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In vivo pharmacological investigations of the crude extracts of *Calamus viminalis* (L.)

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Abstract
The present study was done to evaluate *in vivo* anti-pyretic, gastrointestinal motility, anti-nociceptive, neuropharmacological and acute toxicity effects of different leaf extracts of *Calamus viminalis* in Swiss albino mice following oral administration. *In vivo* gastrointestinal motility test revealed that both the doses of ethanol extracts (100 and 200 mg/kg body weight) and the dose of 200 mg/kg body weight of methanol extract showed maximum charcoal defection time, compared with the effect produced by standard drug. Both methanol 100 and 200 mg/kg and chloroform 200 mg/kg extracts showed significant *in vivo* anti-pyretic effect on mice. Statistically significant ($p<0.01$, $p<0.001$) results were found in case of *in vivo* anti-nociceptive activity test for the 100 and 200 mg/kg methanol extracts when compared to standard diclofenac-Na. In case of neuropharmacological activity, ethanol extract 200 mg/kg body weight showed statistically significant ($p<0.001$) result with highest time of immobility of 191.5 second. None of the extracts showed any significant *in vivo* acute toxicity effect on mice.

Keywords: Calamus viminalis, anti-pyretic, gastrointestinal motility, anti-nociceptive, neuropharmacological study and acute toxicity.

1. Introduction
A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis [1]. Recently World Health Organization suggested that approximately 80% of the rural people across the world confide on herbal remedies and as homeopathic medicines for their primary health care because of their easy availability, efficacy and specially cost effectiveness compared to modern drugs. There are more than 500 medicinal plants growing in our country, however, the inventory is not complete, and many plants with medicinal value are yet to be determined.
Calamus viminalis (*C. viminalis*) is a stems clustered, climbing plant (known as Rattan) with knee, flagellum and grouped leaflets that grows up to a height of 35 m and 4 cm in diameter and belongs to the family of Arecaceae which is one of 370 species[1] in the genus *Calamus* locally known as Khorkoijja bet in Bangladesh. *C. viminalis* is native to Indonesia but distributed in Bangladesh, India, Thailand, Malaysia, Vietnam, Combodia and Australia. It mainly grows in lowland rain forests as well as deciduous forest persisting in cleared areas and often present in villages, at 20-500 m elevation and sometimes planted below 600 m. Leaf sheaths green with dense covering of grayish or brownish hairs, with scattered, greenish or brownish, triangular, flattened spines. Fruits are whitish or yellowish, globose, to 1 cm in diameter, sometimes borne in pairs. About 20% of the known Rattan species are of commercial value. This plant has also been used in traditional medicine for the treatment of dog bite [2], urogenital and gynecological.
As a part of our continuing studies on medicinal plants of Bangladesh the organic soluble materials of the leaf extracts of *C. viminalis* were evaluated for gastrointestinal motility, anti-pyretic activity, anti-nociceptive activity and acute toxicity for the first time [3-7].

2. Materials and Methods
2.1 Collection, Identification and Processing of Plant Samples
The leaves of *C. viminalis* were collected from Dhaka, Bangladesh in October 2015 and then plant sample was submitted to the National Herbarium of Bangladesh, Mirpur-1, Dhaka for its identification and the voucher specimen is as Accession number- 42755. Leaves were sun Dried for seven days in order to remove the moisture contents and then ground into coarse

powder using high capacity grinding machine (Jaipan designer mixer grinder, jaipan, India) which was then stored in air-tight container with necessary markings for identification and kept in cool, dark and dry place for the investigation.

2.2 Extraction Procedure

The powdered plant parts (30 gm) were successively extracted in a soxhlet extractor at elevated temperature using 500 ml of distilled methanol (40-60) ºC which was followed by ethanol, and chloroform. After drying all extracts were labeled and kept in refrigerator at 4 °C for future investigation.

2.3 Experimental Animal

For the experiment Swiss albino mice of either sex, 4-5 weeks of age, weighing between 15-30 gm were collected from ICDDR, B, Mohakhali, Dhaka. Animals were maintained under standard environmental conditions [temperature: (27.0 ±1.0) ºC, relative humidity: (55-65)% and 12 hour light/12 hour dark cycle] and free access to feed and water. The animals were acclimatized to laboratory condition for one week prior to experiments. All protocols for animal experiment were approved by the institutional animal ethical committee.

2.4 Gastrointestinal (GI) Motility Determination

Forty eight Swiss Albino mice, weighing between (15-30) gm were selected and housed properly for 7 days before performing the experiment. On the test day, the animals were divided into 8 groups of 6 mice each. They were weighed and deprived of food, with free access to water. Three hours after food deprivation, the animals in group 1 received orally by gavages 5 ml/kg body weight of 0.9% NaCl (normal saline) as control group, while those in group 2 received 5 mg/kg b.w (body weight) of butapen as standard group. The other six groups received their respective doses as shown in the table 100 mg/kg body weight and 200 mg/kg doses of methanol, ethanol and chloroform extract were used. After 90 min, 0.3 ml of an aqueous suspension of 5% charcoal in normal saline was administered to each animal orally by gavages (time 90 min). Sixty minutes later they had free access to food (time 150 min). The animals were observed at 5 min intervals until feces with charcoal were eliminated (maximum time of observation was 300 min). Charcoal was observed on the feces using normal light when it was easily visible, or using a microscope to help the identification of the black spots. The results were based on the time for the charcoal to be eliminated [8].

2.5 Antipyretic Activity

Forty two Swiss Albino mice of both sexes (15-30) gm were randomly divided into 7 groups and fasted overnight before the experiment with free access to water. The normal body temperature of each mouse was measured rectally at predetermined intervals and recorded. Fever was induced according to the method described by Smith and Hambourger (1935) [9]. A lubricated thermometer probe was inserted 3-4 cm deep into the rectum and fastened to the tail by adhesive tape. Temperature was measured on digital thermometer. After measuring the basal rectal temperature, animals were injected subcutaneously with 10 ml/kg body weight of 20% w/v brewer's yeast in NSS in the dorsum of the mice. Mice were then returned to their housing cages. Eighteen hours after brewer's yeast injection, the animals were again restrained for rectal temperature recording, as described previously. Only mice that showed an increase in temperature of at least 0.5˚ to 1 ˚C were used for this study. The extracts at the doses of 100 & 200 mg/kg body weight were administered orally to four groups of animals. The control group received 10ml/kg body weight dose of vehicle (0.9% NaCl solution) and the standard group received paracetamol (50 mg/kg body weight) orally. Rectal temperature was measured at 1 hr intervals for 4 hr after the extract/drug administration. The rectal temperature of normal mice (normothermic) was also measured at 1 hr. intervals for 7 hr. as stated by Chomchuen *et al*., (2010) [10]. The results are expressed as percentage of the pre-drug temperature recorded for the same animals using the formula of Makonnan *et al*. (2003) [11].

2.6 Anti-nociceptive Activity

Anti-nociceptive activity was evaluated by tail immersion test and acetic acid induced writhing test.

2.6.1 Tail Immersion Test

The tail immersion method was used to evaluate the central mechanism of analgesic activity. Here the painful reactions in animals were produced by thermal stimulus that is by dipping the tip of the tail in hot water [12]. On the test day, Swiss albino mice were divided into 8 groups of 6 mice each. Here diclofenac Na (50 mg/kg) is used as standard drug as well. Animals were fasted for 16hours with free access to water. After administration of standard and test drugs, the basal reaction time was measured by immersing the tail tips of mice (last 1-2 cm) in hot water of water bath, where temperature was previously adjusted at 51 ºC. The actual flick response of mice that is time taken in second to withdraw it from hot water source was calculated and results were compared with control group. The latent period of the tail-flick response was determined at 0, 30, 60, 90 and 120 minute after the administration of drugs.

2.6.2 Acetic Acid Induced Writhing Test

The acetic acid writhing test in mice as described by Koster *et al*., (1959) [13], was employed with slight modification. Mice were divided into 8 groups containing 6 mice in each group. The first group was given 10 ml/kg of 1% Tween 80 intraperitoneally and served as control. Group 2 was served as standard where diclofenac Na has given to mice as dose of 50 mg/kg of body weight. Groups 3, 4 received methanol extract *C. viminalis*100 mg/kg and 200 mg/kg of body weight. Groups 5, 6 received ethanol extract *C. viminalis* 100 mg/kg and 200 mg/kg of body weight. Groups 7, 8 received chloroform extract *C. viminalis* 100 mg/kg and 200 mg/kg of body weight. Thirty minutes later each mouse was injected intraperitoneally with 0.7% acetic acid at doses of 10 ml/kg of body weight. Full writhing was not always completed by the mice. Accordingly, two half writhing were considered as one full writhing. The number of writhing responses was recorded for each mouse during a subsequent 5 min period after 15 min intra peritoneal administration of acetic acid and the mean abdominal writhing for the each group was obtained and recorded.

2.7 Neurophramacological Study
Forced swimming test is carried out in order to check the neurophramacological effects or side-effects of drug.

2.7.1 Forced Swimming Test (FST)
According to Porsolt *et al.* (1978) [14] swimming test was performed with slight modification. Animals were randomly divided into 8 groups with 6 mice on each group. Group 1 was given 10 ml/kg of 1% Tween 80 which served as control and group 2 was given 2 mg of benzyl diazepam per kg of body weight which served as standard. Groups 3, 4 received methanol extracts of *C. viminalis* at 100 and 200 mg/kg of body weight. Groups 5, 6 received ethanol extracts of *C. viminalis* at 100 and 200 mg/kg of body weight. Groups 7, 8 received chloroform extracts of *C. viminalis* at 100 and 200 mg/kg of body weight. The forced swim test was carried out on mice individually forced to swim in an open acquire water tank apparatus (29cm x 19cm x 20cm), containing 9 cm of water at 25±1 °C. The total duration of immobility during the 4-min test was scored as described. Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. The duration of immobility was recorded. Decrease in the duration of immobility during the FST was taken as a measure of antidepressant activity.

2.8 Acute Toxicity Test
The acute toxicity test in mice as described by Ecobichon (1997) [15] was employed with slight modification. Mice were kept fasting for 1-2 hours but water was provided and were divided into 6 groups containing 6 mice in each group. All mice were weighed and kept separated using separate cage. The test samples, i.e. methanol, ethanol and chloroform extracts were administered orally at different doses of 500 mg/kg, 1000 mg/kg, and 2000 mg/kg of body weight of mice. After administration of the extract solutions mortality or sign of any toxicity was observed for one hour and kept under observation for 1 week.

2.9 Statistical Analysis
Data was expressed as Mean ± SEM (Standard error of Mean). The results were analyzed statistically by ANOVA followed by Dunnet's test. Results below *p<0.05, **p<0.01 and ***p<0.001 are considered statistically significant.

3. Results and Discussions
3.1 Gastro Intestinal (GI) Motility Test
In vivo gastrointestinal motility test was conducted on methanol, ethanol and chloroform extracts on the doses of 100 mg/kg and 200 mg/kg. The duration between charcoal administration and charcoal defecation is measured for gastrointestinal motility determination. The present study revealed that, higher dose of methanol extract (200 mg/kg b.w) and the doses of ethanol extract (100 and 200mg/kg b.w) showed significant increase (***p<0.001) in gastrointestinal motility compared to the standard drug butapen (Figure 1) that is maximum defecation time. Among all the test groups, chloroform extract showed lower defecation time with (**p<0.01) than methanol and ethanol extract but higher defecation time than standard. Result indicates that the stimulating effect of the extract dependent on its concentration whereas the presence of Alkaloid in *C. viminalis* may be responsible for this effect [3].

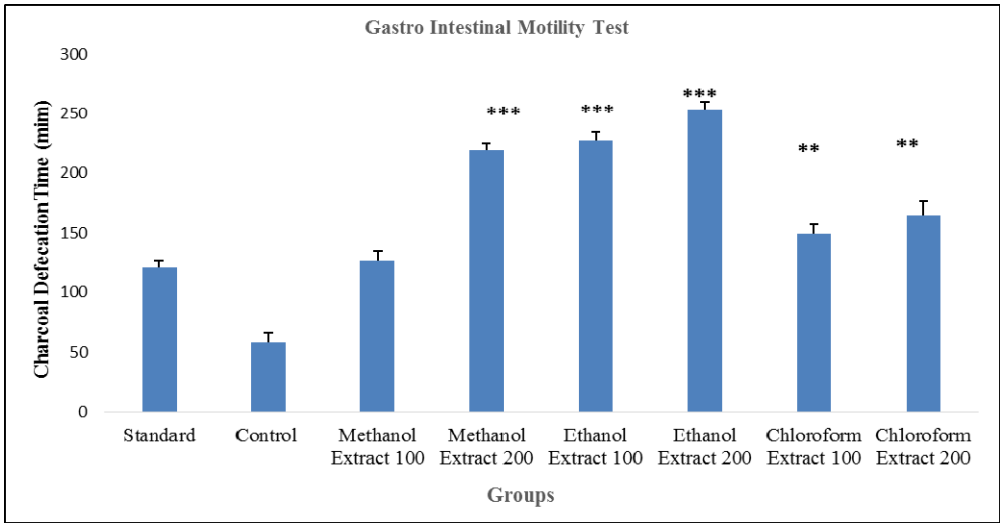

Fig 1: Time of defecation at gastro intestinal motility activity after administration of doses of control, standard and extracts

(Values are expressed as mean ± S.E.M. (n=6), **p<0.01, ***p<0.001significant when compared with the corresponding value of the Standard group, done by independent sample t-test)

3.2 Anti-pyretic Activity Test
The present study with *C. viminalis* leaf revealed that Methanol, ethanol and chloroform extract (100 mg/kg and 200 mg/kg b.w) showed significant (*p<0.05; **p<0.01; ***p<0.001) antipyretic activities in mice, so it can be said that more of active principles responsible for the antipyretic activity might be available in these three extracts (Figure 2).

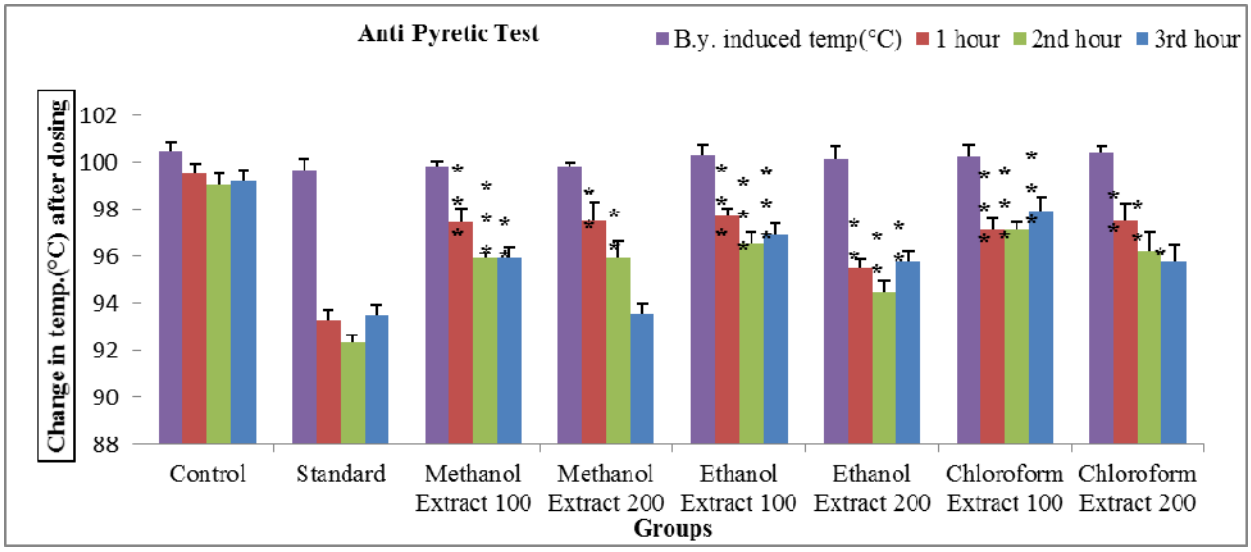

Fig 2: Comparative study of temperature changes at different time intervals using leaf extracts of *C. viminalis*

(Values are expressed as mean ± S.E.M. (n=6), **p*<0.05, ***p*<0.01, ****p*<0.001 significant when compared with the corresponding value of the Standard group, done by independent sample t-test)

3.3 Anti-nociceptive Activity Test

The anti-nociceptive activity of methanol, ethanol and chloroform extract of *C. viminalis* leaf was tested by using two models (acetic acid-induced, tail immersion test) so that both the centrally and peripherally mediated effects could be investigated.

3.3.1 Tail Immersion Test

In tail immersion method the heat itself acts as a source of pain. *In-vivo* anti-nociceptive activity test was done on 100 mg/kg and 200 mg/kg doses of methanol, ethanol and chloroform extracts of *C. viminalis* leaf which is presented in (Figure 3). Both extract of methanol significantly (**p<0.01) raised pain threshold at both doses (100 and 200 mg/kg body weight) from 60 min up to 120 min which is highly comparable with the standard drug diclofenac Na (50 mg/kg body weight). At the same time Chloroform extract of 100 mg/kg body weight with (**p<0.01 at 120 min) showed a significant decrease in tail withdrawal time. In contrast to methanol extract, Chloroform extract showed greater tail withdrawal time but ethanol extract showed greater pain threshold activity than standard at 60 min, 90 min and 120 min.

According to phytochemical screening, this anti-nociceptive effect of the leaf extract can be due to the presence of alkaloid, quinine, coumarin which are known to give analgesic effects *in-vivo* [3].

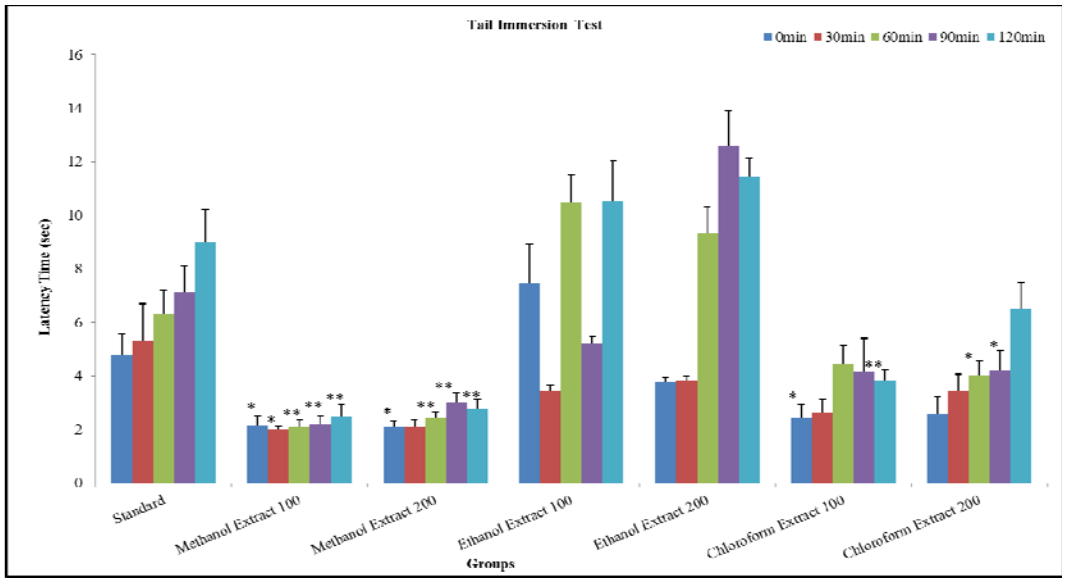

Fig 3: Graphical representation of anti-nociceptive activity through tail immersion test

(Values are expressed as mean ± S.E.M. (n=6), **p*<0.05, ***p*<0.01, ****p*<0.001 significant when compared with the corresponding value of the Standard group, done by independent sample t-test)

3.3.2 Acetic Acid Induced Writhing Test

The result of acetic acid induced writhing test for methanol, ethanol and chloroform extract of *C. viminalis* leaf is presented in (Figure 4). both methanol and ethanol extract inhibited no. of writhing in a dose dependent manner but while comparing with the activity of methanol extract the activity of the ethanol extract at (100 and 200 mg/kg b.w) showed highest inhibition of no. of writhing (***p<0.001) respectably which is even higher than the standard drug. On the other hand 200 mg/kg body weight dose of chloroform extract showed significantly (***p<0.001) decrease in no. of writhing at 4 min and 5 min as compared with the standard drug.

Acetic acid induced writhing response has been a sensitive procedure to evaluate peripherally acting analgesic effect of drugs and chemicals, often advantageous in preclinical investigations of analgesics that represents pain sensation by triggering localized inflammatory response. The acetic acid induced writhing produced an episodes of reaction of abdomen by contraction of abdominal musculature due to the sensation of chemosansative nociceptors that transmit the signal to CNS in response to pain which leads to the release of free arachidonic acid from tissue phospholipids [16].

After injection of acetic acid, increased level of PGE_2 and $PGF_{2\alpha}$ [17, 18] as well as lipoxygenase or cycloxygenases products [19, 20] has been found in the peritoneal fluid as they as they interfering with the mechanism of transduction in primary afferent nociceptor [21].

The analgesic effect of any plant extract reducing the number of writhing will render analgesic effect preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition.

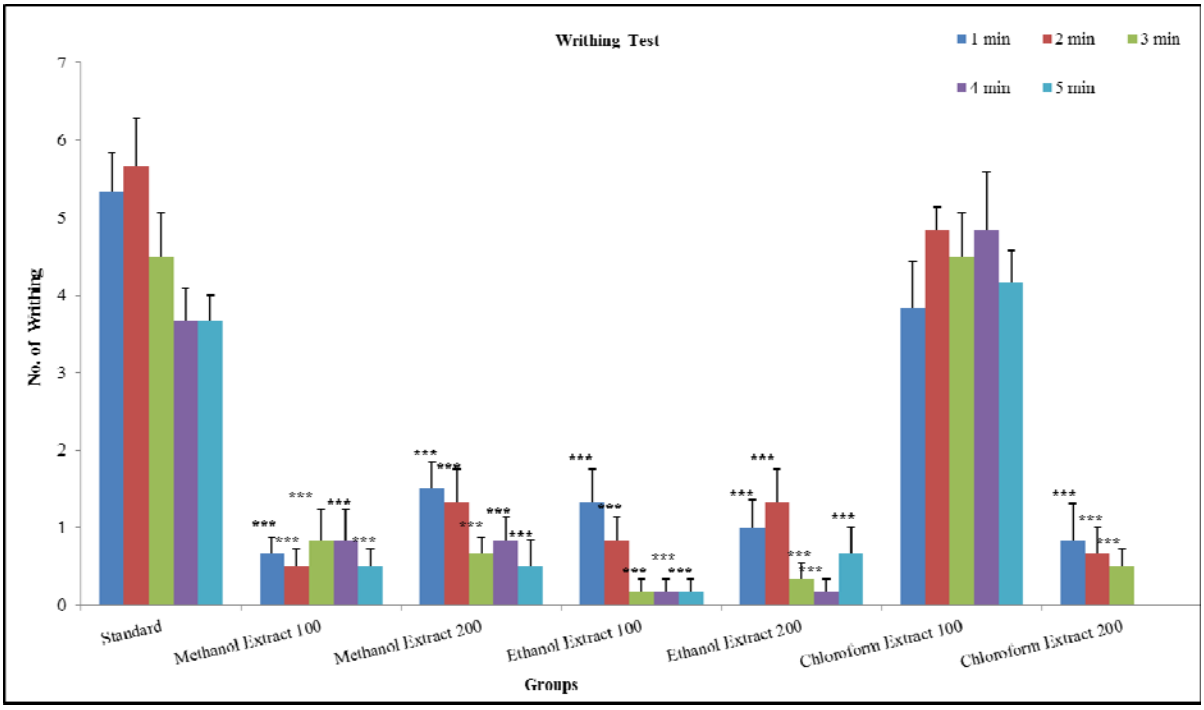

Fig 4: Graphical representation of anti-nociceptive activity through writhing test

(Values are expressed as mean ± S.E.M. (n=6), ***p<0.001 significant when compared with the corresponding value of the Standard group, done by independent sample t-test)

3.4 Neurophramacological Study

3.4.1 Forced Swimming Test (FST)

Forced swimming test (FST) was performed to evaluate the effect of anti-depressant effect of leaf extracts of *C. viminalis* on mice. After investigation of leaf extracts of *C. viminalis*, following data was observed (Figure 5), where the higher doses of methanol and ethanol extract (200 mg/kg body weight) with (**p<0.01) showed greater values compared to standard. Chloroform extract 200 mg/kg body weight showed similar immobility comparing to standard. Ethanol 200 mg/kg body weight with (**p<0.01) gave highest time of immobility of 191.5 sec.

Methanol, ethanol and chloroform extract showed decreased immobility with dose dependently like the standard diazepam which indicates presence of flavonoids and alkaloids because flavonoids are responsible for the decrease in immobile phase in the swim test and so does alkaloids as well [7].The forced-swimming test, the most-widely used tool for assessing antidepressant activity preclinically, is sensitive to the effects of all of the major classes of antidepressant drugs[22], where all mechanisms of action of treatments could be determined, but clinical correlations should be considered very carefully. The behaviors of animal exhibited in the FST could be related to the mood state of the animal [23] and brain levels of noradrenaline, dopamine, serotonin and cholinergic neurotransmitters [24]. When rodents are forced to swim to in a confined place, they tend to become immobile after vigorous activity (Struggling). This stressful inescapable situation can be evaluated by assessing different stress. The development of immobility when the rodents are placed in an inescapable container of water reflects the cessation of persistent escape directed behavior [25].

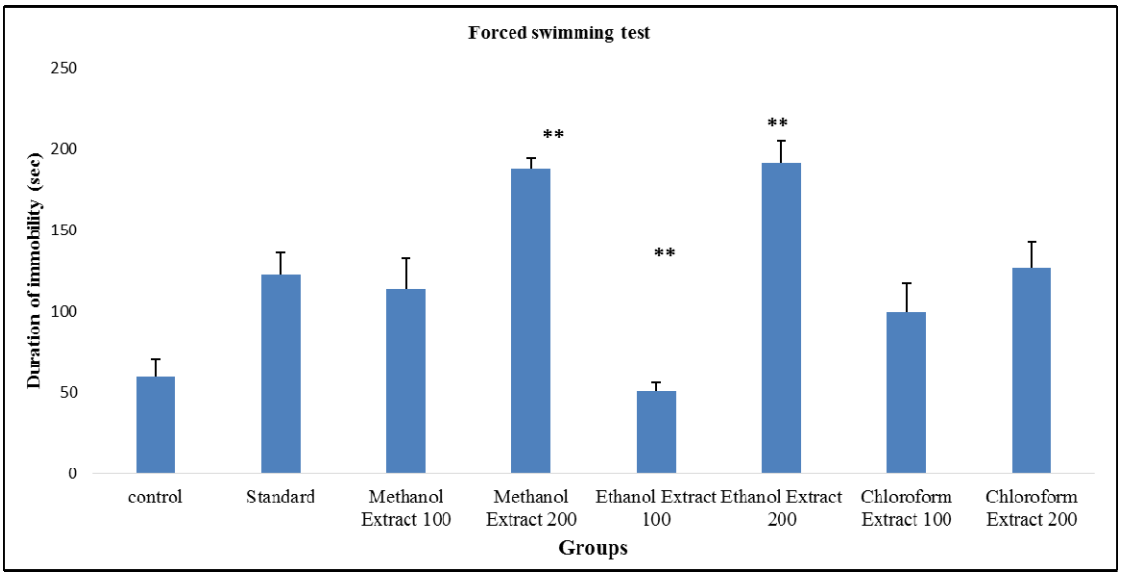

Fig 5: Graphical representation of Forced swimming test

(Values are expressed as mean ± S.E.M. (n=6), $^{**}p<0.01$ significant when compared with the corresponding value of the Standard group, done by independent sample t-test)

3.5 Acute Toxicity Test

No death or toxic reaction was observed in mice during the test period. This indicated the absence of any toxic material in the leaf of *C. viminalis*. The leaf extract of this plant is completely safe up to 2000 mg/kg.

4. Conclusion

Natural products of plant origin have been a promising source of new lead compound for drug discovery for ages. Based on the results of the present study it can be proposed that the leaf extract of *Calamus viminalis* has antidepressant, antipyretic, analgesic properties and reduce gastrointestinal motility to a great extent. Further phytochemical studies can be done for isolation of pure compounds responsible for the specific pharmacological action of this study.

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