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Research Article

Antiangiogenic, antitoxic and antioxidant properties of methanolic extracts of *Caladium bicolor* (Aiton) Venten

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Abstract. Plants as source of cure for many ailments have been the focus of many natural products and drug development research. Their potentials for cancer cure have been exploited for many years and were focused on their antiangiogenic, antioxidant and cytotoxic properties. One of the potential source was *Caladium bicolor* initially found to have many traditional uses. Antiangiogenic, antioxidant and cytotoxic properties of the methanolic extracts of the leaf of the plant was tested using slight modifications of the chorioallantoic membrane (CAM) assay, free radical scavenging and lethal exposures to cultured lymphocytes. Results revealed the antiangiogenic, antioxidant and cytotoxic potential of the methanolic extracts but these were concentration dependent. There is however a need to isolate and characterize the chemicals in the extract and tested individually to be able to identify which specific compound can be of importance in the control of cancer.

Key Words: antiangiogenic, antioxidant, cytotoxic, CAM.

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Introduction

Nature has been a source of medical agents across the globe for thousands of years. This plant-based, traditional medicine system manifest an essential role in human health care with about 80% of the world's inhabitants relying on them (Kensa 2011). Herbal plants possess natural antioxidants, especially phenolics and flavonoids that protect human body from free radicals and retard the progress of cancer (El-Hashash et al 2010). At present, there has been a growing interest in the study of traditional plants for pharmaceutical applications because of its various plant phytochemicals viz phenolic compounds, flavonoids and tannins which were reported to possess significant antioxidant activity against a wide variety of free radicals (Kalita et al 2012). One of the plants argued to have natural properties is *Caladium bicolor* (Ait.) Venten, known as "Corazon de Maria" or "Gabi-gabi" in the Philippines is a tuber-rooted tropical perennial grown for their large and showy leaves (Christman 2003). The most distinct characteristic of the plant is the shape, size and color of the leaf although vary among the hundreds of selections, is like a heart, lance or arrowhead shaped, spotted or streaked with pink, red, gray, or white colors (Fig. 1) . It has injurious properties due to the presence of water-insoluble calcium oxalate raphides and unverified proteinaceous toxin constituents causing painful burning sensation of the lips and mouth upon contact. Inflammatory reaction often with edema and blistering, hoarseness, dysphonia, and dysphagia may also result (Nelson et al 2007). Excessive dosing can cause nausea, vomiting, diarrhea, swelling and redness of the eyes, and swelling of the mouth and tongue. However when cooked, both leaves and bulbs can be eaten as vegetables in tropical America and the West Indies (Stuart and Santiago 2013). Traditionally, there were reports of the plant's folkloric medicinal use in Brazil where heated bulbs are covered with olive oil and applied to tumors. Leaves are used as vermifuge and purgative and are externally used for furunculosis. In Cameroon, decoction of some tubers and leaves are used for vaginal inflammation. They are also used as an antiseptic, anti-tumor, emetic, laxative and can also be used for treating sore throats, constipation, catarrh, wounds, sores, and toothaches. Crushed bulbs are applied to the face, for facial paralysis. In India, decoction of leaves are also used for external cattle festers caused by worms. There were however, no reported folkloric medicinal uses of *C. bicolor* in the Philippines although powdered leaves are used as insecticide (Stuart and Santiago 2013) and as antidote for snake bites (Padal et al 2013). Since the occurrences of lung, breast, colorectum and prostate cancer have been steadily increasing from 1998 to 2002 in the Philippines (Laudico et al 2010) there is therefore a need for more alternatives to anti-cancer drugs and therapies. Exploration of potential natural sources of cancer remedies in plants such as in *C. bicolor* was therefore done.

Studies have shown that *C. bicolor* and those cultivated varieties C. ornamental and C. variegatum have phytochemicals saponins, flavonoids, limonoids, polyphenols, alkaloids, carotenoids, lactones, xanthophs, oxalates, cyanide, and terpenes (Ekanem et al 2013; Stuart and Santiago, 2013). The presences of these biochemical components suggest potential for use in

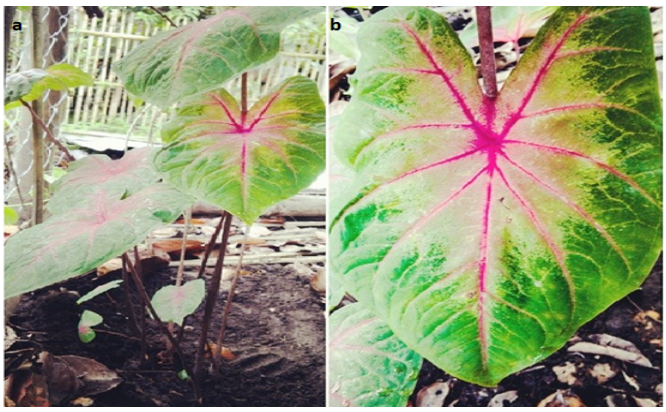

Fig. 1. *Caladium bicolor* (a) whole plant (b) broad heart-shape leaf

cancer cure. In a study conducted on the membrane stabilizing and antimicrobial properties of *C. bicolor* show that the crude methanol extracts well as their pet-ether, carbon tetrachloride, chloroform and aqueous soluble fractions significantly protected the haemolysis of RBC induced by hypotonic solution and heat. It has moderate antimicrobial properties as shown by the degree of inhibition by the carbon tetrachloride and the chloroform soluble fractions which contained the presence of saponins, flavonoids, limonoids, polyphenols, alkaloids, carotenoids, lactones, xanthophs, oxalate, cyanide and terpenes which are argued to be having antitumorogenetic properties (Biswas et al 2013; El-Hashash et al 2010).

In this study we investigated the potential antiangiogenic, antioxidant and cytotoxic properties of the methanolic extracts of *C. bicolor*. Antiangiogenic property evaluation of the extracts may reveal the tumor prevention potential of the plant due to its inhibitory properties of blood vessel formation (Olarte 2007). It is also likely of the potential antioxidant properties of the extracts by interrupting the actions of the harmful free radicals thus whether these will form stable radicals or not will be done in this study (Patel and Jasrai 2013). Finally, since some of the traditional medicine involves the use of crude plant extracts which may have biological effects, determining the toxicity of medicinal plants is important. In vitro toxicity assays are essential for determining the responses of human normal and cancer-derived cells to therapeutic agents and also for the identification and pre-clinical evaluation of new drugs capable of selectively augmenting the susceptibility of cancer cells to conventional therapies (Mirzayans et al 2007) thus was also done in the current study.

Material and methods

Fresh, seven hundred grams (700 g) of leaves of *Caladium bicolor* (Ait.) Vent. (Gabi-gabi) were collected in house backyards at Polomolok, South Cotabato. The plant were botanically authenticated by an authorized plant taxonomist at the National Museum of the Philippines, Manila City. The leaves were washed with distilled water and air dried in shade for about one to two weeks. Then air-dried leaves were cut into smaller pieces and kept (at 20°C) in closed plastic containers. The bulk extraction protocol including solvent partitioning was adapted from the study of Olarte (2007). Fresh leaves of *C. bicolor* were removed from the stems, washed with running water, and air-dried. They were cut into small pieces and homogenized using a blender. The ground sample were soaked in reagent methanol, filtered, concentrated in vacuum using a rotary evaporator (Heidolph) done at a maximum temperature of 68°C. The concentrated methanol extract were the ones tested in the study (Fig 2). The methanol extract was then dissolved in four different concentrations, 25%, 50%, 75%, and 100%.

Antiangiogenic Investigation

The protocol on Antiangiogenic Investigation performed in the study of Olarte (2007) using chorioallantoic membrane (CAM) assay were followed with slight modifications (Fig. 3). The different concentrations of the extract were used for the duck embryo assay. The modified windowing technique was followed. We used thirty pieces of three-day old fertilized duck eggs which were purchased from a local duck breeder at Brgy. Baluan, General Santos City and brought to the Medical Technology/ Pharmacology Laboratory of Notre Dame of Dadiangas University, General Santos City for the biological assay. Each egg was examined ensuring it is fertile and normal.

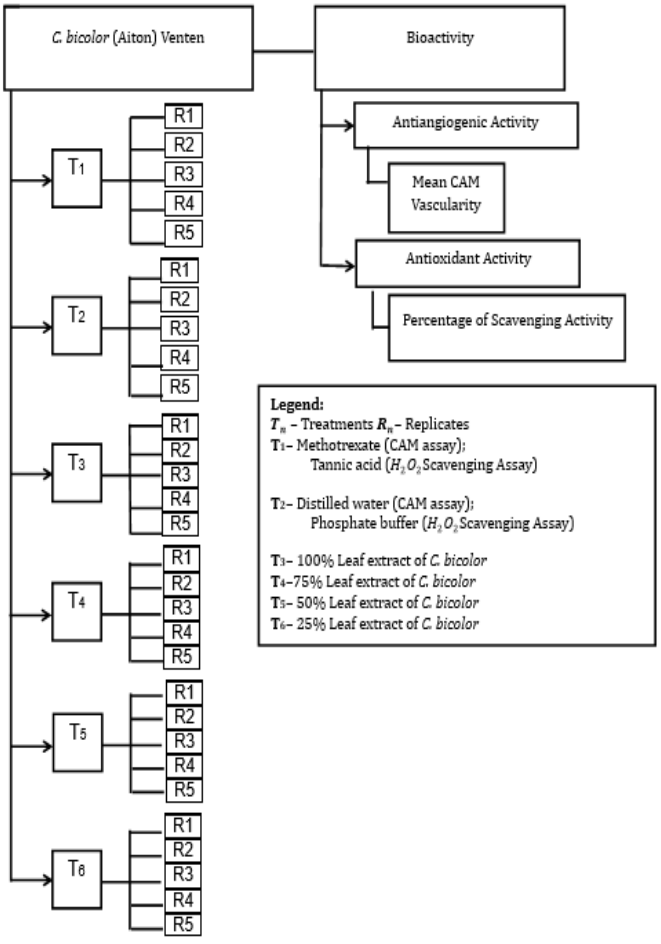

Figure 3. Evaluation of Antiangiogenic and Antioxidant properties of *C. bicolor*.

For the experimental set-up, the eggs containing the three-day old duck embryos were first cleaned by wiping them with paper towels and incubated using an egg incubator maintained at 37°C temperature and constant humidity (Fig. 4a). The test specimens were divided into six groups; two groups for the positive and negative controls consisting of Methotrexate and distilled water. The other groups were those that were treated with different concentrations of the methanol extracts. These are composed

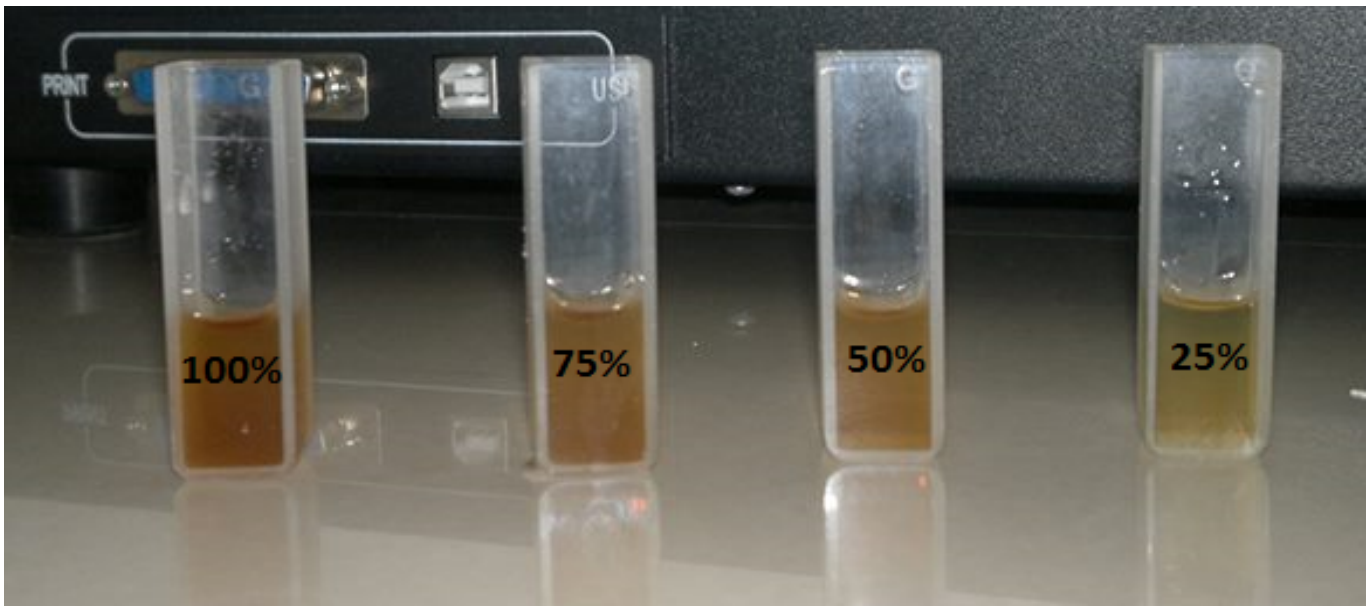

Fig. 2. Extracts from *C. bicolor*

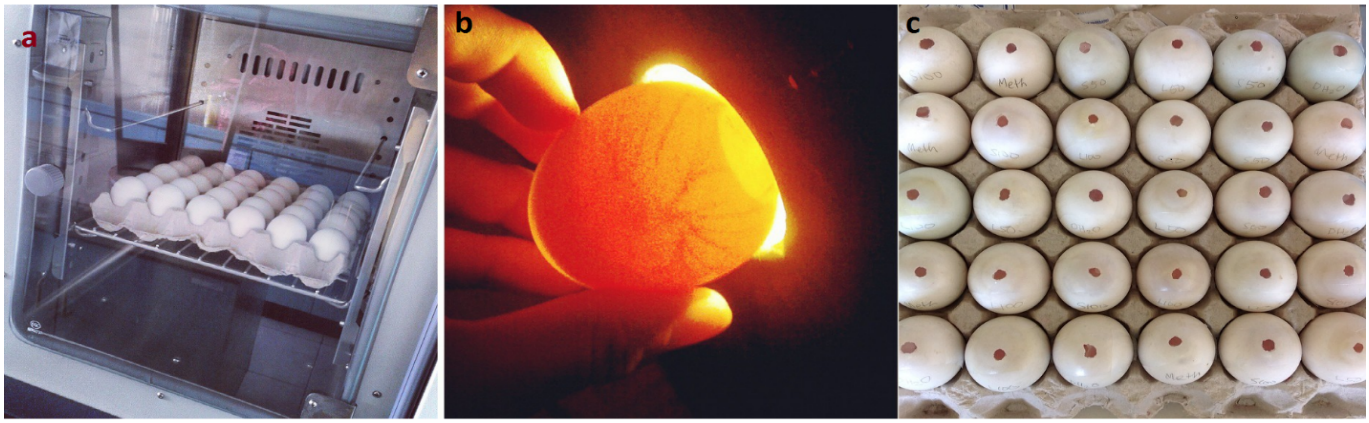

Fig. 4. (a) Duck eggs inside the incubator; (b) fertile eggs for testing and (c) scratched eggs for the introduction of the test extracts

of 5 quality and fit eggs (Fig. 4b). These were acclimatized for one day in the incubator before examined by slightly scratching and puncturing using a sterile probe and a sterile needle (Fig. 4c). The different concentrations of the methanolic extracts were injected directly to the inserted filter disc through the holes or windows that were made on the eggs. The holes were immediately covered with sterile adhesive tapes, This is to protect the treated eggs from being contaminated.

The eggs were only allowed to stay in the incubator for seven days after which they were carefully opened for gross morphological examination of the extra embryonic blood vessels (Fig. 5), the examination of the blood vessels in all treated eggs including the controls were done using a stereomicroscope, photographed and analysed.

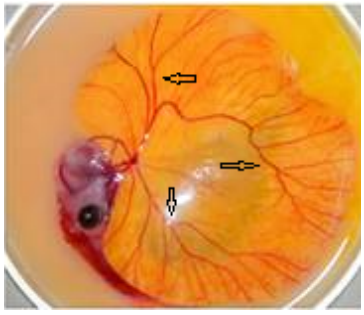

Fig. 5. Extraembryonic blood vessels observed in treated duck eggs

The Antiangiogenic Activity of the extracts were assessed by comparing the degree of proliferation of the extraembryonic blood vessels of the treatment groups compared with those of the controls (Methotrexate and distilled water). The embryos were then photographed for documentation.

Antioxidant Investigation

The ability of the extracts to scavenge hydrogen peroxide were determined (Nabavi et al 2008a, 2009b; Ebrahimzadeh et al 2010) with some modifications.

Hydrogen peroxide (2mM/L) solutions were prepared in phosphate buffer with pH 7.4 the concentration of which were determined at 230 nm using a spectrophotometer. Methanolic extracts (100%, 75%, 50%, and 25%) in distilled water were added to the hydrogen peroxide solution. The concentration used was 0.6 ml from the 2 mM/L solution of the hydroxide. The absorbance of hydrogen peroxide of the different treatments were determined after ten minutes. These were compared to the absorbance of a blank solution containing a phosphate buffer but without adding hydrogen peroxide. The experiment were repeated in five (5) replicates. The scavenging percentage activities were compared against the control (tannic acid) and were calculated using the formula.

$$\text{Scavenging Percentage}(\%S)=(1-(A_0-A_1)/A_0) \times 100\%$$

Where A_0 were the absorbance of the control and A_1 were the absorbance of the sample.

Cytotoxicity Bioassay

The protocol of evaluating the cytotoxic effects of the *C. bicolor* extract was based on the lethal responses of the lymphocytes which was initially cultured following the procedures of Verma and Babu (1995). The various steps in the protocol is summarized in Fig. 6. The leaf extracts (100%, 75%, 50%, and 25%) were filtered through 0.22 µm syringe filter discs and the filtrates were collected in fresh tubes. The following were dispensed into 3 microcentrifuge tubes each: 50 µl supplemented RPMI, 50 µl dH2O, 50 µl phenol (6.4 mg/ml), 50 µl of *C. bicolor* 100% leaf extract filtrate, 50 µl of *C. bicolor* 75% leaf extract filtrate, 50 µl of *C. bicolor* 50% leaf extract filtrate, 50 µl of *C. bicolor* 25% leaf extract filtrate. Four-hundred fifty 450-µl aliquots of lymphocyte culture were added to the microcentrifuge tubes containing the different treatment solutions and the cultures were mixed and incubated at 37^{0}C for 24 hrs. After 24 hrs of incubation, treated cultures were obtained for cell counting. Seven microliters of incubated culture were added to 7 µl of trypan blue, and 7 µl of the mixed solution were placed in a hemocytometer. The number of live lymphocytes and the number of dead lymphocytes were counted in all 25 squares within the 1 mm center grid. Cell density (number of cells per ml) is computed using the following formula:

$$\#\ cells/ml = \frac{\#\ of\ cells\ total}{\#\ of\ 1\ mm^2\ squares} \times 10^4 \times original\ dilution^{\S}$$

§ dilution factor = 2

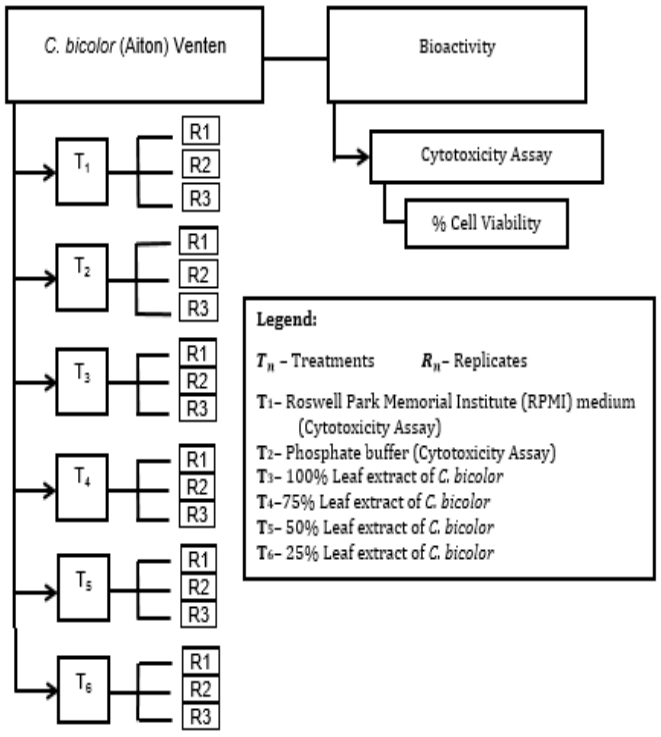

Figure 6. Cytotoxicity testing experimental design

Results and Discussion

Angiogenesis

Results of morphological examination of the duck embryos after treatments are shown in Fig. 7. Counts of blood vessels (Fig. 7) formed per treatment subjected to analysis of variance (Table 1) show there were significant differences between treatments. Comparison of the anti-angiogenetic effects of the different concentrations of the methanolic extract of *C. bicolor* show the optimum concentration of the extracts to effect anti-angionetic effects is at 50% (Table 2).

Table 1. ANOVA of the angiogenetic effects of *C. bicolor* methanolic leaf extract

	Sum of sqrs	df	Mean square	F	p
Between groups	174125	5	34824.9	13.07	<0.001
Within groups:	63942.2	24	2664.26		
Total:	238067	29			

Table 2. Tukey's test on the differences of angiogenetic effects of the different concentrations of methanolic extracts of *C. bicolor*

	25% Extract	50% Extract	75% Extract	100% Extract	Methotrexate
Distilled Water	0.862	0.015	0.0002	0.0002	0.0001
25% Extract		0.165	0.003	0.002	0.001
50% Extract			0.449	0.316	0.227
75% Extract				0.100	0.997
100% Extract					0.100

Table 3. ANOVA results of the antioxidant effects of the *C. bicolor* extracts

	Sum of sqrs	df	Mean square	F	p
Between groups	25008.5	5	5001.7	8.893	<0.001
Within groups	13497.8	24	562.408		
Total	38506.3	29			

Table 4. Tukey's test comparing the antioxidant effects of *C. bicolor* leaf extracts

	Phosphate buffer	25% Leaf Extract	50% Leaf extract	75% Leaf extract	100% Leaf extract
Tannic acid	0.000	0.597	0.718	0.805	0.968
Phosphate buffer		0.012	0.008	0.005	0.000
25% Leaf extract			1	0.999	0.197
50% Leaf extract				1	0.275
75% Leaf extract					0.351

Antioxidant properties

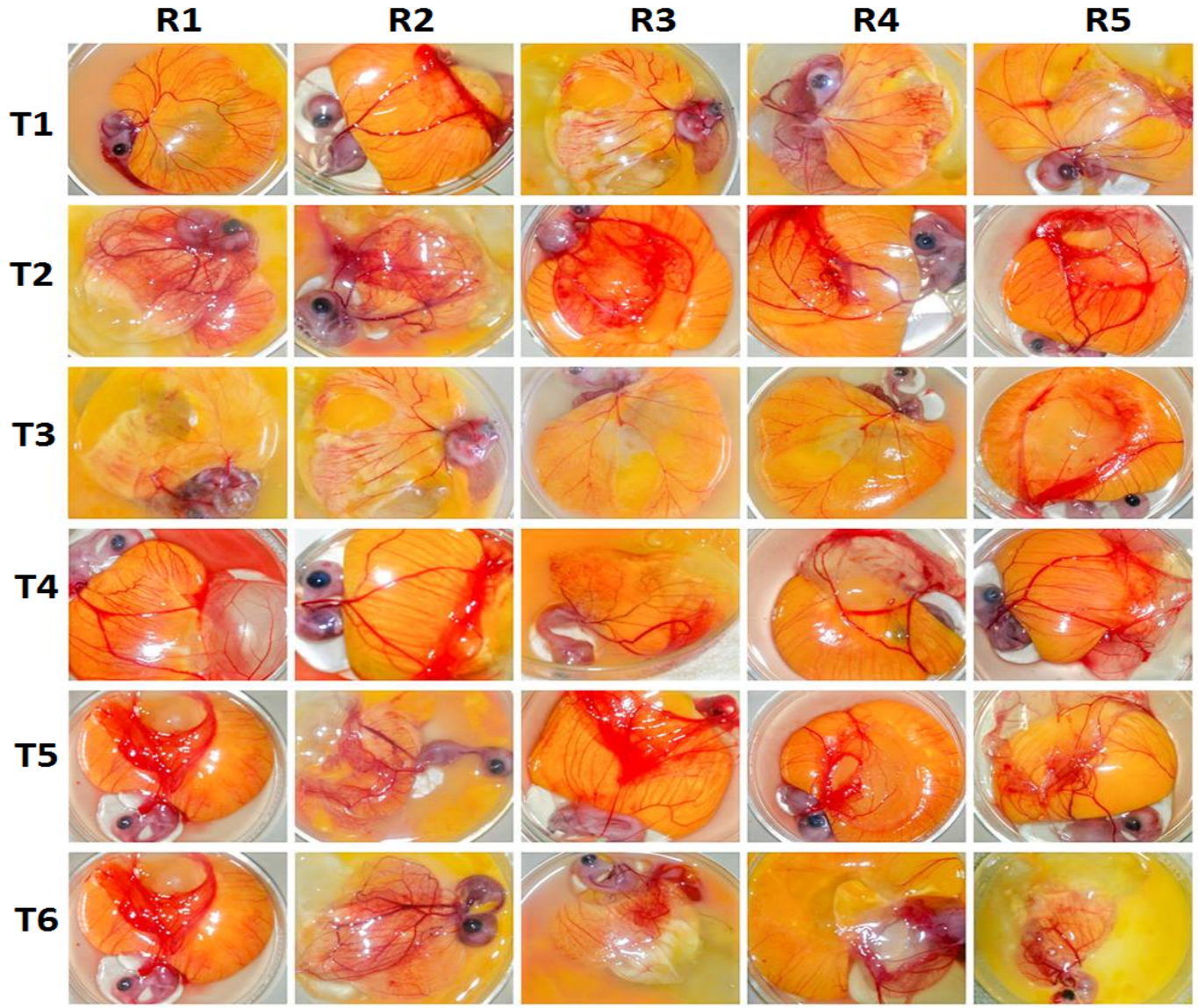

Fig. 7. Angiogenesis response of control and extract group on embryo egg CAM after 3 days incubation

Results of the test of the methanolic extracts show all the concentrations are positive for antioxidant properties (Tables 3, 4). While qualitative evaluation show three concentrations (25-75%) have lower antioxidant activities when compared to 100% which is even higher to the antioxidant properties of tannic acid (control), the differences between concentrations however were not significant.

Cytotoxicity tests

The methanolic extracts of *C. bicolor* are cytotoxic but only on the 75 and 100% concentration (Tables 5 and 6).

Table 5. ANOVA results of the cytotoxicity effects of the *C. bicolor* extracts on human lymphocytes.

	Sum of sqrs	df	Mean square	F	p
Between groups	15455.3	6	2575.88	103.5	<0.001
Within groups	348.527	14	24.895		
Total:	15803.8	20			

Table 6. Tukey's pairwise comparisons of the toxicity of the extracts on human lymphocytes

	Distilled water	Phenol	25% Leaf extract	50% Leaf extract	75% Leaf extract	100% Leaf extract
Supplement RPMI	1	0.0001	0.083	0.146	0.0001	0.0001
Distilled Water		0.0001	0.116	0.200	0.0001	0.00001
Phenol			0.0001	0.0001	0.0002	0.0005
25% Leaf extract				0.100	0.0001	0.0001
50% Leaf extract					0.0001	0.0001
75% Leaf extract						0.944

The results of this study clearly show the potential of *C. bicolor* as a source of compounds that are antiangiogenic, antioxidant

and cytotoxic. Since angiogenesis is a property of most solid tumors necessary for their continued growth, metastasis and cancer progression (Gimbrone 1972; Chaplain 1996; Tufto et al 1998; Rak & Yu 2004; Hoff & Machado 2012), the antiagiogenic properties shown by the methanolic extracts indicate the presence of compounds are that may have suppressed the production of VEGF, a known stimulator of endothelial cell migration (Keshavarz et al 2011). This means that the properties shown are considered to be advantageous for the prevention of tumor growth and metastasis. The antioxidant activity of the methanolic extract of *C. bicolor* was also observed to be increasing with higher concentrations indicating that the chemicals in the extract may also have the potential to interact and neutralize free radicals from causing damage (Diplock et al 1998; Bouayed and Bohn 2011). Likewise, the effects on cellular viability based on the exposure of lymphoblastoid cells at different concentrations of the extract for a period of 24-hour exposure time show only at higher concentrations (75 and 100%) were significant effects can be observed. This means that the compounds present in the extract can have putative cytotoxic effects only at higher concentrations. While all the test results indicate there are potential chemicals isolated by methanol extraction that have antiangiogenic, cytotoxic, and antioxidant properties, there is however a need for the isolation, characterization and further testing of these chemicals.

Conclusion

This study have shown the potential of *Caladium bicolor* as a source of compounds that contains antiangiogenic, antioxidant and cytotoxic properties but these were concentration dependent. There is therefore a need to isolate and characterize the chemicals in the extract and be tested individually to be able to identify which specific compound can be of importance in the control of cancer.

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