Evaluation of Free Radical Scavenging, Anti-inflammatory and Analgesic Potential of Benincasa hispida Seed Extract

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Abstract: The present study was designed to investigate the free radical scavenging, anti-inflammatory and analgesic potential of methanolic extract of Benincasa hispida seeds (MEBH). Free radical scavenging potential of MEBH was evaluated by DPPH (1,1-diphenyl-2-picryl-hydrazyl) and hydrogen peroxide (H$_2$O$_2$) method. The extract showed significant free radical scavenging activity in a dose dependent manner when compared with ascorbic acid. The highest radical scavenging activity of MEBH was found to be 79.8% at concentration of 300 µg mL$^{-1}$. The H$_2$O$_2$ scavenging effect of MEBH was 63.7% at a concentration of 200 (µg mL$^{-1}$). Further, the extract was studied for its anti-inflammatory and analgesic activities at the dose level of 100, 200 and 300 mg kg$^{-1}$. Anti-inflammatory activity was evaluated using carrageenan-induced paw edema in rats. Analgesic activity was evaluated by tail immersion and tail flick methods in mice. The extract showed significant decrease in paw volume (59.7% reduction) and pain at the dose level of 300 mg kg$^{-1}$ when compared with reference drugs diclofenac and morphine, respectively. The MEBH may be useful as a natural antioxidant in the treatment of inflammation and pain.

Key words: Benincasa hispida, DPPH, methanolic extract, pain, inflammation

INTRODUCTION

The natural products today symbolize safety in contrast to the synthetic drugs that are regarded as unsafe to humans and environment. So, people are returning to the natural products with the hope of safety and security. Numerous evidences have shown that increased consumption of fruits and vegetables reduce the risk of various pathological events such as cancer, cardiovascular diseases and cerebrovascular diseases (Goodwin and Brodvick, 1995; Rimm et al., 1996). This is often attributed to the antioxidants in the fruits and vegetables such as vitamin C, E, carotenoids, lycopenes and flavonoids that prevent damages caused by free radicals (Stahelin et al., 1991). Free radicals play an important role in the development of tissue damage and pathological events in living organisms (Kehrer, 1993). Natural medicines with free radical scavenging properties have been used for various purposes and epidemiological data also points at widespread acceptance and use of these agents. Presently, the active constituents from the natural sources are extracted, purified and tested. Results have shown their benefits in prevention of the free radical mediated diseases (Venkta Ratnam et al., 2006).

Many researchers have paid attention towards the Cucurbitaceae family. The seeds and fruits of various plants of the family, such as Momordica charantia, Citrullus colocynthis have been evaluated for their antioxidant, anti inflammatory and analgesic activities (Semiz and Sen, 2007; Kumar et al., 2008; Marzouka et al., 2010). The seeds of Cucumis sativum are reported to possess antioxidant activity (Gill et al., 2009). Benincasa hispida is an important plant of the family. It is a large climbing or trailing herb with stout, angular and hispid stems widely cultivated in tropical Asia. It is called Petha in Hindi and White Gourd, Wax Gourd or Ash Gourd in English. In Ayurveda, Benincasa hispida is recommended for management of peptic ulcer, diabetes mellitus, urinary infection, hemorrhages from internal organs, epilepsy and other nervous disorders (Warrier et al., 1994). The extract of the fruit is reported to possess anti-ulcer (Grover et al., 2001), anti-angiogenic (Lee et al., 2005) and antihistaminic activities (Kumar and Ramu, 2002). The fruit pulp is used in confectionery industry for the preparation of sweets and million tones of seeds are discarded. These seeds have been used traditionally in Ayurveda for treatment of various disorders such as peptic ulcer and as vermifuge (Warrier et al., 1994). The seeds can be used for their therapeutic potential. So our present study was carried out to evaluate the free radical scavenging, anti inflammatory and analgesic potential of Benincasa hispida seeds.

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MATERIALS AND METHODS

Plant material: The seeds were purchased from the Khari Bajoli (spice market) Delhi (India) in August 2009. The healthy looking seeds were selected for authentication and voucher specimen number 0389 has been deposited in the Botanical and Environmental Science Department, Guru Nanak Dev University, Amritsar (India). The seeds were cleaned, washed, dried at low temperature and powered.

Drugs and chemicals: Ascorbic acid and carrageenan were obtained Central Drug House Pvt. Ltd., Mumbai, India. Hydrogen peroxide was obtained from E-Merck Ltd., Mumbai. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Chemical Co., USA. Physician sample of morphine and dieleoflurane sodium was procured from Government Medical College and Hospital, Patiala. All other chemical reagents used were of analytical grade which were procured from different companies (Loba Chem, Mumbai and Merck Limited, Mumbai).

Animals: The wistar albino rats (200-250 g) and swiss albino mice (25-30 g) of either sex were obtained from NIPER Mohali. They were kept at standard laboratory diet, environmental temperature and humidity. A 12 h light and dark cycle was maintained throughout the experimental protocol. The experimental protocol was duly approved by Institutional Animal Ethics Committee (IAEC) and care of the animals was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg No. 874/ac/05/CPCSEA).

Extraction: The powdered seeds were extracted for 72 h with methanol at room temperature. The solvent was filtered off and residue macerated again with the fresh solvent. Both solvents were combined and concentrated under reduced pressure on a rotary evaporator (Hedolph) at 40°C. The concentrated filtrate was suspended in distilled water and partitioned successively with hexane. The aqueous layer was separated and concentrated on water bath. The crude extracts were used for further investigation.

Phytochemical screening: The crude extract was studied for the presence of phytochemicals such as alkaloids, tannins, saponins, flavonoids, steroids, triterpenoids, carbohydrates, proteins and amino acids using standard procedures (Harborne, 1973).

The alkaloids were tested using various reagents such as Dragendorff's reagent (potassium bismuth iodide solution), Hager's reagent (picric acid solution), Mayer's reagent (potassium iodide solution) and Wagner's reagent (solution of iodine in potassium iodide). The sterols were tested by Liebermann's test (blue green color with acetic anhydride plus sulphuric acid). The triterpenoids were tested by Liebermann-Burchard's test. In this test a few mg of the extract was dissolved in chloroform, a few drops of acetic anhydride were added followed by sulphuric acid. Red/pink/purple/violet color confirms the presence of triterpenoids. The ferric chloride test was used for tannins (blue green color with ferric chloride), Molish test for carbohydrates, Ninhydrin reagent test for proteins and amino acids. The flavonoids were tested by concentrated nitric acid (crimson or magenta color with concentrated nitric acid).

Free radical scavenging activity

DPPH radical scavenging activity: The free radical scavenging activity of MBTH was determined by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Sreejavan and Rao, 1997). Briefly, a 0.05 mM solution of DPPH in methanol was prepared and 1.5 mL of this solution was added to 0.5 mL of extract solution in methanol at different concentrations (100-300 µg mL⁻¹). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm using a spectrophotometer (Shimadzu UV-1700 Pharmaspec). A blank without DPPH was used to remove the influence of the color of the samples. A methanolic solution of DPPH was used as negative control. Ascorbic acid was used as a reference drug. All measurements were carried out in triplicate. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The DPPH radical scavenging activity was calculated using the Eq. 1:

\[
\text{Percentage scavenging of DPPH radical} = 100 \times \left( \frac{A_c - A_s}{A_c} \right) \tag{1}
\]

where, \(A_c\) is absorbance of the negative control \(A_s\) is the absorbance of the sample.

Hydrogen peroxide radical scavenging activity: Hydrogen peroxide radical scavenging activity was determined according to the method of Wettasinghe and Shahidi (2000). The extract solution (1.0 mL) in various concentrations (25-200 µg mL⁻¹) was mixed with 2.4 mL of 0.1 M phosphate buffer (pH 7.4) and then 0.6 mL of a 43 mM solution of \(\text{H}_2\text{O}_2\) in the same buffer were added.
After 10 min the absorbance values of the reaction mixtures were recorded against a blank solution containing phosphate buffer without \( \text{H}_2\text{O}_2 \) at 230 nm using a spectrophotometer (Shimadzu UV-1700 Pharma spec). For each concentration, a separate blank sample was used for background subtraction. Ascorbic acid was used as a standard and mixture without sample was taken as a control. The percentage scavenging of \( \text{H}_2\text{O}_2 \) was calculated as:

\[
\text{Percentage scavenging of } \text{H}_2\text{O}_2 = \left( \frac{A_r - A_s}{A_s} \right) \times 100 \quad (2)
\]

where, \( A_r \) is the absorbance of the control and \( A_s \) is the absorbance of the extract/standard. All tests were done in triplicate (Sood et al., 2009).

**Anti-inflammatory activity**

**Carrageenan-induced rat paw edema:** The carrageenan-induced rat paw edema assay was carried out according to Winter et al. (1962). Wistar rats were divided into 5 groups each consisting of 6 animals.

**Group I:** (Disease Control): Carrageenan (1%) was administered in the plantar surface of rat

**Group II:** (Standard): Diclofenac sodium (12.5 mg kg\(^{-1}\), p.o.)

**Group III:** (MEBH 100): Methanolic extract (100 mg kg\(^{-1}\), p.o.)

**Group IV:** (MEBH 200): Methanolic extract (200 mg kg\(^{-1}\), p.o.)

**Group V:** (MEBH 300): Methanolic extract (300 mg kg\(^{-1}\), p.o.)

Edema was induced on the left hind paw of the rats by subplantar injection of 0.1 mL of a solution of 1% (w/v) carrageenin in a 0.9% NaCl (w/v). The paw volume was measured at intervals of 60, 120, 180 min by the mercury displacement method using a plethysmograph after administration of the extract/drug orally. The percentage inhibition of paw edema in drug treated group was compared with the carrageenan control group and calculated according to the following in Eq. 3.

\[
\text{Percentage inhibition} = \left( \frac{V_c - V_t}{V_c} \right) \times 100 \quad (3)
\]

where, \( V_c \) is the inflammatory increase in paw volume of control group of animals and \( V_t \) is the inflammatory increase in paw volume of drug-treated animals.

**Analgesic activity:** Swiss albino mice of either sex were divided into 5 groups each consisting of 6 animals.

**Group I:** (Control): Vehicle (1% CMC, p.o.)

**Group II:** (Standard): Morphine (10 mg kg\(^{-1}\) p.o.)

**Group III:** (MEBH 100): Methanolic extract (100 mg kg\(^{-1}\) p.o.)

**Group IV:** (MEBH 200): Methanolic extract (200 mg kg\(^{-1}\) p.o.)

**Group V:** (MEBH 300): Methanolic extract (300 mg kg\(^{-1}\) p.o.)

**Tail immersion test:** The procedure is based on the observation that morphine like drugs selectively prolongs the reaction time of the typical tail withdrawal reflex in mice (Toma et al., 2003). The tail of mice was immersed in warm water kept constant at 52.5° 0.5°C. The reaction time of the tail-withdrawal response was determined at 0, 30, 60, 90 and 120 min after the administration of drugs. A cut off time of 15 sec was maintained to prevent tissue damage (Grotto and Sulman, 1967).

**Tail flick test:** The tail of mice was placed on the radiant heat source (1 cm distance from the nichrome wire) of an analgesiometer and time taken by the animals to withdraw its tail from the radiant heat source was taken as the reaction time. The reaction time was recorded at 0, 30, 60, 90 and 120 min after the drug administration. A cut off time of 15 sec was maintained to prevent tissue damage. The temperature was maintained at 52° 0.5°C (Davies et al., 1946).

**Statistical analysis:** All the results were expressed as Mean±Standard Error of Means (SEM). The data was statistically analyzed by one way Analysis of Variance (ANOVA) followed by Tukey’s multiple range tests using Sigmastat Version 2.0. Software. The p-value<0.05 was considered to be statistically significant.

**RESULTS AND DISCUSSION**

**Phytochemical screening:** The results of the phytochemical screening of the methanolic extract are shown in Table 1; the results reveal the presence of chemical constituents such as proteins and amino acids, triterpenoids, carbohydrates, sterols and tannins.

**DPPH radical scavenging activity:** DPPH, reacts with antioxidants and gets converted into 1,1-diphenyl-2-picrylhydrazine by accepting a hydrogen atom and hence shows decrease in absorbance. The MEBH showed concentration-dependent DPPH radical scavenging activity. The highest radical scavenging activity of MEBH was found to be 79.8% at concentration of 300 \( \mu \text{g mL}\(^{-1}\), the results are shown in Table 2.
Table 1: Phytochemical chemical screening of MEBH

<table>
<thead>
<tr>
<th>Chemical constituents</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>-</td>
</tr>
<tr>
<td>Protein and amino acid</td>
<td>+</td>
</tr>
<tr>
<td>Triterpenoids</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
</tr>
<tr>
<td>Phytosterols</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
</tbody>
</table>

*: Presence of chemical constituent, - Absence of chemical constituent

Table 2: Percentage scavenging of DPPH radical

<table>
<thead>
<tr>
<th>Conc. of extract (µg mL⁻¹)</th>
<th>Percentage scavenging of DPPH radical</th>
<th>Methanol extract</th>
<th>Ascorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>54.4±0.37</td>
<td>62.0±0.79</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>66.5±0.52</td>
<td>71.2±0.64</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>79.8±0.49</td>
<td>85.3±0.67</td>
<td></td>
</tr>
</tbody>
</table>

Values are the average of triplicate experiments and represented as Mean±SEM.

Table 3: Percentage scavenging of H₂O₂

<table>
<thead>
<tr>
<th>Conc. of extract (µg mL⁻¹)</th>
<th>Percentage scavenging of H₂O₂</th>
<th>Methanol extract</th>
<th>Ascorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>36.2±0.26</td>
<td>41.1±0.37</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>48.1±0.52</td>
<td>57.2±0.26</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>56.3±0.37</td>
<td>66.5±0.34</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>63.7±0.23</td>
<td>74.9±0.26</td>
<td></td>
</tr>
</tbody>
</table>

Values are the average of triplicate experiments and represented as Mean±SEM.

**Hydrogen peroxide radical scavenging activity:** The free radical scavenging activity of the MEBH was evaluated by H₂O₂ scavenging method. The results are shown in Table 3, the MEBH showed concentration dependent activity. The H₂O₂ scavenging effect of MEBH was 63.7% at a concentration of 200 (µg mL⁻¹) which was comparable to the scavenging effect of ascorbic acid.

**Anti-inflammatory activity:** Table 4 shows the results of the anti-edematous effect of orally administered MEBH on carrageenin induced paw edema in rats; The MEBH showed dose dependent anti-inflammatory activity in carrageenin induced paw edema in rats. At dose of 100 mg kg⁻¹, MEBH caused a reduction in paw edema (19.4%) 3 h after the subplantar injection of carrageenin. Doses of 200 and 300 mg kg⁻¹ caused a significant reduction of paw edema (41.7 and 59.7%, respectively) from the first till the third hour compared with the disease control group.

**Analgesic activity**

**Tail immersion test:** Table 5 depicts the analgesic activity shown by MEBH by tail immersion method. MEBH showed dose dependent analgesic activity against conduction of heat-induced algesia in mice. Medium (200 mg kg⁻¹) and high (300 mg kg⁻¹) doses showed significant difference in the analgesic activity when compared with control group. Maximum analgesic effect was observed at 90 min interval.

**Tail flick test:** Table 6 depicts the analgesic activity shown by MEBH by tail flick method. The MEBH showed dose dependent analgesic activity against radiant heat-induced algesia. Medium (200 mg kg⁻¹) and high (300 mg kg⁻¹) doses showed significant difference in the analgesic activity when compared with control group. Maximum analgesic effect was observed at 90 min interval. Hence, it was observed that the MEBH significantly reduced the pain sensation in mice at medium and higher dose.

In the present study, the methanolic extract of *Bennecasa hispida* seeds was evaluated for its free radical scavenging, anti-inflammatory and analgesic potential. Results have revealed that MEBH possess in vitro free radical scavenging activity. Hence, the extract was further evaluated for its in vivo anti-inflammatory and analgesic potential. Carrageenan induced rat paw edema test has been used to assess the anti-edematous effect of natural products. Carrageenan brings about inflammation by the release of mediators of inflammation (prostaglandins, leukotrienes, histamine, bradykinin etc.) (Chunkhorn and Meacock, 1971), also, several free radicals are released during such inflammation (Koblakov, 2001). The MEBH showed significant free radical scavenging activity, so this can be responsible for the reduction of inflammation in the carrageenin-induced paw edema in rats (Cuzzocrea et al., 2001). Tail immersion and tail methods were carried out to evaluate the analgesic potential of seed extract. Significant decrease in the tail withdrawal latency indicated that the methanolic extract of seeds possess analgesic property. This analgesic activity may be due to its free radical scavenging activity as these free radicals are involved during pain stimulation and antioxidants show reduction in such pain (Kim et al., 2004). The literature also reveals the antioxidant capacity of the pumpkin seed extract against DPPH free radical formation (Xanthopoulos et al., 2009). The seeds of *Citrus lanatus* are found to possess antioxidant, anti-inflammatory and analgesic activities (Gill et al., 2010). The seed extract of *Momordica Charantia* normalizes the impaired antioxidant status in streptozotocin induced-diabetes by scavenging of free radicals (Sathishkumar and Subramanian, 2005).

The triterpenoids isolated from various species of cucurbita family have been reported to possess anti-inflammatory activities (Miro, 1995). The cucurbitacin C from *Cucurbita andreama* were evaluated for their anti-inflammatory and inhibitory effects on the
Table 4: Effect of MEBH on carrageenin induced paw edema in rats

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Dose</th>
<th>Mean paw volume (mL)</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
<th>Percentage inhibition of edema</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease control</td>
<td>1% CMC</td>
<td>0.54±0.009</td>
<td>0.62±0.006</td>
<td>0.67±0.005</td>
<td>-</td>
<td>70.1</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>12.5</td>
<td>0.38±0.002</td>
<td>0.35±0.005</td>
<td>0.20±0.005</td>
<td>-</td>
<td>19.4</td>
</tr>
<tr>
<td>MEBH</td>
<td>100</td>
<td>0.48±0.002</td>
<td>0.58±0.005</td>
<td>0.54±0.015</td>
<td>-</td>
<td>19.4</td>
</tr>
<tr>
<td>MEBH</td>
<td>200</td>
<td>0.45±0.009</td>
<td>0.42±0.006</td>
<td>0.39±0.013</td>
<td>-</td>
<td>41.7</td>
</tr>
<tr>
<td>MEBH</td>
<td>300</td>
<td>0.40±0.008</td>
<td>0.35±0.015</td>
<td>0.27±0.007</td>
<td>-</td>
<td>59.7</td>
</tr>
</tbody>
</table>

The values are Mean±SEM of 6 animals. *p<0.05 compared with disease control group, **p<0.05 compared with diclofenac treated group.

Table 5: Analgesic effect of the MEBH by tail immersion test

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>Tail withdrawal latency (time in sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg kg⁻¹) orally</td>
<td>0 min</td>
</tr>
<tr>
<td>Control</td>
<td>1% CMC</td>
<td>3.24±0.04</td>
</tr>
<tr>
<td>Mophine</td>
<td>10</td>
<td>3.23±0.02</td>
</tr>
<tr>
<td>MEBH</td>
<td>100</td>
<td>3.45±0.16</td>
</tr>
<tr>
<td>MEBH</td>
<td>200</td>
<td>3.69±0.05</td>
</tr>
<tr>
<td>MEBH</td>
<td>300</td>
<td>3.54±0.01</td>
</tr>
</tbody>
</table>

Values are Mean±SEM of 6 animals in each group. *p<0.05 compared with control group, **p<0.05 compared with Mophine treated group.

Table 6: Analgesic effect of the MEBH by tail flick test

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>Tail flick latency (time in sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg kg⁻¹) orally</td>
<td>0 min</td>
</tr>
<tr>
<td>Control</td>
<td>1% CMC</td>
<td>2.65±0.01</td>
</tr>
<tr>
<td>Mophine</td>
<td>10</td>
<td>2.54±0.14</td>
</tr>
<tr>
<td>MEBH</td>
<td>100</td>
<td>2.47±0.34</td>
</tr>
<tr>
<td>MEBH</td>
<td>200</td>
<td>2.73±0.01</td>
</tr>
<tr>
<td>MEBH</td>
<td>300</td>
<td>2.56±0.15</td>
</tr>
</tbody>
</table>

Values are Mean±SEM of 6 animals in each group. *p<0.05 compared with control group, **p<0.05 compared with Mophine treated group.

growth of cancer cell lines (Jayaprakasam et al., 2003). Cucurbitacin glucosides are reported to possess antioxidant and free radical scavenging activities (Tamin-Spitz et al., 2007). Thus, the terpenoids might be responsible for the free radical scavenging, analgesic and anti-inflammatory activity.

CONCLUSION

The MEBH was found to have free radical scavenging potential and this may be one of the probable reasons for the reduction in carrageenan-induced paw edema in rats along with decrease in algesia in mice models.

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