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Identification of the Bioactive Compounds Hypotensive Effect in the Ethyl Acetate Extract of *Eribroma oblongum* (Malvaceae) Stem Bark

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Abstract: The stem bark of *Eribroma oblongum* (malvaceae) is used in traditional Cameroonian medicine to treat various metabolic illnesses including the management of hypertension but there is no scientific evidence to how relief is brought about. The present study was to evaluate the effect of the ethyl acetate extract of the dried stem bark of *E. oblongum* on arterial blood pressure and heart rate in normotensive rat (NTR) and their mechanisms of EAEO. The effects of ethyl acetate extract of *Eribroma oblongum* (EAEO; 10, 20, 30 mg/kg; i.v) was tested on systolic blood pressure (SBP) and heart rate (HR) of normotensive rat. The mechanism of EAEO (20mg/kg) was studied in the presence of atropine, yohimbine, propranolol, L-NAME or reserpine. At the end of the experiment, SBP and HR were recorded. EAEO (10-20 mg/kg) induced a significant hypotensive effect of SBP. The hypotensive effects of EAEO (20 mg/kg) were inhibited by pre-treatment of rats with atropine, reserpine, yohimbine and L-NAME. At the end of this study the result demonstrates that the hypotensive as well as the antihypertensive effects of the ethyl acetate extract of the stem bark of *Eribroma oblongum*. Our data validate the use of the extract in traditional medicine against hypertension. The effect on blood pressure is, at least in part, due to a modulation of the orthosympathetic nervous system and to the improvement of the antioxidant status. Further studies are from now needed to study the toxicity of *Eribroma oblongum*.

Keywords: *Eribroma oblongum*, Betulinic Acid, Hypotensive Effect, *Wistar* Rats

1. Introduction

Previous study showed that the hydroethanolic extract of *Eribroma oblongum* possesses antihypertensive and antiatherogenic properties. The present work investigates the hypotensive effect and mechanisms of some compounds from the stem bark ethyl acetate extract of *Eribroma oblongum* in *Wistar* rats. The hypotensive activity of the ethyl acetate extract of the stem bark of *Eribroma oblongum* (Malvaceae) lead to the phytochemical study of this extract which established betulinic acid as the main hypotensive principle. The other compounds isolated: tridecyl 9-hdroxylnonanoate and three others compounds showed few activities. The structure of isolates were established on the basis of NMR inspection, mass spectrometric data and by comparison with those previously reported in the literature. The hypotensive activity was carried out by intravenous injection of different concentration of the extract and natural products using a right carotid receptor attached to a recorder and computer, to monitor the arterial pressure changes of wistar rat.

Hypertension refers to an increase in arterial pressure [1]. It arises from peripheral resistance to blood flow due to increased vasoconstriction and therefore, excess pressure is needed to circulate blood at the normal rate. This has been attributed to the action of norepinephrine and other vasoconstricting hormones [2]. The prevalence in men and women is 18.7-23.8% and 12.7–18.8%, respectively. In this developing country, the cost of modern drug therapy is prohibitive and as such, many patients resort to traditional herbal medicine for treatment [3]. *Eribroma oblongum* is a plant of the Malvaceae family common to the West African society, in the dense humid forest where it grows up to the diameter of 18.6 mm [4]. It is used as timber, for the making of floors, ceiling, and as wood for many other articles [5]. It is commercialised under the name Eyong okoko and Ohaa. In Cameroon, the bark of *Eribroma oblongum* is used for the treatment of cramps, stomach burns, painful menstruation and hypertension. The aim of this study was to evaluate the antihypertensive properties of the extract, fractions and compounds obtain from *E. Oblongum* with the objective of identifying the active principles. To the best of our knowledge, no pharmacological or phytochemical work has been reported from this plant, but previous works of the Malvaceae species led to the isolation of flavonoids, phenols, polyphenols, anthocyanins, tritepenoids and steroids [6], [7], [8]. These compounds have been shown to possess various pharmacological properties.

Plants have proven to be useful in curing diseases and provide an important source of medicine. Plants have great significance to the health of individuals. The medicinal value of these plants lies in some chemicals that produce a specific physiological action on the human body. These major bioactive compounds include saponins, phenols, reducing sugar, and terpenoids. Plants have served as important material for drug development. Plants are now playing an important role in many medicines like allopathic medicine, herbal medicine, homoeopathy and aromatherapy. However, during the last decade, an increase in the use of medicinal plants has been observed in developed countries [9]. Further some synthetic drugs have been suspected to cause undesirable side effects [10], [11]. Globally, herbal medicine is gaining popularity even in region with improved health care systems [12]. The medicinal properties and other properties of some plants have been recognized by various researches.

The aim of the present study was to evaluate the effect of the dried stem bark of fraction of ethyl acetate extract, pure bioactive compounds and their mechanism of action on arterial blood pressure and heart rate in normotensive rats (NTR).

2. Material and Methods

2.1. Chemicals

Urethane was obtained from Prolabo, France. Atropine sulphate, propranolol, yohimbine, reserpine and L-NAME (Nw- L-Nitro Arginine Methyl Ester) from Sigma Chemical, St Louis, MO, USA. Heparine was from Sanofi, France. The drugs were freshly prepared before the experiment. All drugs and the plant extract were dissolved in distilled water.

2.2. Sample Collection and Preparation of the Pure Compound

Fresh stem bark of *Eribroma oblongum* were collected at Eseka, centre province of Cameroon, in August 2013. The plant material was identified at the National Herbarium (HNC) of Yaoundé-Cameroon where a voucher specimen N°27489SRFCam has been deposited. The air-dried and powdered stem bark (2.0 kg) was macerated in hexane for 48h, the resulting extract was filtered, and the solvent removed on a rotary evaporator. This same procedure was repeated trice with hexane (Hex) before proceeding to Ethyl Acetate (EA) and then methanol (MeOH). The Hex, EA and MeOH extracts were respectively 10.0 g, 110.0 g and 180.0 g. Hypotensive activity tests carried on the different extracts highlighted the EA extract as most active and so 90.0 g of the EA extract was subjected to repeated silica gel column chromatography using hexane (Hex), hexane-Ethyl Acetate (EA) and EA- Methanol (MeOH). 143 fractions of 275 mL each was collected, from which betulinic acid (Hex-EA: 70-30, 43.35 mg), tridecyl 9-hydroxynonanoate (Hex-EA: 85-15, 54.25 mg), a fatty acid (Hex-EA: 90-10, 33.82 mg) were

obtained after the solvent evaporated, washing with appropriate solvents and filtration.

2.3. Phytochemical Screening

Phytochemical screening was done as described by [13]. for evaluation of reducing sugars, saponins, flavonoids, tannins, phenols, lipids, steroids, terpenoids, cardiac glycosides, anthraquinones, alkaloids and triterpenes.

2.4. Animal Studies

Male albino Wistar rats of 12 weeks old weighting 180-250 g were used. The animals were maintained on a 12 h light/dark cycle, with free access to water and standard Laboratory diet. Normotensive rats (NTR) were used to evaluate hypotensive effect in the ethyl acetate extract and compounds on arterial blood pressure, heart rate and its mechanisms of action.

2.5. Acute Effect of Eribroma Oblongum on Blood Pressure and Heart Rate of Normotensive Rats

The rats were anaesthetized using an intraperitoneal injection of urethane (1.5 g/kg). The trachea was exposed and cannulated to facilitate spontaneous respiration. The arterial blood pressure was measured from right carotid artery via an arterial cannula connected to a pressure transducer, coupled with a hemodynamic recorder Biopac Student Lab. (MP35) and computer. The animals were allowed to stabilize for at least 30 min before administration of any test substances [14]. The plant extract or drugs were injected via a cannula inserted into the left femoral vein. The dose of 10 mg/kg was used to investigate the hypotensive mechanism of *E. oblongum*. Atropine (1 mg/kg), yohimbine (100µg/kg), Nw-Nitro-L-arginine Methyl Ester (L-NAME, 5 mg/kg) and propranolol (30µg/kg) were injected 5 min before the plant extract. In another set of study, the extract was injected 5 min before L-NAME. Reserpine (5 mg/kg) was given orally to NTR once a day and three days after, the extract (10 mg/kg) was injected to rats after anaesthesia. Blood pressure and heart rate were observed for 1 h after drug administration.

2.6. Statistical Analysis

All results are expressed as mean ± standard error of mean (S.E.M.) and statistical analysis was performed using Graph Pad Instat Software. Data were analysed using one-way analysis of variance ANOVA followed by Tukey post hoc

3. Results

3.1. Phytochemical Screening of Plant Materials

Phytochemical analysis revealed the presence of reducing sugar, triterpene, terpenoids, flavonoids, phenols and saponins. Alkaloids, lipids, steroid cardiac glycosides, anthraquinones, and tannins were absent.

Table 1. *Phytochemical constituents of ethyl acetate extract of Eribroma oblongum.*

Constituents	Ethyl acetate
Test for reducing sugars	++
Test for triterpenes	+
Test for terpenoids	+
Test for flavonoids	+
Test for saponins	+++
Test for phenol	-
Test for anthraquinones	-
Test for cardiac glycosides	-
Test for tannins	-
Test for alkaloids	-
Test for lipid	-
Test for steroids	-

(-): absent; (+): present

3.2. Identification of Compounds 1 and 2

Phytochemical studies of the ethyl acetate extract from the stem bark *E.oblongum* yielded betulinic acid [15], Tridecyl 9-hydroxynonanoate.

Compound 1 was isolated as a white powder and determined to have a molecular formula $C_{30}H_{48}O_3$from its NMR data and ESI-MS which gave a pseudo-molecular ion peak at m/z: 456.06 (calcd $[M]^+$: 456.3623). This composition accounted for seven double bond equivalents. Compound 1 responded positively to the Liebermann–Burchard test indicative of triterpenes. The structure of this compound was determined using its NMR data and by comparison with similar data in literature [15].

Betulinic acid: 3*β*-hydroxylup-20(29)-en-28-oic acid

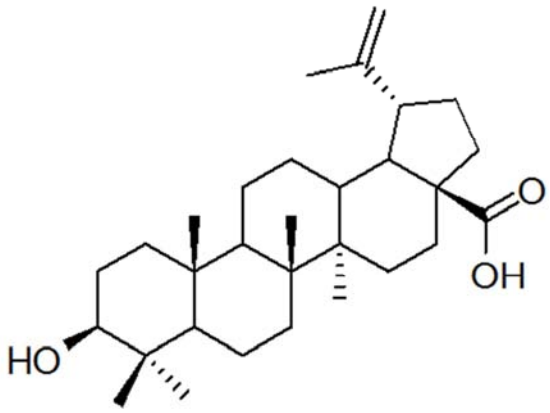


Figure 1. *3β-hydroxylup-20(29)-en-28-oic acid.*

White powder or colourless crystals in methanol, m.p. [280-282°C]

ESI-MS: m/z 456.06 [calc. $C_{30}H_{48}O_3$: 456.3623]

^{13}C-NMR ($CDCl_3$,75 MHz) data: 38.7 (C-1), 27.4 (C-2), 79.0 (C-3), 38.9 (C-4), 55.4 (C-5), 18.4 (C-6), 34.4 (C-7), 40.7 (C-8), 50.7 (C-9), 37.2 (C-10), 20.9 (C-11), 25.5 (C-12), 38.4 (C-13), 42.5 (C-14), 30.6 (C-15), 32.2 (C-16), 56.3 (C-17), 46.9 (C-18), 49.3 (C-19), 150.5 (C-20), 29.7 (C-21), 37.1 (C-22), 28.0 (C-23), 15.4 (C-24), 16.1 (C-25), 16.2 (C-26), 14.7 (C-27), 180.5 (C-28), 109.7 (C-29), 19.4 (C-30).

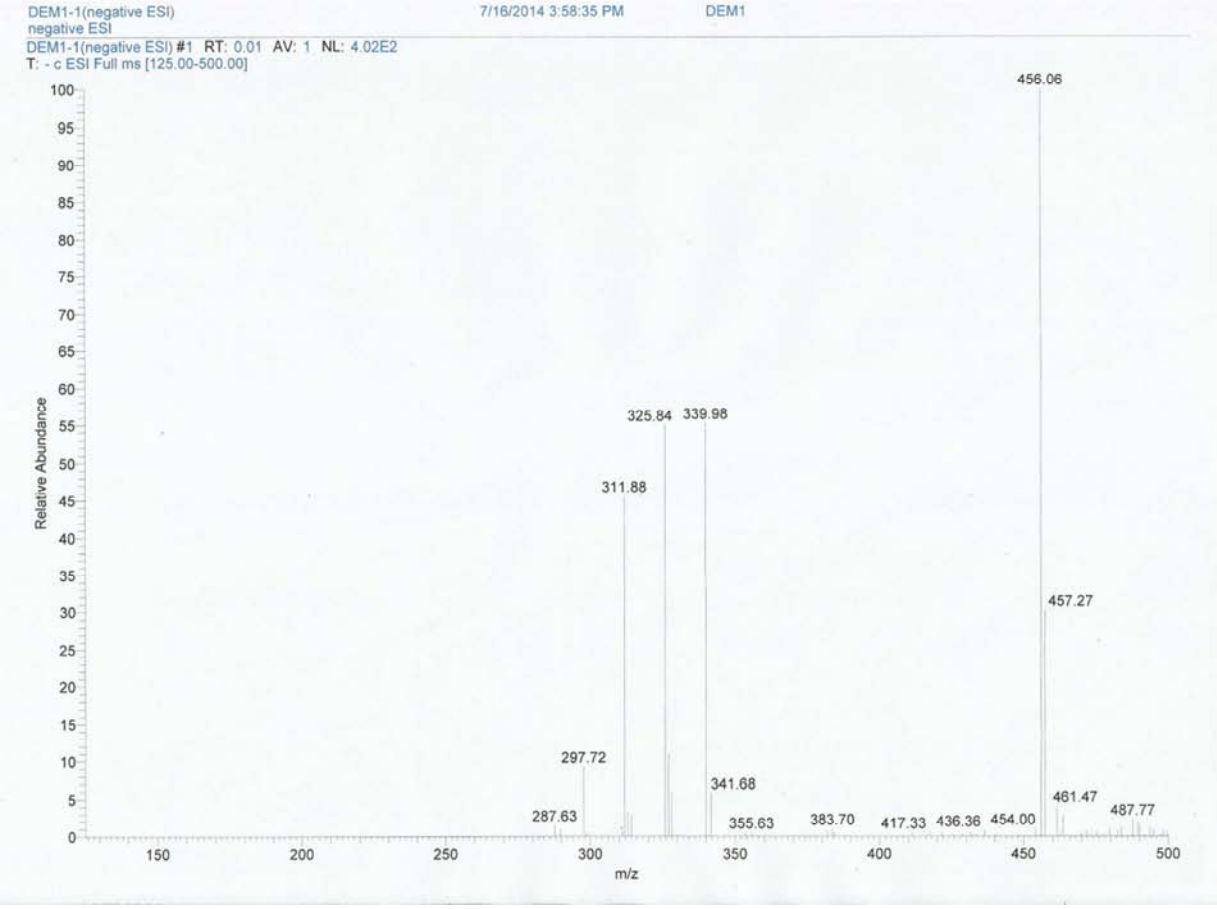


Figure 2. *Mass Spectrum of compound.*

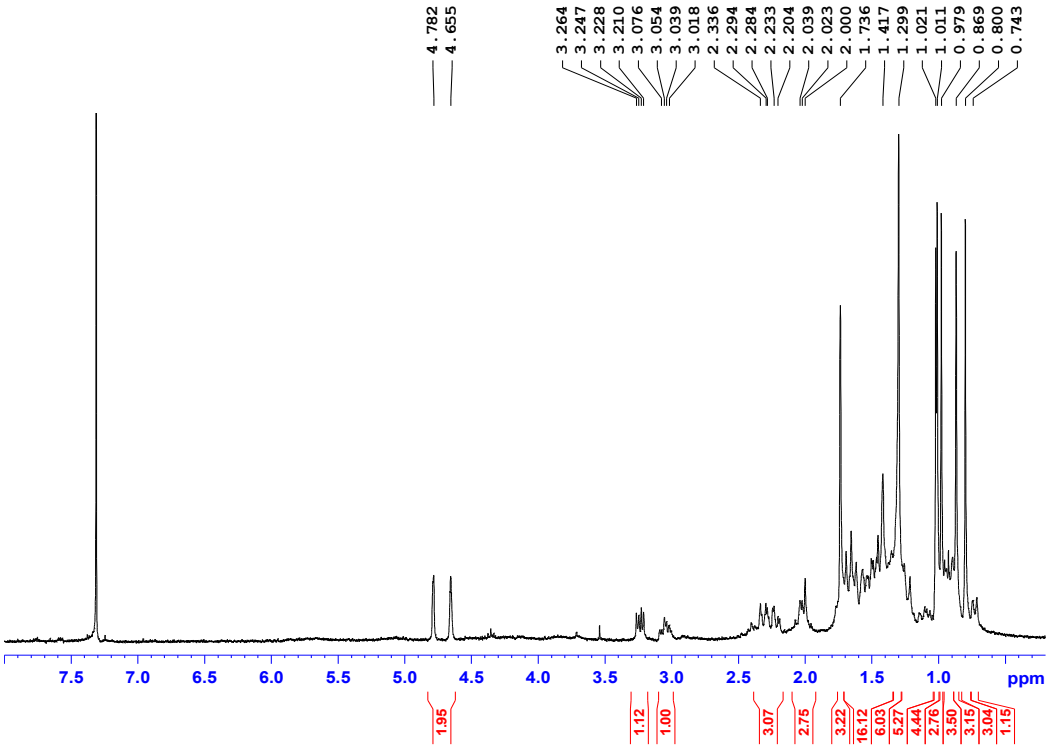


Figure 3. *RMN^{1}H Spectrum (500 MHz, MeOD) of compound.*

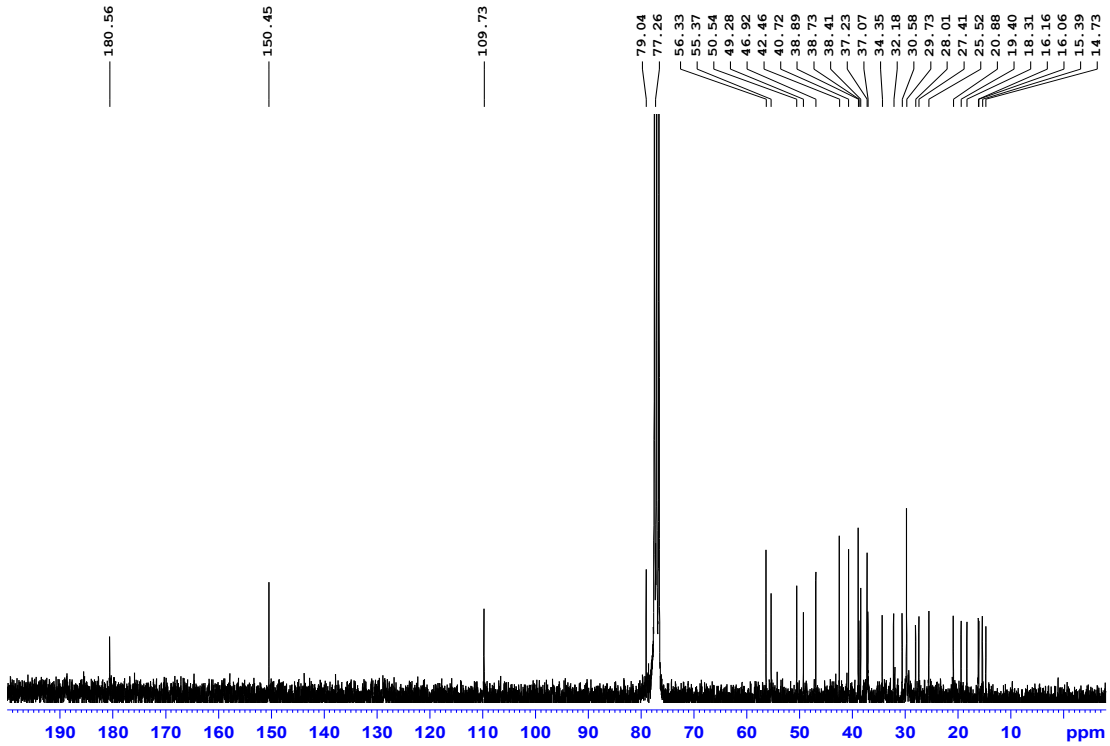


Figure 4. *RMN^{13}C Spectrum (125MHz, MeOD).*

Compound 2 was obtained as a white powder which is soluble in chloroform. Its structure was proposed by use of its NMR data in conjunction with similar data found in the literature.

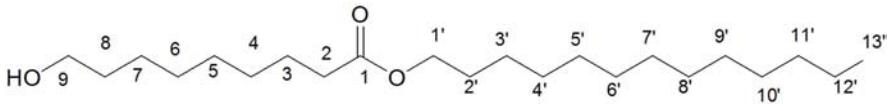
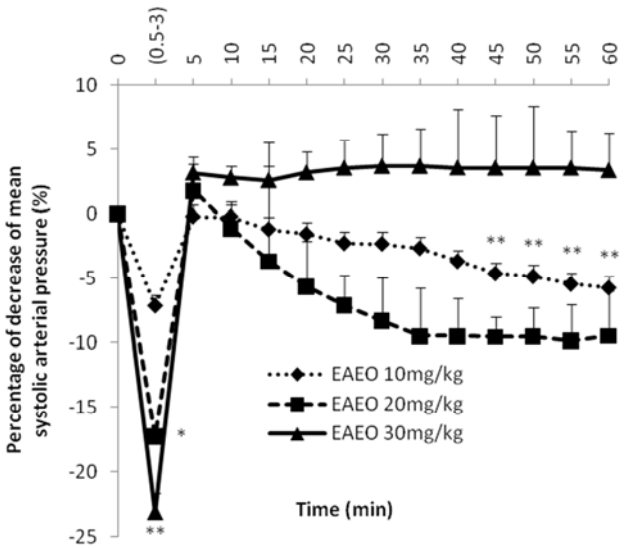


Figure 5. *Chemical structure of Tridecyl 9-hydroxynonanoate.*

White powder,

$C_{22}H_{44}0_3$

^{13}C-NMR data: 174.85 (C-1), 27.21- 31.52 (C-2 to C-8), 65.32 (C-9), 63.52 (C-1'), 27.21- 31.52 (C-2' to C-12'), 14.87 (C-13').



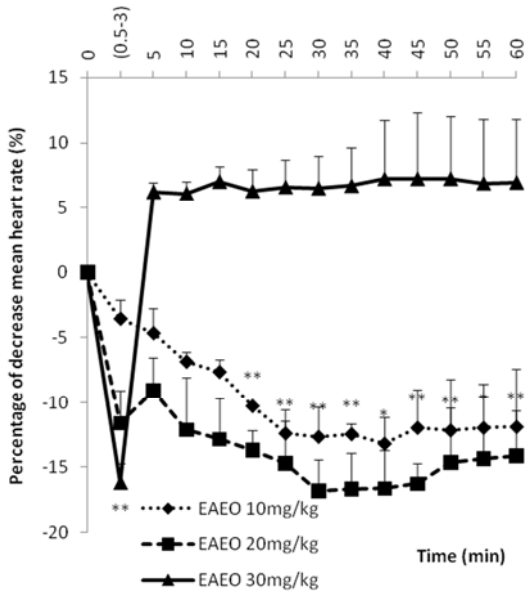


Figure 6. *Changes on systolic arterial pressure (A) and heart rate (B) of anesthetized rats after intravenous administration of EAEO (Ethyl Acetate extract of Eribroma oblongum); n = 5 each bar represents the means ± SEM of group; $^{*}P<0.05$, $^{**}P<0.01$; significantly different compared to initial time t_0.*

3.3. Effects of Acute Injection of Eribroma Oblongum on Blood Pressure and Heart Rate

The injection of the extract of *Eribroma oblongum* in normotensive rats (NTR) resulted in a significant rapid reduction of systolic blood pressure (SBP). As shown in fig. 6 *Eribroma oblongum* reduced the SBP in NTR significantly by 17.29±0.5% ($P<0.05$) and by 23.15±1.47% ($P<0.01$) at the dose 20 and 30mg/kg respectively. The first and rapid hypotensive response was followed by a transient increase of SBP, after that SBP decrease progressively and significantly until the end of the observation period the PAS was at 5.76±0.86% ($P<0.01$), 8.48±3.47% at the dose 10, 20 mg/kg respectively and increase of 3.35±2.92% the dose 30 mg/kg. The intravenous administration of the extract in NTR resulted in a significant $P<0.01$ rapid reducing of heart rate (HR) by 16.14±1.46% at the dose 30mg/kg. The first and rapid decrease of HR response was followed by an increase of HR

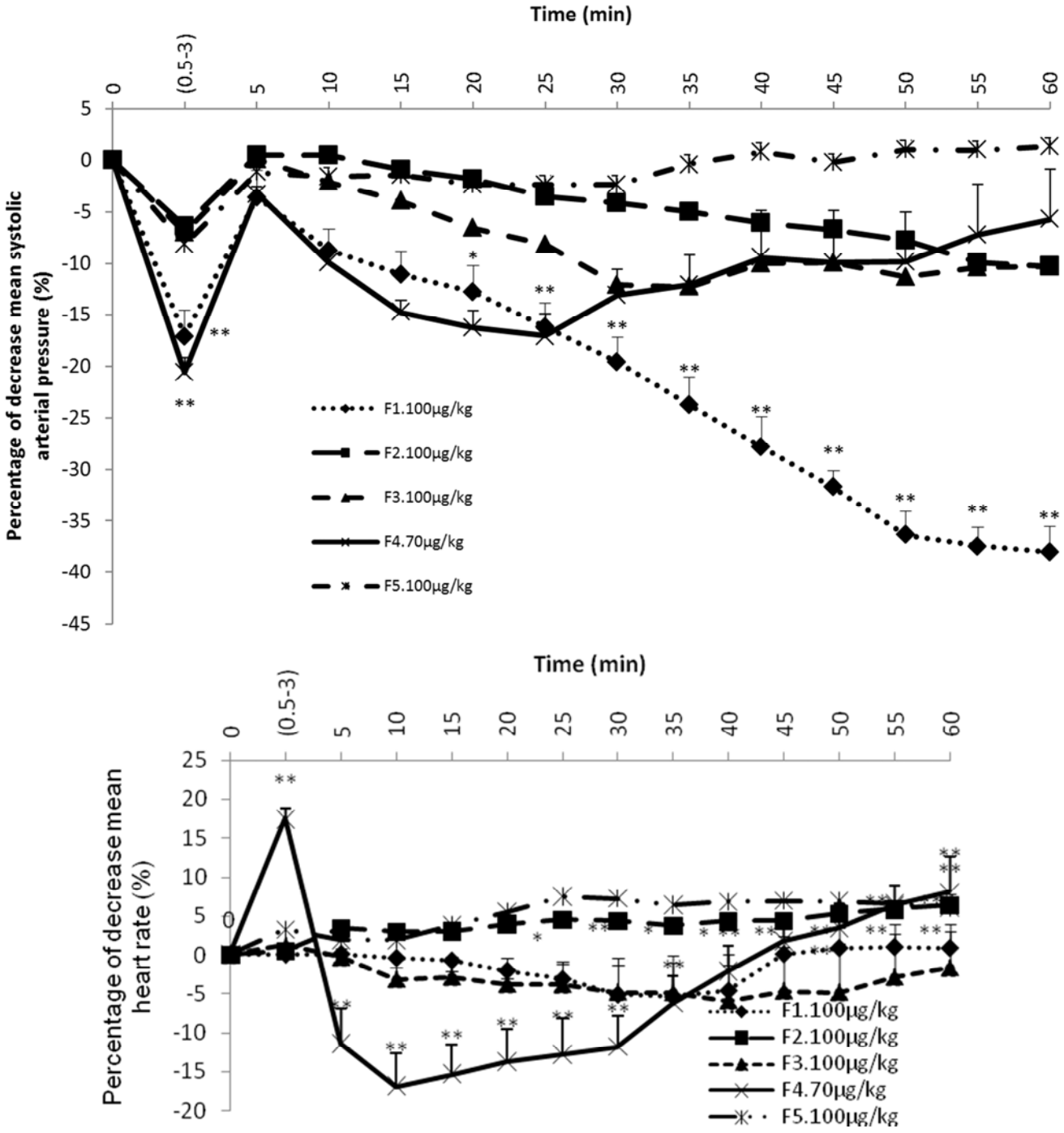


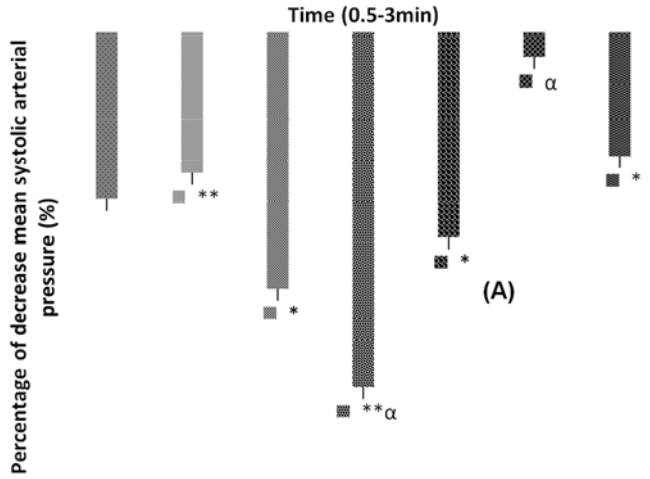
Figure 7. *Changes on systolic arterial pressure (A) and heart rate (B) of anesthetized rats after intravenous administration of F1, F2, F3, F4 or F5 (F = compound of ethyl acetate extract of Eribroma oblongum); n = 5 each bar represents the means ± SEM of group; $^{*}P<0.05$, $^{**}P<0.01$; significantly different compared to initial time t_0.*

3.4. Effects of Acute Injection of Compound 1 on Blood Pressure and Heart Rate

The injection of compound 1 in normotensive rats (NTR) resulted in a significant P<0.01 rapid reduction of systolic blood pressure (SBP). As shown in fig. 7 compound 1 reduced the SBP in NTR by 17.03±2.79% and 38.04±0.07% (P<0.01) representing at the compound 1 (100µg/kg) and compound 4 (70µg/kg) respectively. The first and rapid hypotensive response was followed by a transient increase of systolic blood pressure, after that SBP decrease progressively and significantly P<0.01 until the end of the observation period the SBP was at 38.04±2.24% at the compound 1 (100µg/kg) and increase of 1.33±0.37% at the compound 5 (100µg/kg). The injection of compound 4 (70µg/kg) in NTR resulted in a significant P<0.01 rapid increasing of heart rate by 17.52±0.4%.

3.5. Mechanisms of Hypotensive Effects of Eribroma oblongum Compound 1 in Anesthetized Normotensive Rats

The pretreatment of normotensive rats with reserpine (5mg/kg), NAME (5mg/kg) after compound 1, Propranolol (Prop) (30µg/kg) and yohimbine (Yohim) (100µg/kg) have significantly compared to initial time t_0 reduced the immediate hypotensive response of rats to compound 1 by 11.70±2.45% (P<0.01), 10.28±4.77% (P<0.05), 29.45±2.82% (P<0.01) and 21.30±1.88% (P<0.05) respectively. The pretreatment of normotensive rats with propranolol or NAME prior compound 1 antagonist have significantly P<0.05, compared to compound 1 reduced the immediate hypotensive response of rats to compound 1 by 29.45 ±2.89%; 2.06±3.54%. The later hypotensive response of extract after pretreatment with reserpine, propranolol or NAME prior compound 1 antagonist were significantly reduced by 6.17±6.15% (P<0.01), 17.92±4.05 (P<0.05) and 4.09±1.54% (P<0.05) respectively. NAME after compound 1 and all antagonists used was reduced significantly P<0.01 compared to compound 1 without antagonist (fig. 8).



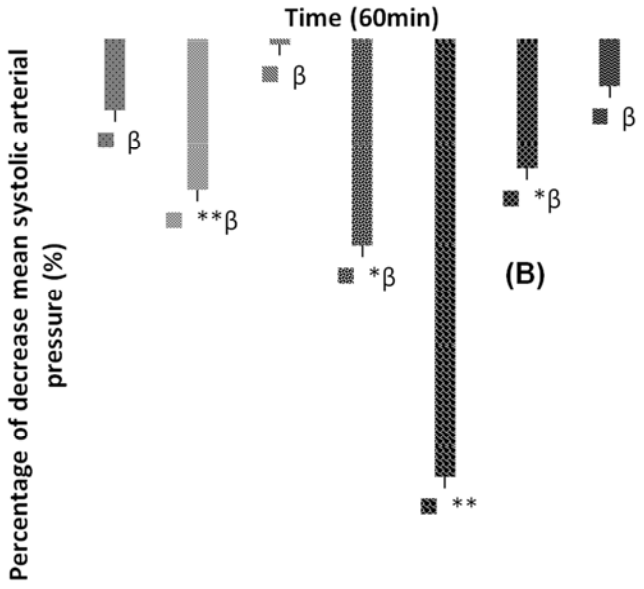


Figure 8. *The maximal immediate changes(time 0.5-3min) (A) and later changes (time 60min) (B) in mean systolic arterial pressure in anesthetized animals that received intravenous injection of F1 100µg/kg (F1= butilinic acid purified from Eribroma oblongum). Some animals received and additional pretreatment of Yoh.(Yohimbine 100µg/kg); Prop. (Propranolol 30µg/kg); Atro. (Atropine 1mg/kg); NAME (Nw-Nitro-L-Arginine Methyl Esther, 5mg/kg) an inhibitor of nitric oxide synthase 5 minutes prior and after the plant extract administration. Res. (Reserpine 5mg/kg) were admisnistered 3 days before the plant extract administration. n = 5 each bar represents the means ± SEM of group; $^{*}P<0.05$, $^{**}P<0.01$; significantly different compared to initial time t_0 and $^{\alpha}P<0.05$, $^{\beta}P<0.01$; significantly different compared to F1 without antagonisms.*

4. Discussion

This study investigated the acute effects of the stem back ethyl acetate extract and the five pure compounds witch were isoled from it. The phytochemical screening of ethyl acetate extract was done as described by [13]. The result revealed the presence of reduced sugar, triterpene, terpenoids, flavonoids, phenols and saponins. Alkaloids, lipids, steroid cardiac glycosides, anthraquinones, and tannins were absent.

In our study ethyl acetate extract of *Eribroma oblongum* reduced the SBP at 5.76±0.86%, 8.48±3.47% respectively at the dose 10 and 20 mg/kg SBP at 38.04±3.17%, 10.27±0.68%, 1.33±7.92%, 5.73±0.12% and 1.33±9.91%. The compound 1 (100 µg/kg) was used to evaluate the mechanisms involved in the hypotensive effects of betillinic acid in normotensive rats.

During all this experimental study, we are revealed a great number of deaths of rats. The percentage of death was gradually higher by 40%, 70% and 80% at the dose 10, 20 and 30mg/kg respectively. Furthermore we have observed these similar results with the compound 4 or compound 5 which have presented 90% and 20% of death at the dose 70 and 100µg/kg respectively. These results are correlated those obtained with to the phytochemical screening which revealed the present of triterpene and terpenoids. These toxicities results could been demonstrated in the work of [16], [17]

which proved that the *in vitro* antitumor cytotoxic activity of BA has been illustrated in broad spectrum of cancer cell lines including those of leukemia, neuroblastoma, colon, breast, melanoma, lung, prostate, and cervical origin.

Six pure compounds were isolated from ethyl acetate fraction; only five of these compounds were investigated in this study. We have identified two of them: betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid) and tridecyl 9-hydroxypentanoate, which is corresponded to compound 1 and compound 2. The compound 1 is a tritertenoid which activities was been demonstrated where as compound 2 is a fatty acid which activities was not been demonstrated now. For the remainder of this study, the fraction F1 is the pure compound 1 who presented the best activity than other.

Betulinic acid is an important natural product widely distributed throughout the plant kingdom [18]. Betulinic acid (BA) exhibits various biological activities, such as anti-HIV, anti-inflammatory, antioxidant, antiretroviral and antibacterial properties [19], [20].

The intravenous administration of ethyl acetate extract or compound 1, 2, 3 and 5 in normotensive anesthetized rats induced a blood pressure lowering effect accompanied by a reduction of heart rate. The hypotensive effect lasted 4 minutes when the heart rate was still significantly low. These results suggested that the hypotensive effect of the extract may be due to its bradycardiac effect. The fall in blood pressure induced by the extract might stimulate the baro-reflexes. Catecholamines are then released to cause a transient rise in pressure due to a vasoconstriction. As against the compound 4 after intravenous administration in normotensive anesthetized rats inducted a blood pressure significant $P<0.01$ lowering effect accompanied by a significant $P<0.01$ increasing of heart rate. The presence of triterpenes in the ethyl acetate extract or pure compound 1 account for its cardiovascular activity. This group of secondary plant metabolites widely occurring in the vegetable kingdom has been shown to display a remarkable array of biochemical and pharmacological actions, including cardiovascular effects [21].

L-NAME, a selective inhibitor of nitric oxide synthase induced 5 minutes prior the compound 1 significantly reduced $P<0.05$ the immediate hypotensive effects of BA. These resultat suggest that BA may act throw the endothelium-mediated/nitric oxide. However propranolol, (the beta blocker may oppose the vascular smooth muscle relaxation induced by the activation of the beta 2 receptor by endogenous epinephrine) appears to potentialize significantly $P<0.05$ the immediate hypotensive effects of BA [22], [23]. The pretreatment of normotensive rats with reserpine (5mg/kg), (inhibitor of vesicular storage of biogenic amines) were admisnistered 3 days before the plant extract administration, yohimbine, (a selective α_2-adrenoceptor) antagonist, atropine, (the muscarinic receptor) antagonist and L-NAME induced 5 minutes after the compound 1 did not modify the immediate hypotensive effect of BA. At the later response, atropine sulfate, reserpine, propranolol or L-NAME were partially blocked the hypotensive effects of BA. Whereas yohimbine, was completely blocked the hypotensive effect of extract. The resultats suggest that the α_2 adrenoceptor system do not participate in hypotensive effect of BA. A dose used in this study (0.1 mg / kg), yohimbine can unlock the presynaptic alpha-2 adrenergic receptor. The effect of yohimbine has been studied in a small dose because high doses of yohimbine increased sympathetic tone and decreased vagal tone [24].

5. Conclusion

At the end of this study the result demonstrates that the hypotensive effects activity of the ethyl acetate extract of the stem bark of *Eribroma oblongum* (Malvaceae) lead to the phytochemical study of this extract which established Betulinic acid as the main hypotensive principle, its activity was 200 times more lofty as crude extract. The other compounds isolated: tridecyl 9- hydroxyloctadecanoate, a fatty acid and 3 compounds showed few activities. The structure of isolates was established on the basis of NMR inspection, mass spectrometric data and by comparison with those previously reported in the literature. Our data validate the use of the extract traditional medicine against hypertension.Further studies are from now evaluated the toxicity of *Eribroma oblongum*.

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Competing Interests

Authors have declared that no competing interests exist.

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