# Evaluation of Antioxidant, Antibacterial and Analgesic Activities of *Syzygium cumini* used in Bangladesh

**Margia Sultana, Jamiuddin Ahmed, Md Abdul Barek, Shaidul Kabir Sohag, Md. Safayet Hossain and Umme Habiba**

Department of Pharmacy, Noakhali Science and Technology University, Noakhali-3814, Bangladesh

**(Received: February 28, 2021; Accepted: February 1, 2022; Published (Web): June 22, 2022)**

**ABSTACT:** *Syzygium cumini* (L.) skeels (Family: Myrtaceae) is a renowned medicinal plant traditionally used in various diseases and available in Bangladesh. This investigation was aimed to explore whether this plant has any potential antioxidant, antibacterial, and analgesic effects of methanolic extracts of *S. cumini* (leaves and bark). Quantitative determination of methanolic extracts of leaves and bark found the total phenolic contents as 199.11 mg GAE/g DW and 204.03 mg GAE/g DW, respectively. The leaves extract possessed mild antibacterial activity (leaves: 7.00 mm at 1 mg/disc, 9.15 mm at 5 mg/disc) against two different bacteria at different concentration whereas the bark extract showed no effect. A significant writhing (leaves: 23.17 ± 1.80, $p \leq 0.001$; bark: 24.33 ± 1.15, $p \leq 0.001$) and licking effects (leaves: 8.17 ± 1.49 sec, $p \leq 0.05$; bark: 9.08 ± 1.96 sec, $p \leq 0.05$) were found at 400 mg/kg of leaves and bark extracts of this plant which is very close to standard drugs mentioned in the tables. Results of this study demonstrated that methanolic extracts of *S. cumini* leaf and bark possessed significant antioxidant, analgesic, and mild antibacterial like activities which tend to suggest medicinal aspects.

**Key words:** Antioxidant, antibacterial, analgesic, writhing, licking, *Syzygium cumini*

## INTRODUCTION

From the very beginning of civilization to till now, nature always plays a great role for the salvation of human beings by providing life-saving oxygen, foods, medicines etc. [1] Due to their curing properties and beneficial effects on health, medicinal plants influence people all over the world from thousand years of human development.[2] Numerous phytochemicals present in different parts of medicinal plants which give beneficial effects for curing of human diseases. Not only in Asia, about 75% people worldwide still rely on medicinal flora in recent days and about 35,000-70,000 plant species are still the main source of drugs for the world's population.[3,4] *S. cumini* is distributed in India, Bangladesh, Myanmar, Nepal, Pakistan, Sri Lanka, Indonesia, Malaysia, USA, Eastern Africa, Madagascar etc.[5]

Biological cells are affected by free radicals which are neutralized by different substances called antioxidants.[6] Natural antioxidants containing low side effects and help to protect from various disease.[7] For this reason, plant materials, such as medicinal plants (herbs, spices, etc.), are considered as a promising source of effective antioxidants. Infectious diseases caused by organisms such as bacteria, virus, fungi, or parasites responsible for premature death in the whole world, killing almost 50,000 people every day.[8] High cost, obnoxious use, adverse effects and resistance of antibiotic drugs are increasing day by day.[9,10] Medicinal plants and phytochemicals are rich sources of antimicrobial agents that have been used for centuries to treat infectious diseases.[11] Pain is an emotional experience and unpleasant sensory perceive during acute or chronic tissue damage.[12] Several studies report that NSAIDs and other painkiller give some side effects and increase the risk of gastrointestinal, kidney, and cardiovascular diseases. Thus, researchers are looking for a new and stronger alternative to treat the pain with less adverse effects.[13]

Traditionally different parts of *S. cumini* (like *e.g.*, bark, leaves, seeds, fruit, etc.) have been used in the medication of different illness condition such as diabetes, inflammation, pain, fever, headache, microbial infection, diarrhea, dysentery, gastric ulcer,

**Correspondence to: Jamiuddin Ahmed**
Tel.: +8801909333648
Email: ajami_ju@yahoo.com

Dhaka Univ. J. Pharm. Sci. **21**(1): 15-24, 2022 (June)
DOI: https://doi.org/10.3329/dujps.v21i1.60392

cancer, hypertension, allergy.[5] Several pharmacological studies of different parts of this plant were also reported for their antioxidant, anti-inflammatory, neurological, antimicrobial, anticancer, antileishmanial, analgesic, antipyretic, antidiarrheal, antifertility, anorexigenic, gastro-protective, anti-ulcerogenic and hepatoprotective and other activities.[14] Several chemical constituents of *S. cumini* have been found already such as quercetin, rutin, 3,5,7,4- tetrahydroxy flavones, caffeic acid, ellagic acid, ferullic acid, albumen, fat, jambosine, ellagic acid , lauric, myristic, palmitic, stearic, oleic acid, linoleic, malvalic and vernolic acid, myricetine, phytosterols, ferrullic acid, catechin, cretegolic acid, n-dotricontanol, myrcetin, mycaminose, quercetin, tannic acid, butylated hydroxyanisole, tocopherol, oleanolic acid, triterpenoid-A, triterpenoid-B.[15] In this study, we have tried to explore the antioxidant, antibacterial, and pain relieving activity of methanolic extracts of leaves and bark of *S. cumini*.

## MATERIALS AND METHODS

**Chemicals and reagents.** Analytical grade chemicals and reagents were used in this whole research work.

**Plant collection.** *S. cumini* was collected from the local region of Laxmipur, Bangladesh in August 2019 and this plant was recognized by Bangladesh National Herbarium, Mirpur, Dhaka. The accession numeral is DACB 46776.

**Plant extract preparation.** Whole plants of *S. cumini* were thoroughly washed with water. After drying and grinding about 400 gm of leaves and 500 gm bark, extracts were prepared by cold extraction method.

**Animals.** Both sexes of Swiss albino mice, aged six-seven weeks, having a mean body weight of 25 ± 5.0 g were collected from the mice lab, Jahangir Nagar University, Savar, Bangladesh. The animals were given free access to water and food and kept in a clean cage filled with saw-dust which was changed four times in a week.

**Phytochemical analysis.** Phytochemical studies of leaves and bark extracts of *S. cumini* were carried out to investigate the existence of alkaloids, cardiac glycosides, steroids, phenol, carbohydrate, polysterol, tannins, saponins, flavonoids and proteins according to the methods described in Trease and Evans.[16]

**Quantitative determination of phenolic compounds of crude extract**

**Analysis of total phenolic content.** The method for detection of total phenolic content using Folin–Ciocalteu assay described by Meda *et al*. (2005), was used for the detection of total phenolic contents of the plant extracts.[17] The absorbance was then quantified at 765 nm using a UV-1601 Shimadzu UV-Vis spectrophotometer and results were defined as milligram gallic acid equivalents per gram of dry weight of the extract (mg GAE/g DW).

**Analysis of total flavonoid content.** The method for detection of total flavonoid content described by Lin and Tang *et al.* (2007) was used for the detection of total flavonoid contents of the plant extracts.[18] 415 nm using for determination of absorbance by using a UV-visible spectrophotometer and the results were defined as mg of quercetin equivalents per gram of dry weight of the extract (mg QE/g DW).

**Analysis of total flavonol content.** The method for detection of total flavonol content described by Kumaran and Karunakaran *et al.* (2007) was used for the detection of total flavonol contents of the plant extracts.[19] The absorbance was calculated at 440 nm. A standard calibration curve (range: 0 to 50 μg/mL) was produced by treated quercetin in the same manner as the sample.

**Antioxidant activities evaluation**

**DPPH antioxidant assay.** The method for detection of DPPH antioxidant assay described by Cavar *et al.* (2012).[20] Percentage of DPPH radical neutralizing activity was calculated as follows:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

$IC_{50}$ was used for the expression of antioxidant activity of the extract.

**Analysis of reducing power assay**. Reducing power assay of the plant extracts and the standard (ascorbic acid) were assessed according to the method described by Oyaizu, (1986)[21] with minor modifications. The results were expressed as median effective concentration i.e., the-$EC_{50}$ values.

**Total antioxidant assay**. The method for detection of total antioxidant activity described by Kubola & Siriamornpun *et al.* (2008).[22] Total

antioxidant activity measured by comparing with the standard (ascorbic acid, 5-500 µg/ml) and the results were assay defined as mg of ascorbic acid equivalent per g dry weight of extract (mg AE/g DW).

**Antibacterial screening**

**Microorganisms.** A total of six different microorganisms were used to examine antibacterial potentials of *Syzygium cumini* methanolic extracts. The test organisms included where three gram-negative bacteria (Escherichia coli ATCC 25922, Pseudomonas acruginose ATCC 27853 and klebsiella pneumonia ATCC 700603 and three gram-positive bacteria (*Bacillus* sp. ATCC 6633, *Staphylococcus aureus* ATCC 25923 and *Enterococcus* sp. ATCC 29212). All microorganisms were obtained from the International Centre for Diarrheal Disease Research, Bangladesh (icddr,b).

**Disc diffusion method.** Disc diffusion method was used for enumerating zone diameter as a measure of antibacterial effect of *S. cumini* methanolic extracts as described by Sacchati.[23] The zone diameter was measured in mm using a ruler.

**Anti-nociceptive effect**

**Acetic acid-induced writhing test.** Acetic acid induced writhing test as described by Koster *et al*. (1959) was used to investigate the anti-nociceptive effect of the plant extract.[24] For this, control (10 mL/kg bw, p.o.), diclofenac sodium (10 mg/kg bw, p.o.) and test samples (200 mg/kg bw & 400 mg/kg bw, p.o.) were administered 30 min before induction of writhing by 0.6% acetic acid (i.p.). For each mouse writhing number was counted and recorded for 20 minutes. Writhing was defined as contractions of the abdomen, elongation of the body, twisting of the trunk and/or pelvises ending with the extension of the limbs. Results were expressed as mean percent inhibition of writhing (PIW):

$$\text{PIW} = \frac{No.\ of\ Writhes\ (control) - No.\ of\ Writhes\ (sample)}{No.\ of\ Writhes\ (control)}\ x\ 100$$

**Formalin induced licking test.** Formalin induced licking test was described by Hishe *et al*. (2018) with minor modifications was used to investigate the anti-nociceptive effect of the plant extract.[25] Control (10 mL/kg bw, p.o.), diclofenac sodium (10 mg/kg bw, p.o.) and test samples (200 mg/kg bw & 400 mg/kg bw, p.o.) were administered 30 minutes prior to 0.02 ml of 2.5% formalin injection into the plain surface (s.c.) of left hind paw of mouse. In this analysis, two phases are involved with this investigation. Phase 1 called the early phase which was recorded during the first 0-5 minutes, while phase 2 named as the late phase (last 20–30 minutes after formalin injection). The experimental results were expressed as percent inhibition of licking response (PIL) in mice:

$$\text{PIL} = \frac{\text{Time spent licking for Control} - \text{Time spent licking for Sample}}{\text{Time spent licking for Control}}\ \text{x}\ 100$$

**Statistical analysis**. All data are expressed as the mean ± SD. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 20.0 software. For calculation of difference between group means, a one-way analysis of variance (ANOVA) followed by Dunnett's 't' test was performed. A probability level of 0.05 or less was considered significant.

## RESULTS AND DISCUSSION

**Phytochemical screening.** The phytochemical analysis of methanolic extracts of leaves and bark of *S. cumini* are summarized in table 1. These chemical tests indicated the existence of alkaloids, carbohydrates glycosides, steroids, saponins, polysterol, phenol, tannins, and flavonoids in both extract leaves and bark except proteins. Phytochemicals like alkaloid, saponin, carbohydrate, glycoside, flavonoid, steroid, tannin, phenolic compound, volatile oil, etc. extracted from numerous species of medicinal plants which have potential therapeutic applications for the different disease.[26] Flavonoids and phenolic compounds give antioxidant, anti-carcinogenic, anti-atherosclerotic, antibacterial,

analgesic, and anti-inflammatory effects.[27] Saponins have been found to have antimicrobial, antidiabetic, cytotoxic, antioxidant and anthelmintic effects.[28,29]

**Estimation of total phenolic, flavonoid and flavanol contents.** Phenolic compounds have multiple biological effects such as the prevention of platelet aggregation and red blood cell damage and are one of the most important phytochemicals. Phenolic compounds also neutralize free radicals by acting as antioxidants, singlet oxygen quenchers and metal chelator.[30] The extract of *S. cumini* leaves and bark contained 199.11 mg GAE/g DW and 204.03 GAE/g DW phenolic compounds that exert their antioxidant properties by a redox reaction. Flavonoids (quercetin, kaempferol, catechins, and anthocyanidins) are the most common group of polyphenolic compounds thought to have a good antioxidant effect.[27] The results revealed that methanolic extract of *S. cumini* leaves and bark contained a considerable amount of phenolic compounds (199.11 mg GAE/g DW, 204.03 mg GAE/g DW respectively). Total flavonoid and total flavonol quantity in leaves were higher than that in methanolic extracts of *S. cumini* bark (table 2). Previous report about different extracts *S. cumini* shows a significant level of total phenol, f**lav**onoid, and flavanol content.

**Table 1. Phytochemical analysis of methanolic extract of *Syzygium cumini*.**

| Phytoconstituent | Methanol extract of *S. cumini* | |
|---|---|---|
| | Leaves | Bark |
| Alkaloids | | |
| - Mayer's test | + | + |
| - Wagner's test | + | + |
| - Dragendorff's test | + | + |
| Cardiac Glycosides | | |
| - Keller Kiliani test | + | + |
| Carbohydrates | | |
| - Molisch's test | + | + |
| Steroids | | |
| - Salkowski's test | + | + |
| - Lieberman-Burchard test | + | + |
| Saponins | | |
| - Frothing test | + | + |
| Phytosterol | | |
| - Liebermann- Burchard test | + | + |
| Phenol | | |
| - Ferric chloride test | + | + |
| Tanins | | |
| - Gelatin test | + | + |
| Flavonoids | | |
| - Lead acetate test | + | + |
| - Alkaline Reagent test | + | + |
| Proteins | | |
| - Xanthoproteic test | - | - |

'+' indicates the presence of constituents and '-' indicates the absence

**Table 2. Result of total phenolic, flavonoid and flavanol contents of methanolic extracts of *S. cumini* leaves and bark.**

| Plant extract | Total phenolic content (mg GAE/g DW)[a] | Total flavonoid content (mg QE/g DW)[b] | Total flavanol content (mg QE/g DW)[b] |
|---|---|---|---|
| Leaves | 199.11 | 16.85 | 48.05 |
| Bark | 204.03 | 7.69 | 14.06 |

[a] Total phenolic content is expressed as mg gallic acid equivalents per g dry weight (mg GAE/g DW).

[b] Total flavonoid and flavanol contents are expressed as mg quercetin equivalents per g DW (mg QE/g DW).

**Antioxidant activities.** The plants play an important pharmacological role against harmful reactive oxygen species by their antioxidant capacity in the human body. We measured the antioxidant capacity of leaves and bark extract of *S. cumini* plant by DPPH, reducing power and total antioxidant capacity assay. These tests evaluate the capability of the extract to neutralize free radicals. The results from these tests showed significant antioxidant activity. In the DPPH study, both plant extracts exhibited to quench DPPH free radicals, as indicated in Figure 1A by comparing their IC50 values to the reference standard ascorbic acid. Highest activity found in standard ascorbic acid (97.20%), than leaves (94.92%) and barks (92.97%). The leaves ($IC_{50}$ = 128.07 µg/ml) and bark ($IC_{50}$ = 120.75 µg/ml) extract

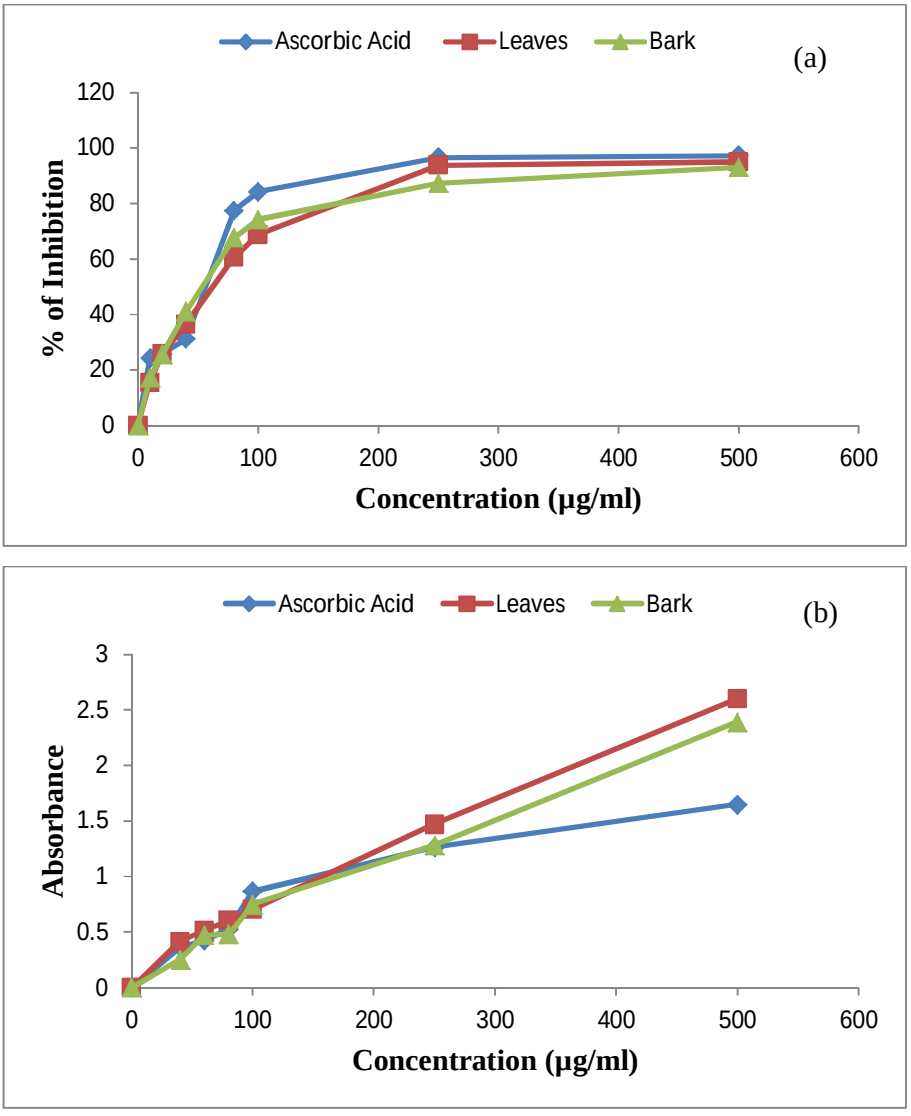


**Figure 1.** Antioxidant effects of methanolic extracts of *S. cumini* leaves and bark. (a) DPPH radical scavenging effect (b) Reducing power effect.

showed lower antioxidant activity than the standards-ascorbic acid ($IC_{50}$ = 97.96 µg/ml) (table 3). The reducing power efficacy (dose dependent response) of *S.cumini* methanolic extracts with reference standard shown in Figure 1B. Significant reducing activity was found in both *S. cumini* plant extracts and standards. The reducing power effect of *S. cumini* methanolic extracts were found to increase with the increasing

concentration. Bark extract showed higher degree of electron donation ($EC_{50}$ = 81.30 µg/ml) than leaves extract ($EC_{50}$ = 65.66 µg/ml) and reference standards-ascorbic acid ($EC_{50}$ = 72.42 µg/ml) (table 3). Calibration curve of total antioxidant assay were shown in figure 2. Leaves extract showed higher antioxidant capacity (214.14 µg/ml) than bark extract (151.36 µg/ml) of the plant. The presence of phenolic chemicals, flavonoids, and vitamins has previously been reported to have powerful antioxidant properties.[31]

**Table 3. Antioxidant test of methanolic extract of leaves and bark of *S. cumini* in DPPH and RP assays.**

| Plant extract | $IC_{50}$ in DPPH radical scavenging analysis (µg/ml) | $EC_{50}$ in reducing power analysis (µg/ml) |
|---|---|---|
| Ascorbic Acid | 97.96 | 72.42 |
| Leaves | 128.07 | 65.66 |
| Bark | 120.75 | 81.30 |

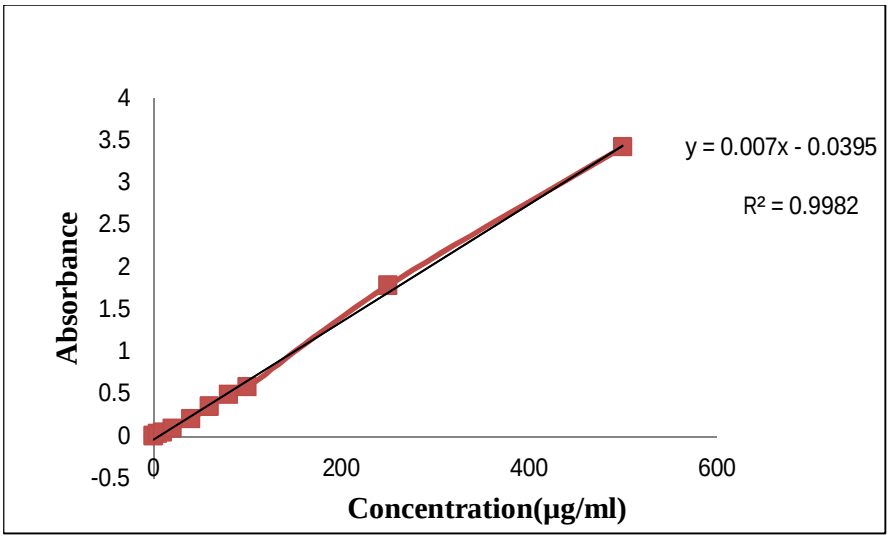


Figure 2. Ascorbic acid standard curve (Abs. vs. Conc.) for determining TAC

**Antibacterial activity.** Antibacterial activity of both parts of *S. cumini* against six pathogens were evaluated by the Disk-diffusion methods. We tested our two methanolic plant at different concentrations (0.1 mg/disc to 5 mg/disc) shown in table 4. No zones of inhibition were found against gram-negative bacteria but the different concentrations of leaves extract give zones of inhibition (varying from 6.5 to 9.15 mm) against *Staphylococcus aureus* are shown table 4. Similarly, no zones of inhibition were identified in bark extract against gram-positive bacteria, however at 5 mg/disc concentrations, *Pseudomonas aeruginosa* showed a zone of inhibition (6.5 mm). Secondary metabolites or some bioactive compounds of plant extracts give antibacterial activities. Several studies found that flavonoids give activity against microorganism by blocking DNA gyrase enzyme.[32] The presence of tannins, saponins also responsible for antimicrobial action.[33]

**Analgesic evaluation.** Both the acetic acid induced writhing test and the formalin induced hind paw licking test were used to evaluate the analgesic efficacy of *S. cumini* leaves and bark extract on the peripheral model of pain. In this test, standard gave 24.50 ± 1.11 ( $p \leq 0.001$) writhes and control mice gave 45.83 ± 2.44 ($p \leq 0.001$) writhes (table 5). Plant extracts of both concentrations significantly decreased writhes compared to control and standard. Specifically, 400 mg/kg of leaves extract showed 23.17 ± 1.80 ($p \leq 0.001$) writhes while 400 mg/kg of bark extract showed 24.33 ± 1.15 ($p \leq 0.001$) writhes. Thus 400 mg/kg of both leaf and bark extract showed significant percentage inhibition of writhing response with standard drug- diclofenac (46.54%). Acetic acid

administration was reported to increase capillary permeability and release certain endogenous mediators like prostaglandin. The released prostaglandin is related to excite pain nerve ending [34, 35], peritoneal mast cells disruption[36] which is the consequence of pain. Common NSAIDs can prevent transduction of primary afferent nociceptors by blocking COX pathway in peripheral tissues. The extract produced better effects in both phases (table 6). Standard group licking duration dropped significantly from the early phase (20.63 ± 1.88 s, $p \leq 0.001$) to the late phase (7.67 ± 2.18 s, $p \leq 0.05$). At the late phase, 400 mg/kg leaf extract showed highest effect (8.17 ± 1.49 s, $p \leq 0.05$) in licking test as showed in figure 3. At the dose of 400 mg/kg and in the late phase, both leaf and bark extracts exhibited 78.55% and 76.16% inhibition, respectively while

**Table 4. Antibacterial effect of methanolic leaves & bark extract of *S. cumini*.**

| Microorganism | Mean inhibition zone (mm) | | | | | |
|---|---|---|---|---|---|---|
| | Standard (Amikacin) | Extract concentrations | | | | |
| | 3 mg/disc | 5 mg/disc | 1 mg/disc | 0.5 mg/disc | 0.25 mg/disc | 0.10 mg/disc |
| **Leaf extract** | | | | | | |
| **Gram Negative** | | | | | | |
| *Pseudomonas aeruginosa* | 8±0.00 | - | - | - | - | - |
| *Escherichia coli* | 11±0.00 | - | - | - | - | - |
| *Klebsiella pneumonia* | 10±0.00 | - | - | - | - | - |
| **Gram Positive** | | | | | | |
| *Staphylococcus aureus* | 13±0.00 | 9.15 ±0.58 | 7±0.00 | 6.5±0.00 | - | - |
| *Bacillus subtilis* | 9±0.00 | - | - | - | - | - |
| *Enterococcus faecalis* | 11±0.00 | - | - | - | - | - |
| **BarkeExtract** | | | | | | |
| **Gram Negative** | | | | | | |
| *Pseudomonas aeruginosa* | 8±0.00 | 6.5±0.00 | - | - | - | - |
| *Escherichia coli* | 11±0.00 | - | - | - | - | - |
| *Klebsiella pneumonia* | 10±0.00 | - | - | - | - | - |
| **Gram Positive** | | - | - | - | - | - |
| *Staphylococcus aureus* | 13±0.00 | - | - | - | - | - |
| *Bacillus subtilis* | 9±0.00 | - | - | - | - | - |
| *Enterococcus faecalis* | 11±0.00 | - | - | - | - | - |

Values are means ± S.E.M ($n = 3$)

**Table 5. Effect of methanolic extract of *S. cumini* on the number of writhing responses in acetic acid induced writhing test.**

| Group | N | Writhing response | Percent inhibition of writhing (PIW) |
|---|---|---|---|
| Control | 6 | $45.83 \pm 2.44^{\gamma}$ | - |
| Standard | 6 | $24.50 \pm 1.11^{c}$ | 46.54% |
| SL200 | 6 | $26.83 \pm 1.90^{b}$ | 41.46% |
| SL400 | 6 | $23.17 \pm 1.80^{c}$ | 49.44% |
| SB200 | 6 | $31.17 \pm 2.55^{a}$ | 31.99% |
| SB400 | 6 | $24.33 \pm 1.15^{c}$ | 46.91% |

'N' indicates the per group mice number; 'SL' stands for *S. cumini* leaves extract and 'SB' stands for *S. cumini* bark extract. Values are expressed as the mean ± SEM and all data were analyzed using ANOVA followed by Bonferroni test for multiple comparisons for means. Significant differences were represented by $^{a}p\leq0.05$, $^{b}p\leq0.01$, $^{c}p\leq0.001$ vs. control and $^{\alpha}p\leq0.05$, $^{\beta}p\leq0.01$, $^{\gamma}p\leq0.001$ vs. diclofenac.

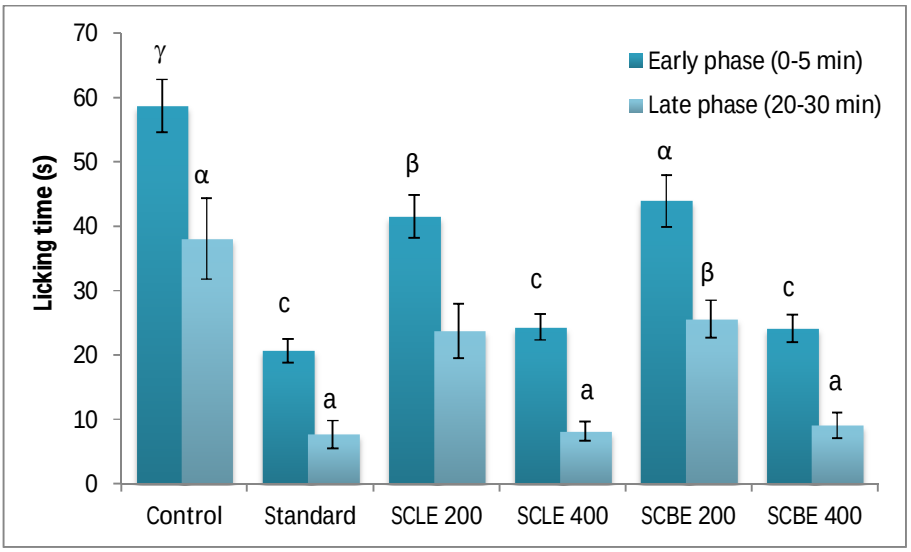


Figure 3. Formalin induced paw licking test response of *S. cumini* methanolic extracts. Values are expressed as the mean ± SEM and all data were analyzed using ANOVA followed by Bonferroni test for multiple comparisons for means. Significant differences were represented by $^{a}p\leq0.05$, $^{b}p\leq0.01$, $^{c}p\leq0.001$ vs. control and $^{\alpha}p\leq0.05$, $^{\beta}p\leq0.01$, $^{\gamma}p\leq0.001$ vs. diclofenac.

**Table 6. Effect of methanolic extract of *S. cumini* on percent inhibition in hind paw licking test.**

| Group | N | Percent inhibition of licking (PIL) | |
|---|---|---|---|
| | | Early phase (0-5 min) | Late phase (20-30 min) |
| Control | 6 | - | - |
| Standard | 6 | 64.84% | 79.86% |
| SL200 | 6 | 29.27% | 37.63% |
| SL400 | 6 | 58.53% | 78.55% |
| SB200 | 6 | 25.14% | 32.83% |
| SB400 | 6 | 58.96% | 76.16% |

'N' indicates the per group mice number; 'SL' stands for *S. cumini* leaves extract and 'SB' stands for *S. cumini* bark extract. Values are expressed as the mean ± SEM and all data were analyzed using ANOVA followed by Bonferroni test for multiple comparisons for means. Significant differences were represented by $^{a}p\leq0.05$, $^{b}p\leq0.01$, $^{c}p\leq0.001$ vs. control and $^{\alpha}p\leq0.05$, $^{\beta}p\leq0.01$, $^{\gamma}p\leq0.001$ vs. diclofenac.

inhibition by the standard was 79.86% in the late phase (20-30 min) (table 6). It has been reported that, some local anesthetic or opiates, which are selective blocker of first phase nociception can act on second phase also.[37,38] Both extracts of the selected plant decreased licking time in both phases probably by blocking the related nociceptive pathways. The presence of Alkaloids, flavonoids, saponins, sterols and so on in extracts of different plants has previously been reported to have powerful analgesic and anti-inflammatory properties.[39-41] Phytochemical screening of *S. cumini* showed the presence of saponins and flavonoids which could be responsible for the anti-nociceptive effect of extracts of *S. cumini* due to the presence of these compounds or in combination.

## CONCLUSION

From present study, it can be concluded that leaves and bark of *S. cumini* possess significant analgesic, anxiolytic activity, and mild antibacterial activities due its phytoconstituents in the extracts. More biochemical and pharmacological investigation, however, are needed in future to establish these pharmacological outcomes.

### Conflict of interest

The authors declare that they have no conflict of interests.

### Authors Contributions

MS and JA designed the study. MS, UH and SKS performed the experiments. JA, MAB and MSH

wrote the manuscript. JA and MAB performed statistical analysis. JA supervised the whole project from experiment to final manuscript. All authors reviews and give approval for the final draft of the manuscript.

**ACKNOWLEDGEMENTS**

All authors are thankful to the Department of Pharmacy, Noakhali Science and Technology University for providing instrumental support and laboratory space. We wish to express our sincere gratitude to the Research Cell of Noakhali Science and Technology University for financial support.



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