INVESTIGATION OF ANTIOXIDANT, CYTOTOXIC AND ANTITUMOR ACTIVITIES OF *Morinda citrifolia* L. FRUIT (YÈ-YO) AND *Catharanthus roseus* L. WHOLE PLANT (THIN-BAW-MA-NYOE)

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Abstract

The present work focused on the investigation of some biological activities such as antioxidant, cytotoxic and antitumor activities on some crude extracts from locally cultivated *Morinda citrifolia* L. fruits (Yè-yo) and *Catharanthus roseus* L. whole plant (Thin-baw-ma-nyoe). The antioxidant activity of ethanol and watery extracts evaluated by DPPH free radical scavenging assay showed that IC_{50} values of EtOH extract (4.395 μg/mL) and watery extract (8.878 μg/mL) from *C. roseus* were repsectively higher than those of EtOH extract (9.347 μg/mL) and watery extract (8.484 μg/mL) from *M. citrifolia*. The ethanol and watery extracts of both plant samples did not exhibit the cytotoxic effect determined by using brine shrimp cytotoxicity bioassay method. Antitumor activity screening determined by potato crown gall test revealed that ethanol and watery extracts of *M. citrifolia* and *C. roseus* can inhibit tumor growth. Tumor inhibition was significantly observed in the concentration of 0.2 g /mL for each extract.

Keywords: *M. citrifolia*, *C. roseus*, antioxidant activity, cytotoxic effect, antitumor activity

Introduction

Natural plant products have served as the basis of man's medicinal arsenal since time immemorial. The history of herbal medicine in the treatment of many diseases and 3,000 plant species that have been used or recommended in various parts of the world for the treatment of cancer. It has been only within the last 20 years that any product from a higher order plant has been successfully used in cancer chemotherapy. *Morinda citrifolia* L. and

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the *Catharanthus roseus* L. have been used as medicinal plants since long ago. *M. citrifolia*, a shrub originating in tropical Asia, has been extensively used in folk medicine and as a dye in Asian countries (Dittmar, 1993). In Myanmar, the fruits are cooked in curries or eaten raw with salt. The fruit juice of *M. citrifolia* is in high demand in alternative medicine for various illnesses, such as arthritis, diabetes, high blood pressure, muscle aches and pains, menstrual difficulties, headaches, heart disease, Acquired Immune Deficiency Syndrome (AIDS) and cancer (Kamiya *et al*., 2004). *C. roseus* is a medicinal herb found in many tropical and subtropical regions around the world (Farah *et al*., 2011). This plant produces a diverse array of secondary metabolites that are pharmaceutically important like vinblastine and vincristine used as chemotherapeutic agents in the treatment of several types of cancers. The plants are also used in treatment of diabetes, fever, malaria and throat infection. The leaf-juices are used in blood dysentery and leaf decoctions are also to babies in gripping pain. The roots of *C. roseus* are used for the treatment in cancerous wounds and septic wounds. The plant bears active phytoconstituents and exhibits various pharmacological activities like anti-diabetic, anti-oxidant, anti-hypertensive, anti-microbial and cytotoxic etc (Mohd *et al*., 2010).

There were some reports on the investigation of some organic constituents and cholestrol lowering effect of locally cultivated *M. citrifolia* (Ye-yo) (Khin Thida Nyo, 2006) and antidiabetic potency of locally grown *C. roseus* (Thin-baw-ma-nyoe) (Myint Myint Khin, 2007). In the present work, some biological activities: antioxidant, cytotoxic and antitumor activities of the fruits of *M. citrifolia* (Ye-yo) and the whole plant of *C. roseus* (Thin-baw-ma-nyoe) could be evaluated.

Materials and Methods

Collection of Plant Materials

The sample of *M. citrifolia* fruits were collected from Kyaung-su Village, Ke-gyi Township, Ayeyarwady Region and whole plants of *C. roseus* were collected from Mingalar-taung-nyot Township, Yangon. The plant samples were identified in Department of Botany, University of Yangon, Myanmar.

The collected samples were cleaned by washing thoroughly with water were then and air-dried at room temperature. The dried samples were cut into small pieces and ground into powder by a grinding machine. These powder samples obtained were separately stored in air-tight container.

Determination of Antioxidant, Cytotoxicity and Antitumor Activities

Preparation of crude extracts from the samples

For antibacterial activity screening, some crude extracts such as EtOAc, 95 % EtOH and H_2O extracts were firstly prepared from *M. citrifolia* and *C. roseus* samples. Dried powdered samples was refluxed with 95 % EtOH and then filtered. This solution was recovered by distillation and evaporation to dryness to give 95 % EtOH crude extracts. The dried powdered samples were extracted with distilled water within 15 min, by using hot extraction. Water solution was concentrated by evaporation to dryness to give water extract. All extracts are stored in desiccators.

Determination of antioxidant activity

DPPH radical scavenging activity was determined by UV-spectrophotometric method (Ashokkumar and Ramaswamy, 2013). The control solution was prepared by mixing 1.5 mL of 60 μM DPPH solution and 1.5 mL of 95 % ethanol with vortex mixer. The sample solution was also prepared by mixing thoroughly 1.5 mL of 60 μM DPPH solutions and 1.5 mL of test sample solution. The solutions were allowed to stand at room temperature for 30 min. After 30 min, measurement of absorbance at 517 nm were made by using spectrophotometer UV 1601 PC (P\N 206 – 6750), Shimadzu corporation. Absorbance measurements were done in triplicate for each solution and the mean value was obtained, and then used to calculate % inhibition of oxidation by the following equation,

$$\% \text{ oxidative inhibition} = \frac{A_c - (A - A_b)}{A_c} \times 100\ \%$$

% oxidative inhibition = % oxidative inhibition of test sample

A_c = absorbance of the control (DPPH alone)

A_b = absorbance of the blank (EtOH + Test sample solution)

A = absorbance of test sample solution

Then IC_{50} (50 % inhibitory concentration) values were also calculated by linear regressive excel program.

Determination of cytotoxicity

Cytotoxicity of 95 % EtOH and H_2O extracts were determined by using brine shrimp bioassay. Firstly, artificial sea water was prepared by dissolving, sodium chloride (38 g) in 1000 mL of distilled water. Brine shrimp cysts (0.5 g) were added to 1 L of artificial sea water. The bottle was placed near a lamp and supplied O_2 for 24 h. After 24 h incubation, hatching of brine shrimp cysts occurred and the alive brine shrimp (napulii) were ready for cytotoxicity test. Test solution (1 mL) was mixed with 9 mL of artificial sea water and placed in the chamber of ice cup. Alive brine shrimp (10 napulli) were taken with pasteur pipette and placed into each chamber which was kept at room temperature for about 24 h. After 24 h incubation, the number of survival brine shrimp was counted and 50 % lethality dose (LD_{50}) was calculated (Ali *et. al*, 2013; Dockery and Tomkins, 2000).

Screening of antitumor activity by potato discs assay method (potato crown gall test)

Antitumor Activity of 95 % EtOH and H_2O extracts of *M. citrifolia* fruits and the whole plant of *C. roseus* were studied by Potato Discs Assay Method. Tumor producing bacteria, *Agrobacterium tumefacien*, isolated from *Sandoricum koetjape* Merr. (Thitto) leaves was used in this study. The bacterial strain had been maintained as solid slants under refrigeration. For inoculation of the potato discs, 48 h broth cultures containing 5×10^7-5×10^9 cell/mL were used. Fresh, disease free potato tubers were obtained from local markets and were used within 48 h of transfer to the laboratory.

Tubers of moderate sizes were surface-sterilized by immersion in 50 % sodium hypochlorite (Clorox) for 20 min. The ends were removed and soaked

for 10 minutes more in Clorox. A core of the tissue was extracted from each tuber by using surface-sterilized (ethanol and flame) 2.5 cm wide cork borer and 2 cm pieces were removed from each end and discarded and the remainder of the cylinder is cut into 1.0 cm thick discs with a surface-sterilized cutter. The discs were then transferred to 1.5 % agar plates (1.5 g of Difco agar was dissolved in 100 mL of distilled water, autoclaved and 20 mL poured into each petri dish). Each plate contained three discs. This procedure was done in the clean bench in the sterile room.

0.1 g and 0.2 g each of the sample was dissolved in EtOH solvent filtered through Millipore filters (0.22 μm) into a sterile tube. 0.5 mL of this solution was added to 1.5 mL of sterile distilled water and 2 mL of broth culture of *A. tumefaciens* strain (48 h culture containing 5×10^7-5×10^9cells/ mL) were added aseptically. Controls were made in this way; 0.5 mL of DMSO and 1.5 mL of sterile distilled water were added to the tube containing 2 mL of broth culture of *A. tumefaciens* (from the same 48 h culture). Using a sterile disposable pipette, 1 drop (0.05 mL) from these tubes was used to inoculate each potato disc, spreading it over the disc, surface. The process of cutting the potatoes and incubation must be conducted within 30 min. The plates were sealed with tape to minimize moisture loss and incubated at room temperature counted with microscope and compared with control. The antitumor activity was examined by observation of tumor produced or not (Collins, 2001).

Results and Discussion

Antioxidant Activity of *M. citrifolia* and *C. roseus*

Antioxidant compounds in plant play an important role as a health-protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Plant source antioxidants like vitamin C, vitamin E, carotenes, phenolic acids and phytoestrogens have been recognized as having the potential to reduce disease risk. The antioxidant activity of *M. citrifolia* and *C. roseus* were evaluated by DPPH (2, 2- diphenyl-1-picrylhydrazyl) radical scavenging assay. The radical scavenging effects were determined for EtOH and watery extracts of two selected plants. The extracts or their constituents when mixed

with DPPH decolorized due to hydrogen donating ability. The antioxdiant activities of crude extracts were expressed in terms of percent radical scavenging activities (%RSA) and IC_{50} (50 % inhibitory concentration). The resultant data from Table 1 and Figure 1, showed that when the concentrations of samples increased the % RSA also increased. It can be suggested that one requires to scavenge effectively radicals the more concentrated crude extracts will be used. These results are shown in Figure 2. Since the lower the IC_{50} values, the higher the antioxidant activity of the samples have, the *C. roseus* EtOH extract (IC_{50} = 4.395 μg/mL) possessed the highest radical scavenging property among the extracts. The IC_{50} values were found to be *C. roseus* (EtOH) (4.395 μg/mL) < *M. citrifolia* (Watery) (8.484 μg/mL) < *C. roseus* (Watery) (8.878 μg/mL) < *M. citrifolia* (EtOH) (9.347 μg/mL), indicating the order of antioxidant activity of the extracts would be in the order of *C. roseus* (EtOH) > *M. citrifolia* (Watery) > *C. roseus* (Watery) > *M. citrifolia* (EtOH) (9.347 μg/mL). However, their antioxidant potency was found to be sligthly weaker than the that of standard ascorbic acid (IC_{50} = 2.2 μg/mL).

Table 1. % Radical Scavenging Activity of Crude Extracts of *M. citrifolia* and *C. roseus* Compared with Standard Ascorbic acid

Test sample	**% RSA ± SD at Different Concentration (μg/mL)**					**IC_{50} (μg/mL)**
	1.25	**2.5**	**5**	**10**	**20**	
M. citrifolia (watery)	7.225 ± 1.388	8.359 ± 2.008	32.650 ± 5.130	57.558 ± 0.4112	72.356 ± 0.823	8.484
M. citrifolia (EtOH)	10.268 ± 1.848	13.095 ± 2.472	24.107 ± 2.839	53.895 ± 0.454	70.498 ± 0.823	9.347
C. roseus (watery)	3.734 ± 1.607	7.467 ± 1.370	30.032 ± 1.035	55.784 ± 0.654	65.786 ± 0.823	8.878
C. roseus (EtOH)	12.748 ± 2.575	25.994 ± 2.576	57.679 ± 0.753	69.536 ± 0.468	70.349 ± 0.823	4.395
Ascorbic acid	41.192 ± 2.443	52.554 ± 0.701	55.382 ± 0.704	56.595 ± 1.001	64.358 ± 1.365	2.200

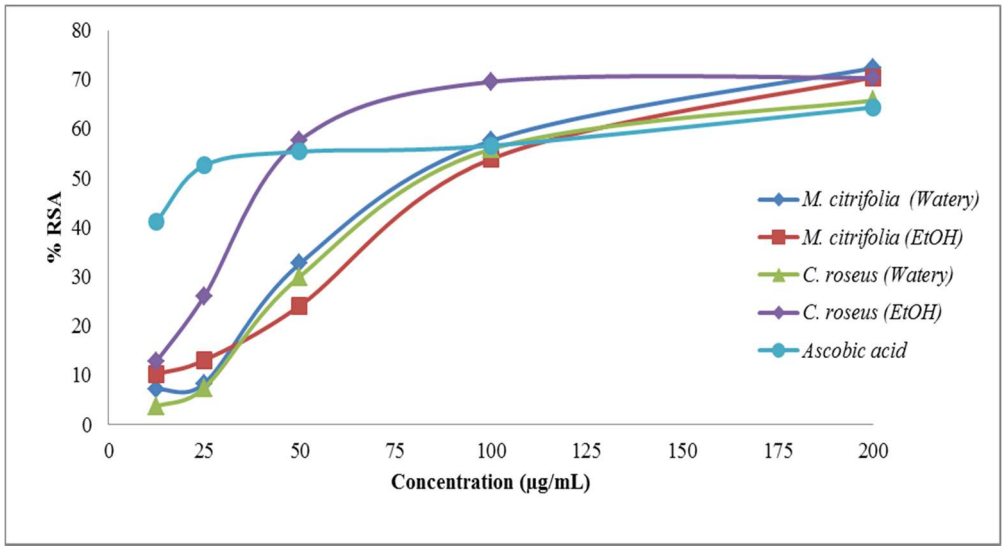


Figure 1: % Radical scavenging activity of crude extract of *M. citrifolia*, *C. roseus* and standard ascorbic acid

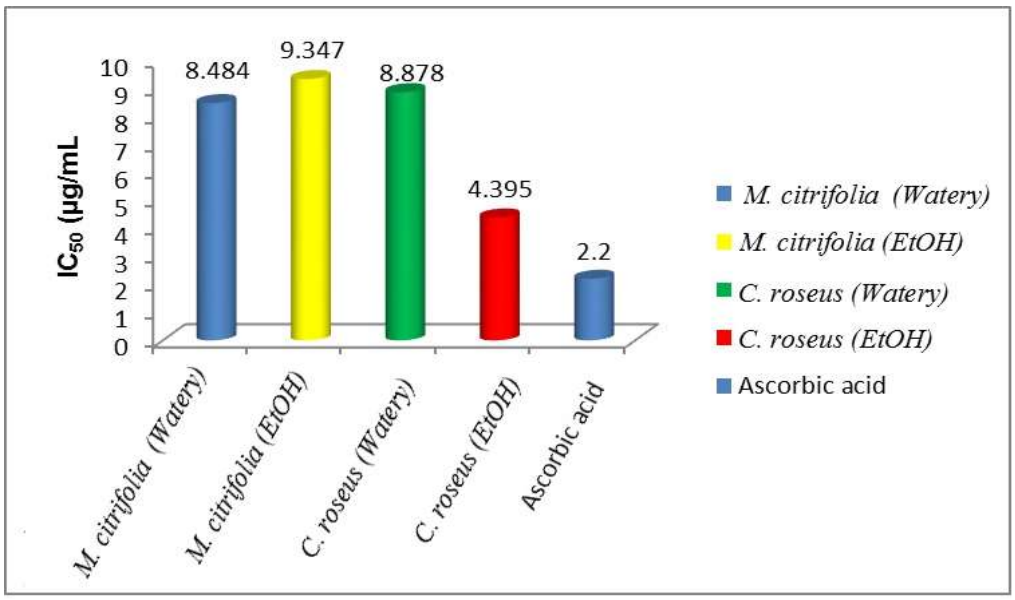


Figure 2: IC_{50} values of crude extracts of *M. citrifolia* and *C. roseus* compared with standard ascorbic acid

Cytotoxicity of *M. citrifolia* and *C. roseus*

The cytotoxicity of watery and ethanol extracts of *M. citrifolia* and *C. roseus* was evaluated by brine shrimp cytotoxicity bioassay. The organisms used were brine shrimp (*Artemia salina*). The cytotoxic effect was expressed as LD_{50} values (50 % Lethality Dose). The resulting cytotoxicity of watery and ethanol extracts of two selected plants are reported in Table 2. According to these results, LD_{50} values could not be detected for both samples until 3200 µg/mL doses. Hence, both samples have no cytotoxic effect compared with $K_2Cr_2O_7$ (LD_{50} = 275 µg/mL) up to the maximum dose of LD_{50} 3200 µg/mL.

Table 2: Cytotoxic Effect of Different Doses of EtOH and Watery Crude Extracts of *M. citrifolia* and *C. roseus*

Samples	No. of Dead Brine shrimp (Mean ± SEM) in various concentration (µg/mL)						LD_{50} (µg/mL)
	100	**200**	**400**	**800**	**1600**	**3200**	
M. citrifolia (Watery)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	1.000 ± 0.000	2.000 ± 0.000	> 3200
M. citrifolia (EtOH)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	2.000 ± 0.000	2.333 ± 0.577	> 3200
C. roseus (Watery)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	1.000 ± 0.000	2.000 ± 0.000	3.000 ± 0.000	> 3200
C. roseus (EtOH)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	1.333 ± 0.577	2.333 ± 0.577	3.000 ± 0.000	> 3200
*$K_2Cr_2O_7$	1.000 ± 0.000	2.000 ± 0.000	10.000 ± 0.000	10.000 ± 0.000	10.000 ± 0.000	10.000 ± 0.000	275

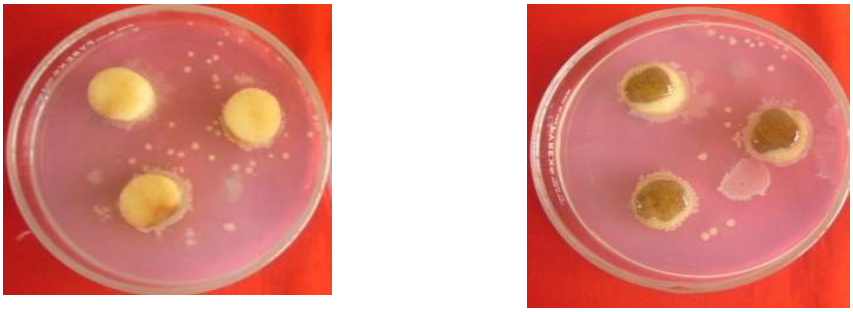
* Used as Cytotoxic Standard ;

** No. of brine shrimp used were 10 for each experiment.

Antitumor Activity of *M. citrifolia* and *C. roseus*

The antitumor activity of EtOH and H_2O extracts of *M. citrifolia* and *C. roseus* was investigated by using PCG test with the isolated tumor producing bacterium *A. tumefaciens.* For inoculation of the potato disc, 48 h broth cultures containing $5x10^9$ cells/mL were used. The tested samples were dissolved in DMSO to dilute and the diluted samples were mixed with the bacterial culture for inoculation. After preparing the inoculums, the bacterial suspension was inoculated on the cleaned and sterilized potato discs, and incubated for 3 days, at room temperature. After that, the tumors were appeared on potato discs and checked by staining the knob with Lugol's (I_2-KI) solution. In the control, the formation of white knob on the blue background indicated the presence of tumor cells because there is no protein in tumor cells. The active test samples did not form any tumors on the potato discs and its surface remained blue as shown in Figures 3 to 5.

Antitumor activity screening revealed that two crude extracts of both samples could inhibit tumor growth. In general, tumor inhibition was significantly observed at the concentration of 0.2 g/mL for each extract.



(a) **(b)**

Figure 3. Photographs showing the control potato disc
(a) Before spraying with iodine
(b) After spraying with iodine

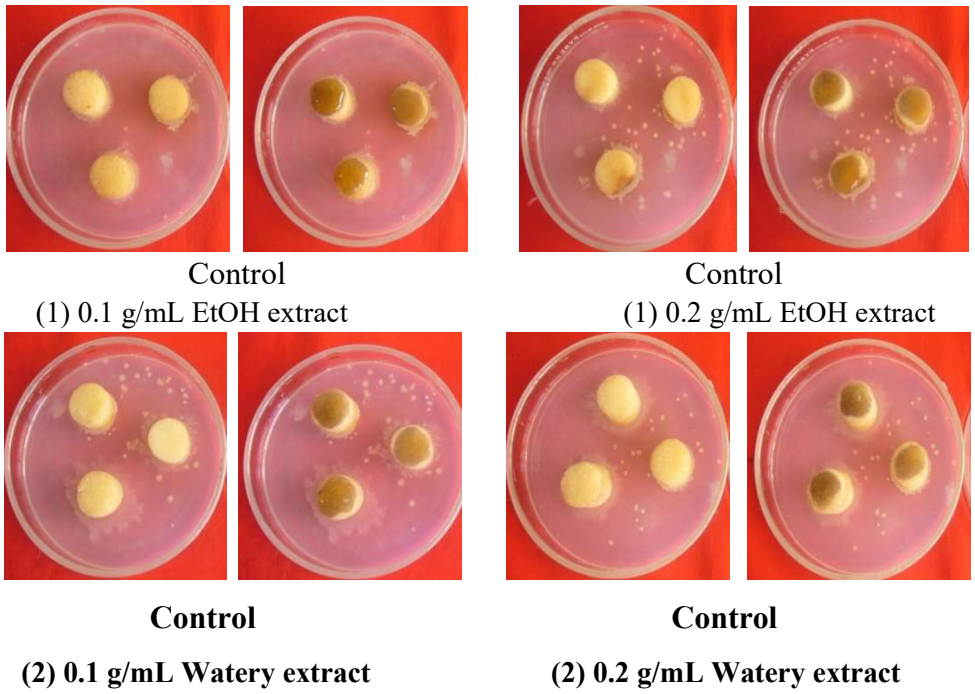
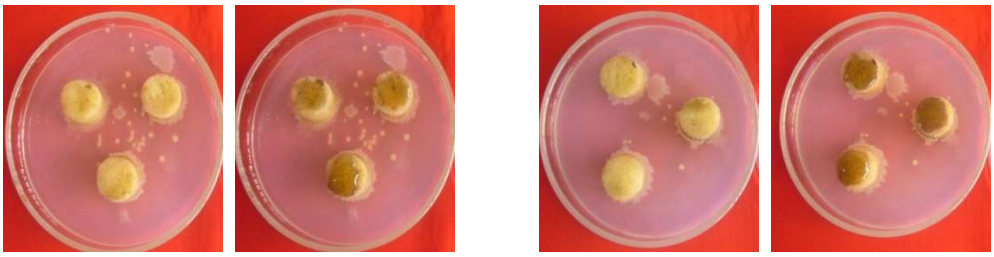


Figure 4. Antitumor assay by potato crown gall test for *M. citrifolia* in different amounts of extracts

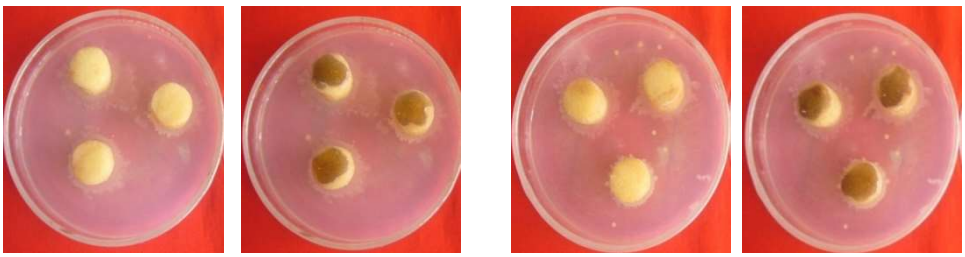
Table 3: Antitumor Activity of Different Crude Extracts from *M. citrifolia*

No.	Test Sample	Concentration of samples g/mL	Tumor
1.	Control	0	+
2.	Ethanol extract	0.1	+
3.	Ethanol extract	0.2	-
4.	Watery extract	0.1	-
5.	Watery extract	0.2	-

(+) Tumor appeared ; (-) No tumor appeared



(1) 0.1 g/mL EtOH extract (1) 0.2 g/mL EtOH extract



(2) 0.1 g/mL Watery extract (2) 0.2 g/mL Watery extract

Figure 5. Antitumor assay by potato crown gall test for *C. roseus* in different amounts of extracts

Table 3: Antitumor Activity of Different Crude Extracts from *C. roseus*

No.	Test Sample	Concentration of samples g/mL	Tumor
1.	Control	0	+
2.	EtOH extract	0.1	-
3.	EtOH extract	0.2	-
4.	Watery extract	0.1	-
5.	Watery extract	0.2	-

(+) Tumor appeared (-) no tumor appeared

Conclusion

From the present study, it can be concluded that *M. citrifolia* fruits and *C. roseus* whole plant possessed the antioxidant activity in the order of *C. roseus* (EtOH extract) > *M. citrifolia* (Watery extract) > *C. roseus* (Watery extract) > *M. citrifolia* (EtOH extract). The EtOH extract of *C. roseus* exhibited the highest antioxidant potency among the extracts. In general, both of the two samples may be useful for the cure of oxidative stress related diseases. The cytotoxicity was not observed in ethanol and watery extracts of *M. citrifolia* and *C. roseus* determined by using brine shrimp cytotoxicity bioassay. Both of the ethanol and watery extracts of *M. citrifolia* and *C. roseus* were found to exhibit the inhibition of tumor growth up to minimum dose of 0.1 g/mL.

These scientific findings from the present work may contribute to the development of Myanmar traditional medicinal in formulation, especially for the disease related to oxidative stress and is expected to inhibit tumor growth in some forms of cancer.

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