

## Vasodilator activity of extracts of field *Alpinia purpurata* (Vieill) K. Schum and *A. zerumbet* (Pers.) Burt et Smith cultured *in vitro*

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Nowadays, the high blood pressure is one of the main causes of death and cardiovascular diseases. Vasodilator drugs are frequently used to treat arterial hypertension. Experiments were undertaken to determine whether hydroalcoholic extracts obtained from leaves of field-grown *Alpinia purpurata* and *A. zerumbet* cultured *in vitro* under different plant growth regulators induce a vasodilator effect on Wistar rat mesenteric vascular bed pre-contracted with norepinephrine. Plant extracts were able to induce a long-lasting endothelium-dependent vasodilation. Efficiency on activity of *A. purpurata* reached 87% at concentration of 60 µg. The extract of *A. zerumbet* maintained in medium containing IAA, induced the relaxation (17.4%) at 90 µg, as compared to the control (MS0) that showed a better vasodilator effect (60%). These results are in agreement with the quantification of phenolic compounds in the extracts, which were 50% lower for those plants cultured in IAA. *A. purpurata* was assayed for the first time in relation to its vasodilator activity. This paper showed a strong probability of correlation between the pharmacological activities of *A. purpurata* with their content in phenolic compounds.

**Uniterms:** *Alpinia purpurata* (Vieill) K. Schum./pharmacognosy. *A. zerumbet* (Pers.) Burt et Smith./pharmacognosy. Natural vasodilators/experimental studies. Plant extracts/vasodilator action. Plant tissue/culture. Endothelium-dependent. Flavonoids. Medicinal Plant. Plant growth/regulators. Zingiberaceae.

Atualmente, a hipertensão arterial é uma das maiores causas de morte e de doenças cardiovasculares. Os vasodilatadores são frequentemente utilizados no tratamento da hipertensão. Extratos hidroalcoólicos de *Alpinia purpurata* de campo e de *A. zerumbet* cultivada *in vitro* sob diferentes reguladores de crescimento vegetal foram ensaiados no leito mesentérico de ratos Wistar. Os extratos de *A. purpurata* e *A. zerumbet* produziram efeito vasodilatador com padrão de resposta dose-dependente de duração prolongada. Extratos da espécie *A. purpurata* tiveram efeito vasodilatador de 87% na dose de 60 µg. O extrato obtido de folhas de *A. zerumbet* oriundas das culturas mantidas em meio contendo AIA (ácido indol acético) inibiu o relaxamento (17,4%) na dose de 90 µg em relação ao controle (MS0), com o qual foi verificado melhor efeito vasodilatador (60%). Estes resultados estão de acordo com a concentração de fenóis totais que foi 50% menor para os extratos de plantas cultivadas *in vitro* em AIA. A espécie *A. purpurata* foi pela primeira vez ensaiada quanto à atividade vasodilatadora. Os resultados obtidos indicaram a presença de substâncias fenólicas provavelmente correlacionadas à ação terapêutica de *A. purpurata*.

**Unitermos:** *Alpinia purpurata* (Vieill) K. Schum./farmacognosia. *Alpinia zerumbet* (Pers.) Burt et Smith./farmacognosia. Vasodilatadores naturais/estudo experimental. Extratos vegetais/ação vasodilatadora. Tecidos vegetais/cultura. Endotélio-dependente. Flavonóides. Plantas medicinais. Crescimento vegetal/reguladores. Zingiberaceae

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## INTRODUCTION

Cardiovascular diseases are the main mortality cause in Brazil. Hypertension is characterized by blood pressure increase, related to heart effort to propel blood and the vascular resistance (Godoy *et al.*, 2007). This disease affects people from different ages and social ranges, and there are several attempts to minimize or prevent hypertension occurrence that, in their majority, involve adequate diet, physical exercises, and medications. In Brazil, several reports have evidenced the use of plants having antihypertensive effects (Rocha *et al.*, 2007). *Alpinia zerumbet*, known as 'colônia', is widely used in northeastern and southeastern Brazil as infusions or decoctions for treatment of arterial hypertension. Hypotensive effects of this species have been justified by composition of hydroalcoholic and aqueous extracts and essential oils (Soares de Moura *et al.*, 1998; Victório *et al.*, 2009a). According to Mendonça *et al.* (1991) a decrease blood pressure in rats and dogs was observed using hydroalcoholic extracts of *A. zerumbet*. Mpalantinos *et al.* (1998) suggested that flavonoids and kavapyrones are responsible for vasodilator activity of aqueous extracts of *A. zerumbet*, while Lahlou *et al.* (2003) attributed it to some essential oil constituents therapeutic effects of *A. zerumbet*. A similarity between *Alpinia purpurata* and *A. zerumbet* was found in phytochemical studies, in terms of essential oils and flavonoids composition (Victório, 2008; Victório *et al.*, 2009b). The presence of flavonoids supported *evidence* for the chemosystematic features of the Zingiberiflorae, Zingiberaceae family (Pugialli *et al.*, 1993; Victório *et al.*, 2009b). Previous studies performed in animals have demonstrated that flavonoids are involved in vasodilator and hypotensive effects of *A. zerumbet* (Da Costa *et al.*, 1998; Mpalantinos *et al.*, 1998; Zheng-Tao *et al.*, 2001; Lahlou *et al.*, 2002; Lahlou *et al.*, 2003; Soares de Moura *et al.*, 2005).

*A. purpurata* is a tropical plant that possesses high value in the international market for cut flowers due to the durability and exuberance of its inflorescences, on the other hand its phytochemical investigation is still scarce and studies about its therapeutic effects are incipient. Recently, some flavonoids isolated from *A. zerumbet* have also been detected in *A. purpurata* extracts, what may corroborate the potential medicinal value of this species in treat arterial hypertension (Mpalantinos *et al.*, 1998; Victório *et al.*, 2007; Victório *et al.*, 2009b). Flavonoids are known to possess cardioprotective properties and their isolation from different medicinal plants has exhibited antihypertensive action. Wang *et al.* (2004) suggested that flavonoids may act in vascular system as antiarrhythmic and vasodilator agents. Some commercialized medicines

available to treat vascular diseases contain purified fractions of flavonoids, as for example, Daflon 500 mg. Flavonoids are antioxidants with health benefits acting in many hypotensive mechanisms such as inhibition of the angiotensin converting enzyme, blocking Ca<sup>2+</sup> channels, inhibition of vasoconstrictor prostanoid synthesis and inducing relaxation elicited by bradikynin (Formica, Regelson, 1995; Pourcel *et al.*, 2006; Havsteen, 2002; Xu *et al.*, 2006).

Production of bioactive metabolites through plant tissue culture techniques has largely been adopted to optimize the content of terpenoids, flavonoids, alkaloids and many phenolic compounds by addition of plant growth regulators in culture media (Canter *et al.*, 2005; Victório *et al.*, 2008). Considering secondary metabolites production, this study also intended to evaluate vasodilator activity of plants obtained from tissue cultures of *A. zerumbet* maintained under growth regulators effects.

Tests utilizing the rat mesenteric bed allow the vasodilator evaluation of plant extracts (McNeill, Jurgens, 2006) and, depending on the results, could even suggest a hypotensive activity. The hypertension has as its main consequence, an inadequate tissue perfusion that increases the risks for cerebral vascular accidents and ischemic heart disease (Kaiser, 2004). The purpose of this work was to evaluate the vasodilator action of plants of *A. purpurata*, collected in Rio de Janeiro, and plants of *A. zerumbet* arose from *in vitro* cultures.

## MATERIAL AND METHODS

### Animals

All procedures concerning animals were carried out in an ethically proper way, by following guidelines as set by the World Health Organization. Experiments were also reviewed and approved by the Ethics Committee of Animal Experiments of the State University of Rio de Janeiro. Male two-month-old Wistar rats, weighing 230-260 g, were kept at constant room temperature (24-26 °C), under a 12-hour standard light/dark cycle, light period starting at 6 a.m., and free access to food and water.

### Plant material and preparation of extracts

Samples of *A. zerumbet* and *A. purpurata* leaves were collected from plants growing in Rio de Janeiro, at *Universidade Federal do Rio de Janeiro* (Rio de Janeiro state, Brazil). Voucher specimen was identified and is deposited at the Herbarium of Rio de Janeiro Botanical Garden, under accession number RB 433485 and RB

433484, respectively. Those *A. zerumbet* plants were used as explants donor for tissue cultures establishment according to Victório, 2008. *A. zerumbet* was cultured in glass bottles containing 60 mL of MS (Murashige, Skoog, 1962) medium, added 30 g.L<sup>-1</sup> of sucrose, vitamins and myo-inositol. The following growth regulator treatments were evaluated for 60 days: MS0 (control); MS + 2 mg.L<sup>-1</sup> indole-3-acetic acid (IAA 2) and; MS + 2 mg.L<sup>-1</sup> IAA + 2 mg.L<sup>-1</sup> thidiazuron (TDZ 2). Cultures were maintained under white light (fluorescent tubs, 20 W, T-12, General Electric®), intensity of 30 µmol.m<sup>-2</sup>.s<sup>-1</sup>, daily photoperiod of 16 hours, at 25 ± 2 °C. The design of the experiments was a complete randomized block, and each experiment consisted of three explants per glass and the minimum of ten replicate culture flasks per plant growth regulator treatments.

Leaves from field-grown donor plants were dried for 3 days in stove (60 °C) and macerated in 50% ethanol (1:20 w/v) using ultrasonic bath (40 kHz, Thornton Unique, model 1400 USC) for 45 min, at 60 °C. Leaves obtained of *in vitro* cultures were dried by lyophilization and extracted in 50% ethanol as described above. All determinations were carried out in triplicates. The crude extracts were filtered in vacuum using a Whatman® filter (110 mm Ø, 1) and dried by rotary evaporation.

### Total phenolics

Total phenolics compounds were determined using the Folin-Ciocalteu method (Singleton, Rossi, 1965). Hydroalcoholic extracts were dissolved in ethanol (70%) at 1mg/mL. An aliquot of 0.5 mL of diluted extract and 2 mL Folin-Ciocalteu reagent (10%) were added, after 3 min, along with 2 mL of 7.5% sodium carbonate, and the contents were mixed. The mixture was homogenized and incubated at 50 °C for 30 min. The absorbance was measured at 740 nm in a spectrophotometer using gallic acid as standard. Two controls were used: (1) Folin-Ciocalteu + sodium carbonate and (2) crude extract solution. The quantification of phenolic compounds in crude extracts was determined from regression equation of calibration curves:  $y = 0.0229x + 0.0968$  ( $R^2 = 0.9993$ ) and expressed as mg gallic acid equivalents (GAE) per 1 g of dried leaves. All determinations were carried out in triplicates.

### High performance liquid chromatography analysis

Crude extracts were filtered in vacuum, dissolved in methanol: Milli-Q water (50%) at 10 mg/mL (field-grown) and 50 mg/mL (*in vitro* cultures). HPLC-UV analyses were performed on an apparatus Shimadzu equipped with SPD-

M10A diode array detector, LC-10AD pump and CBM-10 interface. Data were acquired and processed by a reversed phase column (Lichrosorb® RP-18 25 cm x 4.6 nm, 5 µm), room temperature. Separation was done in the following mobile phase: MilliQ water + 0.1% phosphoric acid (A) and methanol (B): 1-10 min (30% B); 20 min (40% B); 60 min (100% B) (Victório *et al.*, 2009b). The flow rate was kept constant at 1 mL/min and the peaks were detected at 360 nm. All chemical used in analysis as methanol and phosphoric acid were of HPLC grades and were purchased from Merck. MilliQ water was utilized to HPLC mobile phase and sample preparation. Standards were dissolved in methanol 70% at 1 mg/mL and analyzed in the same elution. The injections of 20 µL were repeated in triplicates. Determination of the content of the flavonoids was performed by the external standard method, using authentic standards of rutin and kaempferol-3-*O*-β-D-glucuronide. Quantification of flavonoids in the extracts was obtained against calibration curves of standards.

### Isolated mesenteric vascular bed

Experiments were undertaken to determine whether hydroalcoholic extracts obtained from leaves of field-grown *Alpinia purpurata* and *A. zerumbet* cultured *in vitro* under different plant growth regulators induce a vasodilator effect in the Wistar rat mesenteric vascular bed pre-contracted with norepinephrine. Crude extracts were dissolved in ethanol: Milli-Q water (1:1) at 1 mg/mL concentration.

The rat superior MVB (Mesenteric Vascular Bed) was isolated according to McGregor (1965). Male Wistar rats were killed with inhaled CO<sub>2</sub> and superior MVB was cannulated with a 4 cm polyethylene tube (PE 50, Clay Adams Brand CA – Becton Dickinson) and perfused at a flow rate 4 mL/min with a physiological salt solution (PSS) by pulsatile pump (Life Care Pump, Model 4 – Abbott / Shaw). The PSS had the following composition (mM): NaCl 118.3; KCl 4.7; CaCl<sub>2</sub>.2H<sub>2</sub>O 2.5; MgSO<sub>4</sub>.6H<sub>2</sub>O 1.2; KH<sub>2</sub>PO<sub>4</sub> 1.2; NaHCO<sub>3</sub> 25.0; EDTA 0.026 and glucose 11.1. The PSS (37°C) was bubbled with O<sub>2</sub> 95% and CO<sub>2</sub> 5%. Perfusion pressure (PP) was measured with a transducer connected to a preamplifier (Hewlett Packard - 7754 A) and chart recorded. Drugs were either dissolved in PSS and perfused at the desired concentration or administered as *bolus* injections directly into the perfusion stream (volume <300 µL).

The preparations were left to equilibrate for 40 min, and then injections of 120 µmol of KCl were administered every 10 min until consistent responses were obtained. The basal perfusion pressure after the equilibration period

oscillated between 20 and 25 mmHg. The hydroalcoholic extracts were injected *in bolus* after the PP had been elevated (80-110 mmHg) with norepinephrine (NE, 30  $\mu$ M) added to the perfusion fluid. When the pressor effect of NE reached a plateau, acetylcholine (ACh, 10 pmol) and nitroglycerin (NG, 10 nmol) were injected to test the endothelium-dependent and endothelium-independent vasodilator responses before dose-response curves to crude extracts were obtained. The vasodilator effect of drugs was expressed as a percentage decrease in relation to the pressor effect of NE. Crude extracts were administered *in bolus* in increasing doses 1, 3, 6, 10, 30, 60 and 90  $\mu$ g (n=6).

### Statistical Analysis

The vasodilator effect of drugs was expressed as the percentage decrease in the increase in PP induced by NE. PP variations among experimental groups are presented as average  $\pm$  standard error for the number of rats and were compared using Bonferroni unpaired test. A value of *P* less than 0.05 was used to indicate significance.

## RESULTS AND DISCUSSION

Chromatographic profiles of *A. zerumbet* and *A. purpurata* hydroalcoholic extracts indicated flavonoids which were suggested as contributing for antihypertensive vasodilator effects of *A. zerumbet* (Mpalantinos *et al.*, 1998; Victório *et al.*, 2009b). The content of rutin and kaempferol-3-*O*- $\beta$ -D-glucuronide in dried extracts of *A. zerumbet* and *A. purpurata* leaves is shown in Table I. Higher kaempferol-3-*O*- $\beta$ -D-glucuronide content was found in leaves of *A. zerumbet* (4.74 mg.100 mg<sup>-1</sup> dried extract) compared to *A. purpurata* extracts (0.20 mg.100 mg<sup>-1</sup> dried extract). The increasing in total phenolics was ob-

served in *A. zerumbet* cultured in control medium (66.8  $\mu$ g GAE.mg<sup>-1</sup>), while plantlets maintained under IAA 2 treatment produced lower than 50% of phenolics (26.2  $\mu$ g GAE.mg<sup>-1</sup>) (Table I). These data are in accordance with the high or low effect of *A. zerumbet* extracts from tissue cultures on MVB. The production of phenolic compounds through tissue cultures has been verified in studies with different medicinal plants (Konczak-Islam *et al.*, 2003; Victório *et al.*, 2008). Phenolic compounds are antioxidants responsible for free radical scavenging, capable of minimizing the damaging effects of reactive oxygen species, and considered potential compounds to preventing cardiovascular diseases (Khan, Mukhtar, 2007).

### Vasodilator effect of *Alpinia purpurata* extracts

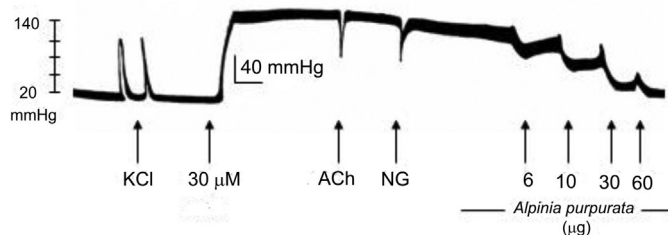
Isolate rat MVB was perfused with PSS solution, at 4 mL/min, and presented basal average perfusion pressure around 25 to 30 mmHg. Functionally, the *in bolus* injection of 120  $\mu$ mol of KCl increased the perfusion pressure up to 80 mmHg and demonstrated the viability of MVB preparation. Addition of 30  $\mu$ M of NE in the perfusion solution induced firstly an increase of perfusion pressure, and then maintained it constant, in the level of 84 to 120 mmHg; which has enabled the study of vasodilator effect of hydroalcoholic extract of *A. purpurata*. The *in bolus* administration of 10 pmol of ACh and 10 nmol of NG decreased the perfusion pressure, confirming the integrity of endothelium and vascular smooth muscle, respectively. The administration of increasing doses of *A. purpurata* extract (3, 6, 10, 30, and 60  $\mu$ g) produced a dose-dependent vasodilator effect of long-lasting duration, similar to patterns of response of *A. zerumbet* extracts (Soares de Moura *et al.*, 2005) (Figure 1). The response risen as the dose increased from 6  $\mu$ g upward (Figure 2). Vasodilator

**TABLE I** - Flavonoids content in hydroalcoholic extracts of leaves of field-grown *A. purpurata* and *Alpinia zerumbet* cultured *in vitro*

<i>Plant species</i>	Origin	Total phenolics* ( $\mu$ g GAE. mg <sup>-1</sup> )	RT (min)	Rutin	Kaempferol-3- <i>O</i> - $\beta$ -D-glucuronide (mg.100 mg <sup>-1</sup> dried extract)
<i>A. zerumbet</i>	Field	58.1 $\pm$ 3.6	31.23 34.77	0.48 -	- 4.74
	In vitro				
	MS0	66.8 $\pm$ 3.4		0.0003	0.028
	IAA 2	26.2 $\pm$ 2.0		0.024	0.018
	IAA 2 + TDZ 2	43.7 $\pm$ 1.4		0.134	0.032
<i>A. purpurata</i>	Field	30.1 $\pm$ 1.8	32.31 35.52	0.58	0.20

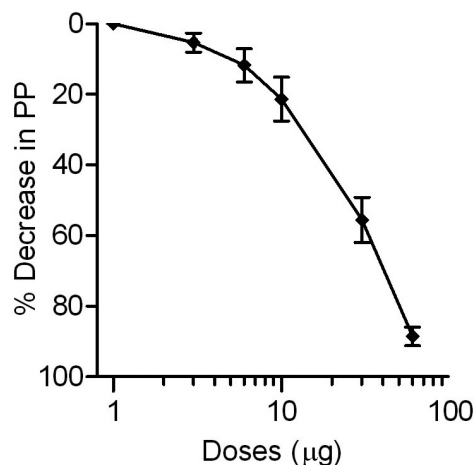
RT – retention time. \*Average  $\pm$  SE.

effect of hydroalcoholic extract of *A. zerumbet* was more effective, than that of *A. purpurata* extract verified in the present study; being reversed more than 50% of constrictive effect of NE, in the dose of 6  $\mu\text{g}$  (Emiliano, 2002). However, *A. purpurata* extracts have shown a rising and continuous relaxation (Figure 2), with the same intensity of results obtained for *A. zerumbet* in the dose of 60  $\mu\text{g}$  (Emiliano, 2002). The effects of *A. purpurata* extracts on MVB reached 87% potency at 60  $\mu\text{g}$  (Figure 2). The similarities respecting to response and vasodilator effect of these species could be related to presence of common secondary metabolites. The *A. purpurata* species, as well as the *A. zerumbet*, are natural sources of flavonoids, such as rutin, derived from quercetin, and the kaempferol-3-*O*- $\beta$ -D-glucuronide. Both are referred for their typical vasodilator and anti-hypertensive actions (Formica, Regelson, 1995; Perez-Vizcaino *et al.*, 2002; Xu *et al.*, 2006). Recent researches conducted by Ferreira *et al.* (2007), showed that rutin is one of the main flavonoids in ethanolic extracts of *Hancornia speciosa* and that this extract has contributed for endothelium-dependent vasodilator activity in the mesenteric artery of rats. The endothelium-independent vasodilator action was also verified in studies with the flavonoid quercetin (Perez-Vizcaino *et al.*, 2002).



**FIGURE 1** - Representative perfusion pressure (mmHg) trace showing the vasodilator effect of *Alpinia purpurata* hydroalcoholic extracts on MVB pre-contracted with NE (30  $\mu\text{M}$ ) added to the perfusion fluid. Acetylcholine (ACh, 10 pmol) and Nitroglycerin (NG, 10 nmol) were also injected to test the endothelium-dependent and independent responses before dose-responses curves to extracts were obtained.

In studies developed by Emiliano (2002), it was suggested that the vasodilator effect of hydroalcoholic extract of *A. zerumbet* is endothelium-dependent, being it related to NO (nitrogen oxide) production, a vasodilator agent that acts increasing the activity of the enzyme soluble guanylate cyclase, which by its time, induces the production of cyclic guanosine monophosphate – the cGMP (Nagao, Vanhoutte, 1991). According to these results, the popular use of *A. zerumbet* in cases of arterial hypertension show positive responses, due to



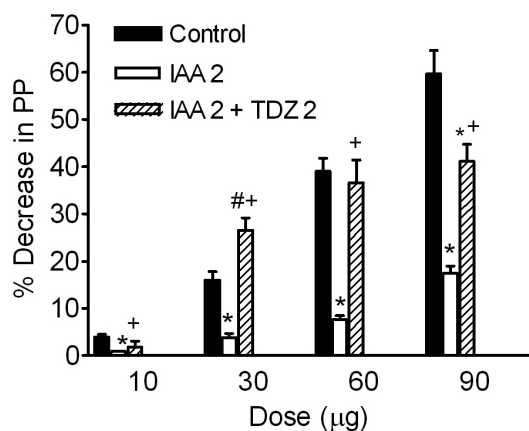
**FIGURE 2** - Vasodilator effect of *Alpinia purpurata* hydroalcoholic extracts on MVB pre-contracted with NE. Each point represents the average  $\pm$  SE,  $n \geq 6$ .

action of the extract for decreasing the peripheral resistance (Emiliano, 2002). Such suggestion could be also conferred for the extract of *A. purpurata*, however, the results obtained are preliminary and other plant material collected from different geographic regions, and biological assays to confirm the species vasodilator effect, would be necessary. Mpalantinos (2001), in studies with aqueous extracts of *A. zerumbet* from different Brazilian regions, has obtained different results in relation to vasodilator activity. Inclusive, for some of these extracts of *A. zerumbet*, the vasodilator activity was lower than that for *A. purpurata* in current study (Figure 2). The environmental conditions exert some influence over the production of secondary metabolites, and this fact, in its turn, alters the dose-effect curves.

### Vasodilator effect of *Alpinia zerumbet* cultured *in vitro*

*A. zerumbet* extracts obtained from *in vitro* cultures have shown different responses on MVB, but always with dose-dependent vasodilator standard of long-lasting duration (Figure 3). The extracts of *A. zerumbet* cultured in media containing plant growth regulators decreased the vasodilator effects compared to plantlets cultured in MSO, except for treatment IAA 2 + TDZ 2 in the doses of 30 and 60  $\mu\text{g}$ . These findings may indicate the inhibitory effect of IAA 2  $\text{mg}\cdot\text{L}^{-1}$  on the production of secondary metabolites, as the isolated administration of IAA 2 resulted in inhibition higher than that of the combination IAA 2 + TDZ 2. Extracts from plantlets maintained in control medium were more effective when inducing the vasodilator response with respect to other *in vitro* treatments, probably,

due to higher concentration of phenolic compounds. The maximum response obtained for the extracts of plantlets from *in vitro* cultures was of 60% de relaxation in the dose of 90 µg, against 95% of field plants at 60 µg. Although tissues culture techniques constitute an important tool for the large scale production of plant raw material and in the optimized production of bioactive compounds as well, the results obtained for the *in vitro* production of secondary metabolites were not improved, even under the effect of growth regulators. However, important results could be suggested, with the inhibition of secondary metabolic pathways by the auxin IAA, in accordance with the decrease of total phenolics in extracts arising from this treatment.



**FIGURE 3** - Vasodilator effect of *Alpinia zerumbet* hydroalcoholic extracts obtained from *in vitro* cultures treated with different plant growth regulators on MVB pre-contracted with NE. \*Value lower than control (MS0), #Value higher than control, +Higher response of IAA 2 + TDZ 2 treatment compared to IAA 2. Each point represents the average  $\pm$  SE,  $n \geq 6$ . Bonferroni,  $P < 0.05$ .

## CONCLUSION

Although the hydroalcoholic extracts of *A. purpurata* have shown vasodilator action, the required efficiency and potency combination to enable the production of a drug was not obtained. The studies are preliminary. However, phenolic compounds and flavonoids were detected for *A. purpurata* species, which has not excluded it as a possible natural source for obtaining bioactive compounds useful against hypertension.

No treatment with plant growth regulators has induced higher production of flavonoids in *A. zerumbet* plantlets, which probably are the responsible agents for the vasodilator effect. But the higher concentration of phenolic compounds verified in leaves of plantlets cultured in MS0 medium has resulted in a better vasodilator effect.

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