

Potential Phytochemical, Analgesic and Anticancerous Activities of *Cymbopogon citratus* Leaf

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Abstract Purpose: The aim of this study was to evaluate phytochemical, analgesic and anticancerous activities of *Cymbopogon citratus*. **Methods:** Phytochemical screening, analgesic test and anticancerous activities were evaluated by the different chemical tests, writhing test and tail immersion test, brine shrimp lethality bioassay. **Results:** In the case of acetic acid induced writhing test, *C. citratus* showed highest percent of inhibition at 400 mg/kg, p.o. which is 49.01% whereas standard diclofenac sodium showed 73.76% at 10 mg/kg, i.p. In the case of tail immersion method, *C. citratus* exhibited its highest activity at 400 mg/kg, p.o. in 90 min which is 5.56±0.88 whereas diclofenac sodium showed 6.71±0.15 at 10 mg/kg, i.p. in 90 min. In the case of anticancerous activity, methanolic leaf extract of *C. citratus* demonstrated significant activity which is 113.74 µg/ml and standard vincristine sulphate showed 12.59 µg/ml. **Conclusions:** The result of phytochemical screening revealed that, methanolic leaf extract of *Cymbopogon citratus* contain alkaloids, steroids, flavonoids, tannins, saponins and carbohydrates. Methanolic leaf extracts of this plant possess moderate analgesic activity as well as exhibited significant anticancerous activity.

Keywords: *cymbopogon citratus*, phytochemical screening, analgesic, diclofenac sodium, acetic acid, writhing test, tail immersion test, brine shrimp

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1. Introduction

Natural products as an alternative and complementary medicine to many ailments have been a major interest among researchers. In addition to documenting the traditional knowledge related to medicinal plants, scientific authentication of these medicinal plants has been an important path of recent research [1]. Epidemiological evidence suggests that consumption of a diet rich in vegetables and fruits has optimistic implications for human health. The World Health Organization reported that 80 % of the world population rely chiefly on indigenous medicine and that the majority of traditional therapies involve the use of plant extracts or of their active constituents [2] and over 25% of modern medicines that are commonly used worldwide contains compounds extracted from medicinal plants [3]. The active principles differ from plants to plants due to their biodiversity and produce a definite physiological action on the human body that develops interest on their medicinal properties [4]. In recent years, there has become a revival in the use of traditional medicinal plants and therefore, pharmaceutical companies are products extracted from plants [5]. In Bangladesh thousands of plant species are considered to

have medicinal value [6] and ninety percent of the medicinal plants are wild sourced [7,8]. *Cymbopogon citratus* is native from the Southwest Asia and, now, it grows spontaneously around the world, mainly in the tropical and savannah regions [9]. Leaf wax contains triterpenes, cymbopogonol and cymbopogone. Rhizome contains alkaloids (hordenine, gramine), saponin glucoside, β-sitosterol, hexacosanol and triacontanol, alkaloid, saponin, glucoside and the phenolic compounds, p-coumaric acid, protocatechuic acid, ferulic acid and phloroglucinol carboxylic acid. An alkaloid with an indole nucleus has been isolated from the rhizome of this plant [10].

2. Materials and Methods

2.1. Plant Material

Cymbopogon citratus was collected from local area of Chittagong district (Bhatiary), Bangladesh and authenticated by the Botanist Dr. Shaikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong, Bangladesh.

2.2. Preparation of Extraction

The leaf was indirectly sun dried by shade and ground. The ground (500 g) were soaked in sufficient amount of

methanol (1:4) for one week at room temperature with occasional shaking and stirring then filtered through a cotton plug followed by Whitman filter paper No. 1. The solvent was evaporated under vacuum at room temperature to yield semisolid. Methanolic leaf extract was then preserved in a refrigerator at 4° C till further use.

2.3. Experimental Animals

Adult Swiss albino mice (both sex) weighing approximately 30-35 g were used for this experiment. The mice were purchased from the animal research branch of the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B). The animals were maintained standard laboratory conditions (25 °C and light/dark cycles i.e. 12/12 h) and provided with standard laboratory food and distilled water ad lib.

2.4. Chemicals and Reagents

Diclofenac sodium, acetic acid were obtained from MERCK, India. 0.9% NaCl saline solution was obtained from Popular Pharmaceuticals Ltd., Bangladesh. All other reagents were of analytical grade. Two more chemicals were purchased from Sigma Aldrich (Munich, Germany), which were vincristine sulfate and 99.5% absolute methanol.

Mayer's Reagent:

1.36 gm mercuric iodide in 60 ml of water was mixed with a solution contains 5 gm of potassium iodide in 20 ml of water.

Dragendorff's Reagent

1.7 gm basic bismuth nitrate and 20 gm tartaric acid were dissolved in 80 ml water. This solution was mixed with a solution contains 16 gm potassium iodide and 40 ml water.

Fehling's Solution A:

34.64 gm copper sulphate was dissolved in a mixture of 0.50 ml of sulfuric acid and sufficient water to produce 500 ml.

Fehling's Solution B:

176 gm of sodium potassium tartarate and 77 gm of sodium hydroxide were dissolved in sufficient water to produce 500 ml. Equal volume of above solution were mixed at the time of use.

Benedict's Reagent:

1.73 gm cupric sulphate, 1.73 gm sodium citrate and 10 gm anhydrous sodium carbonate were dissolved in water and the volume was made up to 100 ml with water.

Molisch Reagent:

2.5 gm of pure α -naphthol was dissolved in 25 ml of ethanol.

2.5. Phytochemical Screening

The following tests were performed for identifying different chemical groups by Trease et al., 1983 and Ghani et al., 1998.

Tests for reducing sugar:

Benedict's test

0.5 ml of aqueous extract of the plant material was taken in a test tube. 5 ml of Benedict's solution was added to the test tube, boiled for 5 minutes and allowed to cool spontaneously. A red color precipitate of cuprous oxide was formed in the presence of a reducing sugar.

Fehling's Test

2 ml of an aqueous extract of the plant material was added 1ml of a mixture of equal volumes of Fehling's

solutions A and B. Boiled for few minutes. A red or brick red color precipitate was formed in the presence of a reducing sugar.

Test for Tannins:

Ferric Chloride Test

5 ml solution of the extract was taken in a test tube. Then 1 ml of 5% Ferric chloride solution was added. Greenish black precipitate was formed and indicated the presence of tannins.

Potassium Dichromate Test

5 ml solution of the extract was taken in a test tube. Then 1 ml of 10% Potassium dichromate solution was added. A yellow precipitate was formed in the presence of tannins.

Test for Flavonoids:

Added a few drops of concentrated hydrochloric acid to a small amount of an alcoholic extract of the plant material. Immediate development of a red color indicates the presence of Flavonoids.

Test for Saponins:

1 ml solution of the extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. One centimeter layer of foam indicates the presence of saponins.

Test for Carbohydrates:

5 ml solution of the extract was taken and then Molish reagent and Sulphuric acid were added. Red-violet ring produced at the junction of two liquids indicate the presence of gums and carbohydrate.

Test for Steroids:

Sulphuric Acid Test

1 ml solution of chloroform extract was taken and then added 1ml Sulphuric acid. Chloroform layer acquired reddish brown color and acid layer showed green fluorescence indicates the presence of steroid.

Test for Alkaloids:

Mayer's test

2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of Mayer's reagent was added. Yellowish buff color precipitate was formed and that was indicated as the presence of alkaloids.

Dragendorff's test

2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of Dragendorff's reagent was added. Orange brown precipitate was formed and that was indicated as the presence of alkaloids.

Wagner's test

2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of iodine solution (Wagner's reagent) was added. Reddish brown precipitate was formed and that was indicated as the presence of alkaloids.

Hager's test

2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of picric acid solution (Hager's reagent) was added. Yellowish precipitate was formed and that was indicated as the presence of alkaloids.

2.6. Tests for Analgesic Activity

The study of analgesic activity of the extract was performed in animal models for both central and

peripheral mechanism of pain. For the screening of analgesic activity against peripheral mechanism of pain acetic acid induced writhing was considered. On the other hand, to evaluate the analgesic activity against centrally mediated pain tail immersion test was done. Beside this, to evaluate the possible mechanism related to the analgesic activity is evaluated by treating with Diclofenac Na. Two methods were employed to study the analgesic effects by using Swiss Albino Mice.

Acetic Acid Induced Writhing Method

Mice of either sex (n = 5) weighing 25-30 gm were used and divided into 4 groups. Group 1 received normal saline (10 ml/kg, p.o.) as control, Group 2 received standard drug diclofenac sodium (10 mg/kg, i.p.) while the remaining groups were received 200 and 400 mg/kg, p.o. of *Cymbopogon citratus* leaf extract. After 30 min of saline, diclofenac sodium, and plant extracts received, the animals were treated i.p. with 1 % (v/v) acetic acid. The number of abdominal constrictions (writhes) was counted after 5 min of acetic acid injection for the period of 10 min and compared to the response in the control group [11]. Antinociceptive activity was calculated as the percentage inhibition of writhing.

Tail Immersion Test

Mice were divided into four groups of five animals each. Group 1 received normal saline (0.9% NaCl, 10 ml/kg, p.o.) as control and group 2 received the standard drug Diclofenac Sodium (10 mg/kg, i.p.) Group 3 and 4 received 200mg/kg and 400mg/kg of methanol extract of *Cymbopogon citratus* orally respectively. The animal withdrawing his tail from hot water within 5 s were selected for the study. The lower 3 cm portion of the tail of mice was dipped in a water bath maintaining at temperature of (55.0±0.5). The time in second (s) for tail withdrawal from the water was taken as the reaction time and recorded by a stopwatch at before 0 and 30, 60 and 90 after the administration of test samples. A maximum immersion time of 15 s was maintained to prevent thermal injury to the animals [12].

Oral Toxicity Test

An acute oral toxicity test was performed according to the "Organization for Environmental Control Development" guidelines (OECD: Guidelines 420; Fixed Dose Method). Swiss Albino mice (n=5) overnight fasted for 18h were used for the study. Different doses of methanolic plant extract were administered orally into the mice. The maximum given dose was 600 mg/kg body weight. Then the animals were observed for the first three hours of administration and mortality recorded within 48 hours.

2.7. Anticancerous Activity

Anticancerous activity of plant extract is evaluated by brine shrimp lethality bioassay, which is widely used for screening bioactive compounds [13,14]. In this study, a simple zoological organism (*Artemia salina*) was used as a convenient monitor for the experiment. The eggs of the

brine shrimp were collected from an aquarium shop (Dhaka, Bangladesh) and hatched in artificial seawater (3.8% NaCl solution) for 48 hrs to develop into larval shrimp called nauplii. The cytotoxicity assay was performed on the brine shrimp nauplii using the Meyer method. The test samples (extract) were prepared by dissolving them in DMSO (not more than 50 µL in 5 mL solution) plus seawater (3.8% NaCl in water) to attain concentrations of 10, 50, 100, 200, 300 and 500 µg/ml. A vial containing 50 µL DMSO diluted to 5 mL was used as a control. Standard vincristine sulfate was used as a positive control. Mature shrimps were placed into each of the experimental vials. After 24 h, the vials were inspected using a magnifying glass, and the number of surviving nauplii in each vial was counted. From these data, the percentage of lethality of the brine shrimp nauplii was calculated for each concentration using the following formula:

$$\% \text{ Mortality} = (\text{Nt}) / \text{No} \times 100\%$$

Where Nt = Number of dead nauplii after a 24-h incubation;

N0 = Number of total nauplii transferred i.e., 10.

The LC50 (median lethal concentration) was determined from the log concentration versus percent mortality curve [15].

3. Results

3.1. Phytochemical Screening

The result of phytochemical screening revealed that, methanolic leaf extract of *Cymbopogon citratus* contain alkaloids, steroids, flavonoids, tannins, saponins and carbohydrates

Table 1. Results of Different Chemical Group Test of the Crude Extracts of the Leaf of *Cymbopogon citratus*

PHYTOCHEMICAL CONTENTS	METHANOL EXTRACT
STEROIDS	+
ALKALOIDS	+
FLAVONOIDS	+
REDUCING SUGAR	-
TANNINS	+
SAPONINS	+
CARBOHYDRATES	+

Present = +; Absent = -.

3.2. Analgesic Test

Writhing test

Analgesic activity was evaluated by acetic acid induced writhing test and tail immersion test. In the case of acetic acid induced writhing test, *C. citratus* showed highest percent of inhibition at 400 mg/kg, p.o. which is 49.01% whereas standard diclofenac sodium showed 73.76% at 10 mg/kg, i.p.

Table 2. Analgesic Activity of methanolic leaf extract of *Cymbopogon citratus* on mice (Mouse Writhing Test)

Group	Treatment	Dose-Route	No. of writhing	Percent Inhibition
Group 1 (Control)	Normal Saline	10 ml/kg, p.o.	83.76±0.96	0.00
Group 2 (Standard)	Diclofenac Sodium	10 mg/kg, i.p.	26.23±0.66**	73.76
Group 3	CC 200	200 mg/kg, p.o.	67.33±1.72**	32.67
Group 4	CC 400	400 mg/kg, p.o.	50.99±0.82**	49.01

Values are expressed as Mean ±SEM (n=5); *P<0.05, **P<0.01 Dunnett's test as compared to control.

Tail Immersion test

In the case of tail immersion method, *C. citratus* exhibited its highest activity at 400 mg/kg, p.o. in 90 min

which is 5.56 ± 0.88 whereas diclofenac sodium showed 6.71 ± 0.15 at 10 mg/kg, i.p. in 90 min.

Table 3. Analgesic Activity of methanolic leaf extract of *Cymbopogon citratus* on mice (Tail Immersion Test)

Group	Treatment	Dose, Route	0 min	30 min	60 min	90 min
Control	Normal Saline	10 ml/kg, p.o.	1.29±0.13	1.76±0.17**	2.37±0.17**	2.02±0.19**
Standard	Diclofenac Sodium	10 mg/kg, i.p.	1.87±0.21	5.33±0.30**	7.15±0.21**	6.71±0.15**
Group-1	CC 200	200 mg/kg, p.o.	1.06±0.33	5.80±0.71*	8.18±0.73**	6.76±1.39
Group-2	CC 400	400 mg/kg, p.o.	2.07±0.15*	4.76±0.90**	5.41±0.59*	5.56±0.88

Values are expressed as Mean ±SEM (n=5); P<0.05, **P<0.01 Dunnett's test as compared to control.

3.3. Anti-cancerous Activity

In the case of anticancerous activity, methanolic leaf extract of *C. citratus* demonstrated significant activity which is 113.74 µg/ml and standard vincristine sulphate showed 12.59 µg/ml.

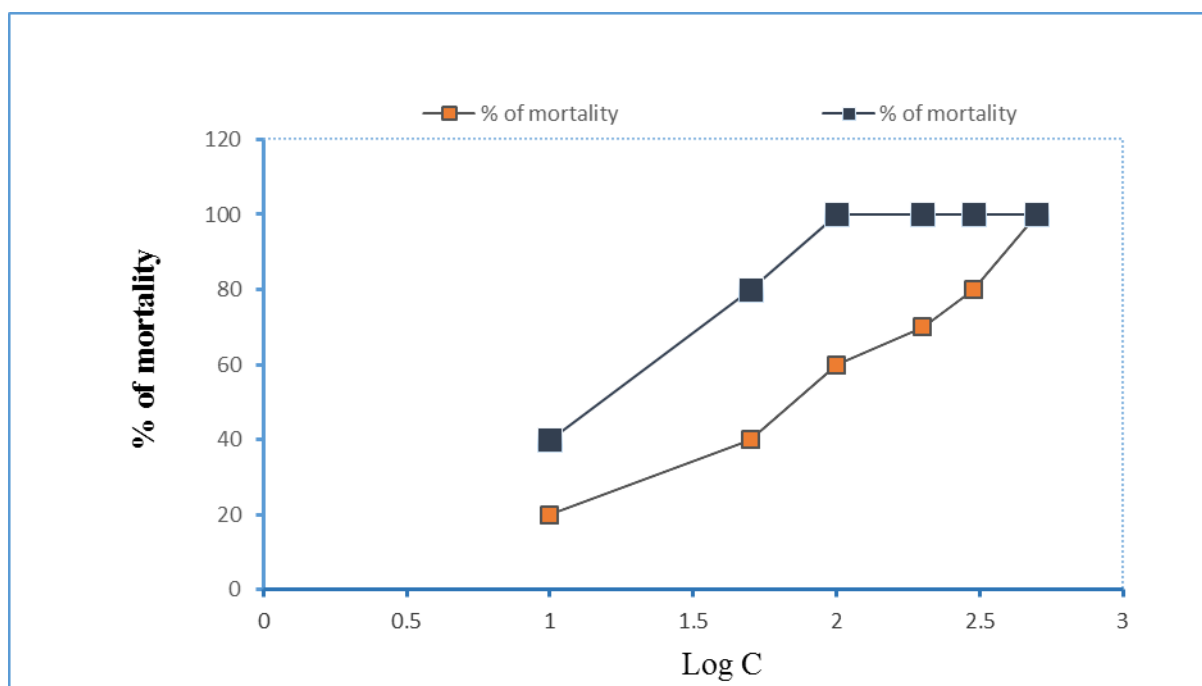


Figure 1. Effect of methanolic leaf extract of *Cymbopogon citratus* and vincristine sulphate on brine shrimp nauplii

Table 4. Percent of mortality of both the extract at six concentrations

Concentration(µg/ml)	LogC	% of mortality	
		CC	Vincristine sulphate
10	1	20	40
50	1.699	40	80
100	2	60	100
200	2.301	70	100
300	2.477	80	100
500	2.699	100	100
LC ₅₀		113.74	12.59

*CC= *Cymbopogon citratus*.

4. Discussion

People on all continents have used hundreds to thousands of indigenous plants for treatment of ailments since prehistoric times. According to World Health Organization, about 80% of the world's population presently uses phytotherapy for some aspect of primary health care system. Approximately 25 percent of modern

drugs used in the United States have been derived from plant origins [16]. So, research on phytotherapy has got great momentum in recent years to find out noble pharmaceuticals. The acetic acid induced writhing response is a sensitive procedure to evaluate peripherally acting analgesic. The response is thought to be mediated by the prostaglandin pathways, peritoneal mast cells and acid sensing ion channels [17, 18, 19]. Therefore, the significant pain reduction of the plant extract may be due to the presence of analgesic principles acting with the prostaglandin pathways or interfering with other mediators responsible for peripheral pain. 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. Analgesic effect against thermal noxious stimuli may be elicited through opioid receptors or through modulation of several neurotransmitters involved in relevant phenomena. But the extend of activity shown by the crude extracts are less than that of the standard drug nalbuphine but many fold more than that of the control group, which justifies its activity. Narcotic

analgesics inhibit both peripheral and central mechanism of pain, while non-steroidal anti-inflammatory drugs inhibit only peripheral pain [20,21]. The extract inhibited both mechanisms of pain, suggesting that the plant extract may act as a narcotic analgesic. The cytotoxic and antitumor activity of plant derived product is either through induction of apoptosis or inhibition of neovascularization [22]. Ideally, any agent useful in the treatment of cancer should not be toxic to normal cell. However, in reality, anticancer agents are often toxic to normal cells, particularly towards rapidly growing cells [23]. It is necessary to test this extract in low concentration to evaluate its potency and also against various cancer cell lines as well as normal cell line so justify the potential to further investigate this plant for anticancer activity.

5. Conclusion

The result of phytochemical screening revealed that, methanolic leaf extract of *Cymbopogon citratus* contain alkaloids, steroids, flavonoids, tannins, saponins and carbohydrates. Methanolic leaf extracts of this plant possess moderate analgesic activity. Writhing test and Tail immersion test proved that this plant has moderate analgesic activity and it is also proved that higher dose is more effective than the lower dose. This plant is also exhibited significant anticancerous activity.

Ethical Approval

The experimental protocol was approved by the P&D committee, Department of Pharmacy, International Islamic University Chittagong, Bangladesh according to governmental guidelines.

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Conflict of Interest Statement

Authors have none to declare.

References

- [1] Uprety Y, Asselin H, Boon EK, Yadav S, Shrestha KK. Indigenous use and bio-efficacy of medicinal plants in the Rasuwa District, Central Nepal. *J Ethnobiol Ethnomed.* 2010; 10(3).
- [2] World Health Organization. Summary of WHO guidelines for the assessment of herbal medicines. *Herbal Gram*, 1993; 28: 13-14.
- [3] Robbers, JE, MK Speedle and VE Tyler. *Pharmacognosy and Pharmacobiotechnology.* Williams and Wilkins, Baltimore, USA, 1996.
- [4] Shammi SJ, Islam R, Zaman AU, Majumder R and Alam B. Comparative Pharmacological Studies of *Abelmoschus sculentus* Linn. *Fruits and Seeds Global J. Pharmacol.* 2014; 8(1): 98-106.
- [5] Shohayeb M, Hameed ESSA, Bazaid SA and Maghrabi I. Antibacterial and Antifungal Activity of *Rosa damascena* MILL. Essential Oil, Different Extracts of Rose Petals. *Global J. Pharmacol.* 2014; 8(1): 01-07.
- [6] Mollika S, Islam N, Parvin N, Kabir A, Sayem MW, Luthfunnesa and Saha R. Evaluation of Analgesic, Anti-Inflammatory and CNS Activities of the Methanolic Extract of *Syzygium samarangense* Leave *Global J Pharmacol.* 2014; 8(1): 39-46.
- [7] Ghani, A. *Medicinal Plants of Bangladesh: Chemical Constituents and Uses.* Asiatic Society of Bangladesh, Dhaka, 1998.
- [8] South Asia Enterprise Development Facility (SEDF) & Intercooperation (IC). *Medicinal Plants Marketing in Bangladesh. A market study report.* SEDF-Intercooperation, Dhaka, 2003.
- [9] Medicinal Plants of Bangladesh, *Cymbopogon citratus.* <http://mpbd.info/plants/cymbopogon-citratus.php>.
- [10] Ghani, A. *Medicinal Plants of Bangladesh with chemical constituents and uses.* 2nd edition, Asiatic Society of Bangladesh, 5 old Secretariate road, Nimtali, Dhaka, Bangladesh, 2003.
- [11] Koster R, Anderson M, Beer DEJ. Acetic acid for analgesic screening. *Proc Soc Exp Biol Med.* 1959; 18:412-5.
- [12] Toma W, Gracioso JS, Hiruma-Lima CA, Andrade FD, Vilegas W, Souza Brito AR. Evaluation of the analgesic and antiedematogenic activities of *Quassia amara* bark extract. *J Ethnopharmacol.* 2003; 85: 19-23.
- [13] Zhao GY, Hui JK, Rupprecht JL, McLaughli KV. Additional bioactive compounds and trilobacin, a novel highly cytotoxic acetogenin, from the bark of *Asimina triloba*. *J Nat Prod.* 1992; 55:347-356.
- [14] Prasad S, Kashyap RS, Deopujari JY, Purohit HJ, Taori GM, Dagainawala HF: Development of an in vitro model to study clot lysis activity of thrombolytic drugs. *Thromb J.* 2006; 4:14.
- [15] Chowdury MIA, Alam MN, Chowdhury S, Biozid MS, Faruk M, Mazumdar MMU, Chowdhury AI. Evaluation of *Ex-vivo* Anti-arthritis, Anti-inflammatory, Anti-cancerous and Thrombolytic Activities of *Mussaenda roxburghii* Leaf. *European Journal of Medicinal Plants.* 2015; 10(4): 1-7.
- [16] WHO. Traditional medicine 2008. <http://www.who.int/mediacentre/factsheets/fs134/en/>.
- [17] Hossain MM, Ali MS, Saha A, Alimuzzaman M. Antinociceptive activity of whole plant extracts of *Paederia foetida*. *Dhaka University Journal of Pharmaceutical Science.* 2006; 5: 67-69.
- [18] Ronaldo AR, Mariana LV, Sara MT, Adriana BPP, Steve P, Ferreira SH and Fernando QC, 2000.
- [19] Voilley N. Acid-Sensing Ion Channels (ASICs): New targets for the analgesic effects of Non- Steroid Anti- inflammatory Drugs (NSAIDs). *Current Drug Targets- Inflammation and Allergy.* 2004; 3: 71-79.
- [20] Elisabetsky E, Amador TA, Albuquerque RR, Nunes DS, Ado CC. Analgesic activity of *Psychotria colorata* (Wild. ex R. and S.) Muell. Arg. alkaloids. *Journal of Ethnop-harmacol.* 1995; 48: 77-83.
- [21] Pal S, Sen T, Chaudhuri AK. Neuropsychopharmacological profile of the methanolic fraction of *Bryophyllum pinnatum* leaf extract. *The Journal of Pharmacy and Pharm-acy.* 1999; 51: 313-518.
- [22] Alam, M.B., I. Sajid, Z. Rashid, M.M. Islam and B.K. Karmaker. Evaluation of Antitumor Effects of the Aerial Parts of *Polygonum viscosum* Linn. *Global J. Pharmacol.* 2014; 8(1): 47-52.
- [23] Priestman, T.. *Cancer Chemotherapy in Clinical Practice.* London. Springer-Verlag, 2008. pp 130-136.