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Anxiolytic, analgesic and anti-inflammatory effects of *Citrus maxima* (Burm.) Merr. Seed extract in Swiss albino mice model

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Abstract

Background *Citrus maxima* (Burm.) Merr. is traditionally used for its diverse pharmacological properties. Therefore, there remains a possibility that the seed extract may contain some bioactive compounds. The present study was carried out to evaluate the anxiolytic, analgesic, and anti-inflammatory effects of methanolic seed extract of *Citrus maxima* (MECM).

Method The effect of MECM on the rodent central nervous system was evaluated using the hole-board and elevated plus-maze method. Analgesic effect was measured with the acetic acid-induced writhing and formalin-induced paw licking method. The anti-inflammatory effect was examined using a formalin and carrageenan-induced mice paw edema model.

Results The MECM at doses of 200 mg/kg and 400 mg/kg significantly ($p < 0.01$) increased the number of head dipping in the hole-board test. Additionally, the test subjects entered into the open arm and spent more time in it at an increased rate ($p < 0.01$) in the elevated plus-maze test. In the acetic acid-induced writhing method, the extract showed high potential ($p < 0.001$) as an analgesic agent. In the case of formalin-induced pain assessment, MECM demonstrated a significant effect ($p < 0.01$) at the early phase as a centrally acting anti-nociceptive agent and very high potential ($p < 0.001$) to reduce pain during the late phase as a peripherally acting analgesic. The extract also showed potency ($p < 0.01$) as an anti-inflammatory agent in formalin and carrageenan-induced mice paw edema test.

Conclusion The findings of the current study indicate that MECM can be a promising new candidate for searching novel anxiolytic, analgesic, and anti-inflammatory compounds through further investigation.

Keywords *Citrus maxima* (Burm.) Merr. Seed, Anxiolytic, Analgesic, Anti-inflammatory, Hole-board, Elevated-plus maze, Writhing, Paw licking, Formalin, Carrageenan

Background

The use of naturally sourced medicine has been traditionally widespread in developing countries such as Bangladesh, China, and India. It is estimated that about a quarter of the medicines prescribed in developing countries consist of plant-derived ingredient [1]. Since developing countries have the larger share of the world population, a sizable portion of the populace depends heavily on naturally sourced medicines for their primary healthcare demand [2]. The medicinal herb is considered

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to be a chemical factory as it contains a multitude of phytochemical compounds known to have beneficial use in industries and medicinal sciences and also exhibit physiological activity. More than 20% of conventional and standardize drugs have a phytochemical backbone [3]. The pharmacological activities of medicinal plants have been attributed to the presence of secondary metabolites including saponins, phenols, flavonoids, tannins, terpenoids and many more [4]. Their traditional uses and medicinal claims are being validated by recent advances in plant-based drug developments. Therefore, there exists a largely untapped market of drugs derived from natural compounds with a superior safety profile. Accredited medicinal plant research can facilitate such exploration into new frontiers of medicine [5].

The Pomelo, Pummelo, or scientifically known as *Citrus maxima* (Burm.) Merr., is the largest citrus fruit of the Rutaceae (citrus) family. *C. maxima,* indigenous to South and Southeastern Asia, known as Jambura in Bangladesh, has many ethnopharmacological uses. Pomelo contains abundant vitamin C like other citrus plants and is generally eaten as fruit. Different parts of this plant contain several organic compounds. Phytochemicals belonging to different chemical classes such as alkaloids, saponins, carbohydrates, phenols, flavonoids, glycosides, anthraquinone, amino acids, carotenoids, and terpenoids are present in different parts of *C. maxima* [6]. The essential oils obtained from the leaves and unripe fruits contain nerolyl acetate, limonin, geraniol, and nerolol [7].

Systemic screening of *C. maxima* literature indicates that it is used in a variety of ailments and is regarded as a high-value medicinal plant. Traditionally, the rinds of *C. maxima* are used for headaches in folk medicine. Moreover, *C. maxima* leaves have been used as an anti-inflammatory agent [8, 9] by applying the hot leaf decoction on swellings or inflamed areas of the body. Nevertheless, efforts to verify the medicinal properties of Pomello claimed by traditional medicine are scarce. Available studies suggest that crude organic extracts of leaves of *C. maxima* have been screened for pharmacological activities and exhibited hepatoprotective, analgesic, anti-tumor, anti-inflammatory, and CNS activity. Hypocholesterolemic and antioxidant activities were found in the juice of *C. maxima*. Additionally, the stem bark of the plant has been found to possess anti-diabetic activity. *C. maxima* peel was investigated for analgesic, hepatoprotective, antibacterial, and anti-inflammatory activity [10]. Aromatic water of *C. maxima* is used as a stress and insomnia reliever [11]. The plant is also said to possess cardiotonic, appetizing, and antitoxic properties [12].

Different parts of the plant- leaves, bark, stem, fruit, and flowers have been investigated for medicinal properties but the seed part is yet to be studied extensively through in vivo and in vitro methods. Thus, in our current study, the crude methanolic seed extract of *C. maxima* (MECM) was investigated for anxiolytic, analgesic, and anti-inflammatory activities.

Methods

Chemical and reagents

Diazepam and Diclofenac sodium were obtained from Square Pharmaceuticals Ltd. (Gazipur, Bangladesh). Indomethacin was collected from Albion laboratories (Chittagong, Bangladesh). Morphine sulfate was procured from Renata Ltd. (Mirpur, Bangladesh). Methanol was purchased from Sigma-Aldrich (Humburg, Germany). Carrageenan, acetic acid, and formalin were sourced from local suppliers. All chemicals and reagents used in this study were of analytical grade.

Collection and extract preparation of plant material

C. maxima seeds were collected from a local plant nursery of Bangladesh Forest Research Institute (BFRI), Chattogram, and authenticated by Dr. Sheikh Bokhtear Uddin, a taxonomist (Department of Botany, University of Chittagong, Chattogram, Bangladesh) with the voucher specimen number of the plant Accr No. SBU 5380. Collected seeds were thoroughly washed with water, shade-dried for 1 week, and ground into a coarse powder using a high-capacity grinding machine. About 1170 g of the powdered seed was taken in two separate flasks and then macerated in 3 l of methanol for 15 days with occasional shaking and stirring. The whole mixture was filtered and then concentrated using a rotary evaporator at 60 °C. The obtained crude extract (MECM) was weighed at 37 g and then preserved in the refrigerator at 4 °C for further studies.

Phytochemical analysis of extract

Testing of different chemical groups present in the extract represents the preliminary phytochemical studies. A small quantity of freshly prepared MECM was subjected to preliminary quantitative investigation for the detection of phytochemicals such as alkaloids, carbohydrates, glycosides, phytosterols, proteins, flavonoids, tannins, saponins, phenols, terpenes, fats, and fixed oils using the standard methods [13].

Test animals

Female Swiss Albino Mice weighing 25-35 g were collected from the animal resources facility of ICDDR, B, Dhaka. They were housed in clean and dry cages with 12 hours light-dark cycle at 25 ± 2 °C in the animal house. The mice were fed with a standard laboratory diet and water ad libitum. The animals were acclimatized for 3–4 days before the experiments were performed. Food

was withdrawn 12 hours before and during the experiment. This study was approved by the Animal Ethics Review Committee (AERB), Faculty of Biological Sciences, University of Chittagong.

Evaluation of anxiolytic potential

Hole-board method

The hole-board test has been used to assess anxiety and responses to stress in animals [14]. The apparatus is composed of a wooden chamber (40x40x25 cm^3) with 16 holes (each of 3 cm diameter) evenly distributed on the floor. The apparatus was elevated to a height of 25 cm from the ground so that the mice could peep through the holes. A total of twenty mice were divided into four groups containing 5 mice in each. Group I served as control that received Tween 80 solution (10 ml/kg) by oral route. Group II was considered as the standard group which was treated with Diazepam 1 mg/kg orally. Group III and group-IV served as test groups which were treated with 200 mg/kg and 400 mg/kg doses of MECM, respectively. A high anxiety state in the animal manifests as the aversion to exploring new places which is correlated with low levels of head dipping. Conversely, increased head dipping represents neophilia or exploratory behavior [15]. After 30 minutes of dose administration, a mouse was placed on the board and allowed to freely explore the apparatus for 5 minutes. The total number of head-dips during a five-minute trial period was recorded [16].

Elevated plus-maze (EPM) method

The elevated plus-maze test has been widely used for studying anxiety response as well as anxiolytic action of a drug in rodents [17, 18]. The apparatus consists of two open arms (30 cm × 5 cm × 0.2 cm) and two enclosed arms with high walls (30 cm × 5 cm × 15 cm), extending from a central platform (5 cm × 5 cm) and elevated to a height of 45 cm above the floor. Each arm of the elevated plus-maze apparatus is positioned at 90° relative to adjacent arms. Similarly, twenty mice were divided into four groups containing 5 mice in each. Group I served as control that received Tween 80 solution (10 ml/kg) by oral route. Group-II was considered as the standard group which was treated with Diazepam 1 mg/kg orally. Group III and group-IV served as test groups which were treated with 200 mg/kg and 400 mg/kg doses of MECM, respectively. After 30 minutes of treatment, animals were placed individually in the center of the maze facing the open arm. The preference of the mouse for open or closed arm, the number of entries and the time spent on open and closed arm were recorded during a 5 minutes test period for each animal [19].

Evaluation of analgesic potential

Acetic acid-induced writhing method

The study was carried out using the method of Koster [20] as modified by Dambisya and Lee [21]. A total of 20 mice were randomly selected for each model and then divided into four groups of five animals. Group I served as control that received Tween 80 solution (10 ml/kg) by oral route. Group II serve the standard group which was treated with Diclofenac sodium (50 mg/kg) orally. Group III and group IV served as test groups which were treated with 200 mg/kg and 400 mg/kg of MECM, respectively. Nociception was induced by an intraperitoneal (i.p.) injection of 0.7% glacial acetic acid (10 ml/kg). Standard group animals were pretreated 15 minutes before acetic acid administration and test groups were pretreated 30 minutes before administration of acetic acid. 5 minutes post-administration of acetic acid, the number of squirms, or writhing were counted and recorded for each mouse for 20 minutes. The percentage inhibition against abdominal writhing was used to assess the degree of analgesia and was calculated using the following formula [21]:

$$\%\ of\ Inhibition = \frac{Nc - Nt}{Nc} \times 100$$

Here,
Nc = Number of writhings in the control group.
Nt = Number of writhings in the test groups.

Formalin-induced paw licking method

Formalin-induced paw licking test was carried out according to the previously described method [22]. Analogous to acetic acid induced writhing method, twenty mice were randomly selected for each model and then divided into four groups of five animals. Group I served as control that received Tween 80 solution (10 ml/kg) by oral route. Group II served the standard group which was treated with Morphine sulfate (10 mg/kg) orally. Group III and group IV served test groups which were treated with 200 mg/kg and 400 mg/kg of MECM, respectively. The 20 µl of 1% formalin was injected subcutaneously into the right hind paw of mice to produce a biphasic pain response 60 minutes post-administration of plant extracts and diclofenac sodium. The time (in seconds) spent licking and biting the injected paw was taken as an indicator of pain response. Responses were measured from 0 to 5 minutes (early phase, neurogenic) and 15–30 minutes after formalin injection (late phase, inflammatory) [23]. The percent pain inhibition was calculated using the following formula:

$$Pain\ inhibition\ (\%) = \frac{Reaction\ time\ (Control) - Reaction\ time\ (Treatment)}{Reaction\ time\ (Control)} \times 100$$

Evaluation of anti-inflammatory potential

Formalin-induced mice paw edema

Formalin-induced mice paw edema is a well-qualified test to evaluate the sub-acute anti-inflammatory effect, closely mimicking human arthritis [24]. The test was carried out following the method described by Hunskaar and Hole [22]. Twenty experimental animals were divided into four groups consisting of 5 mice in each group. Group I served as control that received 10 ml/kg of Tween 80 solution. Group II was considered as standard which was treated with 10 mg/kg of Indomethacin, intraperitoneally. Group III and group IV served as test groups which were treated with 200 mg/kg and 400 mg/kg of MECM, respectively. One hour after the test sample and thirty minutes after standard administration, the paw edema was induced by subcutaneous injection of 50 µl of 5% formalin. The paw circumference (in mm) of mice was measured before and after formalin injection. The percent inhibition of paw edema was calculated as follows:

$$Percent\ inhibition = \frac{(Ct - Co)Control - (Ct - Co)Treated}{(Ct - Co)Control} \times 100 \text{inhibition} = \frac{(\text{Ct} - \text{Co})\text{Control} - (\text{Ct} - \text{Co})\text{Treated}}{(\text{Ct} - \text{Co})\text{Control}} \times 100$$

Here, C_t = Mean paw circumference for each group at a different time interval.

C_o = Mean paw circumference for each group before formalin injection.

Carrageenan-induced mice paw edema

Carrageenan-induced acute inflammation is one of the most effective tests to evaluate the anti-inflammatory potential of pharmacological substances. This test was carried out using carrageenan as a phlogistic agent to induce edema in the right hind limb of mice [25]. Similar to formalin-induced mice paw edema, twenty experimental animals were divided into four groups consisting of 5 mice in each group. Group I served as control that received 10 ml/kg of Tween 80 solution. Group II served as standard which was treated with 10 mg/kg of Indomethacin, intraperitoneally. Group III and group IV served as test groups which were treated with 200 mg/kg and 400 mg/kg of MECM, respectively. One hour after the test sample and thirty minutes after the standard administration, a subcutaneous injection of 100 µl of carrageenan (1% w/v) was given into the right hind paw. Paw circumference (in mm) was measured before the injection of carrageenan and thereafter at the end of the 1st, 2nd, 3rd, and 4th hour. Then the percentage inhibition of edema was calculated by the following formula:

$$Percent\ inhibition = \frac{(Ct - Co)Control - (Ct - Co)Treated}{(Ct - Co)Control} \times 100$$

Here, C_t = Mean paw circumference for each group at a different time interval.

C_o = Mean paw circumference for each group before carrageenan injection.

Statistical analysis

Results of the study were represented by mean ± SEM (Standard Error of the Mean). Data were analyzed by one-way ANOVA followed by post hoc Dunnett's "t" test using Statistical Package for Social Science (SPSS, version 16.0) considering $^{**}p < 0.01$ and $^{***}p < 0.001$ as statistically significant and statistically highly significant, respectively (Tables 1 and 2).

Table 2 Effect of MECM on head dipping of Swiss Albino mice in Hole-board test

Treatment (mg/kg)	Mean ± SEM
Control - Tween 80 solution (10 ml/kg)	20.50 ± 2.44
Standard - Diazepam (1 mg/kg)	56.00 ± 4.15***
MECM (200 mg/kg)	33.80 ± 1.28**
MECM (400 mg/kg)	39.00 ± 1.22**

MECM Methanolic extract of seeds, *SEM* standard error of the mean

All values are expressed as Mean ± SEM ($N = 5$)

$^{**}p < 0.01$, $^{***}p < 0.001$ were considered statistically significant compared to control

Table 1 Phytochemical analysis of methanolic extract of *C. maxima* seed (MECM)

Phytochemicals	Preliminary quantitative result
Terpenoids	+
Flavonoids	+
Tannins	+
Alkaloids	+
Glycosides	+
Resins	+
Fat and fixed oils	+

Results

Phytochemical analysis

In this phytochemical study, MECM showed the presence of terpenoids, flavonoids, tannins, alkaloids, glycosides, resins, fat and fixed oils. It also gave clarification about the absence of saponins, phenols, phlobatanins, steroid, anthraquinones, cardiac glycosides, carbohydrates & proteins.

Anxiolytic effects

In the hole-board method, the tendency of head dipping increased significantly ($p < 0.01$) in mice treated with MECM at 400 mg/kg while compared to the control. In the elevated plus-maze method, administration of the MECM at both doses (200 & 400 mg/kg) caused a significant ($p < 0.01$) increase in the frequency of entry into the open arm and time spent in it compared to the control group. For both tests, the standard group (diazepam 1 mg/kg) showed an increased anti-anxiety effect ($p < 0.001$) than the MECM.

Analgesic effects

In the acetic acid-induced writhing method, the extracts (200 and 400 mg/kg) and the reference drug Diclofenac sodium (50 mg/kg) showed a highly significant reduction ($p < 0.001$) in abdominal writhing when compared to the control (Table 3). In the formalin-induced paw licking test, both doses of MECM significantly decreased ($p < 0.01$) the amount of time spent on licking in the early phase. Subsequently, the time spent licking was significantly reduced ($p < 0.001$) compared to the control group in the late phase (Table 4). The standard group (Diclofenac sodium) showed a similar effect as MECM in pain inhibition.

Anti-inflammatory effects

In the formalin-induced paw edema test, the standard drug Indomethacin (10 mg/kg) caused significant inhibition ($p < 0.01$) of paw edema in the early phase, while it showed maximum inhibition ($p < 0.001$) in the late phase. Both doses of MECM exhibited a similar effect as standard (Table 5). In the carrageenan-induced paw edema test, Indomethacin (10 mg/kg) reduced paw edema significantly ($p < 0.01$) in the late phase. Similarly, MECM also significantly reduced paw edema ($p < 0.01$) in the late phase (Table 6).

Discussion

Before the advent of modern medicine, people depended on plants and their constituents sourced from the immediate vicinity as a major source of medicine to cure

Table 3 Effect of MECM on open and closed arm entry and time spent in both arms

Treatment (mg/kg)	Open arm (OA)		Closed arm (CA)	
	Time spent (sec)	No. of entries	Time spent (sec)	No. of entries
Control-Tween 80 solution (10 ml/kg)	0.50 ± 0.32	0.80 ± 0.37	296.40 ± 2.01	14.60 ± 1.44
Standard-Diazepam (1 mg/kg)	52.65 ± 0.86***	8.60 ± 0.51***	213.78 ± 0.93***	2.80 ± 0.37**
MECM (200 mg/kg)	7.98 ± 1.07**	3.40 ± 0.40**	280.94 ± 0.47**	4.60 ± 0.51**
MECM (400 mg/kg)	8.41 ± 1.16**	3.80 ± 0.374**	283.962 ± 0.78**	4.00 ± 0.45**

MECM Methanolic extract of seeds, *SEM* standard error of the mean

All values are expressed as Mean ± SEM ($N = 5$)

**$p < 0.01$, ***$p < 0.001$ were considered statistically significant compared to control

Table 4 Effect of MECM on acetic acid-induced writhing inhibition in Swiss Albino mice

Treatment (mg/kg)	Number of writhing (Mean ± SEM)	% of inhibition of writhing
Control-Tween 80 solution (10 ml/kg)	41.40 ± 1.08	0
Standard-Diclofenac sodium (50 mg/kg)	17.00 ± 0.71***	58.94
MECM (200 mg/kg)	21.00 ± 0.55***	49.28
MECM (400 mg/kg)	17.60 ± 0.93***	57.49

MECM Methanolic extract of seeds, *SEM* Standard error of the mean

All values are expressed as Mean ± SEM ($N = 5$)

**$p < 0.01$, ***$p < 0.001$ were considered statistically significant compared to control

Table 5 Effect of MECM on formalin-induced pain inhibition in Swiss Albino mice

Treatment (mg/kg)	Mean pain scores			
	Early phase	% Inhibition	Late phase	% Inhibition
Control-Tween 80 solution (10 ml/kg)	52.88 ± 4.49	–	73.00 ± 4.87	–
Standard-Morphine sulfate (10 mg/kg)	8.60 ± 0.69**	83.74	2.12 ± 0.45***	97.09
MECM 200 mg/kg	10.36 ± 4.11**	80.41	2.71 ± 0.82***	96.29
MECM 400 mg/kg	9.19 ± 4.25**	82.62	2.33 ± 1.08***	96.81

MECM Methanolic extract of seeds, *SEM* Standard error of the mean

All values are expressed as Mean ± SEM ($N = 5$)

**$p < 0.01$, ***$p < 0.001$ were considered statistically significant compared to control

Table 6 Effect of MECM on formalin-induced paw edema inhibition

Treatment (mg/kg)	Pre-injection mean paw circumference (mm)	Post-injection mean paw circumference (mm) (% inhibition)			
		1 hour	2 hour	3 hour	4 hour
Control-Tween 80 solution(10 ml/kg)	8.10 ± 0.33	14.40 ± 0.40 -	14.20 ± 0.37 -	13.80 ± 0.20 -	13.60 ± 0.29 -
Standard-Indomethacin(10 mg/kg)	8.90 ± 0.24	11.80 ± 0.34** (53.97%)	11.30 ± 0.34** (60.66%)	9.90 ± 0.19*** (82.46%)	9.40 ± 0.19*** (90.91%)
MECM 200 mg/kg	7.60 ± 0.19	12.00 ± 0.27** (30.16%)	11.40 ± 0.29** (37.70%)	10.80 ± 0.25*** (43.86%)	10.10 ± 0.24*** (54.55%)
MECM 400 mg/kg	7.20 ± 0.25	11.00 ± 0.57** (39.68%)	10.40 ± 0.53** (47.54%)	9.60 ± 0.40*** (57.89%)	9.30 ± 0.41*** (61.82%)

MECM Methanolic extract of seeds, *SEM* Standard error of the mean

All values are expressed as Mean ± SEM ($N = 5$)

**$p < 0.01$, ***$p < 0.001$ were considered statistically significant compared to control

ailments. Such practice established consensus knowledge about the various medicinal properties of different plants and plant components. Plants provide a plethora of rich, complex, highly varied, and biologically important compounds that are of medicinal value. *C. maxima* (Rutaceae) also has various ethnopharmacological use [9]. Although different parts of this plant were investigated before, the seed has not been studied extensively. The current study focuses on the methanolic extracts of *C. maxima* (MECM) to elucidate its pharmacological potency as an anxiolytic, analgesic, and anti-inflammatory agent and indicate possible use in medicine (Table 7).

Depression and anxiety-related disorders are widespread psychiatric ailments [26]. With the advent of modern urban living, causes related to anxiety and depression-associated ailments are increasing exponentially. At present, there are several drugs like

Table 7 Effect of MECM on carrageenan-induced paw edema inhibition

Treatment (mg/kg)	Pre-injection mean paw circumference (mm)	Post-injection mean paw circumference (mm) (% inhibition)			
		1 hour	2 hour	3 hour	4 hour
Control-Tween 80 solution (10 ml/kg)	9.80 ± 0.58	14.60 ± 0.51 -	15.20 ± 0.49 -	14.80 ± 0.37 -	14.20 ± 0.49 -
Standard-Indomethacin (10 mg/kg)	10.20 ± 0.37	13.50 ± 0.35 (31.25%)	13.00 ± 0.35 48.15%	11.20 ± 0.41** (76%)	10.80 ± 0.44** (86.36%)
MECM 200 mg/kg	9.60 ± 0.51	13.90 ± 0.33 (10.41%)	13.40 ± 0.33 (29.63%)	12.40 ± 0.29** (44%)	11.70 ± 0.30** (52.27%)
MECM 400 mg/kg	9.30 ± 0.37	13.30 ± 0.37 (16.67%)	12.80 ± 0.37 35.19%	11.70 ± 0.44** (52%)	11.20 ± 0.34** (56.82%)

MECM Methanolic extract of seeds, *SEM* Standard error of the mean

All values are expressed as Mean ± SEM ($N = 5$)

**$p < 0.01$, ***$p < 0.001$ were considered statistically significant compared to control

Benzodiazepines (BZDs) available on the market to treat anxiety disorders. A benzodiazepine such as Diazepam is a CNS depressant that has a binding site on Gama-amino-butyric acid receptor type-A ionophore complex ($GABA_A$). Gama-amino-butyric acid (GABA) is the major repressive neurochemical within the central nervous system. The binding of GABA reduces neuronal excitability by increasing the action potential threshold resulting in decreased activity, excitement moderation, and calming effect [27–29]. Drugs like diazepam come with associated side effects like insomnia, cardiac arrhythmia, muscle weakness, etc. [30]. Several investigations reported that some plants and plant constituents containing flavonoids, saponins, and tannins can induce anxiolytic activity. These phytoconstituents may act as ligands for neurotransmitter receptors mimicking neuroactive steroids like GABA. Natural compounds showing GABA-mimetic activity may replace synthetic drugs due to their decreased side effects [31, 32]. The study on the anxiolytic activity as measured by the hole-board test and elevated plus-maze method showed that the MECM had significant anxiolytic activity compared to the control group. In the hole-board test, the increased number of head dipping represents neophilia or exploratory behavior. Concurrently, diazepam was used as a reference standard which acted as a highly potent anxiolytic agent. Though, diazepam showed a highly significant increase ($p < 0.001$) in head dipping at a low dose of 1 mg/kg body weight, the potential of MECM as an anti-anxiety agent still cannot be understated as it significantly increased ($p < 0.01$) the number of head dipping of mice compared to the control group. Another widely validated method named the elevated plus-maze (EPM) test is based on the tendency of rodents to avoid novel and potentially dangerous areas in a high anxiety state [33]. The animals usually avoid height and open spaces and have an increased preference for enclosed spaces, therefore, naturally tend to spend more time in the enclosed arm. Upon entering the open arm, the animal shows a fear-like response such as becoming immobile, defecating, etc. [34]. Aversion to the open arm is correlated with the elevated internal anxiety state in rodents. In the current study, MECM markedly increased ($p < 0.01$) the number of entries into the open arm and time spent in it at both doses (200 & 400 mg/kg) while compared to the control. A related study conducted by Sheik et al. (2014) revealed that the orally administered ethanolic leaf extract of *C. maxima* at the doses of 200 and 400 mg/kg body weight was found to have anxiolytic activity in rats [35]. The findings of the current study indicate the potential use of MECM as a mild anxiolytic agent requiring further pharmacological investigations to cement this claim.

Pain is a natural response to noxious stimulants that serve as a warning system and invoke a protective response from the body. Although beneficial, it can cause a lot of discomfort and adverse responses. Analgesics are used as an adjuvant to alleviate discomfort in pain management [36, 37]. Worryingly, frequently prescribed analgesic drugs, particularly opioids and NSAIDs, can only relieve 50% of the pain in about 30% of patients. Moreover, existing analgesics have serious side effects such as ulceration, respiratory distress, gastrointestinal bleeding, drowsiness, nausea, etc. [38, 39]. Based on this information, it is crucial to develop analgesics from natural sources that are more effective and have reduced side effects. In rodents, the acetic acid-induced pain response is manifested via abdominal constriction (writhing) together with stretching of hind limbs. Such pain stimulus triggers localized inflammatory response by release of free arachidonic acid from tissue phospholipids [22, 40]. In the acetic acid-induced writhing method, the decreased number of writhing as well as the increased percentage of writhing inhibition correlates with a higher peripheral analgesic effect in mice. MECM showed notably significant inhibition ($p < 0.001$) in writhing that is comparable to standard drug Diclofenac sodium (50 mg/kg). The inhibitory effects of MECM against acetic acid-induced writhing may be attributable to the retardation of prostaglandins action. Shivananda et al. (2013) found that leaf, stem bark, and fruit peel of *C. maxima* exhibited significant analgesic activity in the acetic acid-induced writhing method [41]. Furthermore, the formalin-induced hind paw licking was used to assess the central and peripheral analgesic effect of the investigated extracts. This test correlates with two well-defined phases of analgesia. In the initial (neurogenic) phase, pain is induced by direct stimulation of sensory nerve fiber by formalin. Subsequent release of inflammatory mediators such as histamine, prostaglandin, and bradykinin marks the pain response in the later (inflammatory) phase [42]. Established data shows that centrally acting analgesics such as narcotics inhibit both phases equally whereas peripheral analgesics such as NSAIDs increasingly inhibit the later phase [43, 44]. In the current study, MECM produced marked analgesia in the early ($p < 0.01$) and late phases ($p < 0.001$) of the test similar to narcotic analgesic morphine (10 mg/kg), suggesting a possible central and peripheral mechanism of the analgesic effect of MECM.

Inflammation is the signaling system to summon the natural defense mechanism of the body to mount resistance against intruding elements. Without inflammation as a physiological response, wounds can fester and infections become lethal [45]. Pain resulting from inflammation is managed by conventional steroidal and non-steroidal anti-inflammatory drugs which have various

adverse effects such as bronchospasm, gastrointestinal ulceration, fluid retention, and prolongation of bleeding time. Several drugs originating from natural sources are being used in the management of pain and inflammation in recent years [46, 47]. Consequently, the current study was conducted to evaluate the anti-inflammatory activity of MECM by using formalin-induced and carrageenan-induced paw edema tests. Formalin-induced inflammation occurs in a biphasic manner. An early neurogenic phase is marked by the release of histamine, 5-HT, and kinin and the later tissue mediated response manifests through the release of prostaglandins [48]. As shown in Table 5, MECM showed significant inhibition ($p < 0.01$) of formalin-induced paw edema at both doses in the early phase and highly significant inhibition ($p < 0.001$) in the late phase of post-injection which is comparable to the standard. Carrageenan-induced inflammation has significant predictive value for anti-inflammatory agents acting through mediators of acute inflammation [49]. Carrageenan, derived from Irish Sea moss Chondrus, is a mucopolysaccharide that is non-antigenic and does not have any systemic effects [25]. The acute inflammation produced by carrageenan is believed to be biphasic where histamine and serotonin predominantly mediate edema production in the early phase (0–2 h after injection) and bradykinin and prostaglandins maintain vascular permeability in the late phase (2–4 hr) [50]. Clinically used anti-inflammatory drugs impacting the second phase of carrageenan-induced edema can be used to assess the antiphlogistic effect of naturally derived products [51, 52]. The intraperitoneal administration of the MECM (200 & 400 mg/kg MECM) caused significant ($P < 0.01$) inhibition of the late phase edema with negligible inhibitory effect in the initial phase of the test. These findings indicate bioactive components in MEMC possibly inhibit the prostaglandin pathway mimicking the activity of clinically used anti-inflammatory drugs that justifies further investigation of MEMC as an anti-inflammatory agent in this regard.

Conclusion

The investigated plant *C. maxima* is a widely used ethnobotanical plant whose methanolic seed extract exhibited potential as an anti-inflammatory, analgesic, and anxiolytic agent. However, this exploratory research work can be further extended to study the mechanism of action of the investigated extracts to identify and isolate the functional compounds that might be responsible for their suggested pharmacological effects. Such investigation may lead to the development of novel drug compounds having superior pharmacological and safety profiles.

Abbreviations

MECM	Methanolic extract of *Citrus Maxima*
CNS	Central nervous system
BFRI	Bangladesh Forest Research Institute
EPM	Elevated plus-maze
IP	Intraperitoneal
SEM	Standard Error of the Mean
ANOVA	Analysis of variance
ICDDR, B	International Centre for Diarrhoeal Disease Research, Bangladesh

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Authors' contributions

MTA designed the experiments and conception. NNM, UT, AAJ conducted the experiments. Data interpretation and analysis were performed by NNM, UT, AAJ and MTA. MTA, NNM, and DUS wrote the manuscript. MTA and DUS made the necessary corrections in the write-up and gave final approval for the submission of the revised version. All authors read and approved the final manuscript.

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Availability of data and materials

All data and materials are contained and described within the manuscript. The data set was deposited in publicly available repositories. The plant's materials for the study were identified and voucher specimens are deposited at Pharmacy Department of University of Chittagong.

Declarations

Ethics approval

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85–23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the Animal Ethics Review Committee (AERB), Faculty of Biological Sciences, University of Chittagong.

Consent for publication

All authors have given their consent to publish this article.

Competing interests

The authors declare that they have no competing interests.

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