ORIGINAL ARTICLE

VASO-RELAXING ACTIVITY OF *Ageratum Conyzoides* Linn. (ASTERACEAE) AERIAL PARTS ON ISOLATED RAT AORTA

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ABSTRACT

Background: *Ageratum conyzoides* (ASTERACEAE) is traditionally used against asthma, articular rheumatism and arterial high blood pressure in Malagasy folk medicine. Few studies are conducted on its antihypertensive activity. **Objectives:** This study aims to validate the use of this plant as antihypertensive through the vaso-relaxing property of the aerial part methanol extract (ME) and the action mechanism of ethyl acetate fraction (EF). **Methods:** Dried powder of *A. conyzoides* aerial part was extracted by maceration in methanol. Methanolic solution was depigmented by activated charcoal then filtered on Whatman's filter paper and evaporated to dryness. This methanol extract was dissolved in distilled water and then successively partitioned with hexane, dichloromethane, ethyl acetate and butanol. The vaso-relaxing activity of extract and fractions was assessed on the phenylephrine pre-contracted isolated rat aorta. Action mechanism of ethyl acetate fraction (EF) was determined using three active reagents including propranolol, indomethacin and L-NAME. Phytochemical screening was assessed in ME with common methods as well as acute toxicity in mice. **Results:** Phytochemical screening shows the presence of phenolic compounds, flavonoids, tannins, saponins, steroids, quinones and anthraquiniones. Tested on isolated rat aorta, ME exhibits a moderate activity (EC_{50} = 383.44±17.34 µg/ml). After bio-guided fractionation, EF, with EC_{50} of 204.07±8.50 µg/ml, was the most active. This activity wasn't modified by propranolol and indomethacin but with L-NAME, the EC_{50} was increased to 584.04±30.98 µg/ml and the tested maximal concentration does not allow achieving its maximum effect. The acute toxicity tests showed that ME is devoid of toxicity. **Conclusion:** The anti-hypertensive activity of *A. conyzoides* is partly the result of the vaso-relaxing effect of bioactive molecules dissolved in ethyl acetate. These results contribute to explain the antihypertensive virtue of *Ageratum conyzoides*.
***Key words**: Ageratum conyzoides, antihypertensive activity, vaso-relaxing activity*

1. INTRODUCTION

Ageratum conyzoides L. (ASTERACEAE) is a tropical plant growing in the western and eastern regions of the African continent, as well as in some regions of Asia and South America [1, 2]. It is known as Ananjazavavy, Hanitrinimpatsaka, Alonimpatsaka or Bemaimbo in different regions of Madagascar and known with other vernacular name from other countries including Ageratum, Billygoat-weed, Goat weed, Chick weed and White weed [3]. In northern of Madagascar, decoction of leaves of *A. conyzoides* is used during difficult childbirth to intimate feminine dressing associated with Romba (*Ocimum gratissimum*) and Fagnivagna (*Aeschynomene sp.*), to wash the woman and child after delivery. This decoction is also used to relieve painful periods and to attenuate vomiting and diarrhea, to clean infected wounds and ulcers of the skin. The decoction of flowers is used to wash eyes and treat conjunctivitis [4]. Decoction of *A. conyzoides* is used to treat asthma and hypertension. The plant is crushed and rubbed on joints to treat rheumatism. In India, flowers are used to treat cough and cold, headache and wormer [5], and roots are antilitic and antidiarrheal [2]. In Central Africa, Brazil and Congo, *A. conyzoides* is used to cure pneumonia, wounds and burns [6], its decoction is given to treat headache, fever and rheumatism [2]. In Cameroon, aqueous extracts of the whole plant are known for their anti-diabetic properties [7]. In Ivory Coast, decoction of leaves is used to treat malaria [8]. In Nigeria, seeds of *A. conyzoides* are anti-hyperglycemia [9]. Secondary metabolites of this plant were widely studied including monoterpenes and sesquiterpenes, triterpenes, steroids, flavonoids, coumarins, tannins and alkaloids [10-16]. Moreira et *al.* (2007) found that methoxyflavone isolated from hexane extract of leaves of *A. conyzoides* had insecticidal activity [12]. Adetutu et *al.* (2012) and Odeleye et *al. (2014)* reported the plant extracts antibacterial activities then Morais et *al.* (2014) highlighted antifungal and Teixeira et *al.* (2014) the anti-parasitic activity [17, 18, 19, 20]. Others properties such as anti-inflammatory [21], antalgic [22], wound healing [23] and cytotoxicity properties [17] have been demonstrated. The essential oil of the leaves and aerial parts of the plant has been widely investigated for its components and biological activities. The major constituents generally found are the chromenes, precocene I and precocene II, and the sesquiterpenes caryophyllene and germacrene-D [1,2]. The main activity described in the literature for the essential oil is the insecticide [15], but *A. conyzoydes* can also exert allelopathic [24] and antifungal activities [25, 26]. Among these biological activities, few studies have been conducted

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regarding its antihypertensive activity. Thus, this work aims to demonstrate the antihypertensive activity of *A. conyzoides* through its vaso-relaxing activity and its mechanism of action.

2. MATERIALS AND METHODS

2.1 Plant material

Aerial parts of *A. conyzoides* were harvested at Ambohimanambola - Antananarivo in December 2018. They were dried in cool and aerated place away of sunlight before to be grinded. The taxonomic botany of the plant was identified and authenticated by *Dr.* Benja Rakotonirina, the botanist of the "*Institut Malgache de Recherches Appliquées*" (IMRA) and the voucher specimen was deposited at the IMRA Botany Department under the identification code NT-009/LPA.

2.2 Animals

Adults males or females wistar rats, weighing between 150-200 g and aged between 5-6 months, are used for *in vitro* pharmacological tests. Male OF_1 mice (25±2 g, aged between 3-4 months) are used for *in vivo* acute toxicity test. Animals are provided from IMRA animal house and allowed free access to standard pellets (1420, Livestock Feed Ltd.) and tap water. They were exposed to day-night light cycle (12h) and room temperature. All experiments were carried out in accordance with the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes (DIRECTIVE 2010/63/EU).

2.3 Extraction and fractionation

Three hundred grams of vegetable material powder of *A. conyzoides* were macerated in methanol for 24 hours with intermittent shaking. The protocol was repeated three times to maximize extraction efficiency. The filtrates were gathered and depigmented through a layer of activated charcoal then depigmented methanolic solution were evaporated to dryness under reduced pressure at a temperature of 40° C, with a rotavapor (Buchi-R114) in order to obtain the methanol extract (ME). Then, 15 g of ME were partitioned by the liquid-liquid fractionation method using distillated water - hexane, dichloromethane and ethyl acetate successively to allow to hexane (HF), dichloromethane (DF), ethyl acetate (EF) and aquoeus (AF) fractions.

2.4 Phytochemical screening

The major classes of secondary metabolites were detected in ME and EF of *A. conyzoides* using specific reagents as described in our previous work [27].

2.5 Pharmacological experiments

2.5.1 Chemicals: All reference products used for pharmacological tests such as phenylephrine, acetylcholine, indomethacin, L-Nitro-Arginine Methyl Ester (L-NAME) and propranolol are purchased from Sigma-Aldrich and all salts including KCl, NaCl, $NaHCO_3$, $MgSO_4$, KH_2PO_4 and $CaCl_2$ and Glucose used to prepare survival solution of Krebs-Heinseleit are purchased from Prolabo.

2.5.2 Organ preparation: Animal was anesthesied with petrolium ether and then exsanguinated by carotid artery transection. The thoracic aorta was removed and carefully cleaned of adhering fat and connective tissue, and cut into rings (2-3 mm length). The rings were then mounted in standard organ baths filled with a physiological salt solution called Krebs-Henseleit solution composed (in mM) by KCl: 4.8; NaCl: 118; $NaHCO_3$: 25; $MgSO_4$: 1.2; KH_2PO_4: 1.2; $CaCl_2$: 1.25 and Glucose: 11, maintained at 37°C and continuously bubbled with carbogen (95% O_2 - 5% CO_2). Resting tension was adjusted to 2 g. Tension developped by the organe trip was measured with an isometric force transducer. After an equilibration period of 90 min, with a renewal of survival medium every 20 min, the vessels were maximally contracted with phenylephrine (10^{-5} M) in order to test their contractile capacity and the integrity of each aorta ring was verified with acetylcholine (10^{-6} M).

2.5.3 Effect of extract and fractions on the pre-contracted wistar rat aorta: Vessels were pre-contracted with 10^{-6} M of Phenylephrine. At the contraction plateau, sample was cumulatively and increasingly tested at different concentrations including 125, 250, 500, 750, and 1000 µg/ml. The relaxing effect of each concentration was calculated and expressed as a percentage by considering the contraction plateau as 100%. The EC_{50} which is the concentration giving 50% of the maximum relaxing effect (E_{max}), was calculated by linear regression.

2.5.4 Effect of propranolol on the vaso-relaxing activity of EF: Isolated organ vessels were divided into two groups. Group I (n = 6) were submerged for 30 min in 10^{-5} M propranolol while group II (n = 6) was submerged in the survival solution. Then, all rings were pre-contracted with 10^{-6} M of phenylephrine. At the plateau contraction, EF was cumulatively and increasingly tested at different concentrations ranging from 125 to 1000 µg/ml. Its EC_{50} was calculated in each condition then compared.

2.5.5 Effect of indomethacin on the vaso-relaxing activity of EF: As previously, the aorta rings were divided into 2 groups so that the first group (n = 6) were in contact for 30 min with 10^{-5} M of indomethacin and the second group (n = 6) without indomethacin. After pre-contraction with 10^{-6} M of phenylephrine, EF was tested at different concentrations ranging from 125 to 1000 µg/ml at the plateau contraction in a cumulative and increasing manner. EC_{50} was calculated for both conditions and compared.

2.5.6 Effect of L-NAME on the vaso-relaxing activity of EF: As previously, the isolated aorta rings were divided into two groups. The first group (n = 6) were in contact for 30 min with 10^{-4} M of L-NAME and the second group were left without L-NAME. After pre-contraction with 10^{-6} M phenylephrine, EF was tested at different concentrations as previously and its EC_{50} was calculated in these two conditions.

2.5.7 Acute toxicity: OF_1 male mice were fasted 12 hours before the beginning of the experiment.

Oral use: Animals were divided into 5 groups of 5 mice. Group A: received only 0.25 ml of distilled water, while group B, C, D and E received respectively 0.5, 1, 1.5, and 2 g/kg of ME administered by gavage at the rate of 0.25 ml/animal. After treatment, animals had free access to water and food. During the 72 hours of observation, all abnormal behavior of the animals relative to the controls and mortalities were noted.

Intra-peritoneal method: Animals were divided into 6 groups of 5 mice. ME was administered by intra-peritoneal injection at a rate of 0.2 ml/animal. Animals of the first group received distilled water, whereas those of the other groups received respectively 0.2, 0.4, 0.6, 0.8, and 1 g/kg of ME. General behaviours of animals as well as the possible mortality were recorded.

2.6 statistical analysis

All the results are expressed as mean ± s.e.m. calculated from the values obtained on n experiments or n animals. The means were compared statistically using the Student's *t*-test. A value of $p < 0.05$ among the degree of freedom used was considered a statistically significant different.

3. RESULTS

3.1 Extraction and bio-guided fractionation

The extraction from drug powder yields 5.82% (17.8 g) of ME which exhibited a moderate activity on pre-contracted isolated rat aorta by phenilephrine with a EC_{50} of 383.44 ± 17.34 µg/ml. After liquid-liquid partition, EF (1.79 g) is the most active with an EC_{50} of 204.07 ± 8.50 µg/ml (table 1, figure 1).

Table 1: The table presents the result of the Yield of extraction and bio-guided fractionation of *A. conyzoides*

Tested fraction	Mass (g)	Yield [a] (%)	EC_{50} values [b] (µg/ml)	E_{max} [b] (%)
ME	17.8	5.82	383.44 ± 17.34	98.73 ± 0.84
HF	7.89	1.58	492.91 ± 29.24 **c,e**	85.01 ± 2.29 **d,e**
DF	1.42	0.28	898.72 ± 100.63 **c,e**	55.78 ± 5.13 **d,e**
EF	1.79	0.36	204.07 ± 8.50 **d**	98.51 ± 1.49
AF	5.85	1.17	< 1000	45.80 ± 2.76 **d,e**

a: yield are relative to plant powder; b: values are expressed as mean ± s.e.m. of 6 independent experiments (n = 6); **c**: $p < 0.01$ *vs* ME ; **d**: $p < 0.001$ *vs* ME ; **e**: $p < 0.001$ *vs* EF.

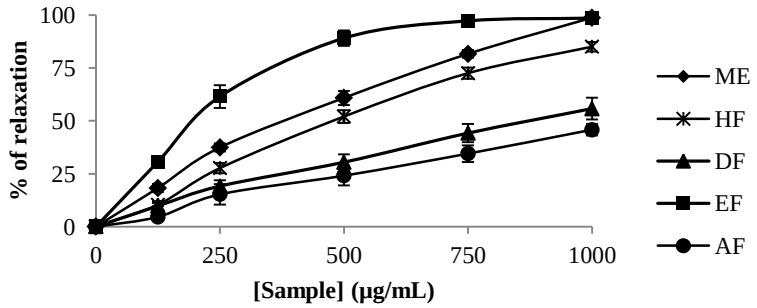

Figure 1: The figure shows the vaso-relaxing effect of the extract and fractions of *A. conyzoides* on the phenylephrine pre-contracted isolated rat aorta (n = 6).

3.2 Phytochemical screening

Phytochemical investigation on ME and EF are reported in table 2. Flavonoids, phenolic compounds and steroids are found in both ME and EF. On the other hand, saponins, tannins, quinones, anthraquinones and cardiac glycosides are only present in ME but not found in EF.

Table 2: The table presents the secondary metabolites in ME and EF.

Secondary metabolite	ME	EF
Flavonoids	+	+
Unsaturated sterols	-	-
Phenolic compounds	+	+
Steroids	+	+
Lactonic steroids	-	-
Cardio-tonic glycosides	+	-
Alkaloids	-	-
Terpenoids	-	-
Anthraquinones	+	-
Quinones	+	-
Tannins	+	-
Saponins	+	-

(+): presence of phytochemical compounds; (-): absence of phytochemical compounds.
ME: methanol extract; **EF**: ethyl acetate fraction.

3.3 Action mechanism study of EF

The EC_{50} and E_{max} of EF of *A. conyzoides* in presence or absence of antagonists are reported in table 3. One the first hand, the presence of propranolol or indomethacin didn't affect significantly the vaso-relaxing activity of EF (figure 2 and 3). On the other hand, the presence of L-NAME increases significantly ($p < 0.001$) the EC_{50} value of EF from 231.15 ± 13.99 µg/ml to 584.04 ± 30.98 µg/ml and decrease significantly ($p < 0.001$) the maximal effect (Emax) of EF of 17.10 % (figure 4) showing its vascular effect in the presence of the e-NOS enzyme inhibitor.

Table 3: The table presents the EC_{50} of EF of *A.conyzoides* in presence or in absence of antagonists

Sample	EC_{50} (µg/mL)	Emax (%)
EF alone	231.15 ± 13.99	97.46 ± 1.15
EF in presence of propranolol	223.94 ± 48.07	98.16 ± 0.88
EF in presence of indomethacin	236.22 ± 10.55	96.37 ± 0.61
EF in presence of L-NAME	584.04 ± 30.98*	80.36 ± 1.15*

Values express the mean ± s.e.m.; *: p < 0.0001 *vs* EF alone. **EF**: Ethyl acetate fraction; **EC_{50}**: median effective concentration**; Emax:** maximal effect

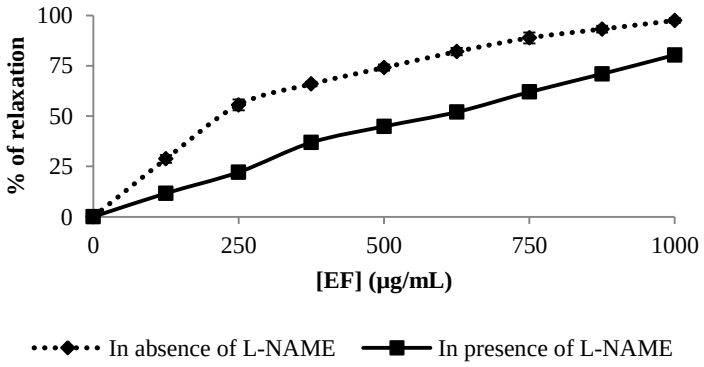

Figure 2: The figure shows the vaso-relaxing effect of EF on the isolated rat aorta pre-contracted to phenylephrine in the absence (♦) and presence (■) of 10^{-4} M L-NAME (n = 6).

3.4 Acute toxicity

During the three consecutive days of behavioral observation of animals treated with different doses of ME, no significant changes were observed concerning the gross behavior of the treated animals compared with the control animals. All tested doses did not cause any mortality in both administration methods. Thus, *A. conyzoides* is not toxic.

5. DISCUSSION

Phytochemical screening methods showed that the aerial part of *A. conyzoides* consists on the following major chemical families: polyphenols, flavonoids, tannins, saponins, steroids and anthraquinone. Several works carried out by Amadi et *al.* (2012) and Odeleye et *al.* (2014) on *A. conyzoides* from Nigeria reported the presence of high concentration of alkaloids and low concentrations of leucoanthocyanins and steroids in the aerial parts extract [28,

18]. Those carried out by Kamboj and Saluja (2008), *A. conyzoides* from India have shown the presence of alkaloids, coumarins, flavonoids, triterpenoids and sterols [29]. In addition of these chemical families, steroids, tannins and phenolic compounds are reported by Dash and Murthy (2011) [30]. The absence of alkaloids in *A. conyzoides* from Madagascar may be due to ecological factors which influences the metabolism of organic molecules of the plant. Presence of phenolic compounds is a promising results for isolation of active compounds from this plant because they constitute a family of organic molecules widely present in the plant kingdom which contribute to the decrease in the incidence of cardiovascular diseases including kaempferol, quercetin [31], gallic acid [27,32] and more others as a vaso-relaxing compounds.

Concerning the vaso-relaxing activity of the ME and fractions, the significant difference between the EC_{50} of ME and EF can be due to the influence of the other secondary metabolites absent in EF however, the maximal effect of has not been influenced. Propranolol is a non-selective β-adrenergic receptor antagonist with no intrinsic sympathomimetic activity [33]. It was used to study the involvement or not of β2-adrenergic receptors in the vascular effect of EF. The vaso-relaxing effect of EF wasn't affected by propranolol indicating that this effect didn't implicate this adrenergic receptor. Indomethacin is an inhibitor of cyclooxygenases (COXs), enzymes responsible for the biosynthesis of prostaglandins including prostacyclin (PGI2), which is one of the endothelial relaxation factors [34]. Thus, it was used to study the involvement of prostacyclin in the vascular effect of EF. The difference of the EF EC_{50} and its maximal effect levels is not statistically significant. It could indicate that PGI2 isn't implicated in its vaso-relaxing activity. L-NAME is an inhibitor of the enzyme e-NOS or endothelial NO synthase [35]. The Nitric oxide (NO) is one of the endothelial relaxation factors [36]. Therefore, L-NAME was used to study the involvement of endothelial NO in the vascular effect of EF. In the presence of this eNOS inhibitor, the pharmacological parameters of the EF vaso-relaxing activity were significantly modified (Fig.2 and Tab 3).

EF of *A. conyzoides* produces a concentration-dependent vaso-relaxing effect whose mechanism of action would involve nitrogen monoxide (NO) which is a vasodilating substance produced by endothelium from L-Arginine under the action of the enzyme nitrogen synthase (NOS). The NO after its intercellular diffusion will stimulate the guanylate cyclase which synthesizes the cyclic guanosine monophosphate (cGMP) in the vascular smooth muscle cells (VSMC). The increase in intracellular cGMP leads to activation of type 1 protein kinase G (PKG) which would reduce the intracellular calcium concentration by opening the membrane potassium channels that would lead to hyperpolarization of the plasmatic membrane and closure of the Ca^{2+} channels type L [37], the same mechanism of action as acetylcholine [38] and bradykinin [39] or by stimulating a serine/threonine protein phosphatase 2A which, in turn, dephosphorylates the Ca^{2+} channel and thus inactivates it [37]. The activated PKG can also activate the calcium/ATP-ases pumps (Ca^{2+}/ATP-ases) for the expulsion of the calcium from the cell through the PMCA and its recovery in the sarcoplasmic reticulum and therefore a decrease in the concentration in intracellular calcium in favor of relaxation [40]. Previous studies have shown that polyphenols such as quercetin, epicatechin increase NO production to improve endothelium-dependent vascular relaxation [41-44], while high-level gallic acid can induce vascular relaxation [27, 32]. In the longer term, polyphenols can increase the level of expression of endothelial NOS, leading to sustain NO formation and therefore persistent vascular protection [45]. Indeed, quercetin induces a rapid phosphorylation of endothelial NOS to serine 1179 (Ser1179) via an Akt-independent pathway and a cyclic adenosine monophosphate/protein kinase A dependent pathway to increase NO production and to promote vasodilation [46]. Epicatechin increases NO in endothelial cells via inhibition of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) [47]. Epicatechin and quercetin can also act as antioxidants by reducing nitrite and nitrate to NO [42,48]. This type of mechanism of action which increases the expression of endothelial NOS and decreases the activity of NADPH oxidase was also observed for the case of the plant *Salva miltorrhiza*. This plant is also used in cases of arterial hypertension due to its vasodilatory properties [49]. Other factors, such as low-density lipoprotein cholesterol (LDL-cholesterol), which are bad cholesterol, could influence the integrity of blood vessels. The accumulation of these lipids in the vessels and arteries causes a vascular restriction, the main cause of hypertension. The presence of phytosterols in particular sitosterol in *A. conyzoides* would be beneficial in hypertensive patients. Because of their very similar structure to that of cholesterol, phytosterols, when present in sufficient amounts in the intestine, compete with cholesterol in the formation of micelles necessary for the absorption of cholesterol [50,51]. Therefore, a 50% reduction in intestinal absorption of cholesterol has been demonstrated with sitosterol [52]. Although sitosterol significantly reduces LDL-cholesterol by 10-20% [53], Becker et *al.,* (1964) reported that taking sitosterol or sitostanol in children with familial hypercholesterolemia increases fecal excretion neutral sterols of 45% and 88% [54]. Heinemann et *al.* (1991) directly measured the effects of sitosterol and sitostanol on endogenous cholesterol absorption in healthy intestinal infusion volunteers and found a 50% reduction in bile cholesterol absorption with sitosterol and 85% Sitostanol [50]. Thus, the anti-hypertensive activity of *A. conyzoides* is the result of the vaso-relaxing effect of polyphenols and flavonoids and the hypocholesterolemic effect of phytosterols.

5. CONCLUSION

Ageratum conyzoides is used by Malagasy traditional healers for the treatment of high blood pressure. The phytochemical screening of the plant revealed the presence of phenolic compounds, flavonoids, steroids, terpenes, saponins and anthraquinones. Results obtained on the ethyl acetate extract of the aerial parts of *Ageratum conyzoides* made it possible to demonstrate its antihypertensive activity dependent-dose on the vascular level via a

vaso-relaxation mechanism dependent on endothelial NO. This activity may be due to the phenolic compounds and phytosterols present in the plant. Its traditional use as an antihypertensive agent is proven; however, this work did not define extactly the action mechanism of EF of *A. conyzoides*. Therefore, further studies are necessary to elucidate this mechanism and isolate the active compounds of this plant. In view of these tremendous activities of *Ageratum conyzoides*, this plant is certainly an interesting medication.

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