again administered after blockage with antagonist (2 mg/kg atropine sulphate), its action on MAP was almost abolished (5.64 ± 0.92 %, p<0.01, WSR-test). Subsequently, the original action of the test dose (100 mg/kg AEEP) on MAP response was reduced (14.15 ± 0.86 %, p<0.01, WSR-test) after blockage. Nevertheless, the time taken for the MAP to return back to its original baseline value remained the same (Figure 5).

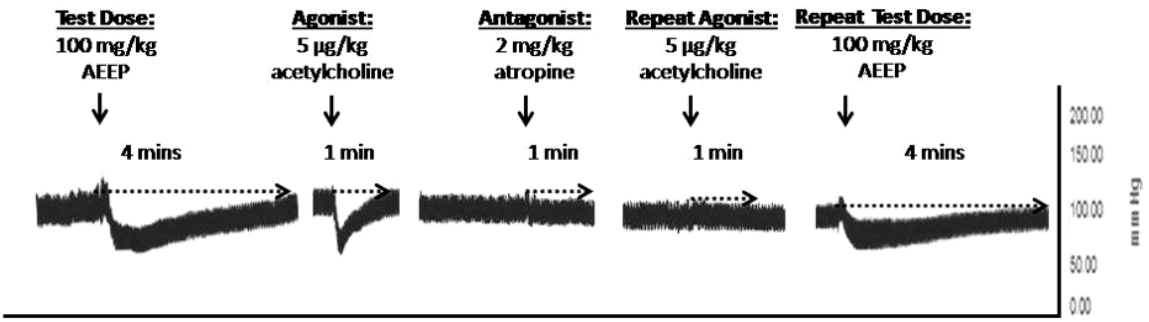

Figure 5: The effect of cholinergic receptor blocking agent (atropine) on the hypotensive effect of AEEP. ▼: represent point of administration. ·····▶ : represent time

Figure 6 shows tabulation of data (n=6) for the effects of atropine on the hypotensive effect of AEEP. The hypotensive effect of 100 mg/kg AEEP was significantly reduced by 54.3 ± 4.3 % (p<0.05, WSR test) after blockage with 2 mg/kg of atropine sulphate whereas similar dose of atropine sulphate has blocked the action of the agonist (5 µg/kg acetylcholine chloride) by 85.45 ± 2.18 % (p<0.05, WSR-test) (Figure 6). Thus, this attenuation suggests that the hypotensive effect of AEEP was partly mediated by cholinergic receptors.

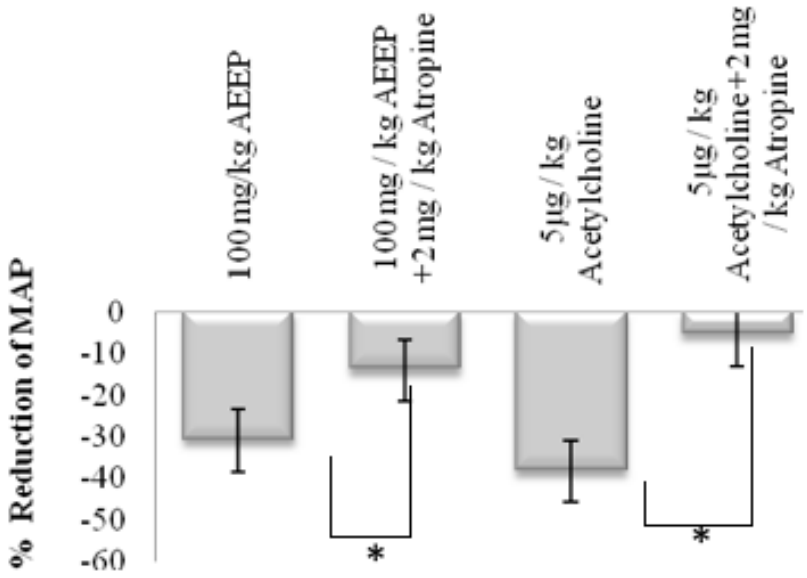

Figure 6: Effects of cholinergic receptor blocking agent (atropine) on the hypotensive effects of AEEP.

Columns represent the average percentage for reduction of mean arterial blood pressure (MAP) of six animals while bars indicate standard error of mean.

*denotes significant difference (WSR test, p<0.05).

CONCLUSIONS

The attenuation on the hypotensive effects of AEEP by atropine, suggests a partial mediation via the cholinergic receptors. Meanwhile, β-adrenergic receptor may not be involved since its blockage with propranolol did not show any significant change in the magnitude of the original hypotensive effects by AEEP. However, further research should be carried out to look for the presence of any other possible mechanisms which may accounts for the cumulative hypotensive effects of AEEP, thus enable us to better understand its complex mechanism of action.

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