STUDY ON THE ANTI-ARTHRITIS PROPERTY OF *CROTON OBLONGIFOLIUS* R. (THETYIN-GYI) LEAVES BY USING PROTEIN DENATURATION METHOD

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Abstract

Leaves of *Croton oblongifolius* R. (Thetyin-gyi) have been known to use in Myanmar traditional medicine concerning antioxidant, anti-arthritis and antimicrobial activities. Therefore, locally grown *C. oblongifolius* has been chosen for this study. This research aimed to investigate the anti-arthritis activities of the leaves of *C. oblongifolius* (Thetyin-gyi). In the present work, anti-arthritis activity and cytotoxicity of the Thetyin-gyi leaves have been determined. The sample was collected from the campus of Inya hostel in Yangon University. The cytotoxicity of watery and ethanol extracts evaluated by brine shrimp cytotoxicity bioassay gave LD_{50} values as 921 µg/mL and 884.5 µg/mL, respectively. The LD_{50} value of $K_2Cr_2O_7$ was 1.5 µg/mL and its cytotoxicity values were between 1 and 10 µg/mL. *In vitro* anti-arthritic activity of ethanol and watery extracts of leaves of Thetyin-gyi was investigated by protein denaturation method by using bovine serum albumin and egg albumin. In both methods, the ethanol extract has shown significant activity at the concentrations of 500 µg/mL and the effects were compared with the standard drug diclofenac potassium. So, the ethanol extract of *C. oblongifolius* have higher anti-arthritic activity than watery extract.

Keywords: *C. oblongifolius*, cytotoxicity, anti-arthritic activity and diclofenac potassium

Introduction

C. oblongifolius (Euphorbiaceae) is a tree available in most places in our country. Traditionally, this plant is employed as wound healing drug in Asia. In Myanmar, the utilization of different parts of several medicinal plants to cure specific aliments has been in vague from ancient times. These systems of drug cater to the requirement of nearly seventy percent of our population residing in the villages. In Homeopathy system, 70 % of the medicines are synthesized from the plants. Extracts of plants from 157 families have been reported to be active against microorganisms. *C. oblongifolius* is extensively used in herbal medicine in South- East Asia. It may be an important herbal drug with some important marker useful to treat some challenging diseases to marking in future life (Mandal and Bose, 2011). The present work is to study the effect of anti-arthritis property of *C. oblongifolius* leaves by protein denaturation method by using bovine serum albumin and egg albumin.

Description and Distribution of *C. Oblongifolius*

C. oblongifolius is a medium sized tree, deciduous, bark brownish, branches lepidote while young. Leaves are alternate, crowded towards the ends of the branchlets (Saleem and Nawaz, 1989). Croton is a genus of Euphorbiaceae comprising around 1300 species, wide spread in tropical regions. Several species have an extended role in traditional medicine in Africa, Asia and South America. *C. oblongifolius* popularly known as 'Thetyin-gyi' in Myanmar and 'Chucka' in Hindi is middle-sized tree belonging to the family Euphorbiaceae. It grows widely in India (Bahar *et al*., 2002).

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Botanical Aspect of *C. oblongifolius* (Thetyin-gyi)

Family	-	Euphorbiaceae
Genus	-	*Croton*
Species	-	*oblongifolius*
Botanical name	-	*Croton oblongifolius* R.
Myanmar name	-	Thetyin-gyi
Common name	-	Chucka
Part used	-	Leaves

Figure 1 Photograph of Plant of *C. oblongifolius* (Thetyin-gyi)

Medicinal Uses and Chemical Constituents of *C. oblongifolius* (Thetyin-gyi)

C. oblongifolius is extensively used in herbal medicine in South-East Asia. *C. oblongifolius* is used to cure liver diseases, sprains, snake bites and as a purgative, insanity, convulsions, asthma, tumors, rheumatism as documented in the Indian Ayurveda medicine system. Bark is used in reducing chronic enlargement of the liver and in remittent fever. It is applied externally to the hepatic region in chronic hepatitis (Julius and Patrick, 1976).

Cembranoid diterpenes, namely crotomembraneic acid, neocrotocembraneic acid, poilaneicacid and their synthetic derivatives including methyl crotocembraneate, crotocembranol, crotocembranol, neocrotocembranal, methyl poilaneate, poilaneol and poilanal were isolated from *C. oblongifolius*. They are approximately 4-fold more active than caffeine which is a known central nervous stimulating agent (Bhowmik *et al*., 2013).

Materials and Methods

Sample Collection and Preparation of *C. oblongifolius* (Thetyin-gyi)

Leaves of *C. oblongifolius* (Thetyin-gyi) were collected from the campus of Inya hostel in Yangon University. Then, the sample was identified at the Department of Botany, University of Yangon. The sample was cleaned by washing with water and air-dried at room temperature. The sample were cut into small pieces and ground into powder by using motor. The powdered samples were stored in air-tight containers.

Preliminary Phytochemical Investigation of Leaves of *C. oblongifolius* R. (Thetyin-gyi).

Phytochemical tests for leaves of *Croton oblongifolius* Roxb. (Thetyin-gyi) was carried out according to the reported methods to investigate the presence and absence of phytochemical constituents such as alkaloids, α-amino acids, carbohydrates, cyanogenic glycosides, organic acids, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, starch, steroids, tannins and terpenoids.

Preparation of Ethanol and Watery Extracts from *C. oblongifolius* R. (Thetyin-gyi)

The dried powder sample (100 g) was percolated with 95 % ethanol (500 mL) for one week and filtered. This procedure was repeated for three times. The combined filtrate containing plant constituents were evaporated under reduced pressure by means of a rotary evaporator. Consequently, 95 % ethanol soluble extract was obtained. Watery extract was prepared by boiling 100 g of sample with 500 mL of distilled water for 6 h and filtered. It was repeated three times and the filtrates were combined followed by heating on water bath and sand bath to give watery extract. Each extract was stored in refrigerator for screening of biological activities.

Determination of Cytotoxicity by Brine Shrimp Lethality Bioassay of *C. oblongifolius* (Thetyin-gyi)

Artificial sea water (9 mL), (1 mL) of different concentrations of samples and standard solutions were added to each chamber of ice tray. Alive brine shrimp (10 nauplii) were taken with pasteur pipette and placed into each chamber. They were incubated at room temperature about 24 h. After 24 h, the number of dead or survive brine shrimp was counted and 50 % of lethality dose (LD_{50}) was calculated (Sahagal *et al*., 2010).

Investigation of Anti-arthritic Activity of Leaves of *C.oblongifolius* (Thetyin-gyi) by Protein Denaturation Method

The *in vitro* anti-arthritic activity was studied by protein denaturation method using Bovine Serum Albumin and Egg Albumin (Rahman *et al*., 2012). Test solution 0.05 mL of different concentrations (500, 250 and 125 μg/mL) and standard drug diclofenac potassium 0.05 mL of different concentrations (500, 250 and 125 μg/mL) were mixed with (0.5 % v/v) aqueous solution of BSA (0.45 mL). Then, the samples were incubated at 37 ºC for 30 min followed by incubation at 57 ºC for 3 min. 2.5 mL of phosphate buffer (pH 6.3) was added to all the above samples after cooling. UV-visible spectrophotometer was used to measure the absorbance at 660 nm. The control represents 100 % protein denaturation. The percentage inhibition of protein denaturation was calculated by the following formula:

$$\text{Percent inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Treated}}}{\text{Abs}_{\text{Control}}} \times 100\ \%$$

The reaction mixture (5 mL) consisted of 0.2 mL of egg albumin, 2.8 mL of phosphate buffered saline (pH 6.4) and test solution of different concentrations (500, 250 and 125 μg/mL) or standard drug diclofenac potassium 0.05 mL of different concentrations (500, 250 and 125 μg/mL) were mixed to form a reaction mixture of 5 mL. Double distilled water of same volume served as control. The samples were incubated at 37±2 ºC in an incubator for 15 min followed by heating at 70 ºC for 5 min. UV-Visible spectrophotometer was used to measure the absorbance at 660 nm. The percentage inhibition of protein denaturation was calculated by the following formula:

$$\text{Percent inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Treated}}}{\text{Abs}_{\text{Control}}} \times 100\ \%$$

Results and Discussion

Preliminary Phytochemical Tests on the Leaves of *Croton oblongifolius* R. (Thetyin-gyi)

A literature survey indicated that a more systematic work needs to be carried out on the preliminary phytochemical studies of the leaves of *Croton oblongifolius* R. (Thetyin-gyi). The results of the preliminary phytochemical screening revealed the presence of alkaloids, α-amino acids, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, starchs, steroids, tannins and terpenoids supporting the reason of its biological activities.

Cytotoxicity of Watery and Ethanol Extracts of Leaves of *C. oblongifolius* (Thetyin-gyi)

The cytotoxicity of watery and ethanol extracts of leaves of *C. oblongifolius* (Thetyin-gyi) was evaluated by brine shrimp cytotoxicity bioassay. This assay is a simple, high throughput cytotoxicity test of bioactive chemicals. It is based on the killing ability of test sample on a simple zoological organism-brine shrimp (*Artemiasalina*). It is a preliminary toxicity screen for further experiments on mammalian animal models. The cytotoxicity of crude extracts was expressed in terms of mean ± SEM (standard error mean) and LD_{50} (50 % Lethality Dose) and the results are shown in Table 1.

In this experiment, potassium dichromate ($K_2Cr_2O_7$) and caffeine were used as standard. Potassium dichromate is generally used as the positive control for this brine shrimp bioassay and caffeine, a natural product, DMSO and artificial sea water, as negative control. The nauplli were counted against a lighted background after 24 h initiation of test. From these results, LD_{50} values of watery and ethanol extracts of Thetyin-gyi were 921 µg/mL and 884.5 µg/mL respectively. Standard caffeine did not show cytotoxicity until 1000 µg/mL concentration whereas LD_{50} of standard $K_2Cr_2O_7$ was 1.5 µg/mL.

Table 1 Cytotoxicity of Different Doses of Watery and Ethanol Extracts of the Leaves of *C. oblongifolius* (Thetyin-gyi)

Sample	Percent Survival of Brine Shrimp (Mean ± SEM) at Various Concentrations (µg/mL)				LD_{50} (µg/mL)
	1	10	100	1000	
watery	26.03 ± 12.33	29.47 ± 6.694	31.11 ± 1.925	51.85 ± 10.5	921
ethanol	27.3 ± 6.757	28.97 ± 3.806	32.82 ± 4.885	53.33 ± 5.774	884.5
*$K_2Cr_2O_7$	48.63 ± 19.19	73.13 ± 4.076	74.67 ± 11.8	100 ± 0	1.5
**Caffeine	0 ± 0	0 ± 0	9.582 ± 0.917	12.73 ± 4.103	>1000

* = Standard for positive control
** = Standard for negative control

In Vitro Anti-arthritic Activity of Leaves of *C. oblongifolius* (Thetyin-gyi)

Arthritis is a type of joint disorder that involves inflammation of one or more joints, accountable for pain, swelling, stiffness, loss of function in joint. One of the main reasons of the arthritis is denaturation of protein. In certain arthritic diseases, auto antigen is produced due to the denaturation of protein. The mechanism of denaturation is probably involved in the alteration of electrostatic hydrogen, hydrophobic and disulphide bonding. In the present study, protein denaturation method (using bovine serum albumin and egg albumin) were selected for *in vitro* assessment of anti-arthritic activity of watery and ethanol extracts of leaves of *C. oblongifolius* (Thetyin-gyi). The standard anti-arthritic activity drug; diclofenac potassium was used for these tests. The absorbance at different concentrations (500, 250 and 125 µg/mL) of tested samples was measured at 660 nm on a UV-visible spectrophotometer.

The *in vitro* anti-arthritic activity of watery and ethanol extracts of *C. oblongifolius* by protein denaturation method using bovine serum albumin was shown in Table 2 and Figure 2. The watery and ethanol extracts of *C. oblongifolius* and diclofenac potassium was tested at different concentrations for anti-arthritic activity and found significant percentage inhibition in protein denaturation. The maximum anti-arthritic activity was observed at the concentration of 500 µg/mL while the minimum activity was observed in the concentration of 125 µg/mL. According to the result, the percentage of arthritic protection was found to be 78.71 % in ethanol, 33.91 % in watery extracts and 83.66 % in diclofenac potassium at the concentration of 500 µg/mL in bovine serum albumin denaturation method. Both extracts exhibited dose dependent response. Similar type of results was observed in the protein denaturation method using egg albumin is shown in Table 3 and Figure 3. According to the result, the inhibition percentage of protein denaturation of egg albumin was found to be 53.02 % in ethanol, 36.12 % in watery extracts and 87.41 % in diclofenac potassium at the concentration of 500 µg/mL.

From this result, it can be stated that these extracts are capable of controlling the production of auto antigen to inhibit the denaturation of protein. The percent inhibition of protein denaturation of leaves of Thetyin-gyi and reference drug with respect to control indicated the stabilization of albumin protein. This anti-denaturation effect was further supported by the change in viscosities. It has been reported that the viscosities of protein solutions increase on denaturation.

Table 2 Anti-arthritic Activity of Watery and Ethanol Extracts of Leaves of *C. oblongifolius* (Thetyin-gyi) by Protein Denaturation Method (Using Bovine Serum Albumin)

Extracts	Concentration (µg/mL)	Absorbance at 660 nm	% Inhibition
Control		0.202	
Watery	125	-	-
	250	0.16	19.8
	500	0.13	33.91
Ethanol	125	0.07	62.62
	250	0.06	69.30
	500	0.04	78.71
Diclofenac potassium	125	-	-
	250	0.08	59.57
	500	0.03	83.66

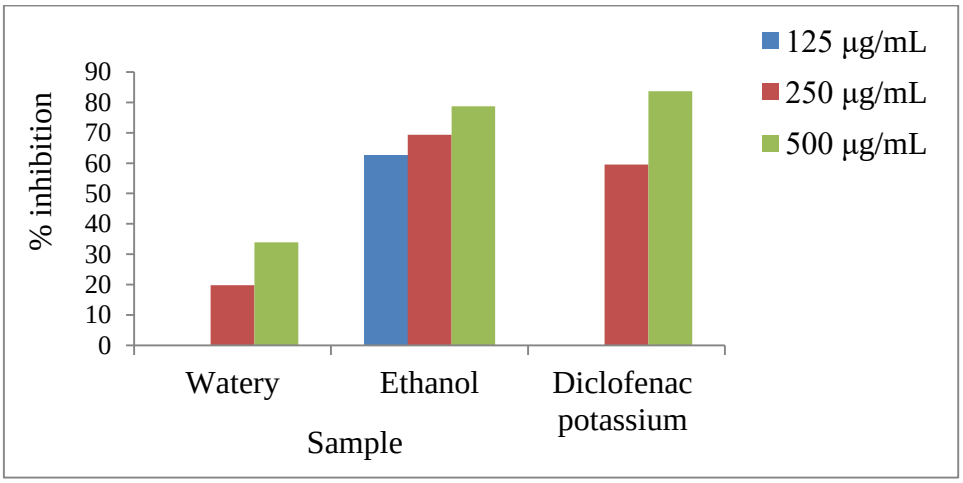

Figure 2 % Inhibition of protein denaturation of leave extracts and standard diclofenac potassium by using bovine serum albumin

Table 3 Anti-arthritic Activity of Watery and Ethanol Extracts of Leaves of *C. oblongifolius* (Thetyin-gyi) by Protein Denaturation Method (Using Egg Albumin)

Extracts	Concentration (µg/mL)	Absorbance at 660 nm	% Inhibition
Control	-	0.596	
Watery	125	-	-
	250	-	-
	500	0.13	36.12
Ethanol	125	0.43	27.01
	250	0.32	44.71
	500	0.28	53.02
Diclofenac potassium	125	0.43	26.84
	250	0.31	46.97
	500	0.07	87.41

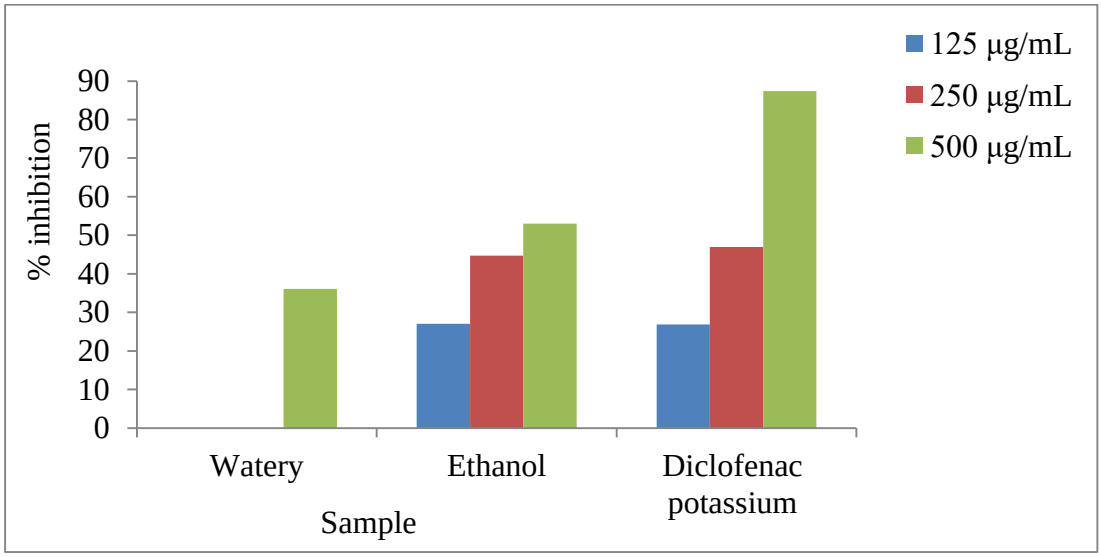

Figure 3 % Inhibition of protein denaturation of leave extracts and standard diclofenac potassium by using egg albumin

Conclusion

The result of preliminary phytochemical screening of different crude extracts of leaves of *C. oblongifolius* R. (Thetyin-gyi) revealed the presence of alkaloids, α-amino acid, glycosides, phenolic compounds, saponins, steroids, tannins, terpenoids and flavonoids supporting the reason of its biological activities. The cytotoxicity of watery and ethanol extracts of leaves of Thetyin-gyi evaluated by brine shrimp cytotoxicity bioassay gave LD_{50} values as 921 µg/mL and 884.5 µg/mL, respectively. The LD_{50} value of $K_2Cr_2O_7$ was 1.5 µg/mL between 1 and 10 µg/mL. Caffeine was not cytotoxic to brine shrimp up to the maximum dose of 1000 µg/mL. Therefore, watery extract showed lower cytotoxicity effect than the ethanol extract. *In vitro* anti-arthritic activity, ethanol and watery extracts of leaves of Thetyin-gyi were investigated by protein denaturation method using bovine serum albumin and egg albumin. In both methods, the ethanol extract showed significant activity at the concentrations of 500 µg/mL and the effects were comparable with the standard drug diclofenac potassium. So, the ethanol extract of *C. oblongifolius* have higher anti-arthritic activity than watery extract

Acknowledgements

The authors would like to thank the Department of Higher Education, Ministry of Education, Yangon, Myanmar, for the permission of doing this research and also to the Myanmar Academy of Arts and Science for allowing the present of this paper.

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