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# Evaluation of *In vitro* Anti-Inflammatory and Antimicrobial Activities of *Abelmoschus moschatus* L. Seed Extracts

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**ABSTRACT**
*Phytochemicals with antioxidant, antimicrobial and anti-inflammatory properties have remarkable potential in suppressing both plant and human diseases. The present study was designed to evaluate the in vitro anti-inflammatory and antimicrobial potential of ethanolic and methanolic seed extracts of Abelmoschus moschatus. Phytochemical analysis showed the presence of phenols, flavonoids, tannins, alkaloids, glycosides and phytosterols. Total phenols, flavonoids and ascorbic acid content of the extracts were determined and the extracts showed remarkable free radical scavenging properties. Methanolic seed extract exhibited strong membrane stabilizing activity and was effective in inhibiting heat induced hemolysis, protein denaturation, COX and LOX activities at a concentration of 500µg/ml. Further, the extracts are effective in inhibiting the growth of both gram positive and gram negative bacterial strains tested. The present study provides a strong evidence to support the synergistic effect of Abelmoschus moschatus seed extracts having potent anti-inflammatory and antimicrobial property, which might serve as an effective drug for various microbial infections and inflammatory disorders.*
***Keywords:*** *Abelmoschus moschatus, Phytochemicals, antioxidant, antimicrobial, anti-inflammatory.*

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## INTRODUCTION

Now-a-days, medicinal plants play a vital for the treatment of various disease conditions, such as diabetes, cancer, inflammatory diseases, arthritis, malaria etc. Plants produce wide range of secondary metabolites such as phenols, flavonoids, quinines, coumarins, alkaloids, terpenoids etc. have been proved for antioxidant activities (1,2). Cell damage caused by free radicals appears to be a major contributor to aging, cancer, cardiovascular disease, cataracts, immune system decline and brain dysfunction (3). Natural compounds are a source of numerous therapeutic agents and recent progress to discover drugs from natural sources has resulted in compounds that are being developed to treat cancer, bacterial and viral diseases and immunosuppressive disorders (4). Inflammation is a complex process, which is frequently associated with pain and involves the occurrence of increase of vascular permeability, increase of protein denaturation and membrane alteration. Leukocytes are the key players of inflammatory response eliminate microbes and dead cells by phagocytosis followed by their destruction. Enzymes and reactive oxygen species may be released into the extracellular environment where they act as mediators of inflammation. Such mediators are mainly arachidonic acid metabolites, generated through Cyclooxygenase and Lipoxygenase pathways. Most of the anti-inflammatory drugs are targeted on these pathways (5).

During inflammation, mainly macrophages, promote the production of pro-inflammatory mediators which include interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, reactive oxygen species (ROS), nitric oxide (NO) and prostaglandins (PGs). However, prolonged chronic inflammation has been linked to the development of various autoimmune diseases including atherosclerosis and type I diabetes (6). Non-steroid anti-inflammatory drugs (NSAIDs) are generally used for slowing down the inflammation process and the use of NSAIDs has many side effects, such as gastrointestinal disorders, water retention, renal failure, bronchospasm, and hypersensitivity reactions (7). Thus, screening of safe and effective sources of potential anti-inflammatory agents and understanding of their modes of action will be essential. The increase in prevalence of multiple drug resistance has slowed down the development of

new synthetic antimicrobial drugs and has necessitated the search for new antimicrobials from alternative sources. Phytochemicals from medicinal plants showing antimicrobial activities have the potential of filling this need, because their structures are different from those of the more studied microbial sources and therefore their mode of action is also very likely to differ. Screening of bioactive compounds from plants has led to the discovery of new medicinal drugs which have efficient protection and treatment roles against various diseases.

Therefore, there is a need and demand for developing natural antioxidants, effective anti-inflammatory and antimicrobial drugs. Hence, the present study was aimed to investigate the antioxidant, anti-inflammatory and antimicrobial properties of ethanolic and methanolic seed extracts of *Abelmoschus moschatus* which belongs to the family, *Malvaceae*, popularly known as Mushkdana/Kasturi bhendi. The seeds are considered as valuable traditional medicine and the aromatic seeds are aphrodisiac, ophthalmic, cardiotonic, antispasmodic and used in the treatment of intestinal complaints. The seeds are used as stimulant, relaxant and also for casting out the poison of snakes.

## MATERIALS AND METHODS

### Source

*Abelmoschus moschatus* plants bearing pods of uniform size were selected in and around Visakhapatnam District. Seeds were removed, shade dried, coarsely powdered and used for phytochemical, anti-inflammatory and antimicrobial studies.

### Preparation of ethanolic and methanolic seeds extracts

250 g of dried and powdered seed sample was soaked in 1000ml of ethanol and methanol separately. After 15 days, the solution was collected through filtration by using cheese cloth and Whatman filter paper. The resulting filtrates were then evaporated in a rotary evaporator below $50^0$C to dryness and thus a concentrated semisolid mass of the extracts was obtained.

### Phytochemical evaluation

Phytochemical tests were checked for the extracts as per the standard methods followed by Roopashree et al. (8) and Obasi et al. (9).

### Quantitative estimation of phytochemicals

### Estimation of total phenolics

The total phenolics were determined using the FolinCio-calteau reagent as reported by Javanmardi et al. (10).

### Determination of Total Flavonoids

Total flavonoid content of extracts was determined according to a modified colorimetric method of Bao et al. (11).

### Estimation of Ascorbic acid

Ascorbic acid content was determined by the procedure given by and Theymoli Balasubramanian and Sadasivam (12).

### Total antioxidant activity (TAA)

The assay was based on the reduction of Mo (VI)-Mo (V) by the extracts and subsequent formation of a green phosphate Mo (V) complex at acidic pH (13).

### Reducing power assay

The reducing power was determined by the $Fe^{3+}$-$Fe^{2+}$ transformation in the presence of extracts as described by method of Ferreira et al. (14).

### Membrane stabilization Assay

The human red blood cell (HRBC) membrane stabilization method has been used to study the anti-inflammatory activity (15).

The percentage of hemolysis of HRBC membrane can be calculated as follows:

% Hemolysis = Optical density of Test sample/Optical density of Control x 100

The percentage of HRBC membrane stabilization can be calculated as follows:

% Protection = 100-Optical density of Test sample/Optical density of Control x 100

### Heat induced hemolysis

Percent membrane stabilization was determined by the method of Shinde et al. (16).

$$\% \text{ Inhibition} = 100-(A1 - A2)/ (A0) \times 100$$

Where

A1 is the absorbance of the sample; A2 is the absorbance of the product control; A0 is the absorbance of the positive control.

### Inhibition of protein denaturation

The percentage inhibition of protein denaturation was carried out by Vallabh et al. (17).

Percentage inhibition = 100-(O.D. of test – O.D. of product control)/ O.D. of Control x 100

**Assay of Cyclooxygenase (COX) inhibition**

The assay mixture consisted of 0.1M Tris-HCl buffer, glutathione, hemoglobin and enzyme. The assay was started by the addition of arachidonic acid and terminated after 20 min incubation at $37^0$C by addition of 0.2 ml of 10% TCA in 1N HCl, mixed and 0.2 ml of Thiobarbituric acid (TBA) was added and contents heated in a boiling water bath for 20 min, cooled and centrifuged at 1000 rpm for 3 min. The supernatant was measured at 632 nm for COX activity (18). For cyclooxygenase inhibition, seed extracts (100 and 500µg/ml) were added to the assay mixture and proceeded as per the test. The results were expressed as % Cyclooxygenase inhibition.

**Assay of 5- Lipoxygenase inhibition**

The reaction was carried out in a quartz cuvette at 25°C with 1cm light path. The assay mixture contains 2.75 ml Tris buffer of pH 7.4, 0.2 ml of sodium linoleate and 50 µl of the enzyme. Optical density was measured in 234 nm (18). For lipooxygenase inhibition, seed extract (100 and 500µg/ml) was added to the assay mixture and proceeded as per the test. The inhibitory effect of seeds extracts tested was expressed as % of enzyme activity inhibited.

**Antibacterial activity**

Antibacterial activity was screened against six bacterial strains by Agar well diffusion method of Murray et al. (19).

**Minimum inhibitory concentration (MIC) assays**

Minimum Inhibitory Concentrations (MIC) of ethanolic and methanolic seed extracts determined according to the method of Elizabeth (20).

## RESULTS

**Phytochemical evaluation of *Abelmoschus moschatus* seed extracts**

Preliminary phytochemical analysis of *Abelmoschus moschatus* seed extracts was done using standard test procedures to confirm the availability of active phytochemicals in the ethanolic and methanolic seed extracts. Phytochemical analysis showed the presence of phenols, flavonoids, tannins, alkaloids, glycosides and phytosterols (Table-1).

**Quantitative estimation of Total phenolic, Flavonoid and Ascorbic acid content**

The results of total phenolic, flavonoid and ascorbic acid content of ethanolic and methanolic seed extracts of *Abelmoschus moschatus* are shown in Table 2. The total phenol content in the ethanolic and methanolic seed extracts expressed as gallic acid equivalent (GAE) was found to be 4.5±0.15 GAE/g extract and 8.6±0.04 GAE/g extract respectively. Total flavonoid content of the seed extracts was recorded in good quantities in quercetin equivalents (QE)/g extract. The ascorbic acid content of the both seed extracts was determined and found to be in satisfactory levels.

**Total antioxidant activity (TAA)**

Total antioxidant activity (TAA) was maximum in the seed extract prepared with Methanol (16.44±0.12 Ascorbic acid equivalents/g extract) followed by Ethanolic extract (8.23±0.68 Ascorbic acid equivalents/g extract). Likewise, reducing power activity was maximum in the extract prepared with Methanol (36.12±0.18 Vit E Equivalents/g extract) (Table-3).

**Table 1. Phytochemical screening of *Abelmoschus moschatus* seed extracts**

| Secondary Metabolite | Ethanolic seed extract | Methanolic seed extract |
|---|---|---|
| Tannins | - | + |
| Alkaloids | - | + |
| Saponins | - | - |
| Terpenoids | + | - |
| Flavanoids | + | + |
| Phenolics | + | + |
| Anthraquinones | - | - |
| Phytosterols | - | + |
| Glycosides | + | + |
| (+) Indicates respective constituent present and (-) Indicates absence of photochemical | | |

**Table 2. Total phenolic, flavonoid and ascorbic acid content in ethanolic and methanolic seed extracts of *Abelmoschus moschatus***

| Extract | Total phenolics GAE/gm extract | Total flavonoids Quercetin Equivalents/gm extract | Ascorbic acid g/gm extract |
|---|---|---|---|
| Ethanolic extract | 4.5±0.15 | 1.22±0.02 | 7.54±0.34 |
| Methanolic extract | 8.6±0.04 | 2.04 ±0.12 | 12.06±0.20 |

**Table 3. Antioxidant ability analysis of ethanolic and methanolic seed extracts of *Abelmoschus moschatus***

| Extract | Total antioxidant activity (Ascorbic acid equivalents/gm extract) | Reducing power (Vit E Equivalents/gm extract) |
|---|---|---|
| Ethanolic extract | 8.23±0.68 | 18.22±0.04 |
| Methanolic extract | 16.44±0.12 | 36.12±0.18 |

***In vitro* anti-inflammatory activity**

**Inhibition of hypotonicity induced hemolysis**

The seed extracts were effective in inhibiting the hypotonicity induced hemolysis at different concentrations (100-500 µg/ml). Both the ethanolic and methanolic seed extracts (100-500µg/ml) inhibited the hypotonicity induced hemolysis of RBCs to varying degree (Figure 1). Of the two seed extracts, methanolic extract showed 75% inhibition at a concentration of 500µg/ml. Diclofenac sodium, standard drug showed the maximum inhibition, 94 % at 100µg/ml.

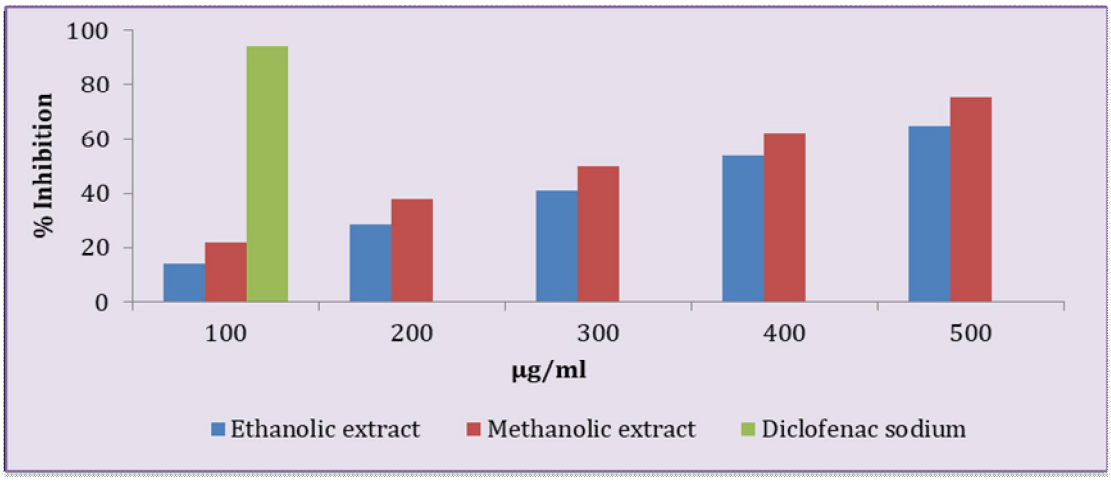


**Figure 1. Effect of ethanolic and methanolic seed extracts of *Abelmoschus moschatus* on RBC membrane stabilization**

**Inhibition of Heat induced hemolysis**

Stabilization of the RBCs membrane was studied further to establish the mechanism of anti-inflammatory action of *Abelmoschus moschatus* seed extracts. The extracts were effective in inhibiting the heat induced hemolysis at different concentrations (100-500 µg/ml). Both the extracts (100-500µg/ml) inhibited the heat induced hemolysis of RBCs to varying degrees (Figure 2). Among the two extracts, methanolic extract showed maximum inhibition (78%) at 500µg/ml concentration.

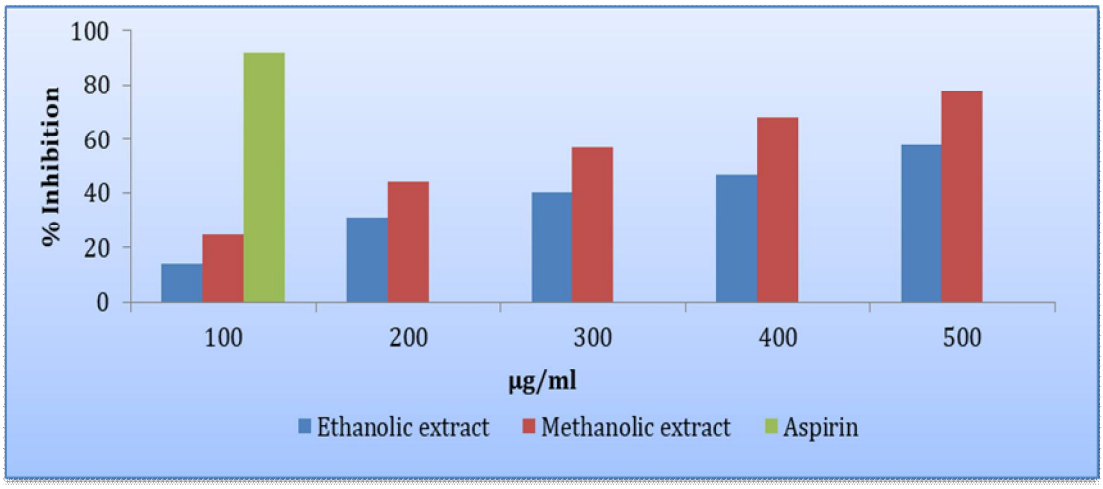


**Figure 2. Effect of ethanolic and methanolic seeds extracts of *Abelmoschus moschatus* on heat induced hemolysis**

**Inhibition of protein denaturation**

Both the seed extracts were effective in inhibiting heat induced albumin denaturation at different concentrations (Figure 3). Maximum inhibition of protein denaturation (82%) was observed with methanolic extract at 500µg/ml. Diclofenac sodium, a standard anti-inflammatory drug showed the maximum inhibition, 95% at 100µg/ml concentration.

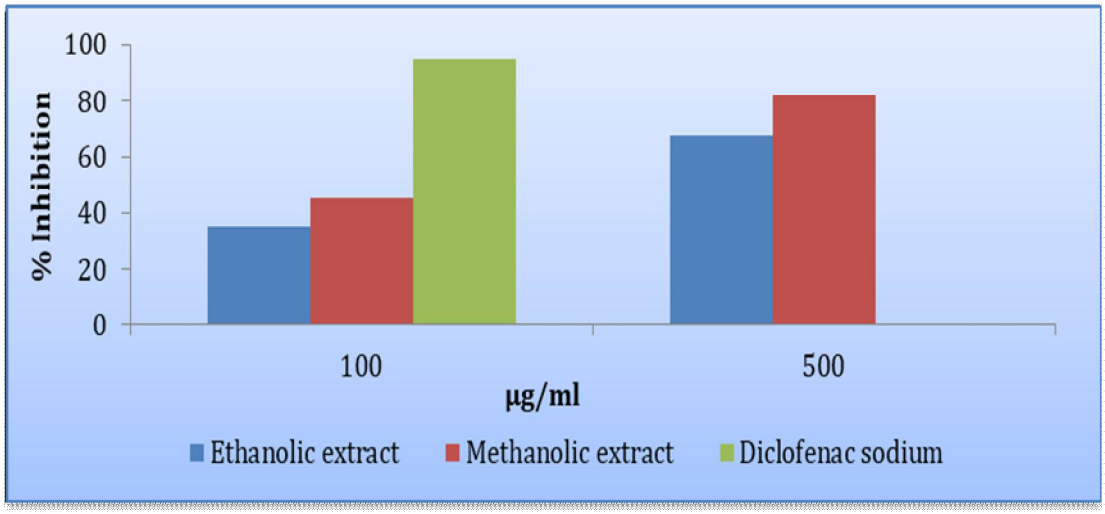


**Figure 3. Effect of ethanolic and methanolic seed extracts of *Abelmoschus moschatus* on protein denaturation**

**Cyclooxygenase (COX) inhibitory assay**

The effect of *Abelmoschus moschatus* seed extracts on production of prostaglandins were determined by the inhibition of Cyclooxygenase activity. From (Figure 4), it is clear that ethanolic and methanolic seed extracts have good cyclooxygenase inhibitory activity in comparision to standard Ibuprofen. Of the two extracts, methanolic seed extract showed maximum inhibition of 80% at 500 µg/ml concentration. The standard Ibuprofen showed 94% inhibition at a concentration of 100µg/ml.

**Assay of 5-Lipoxygenase (LOX) Inhibition**

Ethanolic and methanolic seed extracts were checked at 100 and 500µg/ml for anti-lipoxygenase activity. Methanolic seed extract showed maximum inhibition of 76% at 500 µg/ml than the ethanolic extract (Figure 5). The standard Ibuprofen showed 95% inhibition at a concentration of 100µg/ml. The results obtained from the present study support anti-inflammatory potential of ethanolic and methanolic seed extracts.

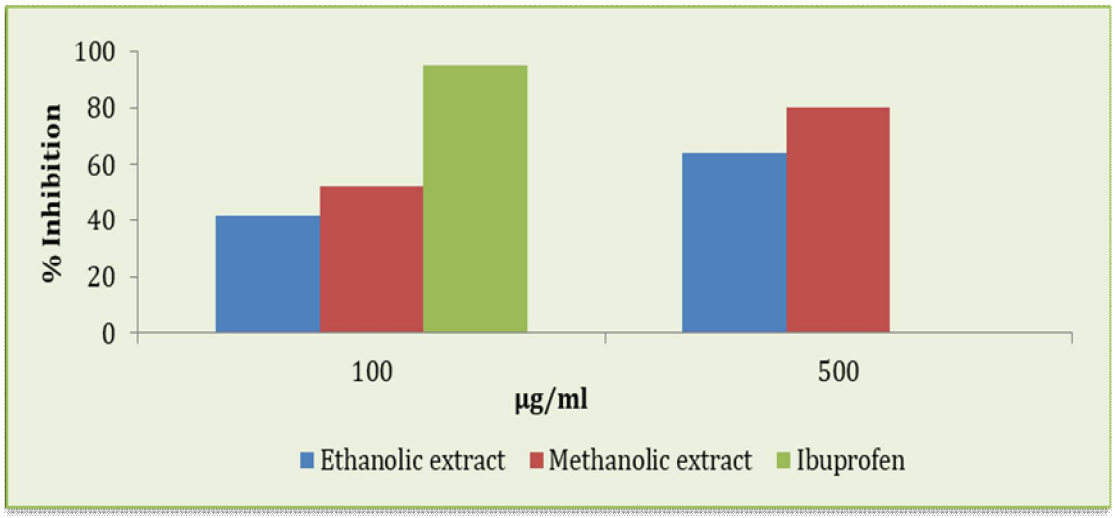


**Figure 4.Effect of ethanolic and methanolic seed extracts of *Abelmoschus moschatus* on Cyclooxygenase inhibition**

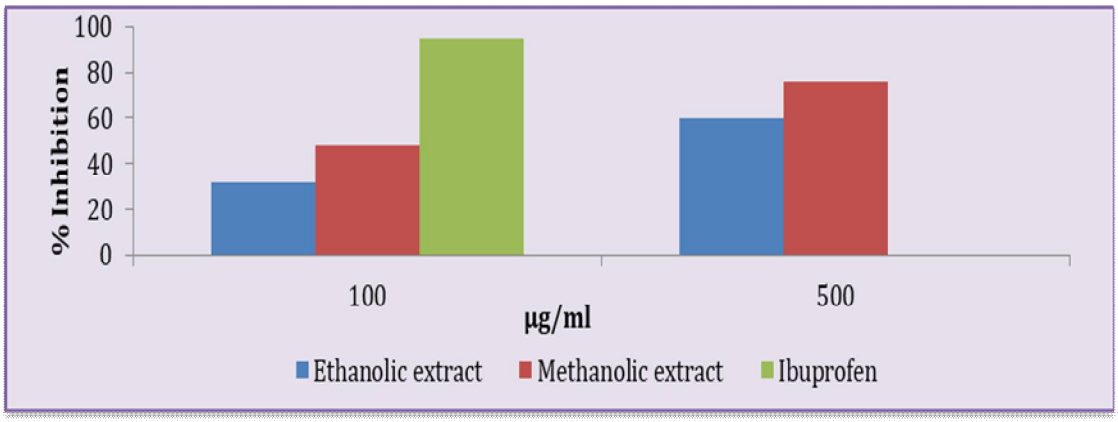


**Figure 5.Effect of ethanolic and methanolic seed extracts of *Abelmoschus moschatus* on 5-Lipoxygenase inhibition**

**Antibacterial activity**

The antibacterial properties of the ethanolic and methanolic seed extracts of *Abelmoschus moschatus* were evaluated against three Gram positive and three Gram negative bacterial strains using agar well diffusion method. Ethanolic and methanolic seed extracts exhibited significant antibacterial activity on Gram positive bacteria than Gram negative bacteria. Of the two extracts, methanolic seed extract showed potent growth inhibition than ethanolic extract (Table 4). Methanolic seed extract strongly inhibited Gram positive bacteria-*Bacillus subtilis* (20mm), *Staphylococcus aureus* (19mm), *Streptococcus pneumoniae* (18mm) and Gram negative–*Pseudomonas aeruginosa* (17mm) and *E.coli* (16mm) at a concentration of 10mg/ml whereas ethanolic extract moderately inhibited the growth of both gram positive and gram negative strains at the same concentration. The minimum inhibitory concentrations (MIC) of both the extracts against tested pathogenic bacteria are presented in Table 5.

**Table 4.Effect of ethanolic and methanolic seed extracts of *Abelmoschus moschatus* on the growth of bacteria**

| Name of the Bacterial strain | Zone of Inhibition (Diameter in mm) | | | | |
|---|---|---|---|---|---|
| | Ethanolic extract | | Methanolic extract | | Rifampicin (20 µg) |
| | 5mg/ml | 10mg/ml | 5mg/ml | 10mg/ml | |
| ***Gram positive bacteria*** | | | | | |
| *Bacillus subtilis* | 11 | 18 | 17 | 20 | 26 |
| *Staphylococcus aureus* | 13 | 16 | 14 | 19 | 25 |
| *Streptococcus pneumoniae* | 11 | 17 | 11 | 18 | 25 |
| ***Gram negative bacteria*** | | | | | |
| *Escherichia coli* | 9 | 12 | 10 | 16 | 26 |
| *Klebsiella pneumoniae* | 10 | 10 | 12 | 14 | 25 |
| *Pseudomonas aeruginosa* | 10 | 10 | 15 | 17 | 25 |

**Table 5.Minimum Inhibitory concentrations (MIC) of ethanolic and methanolic seed extracts of *Abelmoschus moschatus* on bacterial growth**

| Name of the bacterial strain | MIC of Ethanolic extract (mg/ml) | MIC of Methanolic extract (mg/ml) |
|---|---|---|
| *Bacillus subtilis* | 2.5 | 2 |
| *Streptococcus pneumoniae* | 3 | 3 |
| *Staphylococcus aureus* | 3 | 3 |
| *Escherichia coli* | 4 | 3.5 |
| *Klebsiella pneumoniae* | 5 | 4 |
| *Pseudomonas aeruginosa* | 5 | 3.5 |

## DISCUSSION

Natural antioxidants, which are ever-present in medicinal plants have gained immense attention and have been studied extensively, since they are efficient free radical scavengers being involved in the anti-inflammatory response and are assumed to be less toxic than synthetic antioxidants (21). The preliminary phytochemical analysis of ethanolic and methanolic seed extracts showed the presence of phenols, flavonoids, tannins, alkaloids, glycosides and sterols. The total phenolic content in both the seed extracts was found to be high. Both the extracts exhibited potent total antioxidant and Ferric reducing power activities. It has been found that the highest antioxidant activity, measured as total antioxidant activity (TAA) depend on total phenol content in the extracts. The results of the present study reveal that there is a strong coincidence between antioxidant activity and total phenolic content. Similar results have been reported earlier as well suggest a contributory relationship between total phenol content and antioxidant activity (22,23,24).

The HRBC membrane stabilization has been used as a method to study the *in vitro* anti-inflammatory activity of seed extracts because the erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may well stabilize the lysosomal membrane. The extracts exhibited membrane stabilization effect by inhibiting hypotonicity and heat induced lyses of erythrocyte membrane at different concentrations (100-500 µg/ml). The present results provide evidence for membrane stabilization as an additional mechanism of their anti-inflammatory effect. The extracts may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. These neutrophil lysosomal constituents include bactericidal enzymes and protease, which upon extracellular release cause further tissue inflammation and damage (25).

Protein denaturation is one of the important known causes of certain anti-inflammatory diseases where electrostatic, hydrogen and disulphide bonding were altered in denaturation mechanism. Both the seed extracts were effective in inhibiting heat induced albumin denaturation at different concentrations and maximum inhibition of protein denaturation was observed with methanolic extract at 500µg/ml and comparing its effect with diclofenac sodium as a standard drug. Phenolic content of the seed was responsible for anti-inflammatory activity as these compounds have a capacity to inhibit either the production or the action of pro-inflammatory mediators resulting in anti-inflammatory capacity (26). It has been reported that *Cydonia oblonga* seeds are good source of important phytochemical compounds and have significant antioxidant and anti-inflammatory activities (27).

Both the ethanolic and methanolic seeds extracts of *Abelmoschus moschatus* have good cyclooxygenase and lipoxygenase inhibitory activity at 500 µg/ml concentration in comparision to standard Ibuprofen. Further, flavonoid compounds may exert protection against heart disease through the inhibition of cyclooxygenase and lipoxygenase activities in platelets and macrophages. Lipoxygenases are the key enzymes in the biosynthesis of leukotrienes which play an important role in several inflammatory diseases such as arthritis, asthma, cancer and allergic diseases. In this process, arachidonic acid gets converted to leukotrienes and prostaglandins through lipoxygenase and cyclooxygenase pathways respectively (28,29).

The ethanolic and methanolic seed extracts of *Abelmoschus moschatus* were also tested for their antimicrobial activity against a panel of six pathogenic bacteria. The results indicate that the extracts exhibited antimicrobial property in a dose dependent manner and methanolic seed extract was found to be more effective which signifies the antibiotic nature of these extracts. It has been reported that the different extracts of *A. moschatus* exhibited antimicrobial activity (24) and stated that organic solvent extract of leaf is more efficient than other aqueous extracts in exhibiting antibacterial activity.

The results of the present study indicate that the ethanolic and methanolic extracts of *Abelmoschus moschatus* possess antioxidant, anti-inflammatory and antimicrobial activities and these effects could be attributed to the presence of phytoconstituents in the seed extracts which may be responsible for its ethnomedicinal use as a promising anti-inflammatory and antimicrobial drug.

## CONCLUSION

In the present study, ethanolic and methanolic seed extracts of *Abelmoschus moschatus* were quantified for main phytochemicals and the presence of various phenolics and non-phenolic phyto-compounds indicates the medicinal importance of the seed extract. The methanolic seed extract showed potent anti-inflammatory activity by inhibiting protein denaturation, COX inhibition and 5-LOX inhibition. These activities may be due to the strong occurrence of polyphenolic compounds in the seed extracts. The seed extracts contain maximum polyphenolics, flavonoids and terpenoids, thus serve as free radical scavengers and act possibly as primary oxidant thereby inhibiting inflammation. The seed extracts also inhibited the growth of bacteria tested. *In vitro* anti-inflammatory and antimicrobial property of the seed extracts

establish the therapeutic applications and their use as herbal medicine for the prevention of inflammation and bacterial diseases.

## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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