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Evaluation of Analgesic Activity of Ethanolic, Hydroethanolic, Aqueous and Chloroform Extracts of *Nyctanthes arbortristis* Leaves

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ABSTRACT: Allopathic antibiotic and anthelmintic overuse leads to the development of antimicrobial and anthelmintic resistance in both humans and animals, while employing plants for treatment that have phytotherapeutic characteristics reduces the likelihood of developing such resistance. Historically, medicinal plants have been used to cure a variety of conditions. Active compounds that are sources of healing can be found in medicinal plants. The current study sought to assess the analgesic potential of a leaf extract from *Nyctanthes arbor-tristis*. Melonex and tramadol hydrochloride were employed as standard drug for Eddy's hot plate, tail clip, and acetic acid-induced writhing methods of evaluating analgesic activity. Eddy's hot plate, tail clip, and acetic acid-induced writhing procedures were used to test all four extracts (ethanolic, hydroethanolic, aqueous, and chloroform) of *Nyctanthes arbor-tristis* at doses of 250, 500, and 1000 mg/kg.

Keywords: *Nyctanthes arbor-tristis*, Analgesic, Eddy's hot plate method, Tail clip method, Acetic acid induced writhing method

INTRODUCTION

Pain occurs when a noxious stimulus is applied to the body (Mersky, 1979). When a nociceptive stimulus is applied to the body, pain is produced. Endogenous pain-producing chemicals such as histamine, 5-HT, quinine, acetylcholine, lactate, K+, and pain-modulating agents such as prostaglandins and leukotrienes are released into the injured tissue (Camillo *et al*., 1954). Parijat is the local name for *Nyctanthes arbor-tristis*. The plant is native to South Asia, northern Pakistan, southern Nepal, northern India, and southeastern Thailand (Sharma *et al*., 2002). The leaves are also used as a cough suppressant. Cough is treated by mixing the juice of the leaves with honey and taking it three times a day. Fever, high blood pressure and diabetes can also be treated with a paste made from the leaves and honey.

It can also be achieved with the help of leaves: leaves of *N. arbor-tristis* is involved in central nervous system activities such as hypnotics, sedatives and local anaesthetics (Saxena *et al*., 2002 ; Ratanasooriya *et al.,* 2005; Das *et al.,* 2008). *N. arbortristis* leaves show antifungal activity against *Alrernaria alternates* (Chauhan and Saraswat 1978). A water extract of the leaves has been shown to have hepatoprotective effects (Chauhan and Saraswat 1978). Kiew and Baas (1984) isolated an alkaloid component called nictanthine from leaves. Iridoid glycosides have been isolated from plants and are effective against Leishmania (Tandon *et al*., 1991). New iridoid glycosides have been isolated from plants along with nictantic acid, oleanolic acid, friedelin, 6-β-hydroxyloganine, and albortolystside A (Rathore *et al*., 1990). A smaller iridoid glucoside, arboside D, and its acetyl derivative have been identified in plants (Singh and Jindal 1965) . The leaf of the plant along with the leaves of *Hygrophila auriculata* and *Achyranthes aspera* are crushed together and consumed daily for getting relief from spleen enlargement (Kumar *et al*., 2020). The water-soluble portion of the alcoholic extract of *N. Arbor tristis* leaves have been reported to have anti-inflammatory activity in various experimental models (Saxena *et al*., 1984). This study aimed to evaluate the analgesic activity of ethanolic, hydroethanolic, aqueous, and chloroform

extracts from leaves of *Nyctanthes arbor-tristis* in experimental animals.

Nyctanthes arbor-tristis.

MATERIALS AND METHODS

Plant material. The fresh leaves of *N. arbor – tristis* will be collected from in an around Khanapara campus in the month of July to September for pharmacological experimental purpose. Leaves were identified and authenticated by Botanical Survey of India (BSI), Eastern Regional Centre, Shillong.

Processing of Plant Materials. After identification and characterisation by BSI, leaves were further collected. The collected leaves were gently washed with fresh water to remove soil and dust particles. Leaves were then shade dried at room temperature for about 7-10 days. They were regularly turned over, to prevent fermentation and rot. Dried leaves were then grounded or pulverised to powder by Laboratory Willey Mill and kept at room temperature in air tight containers after proper labelling until preparation of extracts.

Preparation of Ethanolic, Hydroethanolic, Aqueous and Chloroform Extracts. Powdered plant materials were extracted with ethanol, hydroethanol (1:1) and distilled water respectively as per the procedure of Prasad (1965). Finely powdered plant powders were soaked with individually for 72 hours, three times, with intermittent agtitation. The extracts were then double filtered using muslin cloth and Whatman No.1 filter paper. The filtrate obtained was concentrated in rotary evaporator and completely dried over regulated water bath maintained at 50ºC. The extracts were refrigerated at 4ºC until the experiments for screening was done. Standard procedures (Lateef *et al*., 2003); Sujon *et al.* (2008) were used with a few modifications.

Phytochemical Analysis of Extracts. The ethanolic, hydroethanolic, aqueous & chloroform extracts of *Nyctanthes arbor-tristis* were subjected to phytochemical analysis for the presence of various active principles namely steroids, alkaloids, phenolic compounds, tannins, flavonoids, glycosides, triterpenes and saponins as per the procedure described by Harborne (1991).

Experimental Animals. Wistar albino mice weighing 30-40g were taken from the Department of Veterinary Pharmacology and Toxicology, Assam Agricultural University, Khanapara. All the mice were kept in polypropylene cages and they were divided into groups of 6 mice each. Paddy husk was used as litter material which was regularly changed every week. All the animals were provided with a balanced ration and clean drinking water *ad libitum* and were maintained in standard laboratory conditions (12:12 hour light/dark) cycle at an ambient temperature ranging between (22-27°C). The animal experimentation was carried out according to the Committee for the Purpose of Control and Supervision of experimental animals (CPCSEA) guideline and Institutional Animal Ethical Committee Approved all the procedure for investing experimental pain in conscious animals.

Acute Toxicity Study. The study was carried out according to OECD (Organization for Economic Co-operation and Development) 425 guidelines. For acute toxicity study, nulliparous and non-pregnant female albino mice, weighing 30-40g, were randomly selected. Ethanolic, hydroethanolic, aqueous and chloroform extracts of the leaves of *Nyctanthes arbor-tristis* was administered orally to the mice. Limit test was performed. Prior to administration of the test extracts animals were fasted overnight but given water *ad libitum.* Group-I served as vehicle control (20% Tween-80) and Group II-V kept ethanolic, hydroethanolic, aqueous and chloroform leaf extract of *Nyctanthes arbor-tristis* @ 2000 mg/kg orally, as single dose. The animals were closely observed for behavioral changes, toxicity and mortality upto 72 hours and animals were further observed for 14 days to record mortality if any. Based on acute toxicity study three doses were selected and used for evaluation of analgesic activity in mice with six animals in each group for each of the following tests.

Analgesic Activity

Eddy's hot plate method. The Hot Plate test was carried out according to the method described by Eddy and Leimbach (1953) in mice of either sex. The responses taken are jumping, withdrawal of the paws and licking of the paws. The hot plate, which is commercially available, consists of a electrically heated surface. The mice were placed on the Hot Plate maintained at 55˚C± 1˚C and the time between placement on the hot plate and the occurrence of either licking of the paws or jumping off from the plate or withdrawal of the paw was recorded as the reaction time (sec). The mice with reaction time of more than 10 sec were not included in the study. The reaction time of each mouse was recorded at 0, 15, 30, 45, 60, 90, 120 and 150 min after administration of the test extract and standard drug (Tramadol hydrochloride) administration with a cut off time of 30 sec.

Tail clip method. In this study, the procedure described by Camillo (1929) was used to assess the

central analgesic activity. A metal artery clip was used to screen all of the mice at the base of the tail. The clip's pressure was adjusted to the point where it was just enough to elicit a response from all of the control mice. The animals that did not try to dislodge the clip within 5 seconds were not employed in the study. For the experiment, the responding mice were divided into fourteen groups. Group I served as vehicle control, Group II served as standard Tramadol hydrochloride @ 30mg/kg i.p. The ethanolic, hydroethanolic, aqueous, and chloroform leaf extracts of *Nyctanthes arbor-tristis* were given orally at dose rates of 250, 500, and 1000 mg/kg body weight to groups III-XIV, respectively. Analgesic activity was measured at 0, 30, 60, and 90 minutes after the test extracts were administered. If there was no attempt to dislodge the clip within 5 seconds, it was considered a favourable analgesic reaction. At various intervals, the reaction time was noted and documented.

Acetic acid induced writhing method. This test was performed in mice of either sex according to the method of Witkin *et al.* (1961). Adult albino mice which had shown at least 40 to 45 stretching episodes in 30 minutes after i.p. administration of acetic acid 0.5% solution at the dose rate of 10ml/kg body weight were selected for the study. The mice were divided into 14 groups of 6 animals each. Group I was kept as vehicle control and Group II received Standard drug Melonex suspension @ 5 mg/kg orally. Group III-XIV received ethanolic, hydroethanolic, aqueous and chloroform leaf extract of *Nyctanthes arbor-tristis* orally at the dose rate of 250, 500, 1000 mg/kg body weight respectively. After 30 minutes of pre-treatment with standard drug and test extracts mice were injected with acetic acid (0.5% i.p). Each mouse was put into a polypropylene cage and total numbers of stretching episodes for 15 and 30 minutes were recorded. The percent inhibition of each of the extracts and the standard drug were calculated and noted.

Statistical Analysis. Results were expressed as Mean ± S.E.M. Statistical analysis was performed by MS-excel to calculate mean, standard error of mean (SEM), analysis of variance (ANOVA), and co-efficient of correlation (r) values as per standard method Snedecor and Cochran (2004).

RESULT

Phytochemical Analysis. Various phytochemical tests were done using ethanolic, hydroethanolic, aqueous and chloroform extracts of *Nyctanthes arbor-tristis* leaves. They were subjected to phytochemical analysis for the presence of various active principles or phytochemical constituents namely steroids, alkaloids, phenolic compounds, tannins, flavonoids, glycosides, triterpenes, diterpenes and saponins. Ethanolic extract of *Nyctanthes arbor-tristis* leaves showed presence of steroids, alkaloids, phenolic compounds, glycosides, flavonoids, diterpenes, triterpenes and absence of tannins and saponins. The hydroethanolic extract of *Nyctanthes arbor-tristis* leaves showed presence of steroids, alkaloids, phenolic compounds, glycosides, flavonoids,tannin, diterpenes, triterpenes and saponins. The aqueous extract of *Nyctanthes arbor-tristis* leaves showed presence of steroids, alkaloids, phenolic compounds, flavonoids, glycosides, diterpenes , triterpenes and saponins and absence of tannins. The chloroform extract of *Nyctanthes arbor-tristis* leaves showed presence of steroids, phenolic compounds, flavonoids, diterpenes and triterpenes and absence of alkaloids, glycosides, tannins and saponins.

Acute Toxicity Study. Upto 14 days of observation period ethanolic, hydroethanolic, aqueous, and chloroform extracts of *Nyctanthes arbor-tristis* leaves did not show any behavioural alteration, gross abnormality or symptoms of toxicity. And mortality was absent within 48 hours for each of the extract administered @ 2000 mg/kg body weight. The extracts were considered to be safe up to a maximum dose of 2000 mg/kg.

Eddy's Hot Plate Method. The analgesic activity of the ethanolic, hydroethanolic, aqueous and chloroform extracts of the leaves of *Nyctanthes arbor-tristis* against thermally induced pain was evaluated using Eddy's hot plate analgesiometer. Mean reaction time at 0, 15, 30, 45, 60, 90, 120 and 150 min was calculated after giving 250, 500, 1000 mg/kg body weight of ethanolic, hydroethanolic, aqueous and chloroform leaf extracts of *Nyctanthes arbor-tristis*. There was variable but significant ($p<0.05$) increase in reaction time in mice after treatment with all the leaf extracts of *Nyctanthes arbor-tristis* when compared with the control. All the three doses of NAEE, NAHE, NAAE and NACE showed significant ($p<0.05$) increase in reaction time at 90 min which was comparable to the standard drug. But after 90 min upto 150 min of observation there was decrease in reaction time in all the three doses of NAEE, NAHE, NAAE, NACE and standard drug NAEE at 250, 500 and 1000 mg/kg body weight showed maximum reaction time at 90 min *i.e.* $21.67_{c}^{AC}\pm0.56$, $12.00_{bc}^{EF}\pm0.58$ and $24.33_{d}^{AC}\pm0.80$ respectively. The standard drug (Tramadol hydrochloride) also showed maximum reaction time at 90 min i.e. $43.67_{e}^{B}\pm9.15$. NAEE at 250 and 500 mg/kg body weight showed significant difference in reaction time at 90 min, whereas the difference in reaction time was non-significant with 1000 mg/kg body weight. However, NAEE at all the doses used showed significant difference with standard drug. The reaction time was not dose dependent in NAEE treated group. NAHE at 250, 500 and 1000 mg/kg body weight showed maximum reaction time at 90 min *i.e.* $27.67_{e}^{C}\pm1.99$, $44.33_{e}^{B}\pm1.99$ and $76.17_{e}^{D}\pm2.39$ respectively. NAHE at 250, 500 and 1000 mg/kg body weight showed significant ($P<0.05$) difference in reaction time at 90 min whereas NAHE at 500 mg/kg body weight showed non-significant reaction time at 90

min with standard drug and at 1000 mg/kg body weight showed maximum reaction time of 76.17_{e}^{D}±2.39 which was comparatively higher to the standard drug. The increase in reaction time was dose dependent in NAHE treated group. NAAE at 250, 500 and 1000 mg/kg body weight showed maximum reaction time at 90 min *i.e.* 18.50_{c}^{AEF}±0.56, 21.33_{d}^{AC}±0.42 and 22.83_{e}^{AC}±0.48 respectively. The increase in reaction time was dose dependent in NAAE treated group. NACE at 250, 500 and 1000 mg/kg body weight showed maximum reaction time at 90 min *i.e.* 21.50_{c}^{AC}±0.43, 11.50_{bce}^{F}±0.43 and 23.83_{d}^{AC}±0.87 respectively. NACE at 250 and 1000 mg/kg body weight showed non-significant reaction time at 90 min whereas at 500 mg/kg body weight showed significant (P<0.05) reaction time with 250 and 1000 mg/kg doses. The increase in reaction time was not dose dependent in NACE treated group. Increase in reaction time indicated increase in analgesic activity.

Tail clip method. The analgesic activity of the ethanolic, hydroethanolic, aqueous and chloroform extracts of *Nyctanthes arbor-tristis* leaf extracts was evaluated using tail clip method. The leaf extract of NAEE, NAHE,NAAE and NACE at 250 , 500 & 1000 mg/kg body weight showed significant (P<0.05) increase in reaction time upto 90 min of observation period as compared to control. NAEE at 250, 500 and 1000 mg/kg body weight showed maximum reaction time at 90 min *i.e.* 8.50_{d}^{EFG}±0.56, 9.83_{d}^{BC}±0.31 and 8.83_{d}^{CEF}±0.40 respectively. The standard drug (Tramadol hydrochloride) also showed maximum reaction time at 90 min *i.e.* 10.67_{d}^{B}±0.33. NAEE at 250 and 1000 mg/kg body weight showed non-significant reaction time at 90 min whereas at 500 mg/kg body weight it showed significant (P< 0.05) reaction time with 250 mg/kg body weight but non-significant reaction time with 1000 mg/kg body weight. NAEE at 500 mg/kg body weight showed maximum increase in reaction time which was non-significant with the standard drug at 90 min of observation period. NAHE at 250, 500 and 1000 mg/kg body weight showed maximum reaction time at 90 min *i.e.* 9.33_{d}^{CE} ±0.49, 12.17_{d}^{D}±0.95 and 9.67_{d}^{BC}±0.33 respectively. NAHE at 250 and 1000 mg/kg body weight showed non-significant reaction time at 90 min whereas at 500 mg/kg body weight showed significant (P<0.05) reaction time with 250 and 1000 mg/kg doses. NAEE at 500 mg/kg body weight showed maximum increase in reaction time which was non-significant to the standard drug at 90 min of observation period. NAAE at 250, 500 and 1000 mg/kg body weight showed maximum reaction time at 90 min *i.e.* 6.17_{d}^{H} ±0.17, 7.50_{d}^{GIJ} ±0.22 and 6.67_{d}^{HI}±0.33 respectively. NAAE at 250, 500 and 1000 mg/kg body weight showed non-significant reaction time at 90 min of observation period. NACE at 250, 500 and 1000 mg/kg body weight showed maximum reaction time at 90 min *i.e* 8.50_{d}^{EJK}±0.22, 9.17_{d}^{CFK} ±0.31 and 9.83_{d}^{BC}±0.48 respectively. NACE at 250 and 500 mg/kg body weight showed non-significant reaction time at 90 min whereas at 250 mg/kg body weight showed significant (P<0.05) reaction time with 1000 mg/kg body weight but 500 mg/kg body weight showed non- significant reaction time with 1000 mg/kg body weight. However, increase in reaction time of all the group was significant (p<0.05) upto 90 min of observation period. Increase in reaction time indicated increase in analgesic activity. The increase in reaction time was not dose dependent in NAEE, NAHE, NAAE treated group, but it was dose dependent in NACE treated group.

Acetic acid induced writhing method. The analgesic activity of the ethanolic, hydroethanolic, aqueous and chloroform extracts of *Nyctanthes arbor-tristis* against chemically induced pain was evaluated using acetic acid induced writhing method. Leaf extracts of NAEE, NAHE, NAAE and NACE at 1000 mg/kg body weight showed significant (p<0.05) reduction in writhing at 15 min after administration of acetic acid intraperitoneally as compared to control and 250 and 500 mg/kg doses. The control and standard showed 32.33_{a}^{AC} ±1.99 and 10.50_{a}^{B} ±1.67 number of writhing's respectively at 15 min. The mean inhibition number of writhing of NAEE, NAHE, NAAE and NACE at 1000 mg/kg body weight were recorded as 5.17_{a}^{FI} ±1.05, 6.33_{a}^{BF}±1.12,8.17_{a}^{BI} ±0.79 and 5.00_{a}^{FI} ±1.13 respectively at 15 min. All the four extracts of *Nyctanthes arbor-tristis* at 1000 mg/kg body weight showed significant (P<0.05) inhibition of writhing at 15 min which was comparable to the standard drug Melonex. Analgesic activity of different extracts of *Nyctanthes arbor-tristis* in different method is displayed in Table 1-12.

Table 1 : Analgesic activity of ethanolic leaf extract of *nyctanthes arbor-tristis* by Eddy's hot plate method.

Group	Time (min)							
	0	15	30	45	60	90	120	150
Control	7.83_{a}^{AB}±1.14	10.83_{ab}^{A}±4.93	9.83_{acd}^{AD}±2.06	20.83_{e}^{A}±2.63	16.17_{bce}^{AF}±3.61	19.33_{e}^{AE}±3.71	15.83_{bde}^{AB}±1.78	16.00_{bde}^{AB}±0.97
Standard (Tramadol hydrochloride)	13.67_{a}^{A}±1.09	32.50_{b}^{B}±7.97	32.34_{b}^{B}±8.02	17.20_{ac}^{AB}±8.03	28.76_{bd}^{B}±10.89	43.67_{e}^{B}±9.15	19.61_{af}^{A}±6.70	21.56_{cdf}^{A}±8.52
NAEE (250 mg/kg)	3.50_{a}^{AC}±0.56	10.00_{ab}^{A}±0.86	16.17_{bc}^{A}±0.91	18.50_{c}^{AF}±0.76	19.83_{c}^{AC}±0.60	21.67_{c}^{AC}±0.56	8.50_{d}^{BE}±0.76	4.00_{d}^{D}±0.58
NAEE (500 mg/kg)	2.83_{a}^{BC}±0.60	5.00_{ab}^{A}±0.58	7.17_{ab}^{D}±0.60	8.67_{a}^{G}±0.42	10.33_{a}^{F}±0.42	12.00_{bc}^{EF}±0.58	6.67_{ac}^{E}±0.67	3.17_{a}^{D}±0.31
NAEE (1000 mg/kg)	3.17_{a}^{BC}±0.48	5.33_{ab}^{A}±0.42	12.17_{bce}^{AD}±0.70	19.00_{cdf}^{AH}±0.58	21.33_{d}^{AB}±0.61	24.33_{d}^{AC}±0.80	12.33_{bfg}^{AEB}±0.84	5.67_{aeg}^{D}±0.56

Table 2: Analgesic activity of hydroethanolic leaf extract of *Nyctanthes arbor-tristis* by Eddy's hot plate method.

Group	Time (min)							
	0	15	30	45	60	90	120	150
Control	7.83_{a}^{AB}±1.14	10.83_{ab}^{A}±4.93	9.83_{acd}^{AD}±2.06	20.83_{e}^{A}±2.63	16.17_{bce}^{AF}±3.61	19.33_{e}^{AE}±3.71	15.83_{bde}^{AB}±1.78	16.00_{bde}^{AB}±0.97
Standard (Tramadol hydrochloride)	13.67_{a}^{A}±1.09	32.50_{b}^{B}±7.97	32.34_{b}^{B}±8.02	17.20_{ac}^{AB}±8.03	28.76_{bd}^{B}±10.89	43.67_{e}^{B}±9.15	19.61_{af}^{A}±6.70	21.56_{cdf}^{A}±8.52
NAHE (250 mg/kg)	4.67_{a}^{BC}±0.67	11.83_{ab}^{A}±1.62	15.00_{bc}^{AC}±8.80	18.67_{bd}^{AC}±1.98	24.50_{def}^{BC}±2.17	27.67_{e}^{C}±1.99	16.83_{b}^{A}±1.85	9.00_{acf}^{B}±1.24
NAHE (500 mg/kg)	8.17_{a}^{C}±1.66	10.50_{ab}^{A}±1.23	18.00_{bc}^{C}±2.14	29.17_{d}^{D}±2.98	38.00_{e}^{D}±2.39	44.33_{e}^{B}±1.99	30.00_{d}^{C}±2.48	14.50_{ac}^{AB}±2.36
NAHE (1000 mg/kg)	10.00_{a}^{AC}±2.57	32.67_{b}^{B}±5.68	54.67_{c}^{E}±5.71	63.17_{d}^{E}±5.10	69.17_{def}^{E}±4.16	76.17_{e}^{D}±2.39	62.17_{cdf}^{D}±3.34	37.50_{ef}^{C}±5.74

Table 3: Analgesic activity of aqueous leaf extract of *Nyctanthes arbor-tristis* by Eddy's hot plate method.

Group	Time (min)							
	0	15	30	45	60	90	120	150
Control	7.83_{a}^{AB}±1.14	10.83_{ab}^{A}±4.93	9.83_{acd}^{AD}±2.06	20.83_{e}^{A}±2.63	16.17_{bce}^{AF}±3.61	19.33_{e}^{AE}±3.71	15.83_{bde}^{AB}±1.78	16.00_{bde}^{AB}±0.97
Standard (Tramadol hydrochloride)	13.67_{a}^{A}±1.09	32.50_{b}^{B}±7.97	32.34_{b}^{B}±8.02	17.20_{ac}^{AB}±8.03	28.76_{bd}^{B}±10.89	43.67_{e}^{B}±9.15	19.61_{af}^{A}±6.70	21.56_{cdf}^{A}±8.52
NAAE (250 mg/kg)	2.67_{a}^{BC}±0.33	5.00_{ab}^{A}±0.58	7.00_{ab}^{D}±0.58	11.33_{bcd}^{BCGI}±0.67	14.83_{cd}^{AF}±0.70	18.50_{c}^{AEF}±0.56	7.67_{abd}^{BF}±0.67	3.67_{ab}^{D}±0.49
NAAE (500 mg/kg)	3.83_{a}^{BC}±0.60	6.67_{ab}^{A}±0.67	9.17_{ac}^{AD}±0.31	12.67_{bc}^{BCFGH}±0.67	15.83_{cd}^{AFG}±0.91	21.33_{d}^{AC}±0.42	9.00_{ac}^{BF}±0.58	5.00_{a}^{D}±0.58
NAAE (1000 mg/kg)	2.83_{a}^{BC}±0.40	5.17_{ab}^{A}±0.31	11.00_{bc}^{AD}±0.58	15.00_{cd}^{AFG}±0.58	20.17_{de}^{AC}±0.60	22.83_{e}^{AC}±0.48	12.17_{bdf}^{AEF}±0.79	5.17_{acf}^{D}±0.60

Table 4: Analgesic activity of chloroform leaf extract of *Nyctanthes arbor-tristis* by Eddy's hot plate method.

Group	Time (min)							
	0	15	30	45	60	90	120	150
Control	7.83_{a}^{AB}±1.14	10.83_{ab}^{A}±4.93	9.83_{acd}^{AD}±2.06	20.83_{e}^{A}±2.63	16.17_{bce}^{AF}±3.61	19.33_{e}^{AE}±3.71	15.83_{bde}^{AB}±1.78	16.00_{bde}^{AB}±0.97
Standard (Tramadol hydrochloride)	13.67_{a}^{A}±1.09	32.50_{b}^{B}±7.97	32.34_{b}^{B}±8.02	17.20_{ac}^{AB}±8.03	28.76_{bd}^{B}±10.89	43.67_{e}^{B}±9.15	19.61_{af}^{A}±6.70	21.56_{cdf}^{A}±8.52
NACE (250 mg/kg)	3.33_{a}^{BC}±0.49	10.33_{ab}^{A}±0.80	16.17_{bc}^{A}±0.91	18.33_{c}^{AFI}±0.71	20.17_{c}^{AC}±0.87	21.50_{c}^{AC}±0.43	8.33_{ab}^{BF}±0.84	8.50_{d}^{EJK}±0.22
NACE (500 mg/kg)	2.50_{a}^{BC}±0.43	5.00_{ab}^{A}±0.58	6.83_{ac}^{D}±1.37	9.00_{ac}^{G}±0.58	10.50_{bcd}^{F}±0.43	11.50_{bce}^{F}±0.43	6.67_{ade}^{EF}±0.67	9.17_{d}^{CFK}±0.31
NACE (1000 mg/kg)	2.83_{a}^{BC}±0.40	5.00_{ab}^{A}±0.26	12.17_{bc}^{AD}±0.79	18.83_{cde}^{AFI}±0.48	21.33_{d}^{ABG}±0.49	23.83_{d}^{AC}±0.87	12.00_{be}^{AEF}±0.86	5.83_{ab}^{D}±0.48

Table 5: Analgesic activity of ethanolic leaf extract of *Nyctanthes arbor-tristis* by tail clip method.

Group	Time (min)			
	0	30	60	90
Control	2.50_{a}^{A} ±0.43	3.17_{ab}^{AF} ±0.48	4.17_{b}^{A} ±0.60	3.17_{ab}^{A} ±0.48
Standard (Tramadol hydrochloride)	1.83_{a}^{AB} ±0.31	4.83_{b}^{B} ±0.31	8.50_{c}^{B} ±0.56	10.67_{d}^{B}±0.33
NAEE (250 mg/kg)	1.83_{a}^{AB}±0.40	4.67_{b}^{BD} ±0.33	6.67_{c}^{DE} ±0.67	8.50_{d}^{EFG}±0.56
NAEE (500 mg/kg)	1.67_{a}^{AB}±0.33	4.83_{b}^{B} ±0.31	7.00_{c}^{EF}±0.37	9.83_{d}^{BC}±0.31
NAEE (1000mg/kg)	1.17_{a}^{B}±0.17	4.67_{b}^{BD}±0.33	7.67_{c}^{BCEI} ±0.33	8.83_{d}^{CEF}±0.40

Table 6: Analgesic activity of hydroethanolic leaf extract of *Nyctanthes arbor-tristis* by tail clip method.

Group	Time (min)			
	0	30	60	90
Control	2.50_{a}^{A} ±0.43	3.17_{ab}^{AF} ±0.48	4.17_{b}^{A} ±0.60	3.17_{ab}^{A} ±0.48
Standard (Tramadol hydrochloride)	1.83_{a}^{AB} ±0.31	4.83_{b}^{B} ±0.31	8.50_{c}^{B} ±0.56	10.67_{d}^{B}±0.33
NAHE (250 mg/kg)	2.17_{a}^{AB}±0.48	4.83_{b}^{BC} ±0.31	7.00_{c}^{CD}±0.37	9.33_{d}^{CE} ±0.49
NAHE (500 mg/kg)	2.00_{a}^{AB} ±0.37	5.50_{b}^{B} ±0.43	9.67_{c}^{I} ±0.88	12.17_{d}^{D}±0.95
NAHE (1000 mg/kg)	1.83_{a}^{AB}±0.31	5.67_{b}^{B}±0.49	7.83_{c}^{BCI}±0.31	9.67_{d}^{BC}±0.33

Table 7: Analgesic activity of aqueous leaf extract of *Nyctanthes arbor-tristis* by tail clip method.

Group	Time (min)			
	0	30	60	90
Control	2.50_a^{A} ±0.43	3.17_{ab}^{AF} ±0.48	4.17_b^{A} ±0.60	3.17_{ab}^{A} ±0.48
Standard (Tramadol hydrochloride)	1.83_a^{AB} ±0.31	4.83_b^{B} ±0.31	8.50_c^{B} ±0.56	10.67_d^{B}±0.33
NAAE (250 mg/kg)	1.33_a^{B} ±0.21	3.33_b^{AF} ±0.21	4.50_c^{AG} ±0.22	6.17_d^{H} ±0.17
NAAE (500 mg/kg)	1.33_a^{B}±0.21	3.50_b^{AF}±0.22	5.50_c^{GH} ±0.22	7.50_d^{GIJ} ±0.22
NAAE (1000 mg/kg)	1.17_a^{B}±0.17	2.83_b^{A} ±0.31	4.17_c^{A} ±0.31	6.67_d^{HI}±0.33

Table 8: Analgesic activity of chloroform leaf extract of *Nyctanthes arbor-tristis* by tail clip method.

Group	Time (min)			
	0	30	60	90
Control	2.50_a^{A} ±0.43	3.17_{ab}^{AF}±0.48	4.17_b^{A} ±0.60	3.17_{ab}^{A} ±0.48
Standard (Tramadol hydrochloride)	1.83_a^{AB} ±0.31	4.83_b^{B} ±0.31	8.50_c^{B} ±0.56	10.67_d^{B}±0.33
NACE (250 mg/kg)	1.17_a^{B} ±0.17	3.33_b^{AF} ±0.21	6.17_c^{DFG}±0.31	8.50_d^{EJK}±0.22
NACE (500 mg/kg)	1.17_a^{B}±0.17	4.33_b^{CDF}±0.21	6.50_c^{DFH}±0.22	9.17_d^{CFK} ±0.31
NACE (1000 mg/kg)	1.33_a^{B}±0.21	3.83_b^{ACD}±0.31	6.33_c^{DFH}±0.33	9.83_d^{BC}±0.48

Table 9: Analgesic activity of ethanolic leaf extract of *Nyctanthes arbor-tristis* by acetic acid induced writhing method.

Group	Time (min)	
	15 min	30 min
Control	32.33_a^{AC} ±1.99	81.17_b^{A} ±0.70
Standard(Melonex)	10.50_a^{B} ±1.67	21.33_b^{B} ±0.71
NAEE (250 mg/kg)	31.00_a^{CG} ±1.06	64.50_b^{C} ±1.67
NAEE (500 mg/kg)	16.83_a^{H} ± 1.17	42.17_b^{EH} ±1.25
NAEE (1000 mg/kg)	5.17_a^{FI} ±1.05	20.00_b^{BF} ±1.77

Table 10: Analgesic activity of hydroethanolic leaf extract of *Nyctanthes arbor-tristis* by acetic acid induced writhing method.

Group	Time (min)	
	15	30
Control	32.33_a^{AC} ±1.99	81.17_b^{A} ±0.70
Standard (Melonex)	10.50_a^{B} ±1.67	21.33_b^{B} ±0.71
NAHE (250 mg/kg)	36.83_a^{AD} ±1.89	64.33_b^{C}±1.50
NAHE (500 mg/kg)	24.17 a^{E} ±1.08	64.33_b^{C}±1.50
NAHE(1000 mg/kg)	6.33_a^{BF} ±1.12	36.00_b^{D}±2.05

Table 11: Analgesic activity of aqueous leaf extract of *Nyctanthes arbor-tristis* by acetic acid induced writhing method.

Group	Time (min)	
	15	30
Control	32.33±1.99	81.17 ±0.70
Standard (Melonex)	10.50±1.67	21.33±0.71
NAAE (250 mg/kg)	37.33±1.84	75.17 ±2.15
NAAE (500 mg/kg)	15.83±1.60	42.50±2.06
NAAE (1000 mg/kg)	8.17 ±0.79	16.00±0.97

Table 12: Analgesic activity of chloroform leaf extract of *Nyctanthes arbor-tristis* by acetic acid induced writhing method.

Group	Time (min)	
	15	30
Control	32.33_a^{AC} ±1.99	81.17_b^{A} ±0.70
Standard (Melonex)	10.50_a^{B} ±1.67	21.33_b^{B} ±0.71
NAAE (250 mg/kg)	37.33_a^{D} ±1.84	75.17_b^{G} ±2.15
NAAE (500 mg/kg)	15.83_a^{H} ±1.60	42.50_b^{E} ±2.06
NAAE (1000 mg/kg)	8.17_a^{BI} ±0.79	16.00_b^{F} ±0.97

Table 13: Analgesic activity of chloroform leaf extract of *Nyctanthes arbor-tristis* by acetic acid induced writhing method.

Group	Time (min)	
	15	30
Control	32.33_a^{AC} ±1.99	81.17_b^{A} ±0.70
Standard (Melonex)	10.50_a^{B} ±1.67	21.33_b^{B} ±0.71
NACE (250 mg/kg)	26.50_a^{EG}±2.88	64.17_b^{CH}±2.98
NACE (500 mg/kg)	23.83_a^{E} ±1.14	34.83_b^{D}±1.60
NACE (1000 mg/kg)	5.00_a^{FI} ±1.13	17.00_b^{BF} ±1.46

N.B.: *All values are Mean ± S.E. (n=6)

* Means with different superscripts (capital letters) within a column and different subscripts within rows (small letters) differ significantly (P< 0.05)

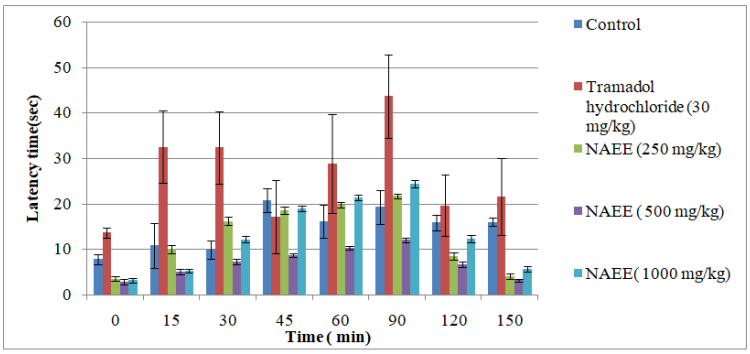

Fig. 1. Graph representing latency time of mice treated with ethanolic leaf extract of *Nyctanthes arbor-tristi*s and tramadol hydrochloride by eddy's hot plate method.

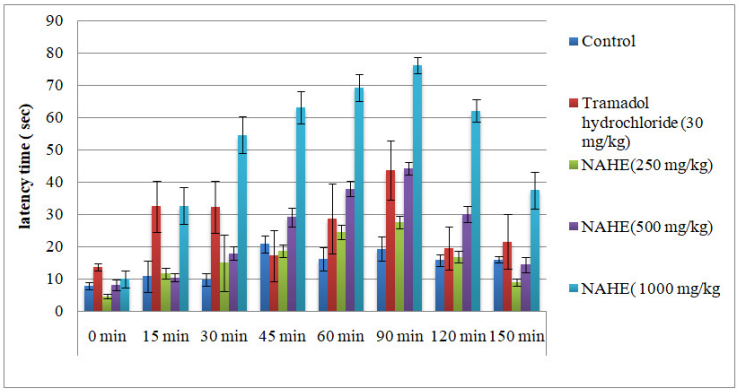

Fig. 2. Graph representing latency time of mice treated with hydroethanolic leaf extract of *Nyctanthes arbor-tristi*s and tramadol hydrochloride by eddy's hot plate method.

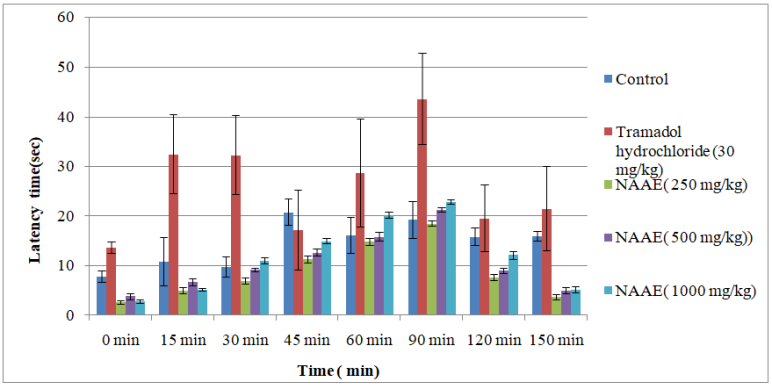

Fig. 3. Graph representing latency time of mice treated with aqueous leaf extract of *Nyctanthes arbor-tristi*s and tramadol hydrochloride by eddy's hot plate method.

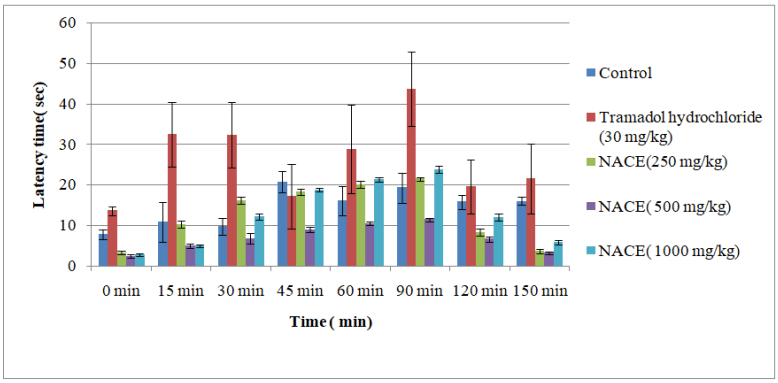

Fig. 4. Graph representing latency time of mice treated with chloroform leaf extract of *Nyctanthes arbor-tristi*s and tramadol hydrochloride by eddy's hot plate method.

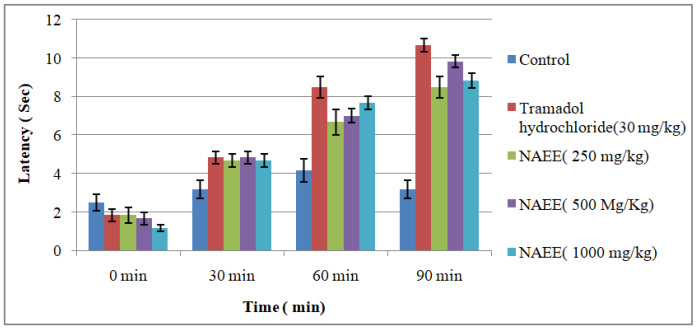

Fig. 5. Graph representing latency time of mice treated with ethanolic leaf extract of *Nyctanthes arbor-tristi*s and tramadol hydrochloride by tail clip method.

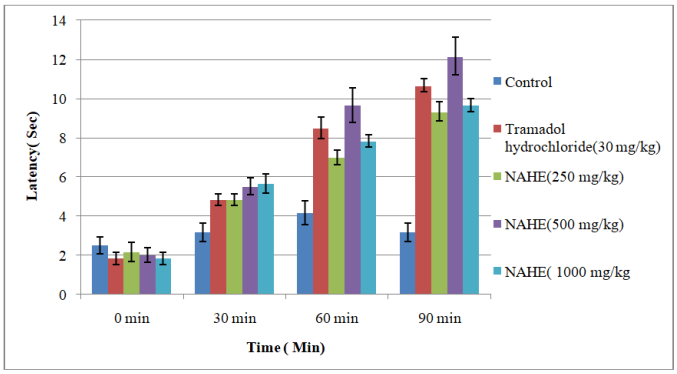

Fig. 6. Graph representing latency time of mice treated with hydroethanolic leaf extract of *Nyctanthes arbor-tristi*s and tramadol hydrochloride by tail clip method.

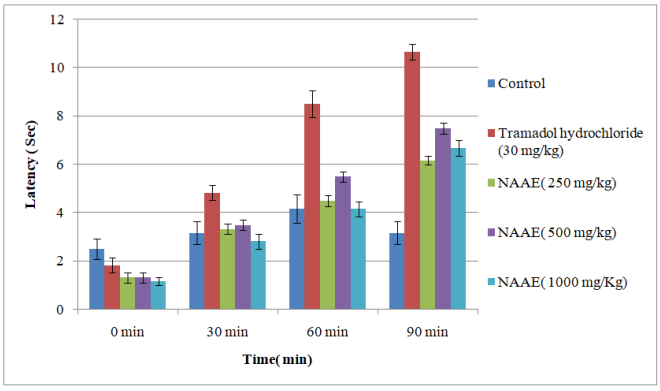

Fig. 7. Graph representing latency time of mice treated with aqueous leaf extract of *Nyctanthes arbor-tristi*s and tramadol hydrochloride by tail clip method.

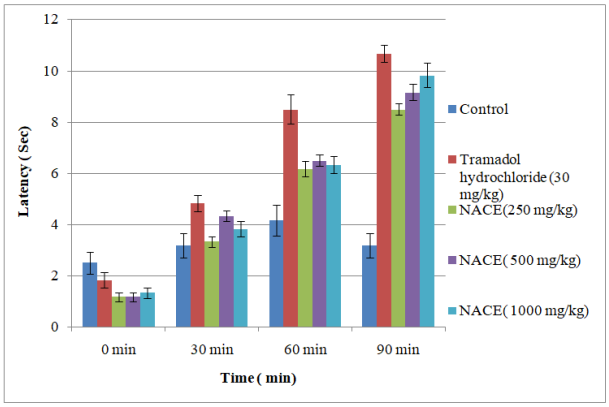

Fig. 8. Graph representing latency time of mice treated with chloroform leaf extract of *Nyctanthes arbor-tristi*s and tramadol hydrochloride by tail clip method.

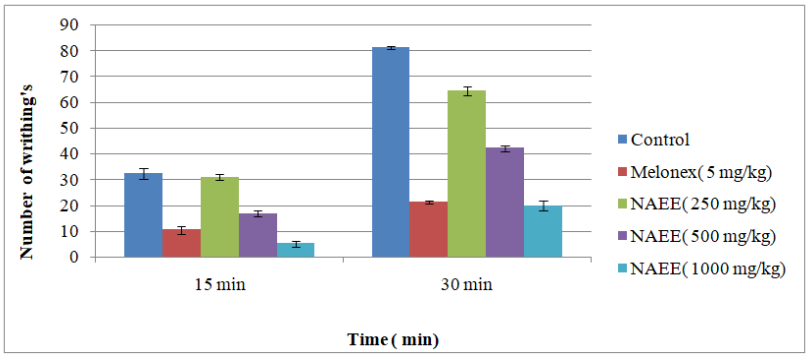

Fig. 9. Graph representing mean number of writhing in mice treated with ethanolic leaf extract of *Nyctanthes arbor-tristis* and melonex in acetic acid-induced writhing test.

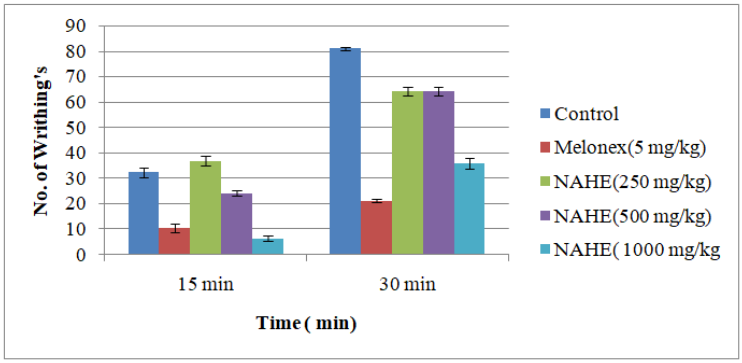

Fig. 10. Graph representing mean number of writhing in mice treated with hydroethanolic leaf extract of *Nyctanthes arbor-tristis* and melonex in acetic acid-induced writhing test**.**

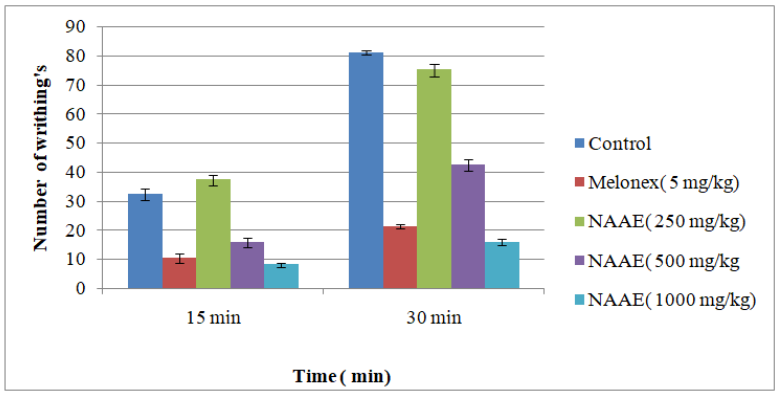

Fig. 11. Graph representing mean number of writhing in mice treated with aqueous leaf extract of *Nyctanthes arbor-tristis* and melonex in acetic acid-induced writhing test.

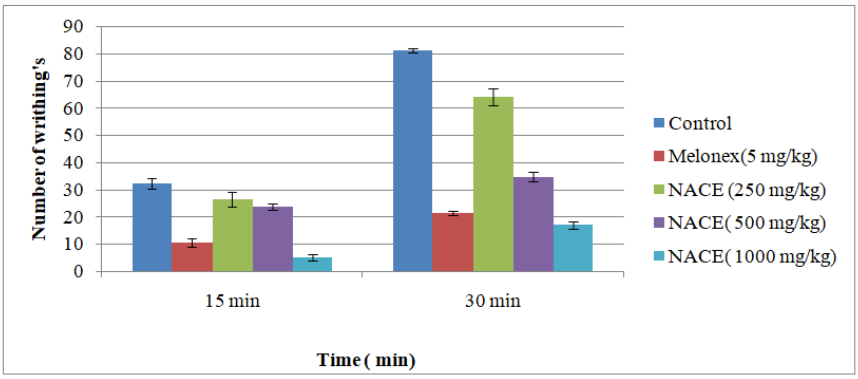

Fig. 12. Graph representing mean number of writhing in mice treated with chloroform leaf extract of *Nyctanthes arbor-tristis* and melonex in acetic acid-induced writhing test.

DISCUSSION

Plants are known to have a range of chemical compounds with a wide range of biological activity (Klocke, 1989). Some of the chemicals have long been used in animal and human medical systems due to their specific and unique qualities. Natural plant chemicals, which influence metabolic pathways, are still the most common source of medicine for animal and human health management.In the present study qualitative phytochemical tests, acute toxicity study, analgesic study was carried out with ethanolic, hydroethanolic, aqueous and chloroform extracts of the leaves of *Nyctanthes arbor-tristis*. Preliminary phytochemical screening confirmed the presence or absence of some important secondary metabolites *viz.* alkaloids, steroids, saponins, tannins,flavonoids, terpenoids, diterpenes, triterpenes, phenol and glycosides in leaves by specific qualitative tests. Presence of alkaloids was determined by the formation of precipitates by Mayer's, Wagner's and Hager's test. Extracts were screened for presence of steroids, glycosides, phenol, tannins, flavonoids, diterpenes, triterpenes, saponins using Salkowski test, Sodium hydroxide test, Benedict's test, Phenolic test, Ferric chloride test, Lead acetate test, Diterpenes, Salkowski test, Liberman Burchardts test and Foam test. The ethanolic extract of *Nyctanthes arbor-tristis* leaves showed presence of glycosides, steroid, flavonoids, diterpenes, triterpenes, phenol and saponin. Presence of alkaloids was found in Hager's test and absence in Mayer's, Wagner's and Dragendroff's test. Presence of steroids, phenol, alkaloids, saponin, tannin, terpenoid and flavonoids in leaf extracts had already been confirmed by Ramachandran *et al.* (2014); Chidi *et al*. (2015). Sathiya *et al*. (2008); Hazarika (2019) also reported the presence of steroids, alkaloids, phenolic compounds, flavonoids and glycosides in ethanolic extract of the leaves of *Nyctanthes arbor-tristis.* Presence of alkaloids was found in Mayer's test and was absent in Hager's Wagner's and Dragendroff's test. The hydroethanolic extract of the leaves showed presence of almost all types of phytoconstituents. A similar finding was also observed by Shruti *et al.*(2009); Hazarika (2019), where they reported presence of hydroethanolic leaf extract showed the presence of carbohydrates, flavonoids, phenolic compounds, steroids, alkaloids, triterpenes and tannins in the hydroethanolic extract of *Nyctanthes arbor-tristis*. The aqueous extract of *Nyctanthes arbortristis* leaves showed presence of steroid, glycoside, flavonoids, diterpenes, triterpenes and saponins and absence of tannins. Presence of alkaloids was found in Mayer's test and was absent in Hager's Wagner's and Dragendroff's test. Sharma *et al.* (2019); Hazarika (2019) also confirmed the presence of alkaloids, glycosides, flavonoids and terpenoids in the aqueous leaf extract of *Nyctanthes arbor-tristis.* The chloroform extract of *Nyctanthes arbor-tristis* leaves showed presence of flavonoids, diterpenes and triterpenes and absence of steroids, alkaloids, glycosides, tannins and saponins. Some of these phytoconstituents may be responsible for potent analgesic activity. Acute oral toxicity study was conducted according to OECD Test Guidelines 425 in female mice for all the four type of leaf extracts (ethanolic, hydroethanolic, aqueous and chloroform) at 2000 mg/kg body weight, as single oral dose. There was no mortality, no change in behavior and body weight in the 14 days observation period. Khan *et al.* (2017) in their study on *Nyctanthes arbor-tristis* found that ethanolic and methanolic extract at the doses of 50 – 3000 mg/kg orally was not toxic in mice and did not show any signs of abnormality and the LD50 was found to be more than 3000 mg/kg. Omkar *et al.* (2006) studied the LD50 of the ethanolic extract from the orange tubular calyx of the *Nyctanthes arbor-tristis* flower to be 1500 mg/kg. In a similar acute toxicity study by Sasmal *et al*., 2007, the ethanol extract of leaves, seeds and flowers were also found to be safe @2000 mg/kg upon intraperitoneal administration in rats . From the present study, it can be concluded that *N.arbor-tristis* leaf extract is safe for oral administration without any toxic symptoms. The *Nyctanthes arbor-tristis* leaf extracts showed significant ($p<0.05$) analgesia, both centrally and peripherally. NAEE, NAHE, NAAE and NACE at 250,500 and 1000 mg/kg body weight showed increase in analgesic activity in Eddy's hot plate method, Tail clip method and Acetic acid writhing method. In eddy's hot plate method all the four types of extracts of *Nyctanthes arbor-tristis* at 1000 mg/kg body weight showed significant increase in reaction time at 90 min. In tail clip method NAEE, NAHE and NACE at 500 mg/kg body weight showed significant increase in reaction time compared to the 1000 mg/kg body weight at 90 min. But NACE at 500 and 1000 mg/kg body weight showed similar increase in reaction time. In Acetic acid induced writhing method NAEE, NAHE, NAAE and NACE at 1000 mg/kg body weight showed significant ($p<0.05$) inhibition of writhing response at 15 min which was comparable to the standard drug Melonex. Bordoloi *et al.* (2016) found that ethanolic leaf extract of *Nyctanthes arbor-tristis* produced significant analgesia, both centrally and peripherally. He observed that the ethanolic extract of *Nyctanthes arbor-tristis* leaves at doses of 200 mg/kg and 400 mg/kg increased the pain threshold significantly at 30, 60, 90, and 120 min of administration when compared to the control group. In acetic acid induced writhing in mice pretreated with 100, 200 and 400 mg/kg ethanolic extract of *Nyctanthes arbor-tristis* significantly reduced abdominal writhing in mice when compared to the negative control group reducing the mean number of writhing from 30.5± 7.46 in the negative group to 9.0± 4.29 at the dose of 400 mg/kg. These findings are in agreement to the present study wherein all the doses of all the extracts used in hot plate method and tail clip method showed significant increase in analgesic activity which was maximum at 90 min of observation

period. Also in acetic-acid induced writhing method *Nyctanthes arbor-tristis* leaf extracts could effectively reduce the abdominal writhing in mice at 15 min post administration of acetic acid. This indicates that the leaf extracts of *Nyctanthes arbor-tristis* possess central as well as peripheral analgesic activity. A similar study carried out by Saxena and his colleagues in 1987 found that water soluble portion of an ethanolic extract of *Nyctanthes arbor-tristis* was virtually devoid of analgesic activity in models where superficial nociceptors were stimulated, *i.e*. the tail-flick and tail-clip methods. However, it was interesting to note that *Nyctanthes arbor-tristis* exhibited significant antinociceptive activity in the mouse writhing test which is particularly sensitive to analgesic-antipyretic agents. Analgesia produced by aspirin has been explained by inhibition of the synthesis of endogenous prostaglandins normally produced as a result of mechanical or chemical stimulation (Ferreira, 1972). Thus, it seems likely that *Nyctanthes arbor-tristis* may be acting through a similar mechanism. Pattanayak *et al.* (2013) revealed that ethanolic extract showed significant central as well as peripheral analgesic activity. Peripheral analgesic activity of *Nyctanthes arbortristis* leaves extract at 200mg/kg and 400 mg/kg doses with significant increase in the efficacy with increased dose (400 mg/kg) as compared to lower dose (200 mg/kg), in a dose dependent manner. For central analgesia also, the minimum effective dose was found to be 200 mg/kg body weight. With increase in the dose (400 mg/kg), significant increase in the analgesic activity was seen after two hours of injecting the drug. Presence of flavonoids has been reported in *Nyctanthes arbor-tristis* and flavonoids are known to inhibit prostaglandin synthesis by inhibiting cyclooxygenase enzyme. Since prostaglandins are involved in pain perception and are inhibited by flavonoids, it would be suggested that reduced availability of prostaglandins by flavonoids of *Nyctanthes arbor-tristis* ethanolic leaf extract might be responsible for its analgesic effect. This is in agreement with the findings of the present study where NAEE, NAHE, NAAE and NACE at 250, 500 and 1000 mg/kg body weight showed significant increase in analgesic activity in a dose dependent manner.

CONCLUSION

Ethanolic, hydroethanolic, aqueous and chloroform leaf extracts of *Nyctanthes arbor-tristis* was found to possess significant narcotic analgesic activity when tested by eddy's hot plate and tail clip method. The four leaf extracts was also found to possess significant non-narcotic analgesic activity in Acetic acid induced writhing test. Among all the four extracts (*i.e*. ethanolic, hydroethanolic, aqueous and chloroform extracts) of *Nyctanthes arbor-tristis* under study. Hydroethanolic extract @ 1000mg/kg body weight showed better analgesic activity in comparison to other three extracts in the present study. It may be due to presence of flavonoids and steroids in the extract which inhibit prostaglandin synthesis. The present study indicated that leaves of *Nyctanthes arbor-tristis* can be used as an alternative to analgesic drug. However further studies are necessary to isolate active ingredients responsible for therapeutic effect and dose determination.

FUTURE SCOPE

1. This study was the preliminary step towards screening of *Nyctanthes arbor-tristis* plant and it paves the way for further attention and research to identify the active compounds responsible for biological/pharmacological activities.
2. Further investigations are needed to find out the actual molecular mechanism of the active constituents present in the leaves of *Nyctanthes arbor-tristis.*

Abbreviation. NAEE: *Nyctanthes arbor-tristis* ethanolic extract NAHE: *Nyctanthes arbor-tristis* hydroethanolic extract NAAE: *Nyctanthes arbor-tristis* aqueous extract NACE: *Nyctanthes arbor-tristis* chloroform extract NSAIDs: Non-steroidal Anti-inflammatory Drug OECD: Organization for Economic Co-operation and Development

Animal welfare and ethics statement. The animal experimentation was carried out according to the Committee for the Purpose of Control and Supervision of experimental animals (CPCSEA) guideline and Institutional Animal Ethical Committee Approved all the procedure for investing experimental pain in conscious animals.

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Conflict of Interest. None.

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