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Sedative, Analgesic and Cytotoxic activities of *Nyctanthes arbor-tristis* L.

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Abstract: *Nyctanthes arbor-tristis L. is a flowering plant widely distributed in Bangladesh as well as all over tropical and subtropical regions of Southeast Asia. It has been used in traditional medicine for its various pharmacological activities. This study evaluated the sedative, analgesic and cytotoxic activities of the ethanolic extract (NAE) and methanol extract (NAM) of Nyctanthes arbor-tristis L. leaves. Both the extracts showed significant sedative depressant property when assessed by thiopental sodium-induced sleeping time study and open field test at the doses 200 and 400 mg/kg (p.o.). Diazepam (1mg/kg, i.p.) was used as reference in both the experiments. The extracts also showed antinociceptive activity when determined using acetic acid-induced writhing test and formalin-induced paw licking test in mice at the doses 250 and 500 mg/kg (p.o.). Diclofenac sodium (40 mg/kg, p.o.) was used as reference analgesic drug. Brine shrimp lethality bioassay revealed that both extracts possess dose dependent cytotoxic activity. The ethanolic extract showed greater sedative, analgesic and cytotoxic activities than methanolic extract at all doses.*

Keywords: *Nyctanthes arbor-tristis L., sedative, writhing and cytotoxicity*

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I. Introduction

Over many decades researchers across the globe have been carrying out investigations to unveil the pharmacological actions of plants used in traditional medicine. Plants are the largest reservoirs of secondary metabolites which can be isolated, purified and analyzed to detect their medicinal properties. These secondary metabolites which possess pharmacological properties are thus useful in the drug discovery process [1,2]. Bioactive compounds isolated from plants have been proved to contain low animal and human toxicity. According to the World Health Organization (WHO), about 80% of the world population depends mainly on plant-based drugs [3]. Based on documented reports of safety advantage of medicinal plants, there is huge scope for research in the field of ethnopharmacology.

Nyctanthes arbor-tristis L. is a medicinal plant which is widely distributed in Bangladesh. It is also found widely in India, Nepal and other tropical and sub tropical regions of South East Asia. It is a small flowering shrub that grows well in secluded and semi-shady place. This woody shrub has a maximum life span of 20 years and is used mostly in Indian Systems of medicines such as Ayurveda [4]. It is also used extensively by Unani and Sidhi Practitioners [5,6]. Various parts of this plant are used by traditional medicine practitioners for various pharmacological actions like anti-leishmaniasis, antifungal, antipyretic, antihistaminic, anti-oxidant, and anti-inflammatory activities [7]. The leaves are used as cholagogue, laxative, diaphoretic and diuretic agents. The bitter leaf juice is used treat liver disorders, biliary disorders, and chronic fever. A decoction of the leaves is widely used in Ayurvedic medicine to treat arthritis and malaria [8]. The seed powder is used for the treatment of scurvy, alopecia, and also as anthelmintics. The bark has been found useful for the treatment of bronchitis, while its roots are used as anthelmintics [9]. The hot flower infusion was used in Srilanka as a sedative [10]. Based on the traditional evidence, and past reports, it has been noted that this plant possesses analgesic, CNS depressant, and cytotoxic potential. In this investigation, the methanolic and ethanol extracts of *Nyctanthes arbor-tristis* leaves were evaluated for cytotoxic, sedative analgesic activities in mice.

II. Materials And Methods

2.1 Collection of Sample

The fresh leaves of *Nyctanthes arbor-tristis* L. were collected from Savar Dhaka in June 2016. The identity of the plant was authenticated from the Bangladesh National Herbarium, Dhaka where a voucher specimen was deposited having different accession number as given in parenthesis: *Nyctanthes arbor-tristis* L. *(DACB 45114).*

2.2 Preparation of extracts

The fresh leaves of *Nyctanthes arbor-tristis L.* were shade dried for 7 days and pulverized into fine powder. For the preparation of the extracts, 100 g leaf powder was macerated in 500 ml methanol and ethanol separately, at room temperature for a period of 10 days with occasional stirring. Each of the extracts was then filtered through Whatman No.1 filter paper and the filtrate obtained was concentrated using a rotary evaporator to obtain a thick viscous mass of extract. The extractive value was determined for each extract. 16.5% yield and 14.3% yield were obtained from ethanolic extract (NAE) and methanolic extract (NAM) respectively. Each crude extract was taken in a light proof airtight container and stored in the refrigerator at 4^{o}C.

2.3 Experimental animals:

Swiss Albino male mice weighing 25-30 g were collected from the animal house of the Department of Pharmacy, Jahangirnagar University, Savar, Bangladesh. The animals were kept in standard environmental conditions (at 23.0±3°C temperature & 55-65% relative humidity and 12-hour light/12-hour dark cycle) for one week for acclimation. The animals were provided with free access to food (standard laboratory rodent's food) and water ad libitum at the Laboratory Animal House, Department of Pharmacy, Primeasia University, Bangladesh. The ethical guidelines for the investigation of experimental animals were followed in all tests.

2.4 Drugs and Chemicals

Diclofenac Sodium and Diazepam were obtained from Square Pharmaceuticals Ltd., Bangladesh. Thiopental sodium was obtained from Gonoshashthay Pharmaceutical Ltd., Bangladesh. Acetic Acid (Merck, Germany) and Tween 80 (Merck) were purchased.

2.5 Acute toxicity test

The Acute toxicity studies were performed on mice according to the method proposed by Ghosh [11]. The extracts were administered orally at the doses of 50,100, 300, 1000, and 3000 mg/kg body weight to separate groups of the mice (n = 6) after overnight fasting. The mice were observed closely for the first 3 h for toxic manifestations such as salivation, convulsion, increased motor activity, coma, and death. The observations were made at regular intervals of 24 h. The mice were closely monitored for one week and any abnormality was noted [11].

2.6 Thiopental sodium-induced sleeping time test

The thiopental induced sleep time study as described by Turner [12] was used to detect the sedative activity of *Nyctanthes arbor-tristis*. The mice were divided into six groups each of six mice. Group I served as the negative control and received orally (p.o.) 1% tween 80 solution at a dose of 10 ml/kg body weight. Group II served as positive control and was treated with diazepam (1 mg/kg, i.p.). Group III and Group IV received orally NAE at a dose of 200 mg/kg and 400 mg/kg respectively, while Group V and Group VI orally received NAM at the same dose of 200 mg/kg and 400 mg/kg body weight respectively. Thirty minutes after oral dose administration, Thiopental sodium (40 mg/kg) was administered intraperitoneally (i.p.) to each mice to induce sleep. The animals were observed for the latent period (time between thiopental sodium administration to loss of righting reflex) and duration of sleep (time between the loss and recovery of reflex).

2.7 Open field test

The open field test was carried out using mice as described by Gupta *et al.* [13]. The animals were divided into Control, standard and experimental groups containing six mice each. The experimental groups received Ethanolic (NAE) and Methanolic (NAM) extracts at the doses 200 and 400 mg/kg. The standard group received diazepam at a dose 2 mg/kg. The floor of an open field of half square meter was divided into a series of squares. The height of the apparatus was 40 cm. The number of squares visited by the mouse was counted for 5 min at 0, 30, 60, 90, and 120 min after oral administration of each of the doses.

2.8 Acetic acid-induced writhing test

The method described by Sulaiman *et al.* [14] was used to study the analgesic activity of the extract by acetic acid induced writhing test. In this study, albino mice (20-25 g) were divided into eight groups each consisting of six mice. Group I served as negative control (received 10 ml/kg body weight distilled water), Group II served as positive control and was treated orally with diclofenac sodium (40 mg/kg). The test groups III and IV received NAE at the dose of 250 mg and 500 mg per kg body weight respectively while the test groups V and VI received NAM at the dose of 250 and 500 mg per kg body weight respectively. Thirty minutes after treating with the drug, 1% acetic acid (10 ml/kg) was administered intraperitoneally (i.p.) to induce pain. The writhing response in mice was noted from 5 min after injection of acetic acid for up to 20 min.

The inhibition of writhing response for each of the test sample was calculated according to the following formulae:

% Inhibition of writhing= (M_c-M_t)x100/Mc

Where M_c = *Mean number of writhing in the control group and* M_t = *Mean number of writhing in treated group*

2.9 Formalin Induced Paw licking test

The mice were divided into 6 groups each of six mice. The method of Hunskaar and Hole [24] was used for this study. Group I received 1% Tween 80 solution (dose 10 ml/kg). Group II received diclofenac sodium at a dose 40 mg/kg. Group III and IV received NAE (250 and 500 mg/kg respectively) while Group V and VI received NAM (250 mg/kg and 500 mg/kg respectively). 2.5% Formalin was injected after 1 hr of dose administration into the dorsal surface of the right hind paw. The time spent licking the injected paw was recorded for the first 5 min post formalin injection (acute phase) and for 5 min starting at the 20th min post formalin injection (late phase).

2.10 Brine shrimp lethality bioassay

The brine shrimp lethality bioassay as proposed by Meyer *et al.* [15] used to predict the cytotoxic activity of the ethanolic and methanolic extracts of *Nyctanthes arbor-tristis* [15]. For this experiment, 4 mg of each of the extracts was dissolved in dimethyl sulfoxide (DMSO) and solutions of varying concentrations (400, 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 mg/ml) were obtained by the serial dilution technique using simulated seawater. The solutions were then added to the pre-marked vials containing 10 live brine shrimp nauplii in 5 ml simulated seawater. After 24 h, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. The percent of lethality of the brine shrimp nauplii for each concentration and control was calculated. The median lethal concentration LC_{50} of the test samples after 24 hours was obtained by a plot of percentage of the shrimps killed against the logarithm of the extract concentration (LC). The best-fit line was obtained from the curve data by means of regression analysis using Microsoft Excel 2010.Vincristine sulfate was used as positive control.

3.7 Statistical analysis

Data were represented as Mean ± SEM (n=6, in each group). The statistical significance between treated and control groups were analyzed by ANOVA following Dunnett's test ($P<0.05$ was considered statistically significant).

III. Results

3.1 Acute toxicity test

The mice survived the test period and did not show any sign of toxicity or abnormality at the doses of 50–3000 mg/kg. No abnormal behavior or body weight changes were observed for a period of 7 days. It indicated that both NAE and NAM have low toxicity profile and the LD_{50} is more than 3000 mg/kg.

3.2 Thiopental induced-sleep time study

When compared to control, there was a significant dose dependant increase in sleep time for both NAE and NAM treatments. The latency period (onset of sleep) for the extract and the control was similar and showed no significant changes. NAE extract treatment showed an increase in duration of sleep from 88.67 min to 115.2 min when the dose is increased from 200 mg/kg to 400 mg/kg. The NAM treatment also caused increase in sleep duration but it was lower than that caused by NAE at both the doses of the extract (Table 1).

Table 1: Mean Onset and duration of sleep time for Thiopental induced sleep time study (Results are expressed in Mean±SEM)

Treatment	Dose	Onset of sleep (Min.)	Duration of sleep (Min.)
Control (1% Tween 80)	10 ml/kg	20±1.506	47±2.352
Diazpem	1 mg/kg	6.5±0.5 *	132.7±3.537**
NAE	200 mg/kg	21.5±2.363	88.67±3.383**
	400 mg/kg	21.3±1.145	115.2±4.7**
NAM	200 mg/kg	23.83±1.797	77±4.028**
	400 mg/kg	22.5±2.291	96±3.945**

The values are mean ± SEM, (n = 6); * $p < 0.05$, ** $p < 0.01$, *Dunnett t-test* as compared to control. **NAE** = Ethanolic extract of *Nycthanthes arbor-tristis* leaves, **NAM** = Methanolic extract of *Nyctanthes arbor-tristis* leaves

3.3 Open field test:

There is a significant dose-dependent decrease in locomotion caused by NAE and NAM from 0 min to 120 min (Table 2).

Table 2: Number of Movements in Open field test

Treatment	Dose (mg/kg)	No. of movement				
		0 mins	30 mins	60 mins	90 mins	120 mins
Control	0	129.33±0.99	129.16±0.95	128.5±0.62**	126.17±0.66**	123.83±1.33**
Diazepam	1	130.5±0.85	73.83±1.05**	64.67±0.50**	33.5±0.67**	15±0.45**
NAE	200	130.16±1.20	88.5±1.12**	72.5±1.23**	42.33±0.62**	23.83±0.48**
NAE	400	131.67±1.09	82.33±0.33**	65.5±0.76**	33.5±1.057**	16.83±0.60**
NAM	200	129.17±1.32	100±1.16**	73.33±1.73**	56±0.68**	36.83±0.48**
NAM	400	128.33±0.95	85.5±0.43**	65.17±0.40**	50±1.03**	26.83±0.70**

The values are Mean ± SEM, (n = 6); * $p < 0.05$, ** $p < 0.01$, *Dunnett t-test* as compared to control. **NAE** = Ethanol extract of *Nycthanthes arbor-tristis* leaves, **NAM** = Methanol extract of *Nyctanthes arbor-tristis* leaves

3.4 Acetic acid-induced writhing test:

The extracts of NAE and NAM treatments showed significant dose-dependent inhibition of writhing. The extract of NAE demonstrated significant analgesic effect at the dose 500mg/kg (TABLE 3). The water soluble portion of NAE shows good analgesic property while NAM showed slight antinoceptive activity when tested by acetic acid induced writhing test in mice. The percentage inhibitions of writhing at 250 and 500 mg/kg dose of ethanolic extract are 59.9% and 68.7% and NAM gave 29.3% and 49.4% inhibition of writhing. The NAE showed potential of good analgesic property when compared to standard drug diclofenac sodium which has 81.9% inhibition of writhing at 40 mg/kg dose.

Table 3: Percent inhibition of writhing following dose administration

Treatment	Dose (mg/kg)	Mean Writhing	% inhibition of writhing
Control (1% Tween 80)	10 ml/kg	34.8±1.759	---
Diclofenac sodium	40 mg/kg	6.3±0.511**	81.9
NAE	250 mg/kg	20.7±0.9811**	59.9
	500 mg/kg	10.9±0. 92**	68.7
NAM	250 mg/kg	24.6±0.5974**	29.3
	500 mg/kg	17.6±0.6379**	49.4

All the values are expressed as mean ± SEM (n=6); One way Analysis of Variance (ANOVA) followed by Dunnett's test, **p<0.01 significant compared to control.

3.5 Formalin induced paw lick test:

TABLE 4 shows the results for paw licking test. The result shows that there is a significant (p< 0.05) decrease in paw licking time in both acute and late phase for both the extracts.

Table 4: Formalin Induced Paw lick test

Treatment	Dose	Acute phase Paw licking time/secs	Late Phase Paw licking time/secs
Control (1% Tween 80)	10 ml/kg	105.83±5.02	79.21±3.321
Diclofenac sodium	40 mg/kg	62.67±2.57**	35±1.183**
NAE	250 mg/kg	79.63±2.67**	52.67±0.9545**
	500 mg/kg	38.5±1.92**	30.5±0.8851**
NAM	250 mg/kg	76.83±2.87**	63.17±0.7923**
	500 mg/kg	72.33±2.99**	51.33±1.116**

All the values are expressed as mean ± SEM (n=6); One way Analysis of Variance (ANOVA) followed by Dunnett's test. **P<0.01, significant compared to control.

3.6 Brine Shrimp Lethality Bioassay

Table 5 shows the results of the brine shrimp lethality after 24-hour exposure to all the samples and vincristine sulphate (positive control) [15]. The positive control (Vincristine sulphate) as compared to the negative control (sea water) was found to be lethal, giving significant mortality of the shrimp nauplii. The degree of lethality was directly proportional to the concentration of the extract ranging from significant with the lowest concentration (1.56 µg/ml) to highly significant with the highest concentration (400 µg/ml). In other

words, mortality increased with the increase of the concentration of the test samples. LC_{50} doses obtained from the best-fit line were 4.97 µg/ml and 5.26 µg/ml for NAE and NAM respectively. In comparison with positive control (Vincristine Sulphate), the cytotoxicity exhibited by NAE and NAM was promising.

Table 5: Cytotoxic activity of of NAE, NAM and Vincristine sulphate

Concentration of extracts (µg/ml)		Log C	% Mortality		Vincristine sulphate		
NAE	NAM		NAE	NAM	Concentration(µg/ml)	Log C	%Mortality
400	400	2.6	100	83.3	40	1.602	100
200	200	2.3	100	79.5	20	1.301	100
100	100	2	93.3	76.6	10	1	100
50	50	1.69	83.3	63.3	5	0.699	70
25	25	1.49	73.3	56.6	2.5	0.398	60
12.5	12.5	1.09	53.3	53.3	1.25	0.097	60
6.25	6.25	0.79	50	51.3	0.625	-0.201	70
3.125	3.125	0.49	46.7	48.4	0.3125	-0.509	70
1.56	1.56	0.19	36.7	43.3	0.156	-0.796	60

Table 6: The LC_{50} values of NAE, NAM and Vincristine sulphate

Sample	LC_{50}(µg/ml)	Regression Equation	R^2
Vincristine Sulphate (Std.)	0.12 µg/ml	Y = 33.13x + 59.57	0.954
NAE	4.97 µg/ml	Y=29.287x+29.601	0.964
NAM	5.26 µg/ml	Y=17.153x+37.639	0.940

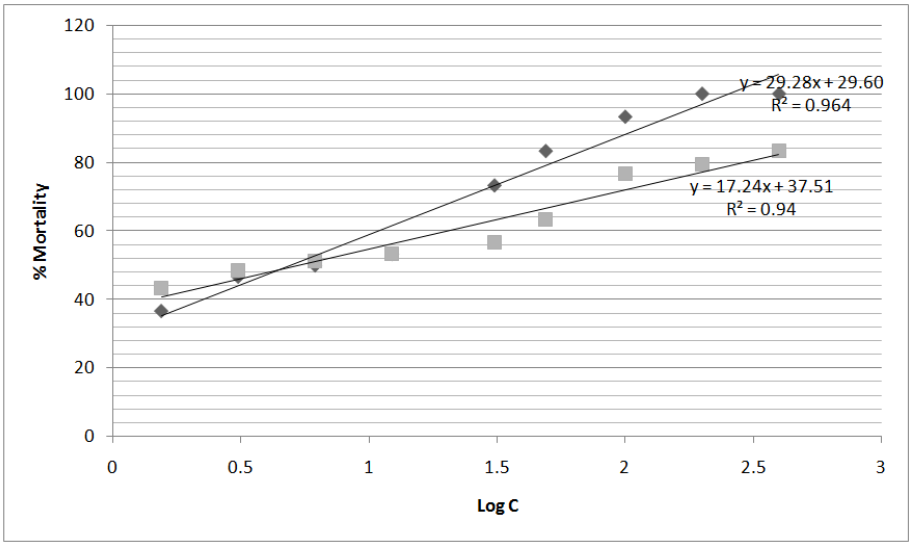

Figure 1: Plot of log concentration (Log C) of NAE (—♦—), and NAM (— ■ —) versus percent shrimp mortality after 24h exposure

IV. Discussion

In this study, the sedative, analgesic and cytotoxic activities of the ethanolic (NAE) and methanolic extracts (NAM) of *Nyctanthes arbor-tristis* L. was evaluated. Thiopental induced sleeping time study was used to detect the sedative potential of the plant. The classic parameters used to relate the action of CNS depressants are reduction of onset of sleep and increase in total sleep time [16]. The prolongation of sleep time for each of the extracts was compared with that for control group (10 ml 1% Tween 80 solution/kg) and also for standard reference drug (diazepam 1 mg/kg). It has been reported that the elderly Sri Lankan Buddhist monks used the hot infusion of the flowers of *Ncytanthes arbor-tristis* as a sedative [17]. Das *et al.* [18] detected that the ethanolic extracts of flowers, seed, stem, and leaves have significant CNS depressant activity using phenobarbitone induced sleep time study. In this study, it was observed that both the extracts produced a significant increase in sleeping time in thiopental induced sleep time study (Table 1). Sedation induced by thiopental are principally mediated in the CNS by the $GABA_A$ receptor complex. Thiopental sodium interacts

with GABA and potentiates GABA activity and permits the entry of chloride into the neuron by prolonging the duration of chloride channel opening [19]. Thiopental can also block excitatory glutamate receptors and induces CNS depression. Therefore, the crude extracts which possess sedative property may follow similar molecular activity to prolong sleeping time. The prolongation of sedation for NAE was higher than that for NAM. The extracts had no significant effect on the onset of sleep (Table 1) suggesting that the plant is not potent to induce sedation but has sufficient tranquilizing effect with increasing dose of the extract.

The open field test is used to study anxiety, depression and sedative action [13]. The decrease in movement based on the number of squares explored by the animal in the open field correlates with an increase in CNS depressant activity. Both the plant extracts caused significant ($p<0.05$) decrease in movement with an increase in dose of the plant extract (Table 2). Thus, the results indicate the presence of sedative activity of *Nyctanthes arbor-tristis.* The data obtained from this study support previous findings and the leaves possess sufficient tranquilizing effect and ethanolic extract is more potent at 400 mg/kg to depress the CNS than methanolic extract (Table 1 & 2).

The leaves of *Nyctanthes arbor-tristis*. had been advocated for arthritis and painful conditions by Ayurvedic practitioners. In 1987, Saxena and his colleagues reported that the water soluble ethanolic extract of *Nyctanthes arbor-tristis* exhibited significant aspirin-like antinociceptive activity but failed to produce morphine-like analgesia [20]. In this study, the analgesic activity of the ethanol and the methanolic extracts was determined by chemically induced *in vivo* tissue damage animal model [21], namely the acetic acid induced writhing test and formalin induced paw lick test. The result obtained suggested that both the extracts can significantly ($p<0.05$) reduce pain at the dose 250 mg/kg and 500 mg/kg body weight (Table 3). The ethanolic extract at dose 500mg/kg is more potent and can inhibit writhing by 76%. Both the extracts exhibited good antinociceptive property when compared to standard drug diclofenac sodium. The results indicate that the extraction in polar solvents has significant antinociceptive property. The formalin induced paw lick test is a highly reliable model for studying analgesic activity [22,23]. This is a biphasic test which measures both neurogenic pain and pain associated with inflammatory origin. The first phase 0 to 5mins is a result of the direct stimulation of the nociceptors by formalin and results from centrally mediated effects and is insensitive to anti-inflammatory agents. The second phase (15 to 30mins) is dependent on peripheral inflammation and changes in central procession due to chemical mediator released from damaged cells that stimulated nociception and thus induced pain [24]. A suppression of pain in both the phases was observed following administration of NAE and NAM at doses 250 and 500 mg/kg (Table 4).

The Brine Shrimp Lethality Bioassay (BSLB) as proposed by Meyer *et al*. [15] was performed to study the cytotoxic property of the leaf extracts. It is a very useful tool to screen a wide range of chemical compounds for their various bioactivities. It has been observed that BSLB correlates reasonably well with cytotoxic and other biological properties. The significant correlation between the Brine shrimp lethality bioassay and *in vitro* growth inhibition of human solid tumor cell lines demonstrated by the National Cancer Institute (NCI), USA is significant because it shows the value of this bioassay as a pre-screening tool for antitumor drug research [25]. In toxicity evaluation of plant extracts by Brine shrimp lethality bioassay, LC_{50} values lower than 1000 µg/ml are considered bioactive [15]. The brine shrimp lethality bioassay of NAE and NAM (Table 5) showed that mortality of nauplii increases with the increase in the concentration of extract. Thus it is clear that there is a linear dose-effect relationship between extract concentrations and LC_{50} values, and the degree of lethality is found to be directly proportional to the concentration of the extract. The NAE and NAM have shown the LC_{50} values of 4.97 µg/ml and 5.26 µg/ml respectively while the LC_{50} value of Vincristine sulphate was found to be 0.12 µg/ml. Results showed that ethanolic and methanolic extracts of *Nyctanthes arbor-tristis* has potent cytotoxic effect comparable to that of standard drug Vincristine sulphate.

V. Conclusion

This study proves that *Nyctanthes arbor-tristis* L. has significant sedative, analgesic, and cytotoxic property. The ethanolic extract showed greater sedative analgesic and cytotoxic potential than methanolic extract at all doses used in this investigation. Results of the present study tend to substantiate the use of this plant in various disease conditions by Ayurvedic and traditional medicine practitioners. This study also indicates that there is a scope for further investigation on this plant extract in order to identify the active principles responsible for medicinal values.

Acknowledgments

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VI. Competing Interests

The authors declare that they have no competing interests

VII. Consent For Publication

We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the manuscript and agreed with submission to the IOSR Journal of Pharmacy and Biological Sciences.

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