Evaluation of Phytochemical and Pharmacological Activity of Carissa carandas L. Fruits at Three Different Stages of Maturation

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Abstract
Inflammation plays an important role in various diseases with high prevalence within populations such as rheumatoid arthritis, ulcer, atherosclerosis and asthma. Many drugs are available in the market for inflammatory diseases but they exhibit several unwanted side effects. Therefore, alternative treatments with safer compounds are needed. The plant Carissa carandas L. plant is used traditionally for the treatment of various diseases. Hence to validate its traditional use, the present study has envisaged screening different solvents extract of Carissa carandas fruit for their phytochemical and pharmacological activity especially the anti-inflammatory activity of the fruits at 3 different stages of maturation. The n-hexane and chloroform extracts of immature, mature and ripe fruits showed positive tests for steroids and triterpenoids, whereas acetone extract showed positive tests for steroids, triterpenoids, alkaloids, tannins, sugar, saponins except for triterpenoids in immature fruits. The hydroalcoholic extract showed presence of alkaloids, tannins, sugars, saponin and flavonoids. The highest concentration of phenol, flavonoids and ascorbic acid were found to be more in acetone extract of mature fruits and of carbohydrates in ripe fruits. The hydroalcoholic extract also exhibited similar pattern. The anti-inflammatory property was evaluated by using different models like carrageenan induced paw edema in Wistar rats and cotton pellets induced granuloma. There was a consistent increase in % inhibition of inflammation at concentrations of 100 and 200 mg/kg up to 3 h. The highest activity was at 3 h with 200 mg/kg dose. Thus the present work has clearly proved that the acetone extract of mature fruits have considerable anti-inflammatory activity.

Introduction
Inflammation is the complex biological response of vascular tissues to harmful stimuli such as pathogens, damaged cells, or irritants. It is a protective attempt by the organism to remove the injurious stimuli as well as initiate the healing process for the tissue. In the absence of inflammation, wounds and infections would never heal and progressive destruction of the tissue would compromise the survival of the organism [1]. Essentially, there are 2 types of inflammation: acute and chronic. However a large variety of proteins are involved in inflammation and any one of them is open to a genetic mutation which impairs the normal function and expression of that protein [2] which leads to inflammatory disorders; such as acne vulgaris, asthma, autoimmune diseases, chronic inflammation, chronic prostatitis, glomerulonephritis, hypersensitivities, inflammatory bowel diseases, pelvic inflammatory disease, reperfusion injury, rheumatoid arthritis, transplant rejection, vasculitis, interstitial cystitis and allergies [3].

There are many drugs available in the market, mainly nonsteroidal anti-inflammatory drugs and steroidal drugs for the treatment of inflammatory diseases but they exhibit several unwanted side effects to humans [4]. Therefore, alternative treatments with safer compounds are needed. Carissa carandas L. (Apocynaceae), commonly known as karaunda, is a widely used traditional medicinal plant. Ethnomedically, the fruits, leaves, barks, and roots of C. carandas have been used in ethnomedicine for the treatment of various diseases such as diarrhea, stomatchic, anorexia, intermittent...
fever, mouth ulcer and sore throat, syphilitic pain, burning sensation, scabies, and epilepsy [5]. The prominent biological activities reported include antidiabetic, antimicrobial, cytotoxicity, anticonvulsant, hepatoprotective, antihyperlipidaemic, cardiac depressant, analgesic, anti-inflammatory, antipyretic, and antiviral properties [6–11]. Hence, the objective of present study was procurement of fruits at 3 stages of maturation of Carissa carandas, phytochemical evaluation and their pharmacological evaluation especially anti-inflammatory activity.

### Experimental Work

#### Plant Material

The immature, mature and ripe fruits of C. carandas were collected locally, from Telankhedi region of Nagpur. The plant specimen was dried, mounted on the herbarium sheet and was authenticated at Department of Botany, R.T.M. Nagpur University, Nagpur, Maharashtra, India. The specimen voucher number is 9552.

#### Phytochemical analysis

**Preparation of extract and preliminary phytochemical screening**  The fresh fruits of C. carandas were crushed to obtain powder and vacuum dried- 100 gm each of immature and mature and 50 gm of ripe fruit powders were defatted separately with n-hexane in soxhlet apparatus. Initially the plant extract is defatted with petroleum ether and after defatting, it was successively extracted with the increasing order of polarity of solvent such as chloroform, petroleum ether and after defatting, it was successively extracted with the probable number of compounds present in extracts. The details of TLC are Adsorbent: Silica gel GF 254 (activated); Thickness: 0.4 mm; Plate size: 10 × 20 cm; Activation temp: 110 °C for 1 hr; Volume of spot: 20 μl. (▶ Table 2 and ▶ Fig. 1).

#### Column Chromatography of Chloroform Fraction

Column chromatography was carried out for separation of different chemical constituents in chloroform fraction of acetone extract. The details of column chromatography are- fractionating column of size 45 cm × 2.5 cm, column chromatographic grade Silica gel (60–120 mesh size) was used as stationary phase and different distilled solvents were used as eluents. The different fractions collected were analyzed by TLC using 1 % vanillin sulphuric acid spray and anisaldehyde [17, 21] and plates were observed in UV exposed to iodine fumes in chamber.

#### Pharmacological studies

**Procurement of Experimental Animals**  Male Wistar rats (150–250 gm) of approximately same age were used for the evaluation of anti-inflammatory activity. The animals were maintained on the suitable nutritional and environmental conditions throughout the experiment as per the rules and regulations set by the Institutional Animal Ethics Committee. Experimental protocols for the pharmacological and toxicity studies were reviewed and approved by the Institutional Animal Ethics Committee (IAEC No.-648/02/c/CPCSEA) after scrutiny.

#### Acute Toxicity Studies

An acute toxicity study was performed to determine LD50 using different doses of the extracts according to the method described under OECD 423 guidelines [22]. The overnight fasted rats were weighed and divided into 5 groups of 6 in each. AEIF (Acetone extract of immature fruits), AEMF (Acetone extract of mature fruits), AERF (Acetone extract of ripe fruits) given in various doses (500–5000 mg/kg body weight) by oral route. After administration of the extract suspension, the animals were observed continuously for 24 h for the death due to acute toxicity. The number of deaths within this period was recorded. In acute toxicity study, extract suspension did not show lethality up to the dose level of 2000 mg/kg, which indicates as a safe drug [22].

#### Thin layer chromatography

The different solvent extracts were subjected to thin layer chromatographic studies [21] to find out the probable number of compounds present in extracts. The details of TLC are Adsorbent: Silica gel GF 254 (activated); Thickness: 0.4 mm; Plate size: 10 × 20 cm; Activation temp: 110 °C for 1 hr; Volume of spot: 20 μl. (▶ Table 2 and ▶ Fig. 1).

### Table 1  Preliminary phytochemical screening (qualitative) of C. carandas fruit extracts.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Sterols</th>
<th>Triterpenoids</th>
<th>Alkaloids</th>
<th>Glycoside</th>
<th>Tannins</th>
<th>Flavonoids</th>
<th>Sugars</th>
<th>Proteins</th>
<th>Amino acids</th>
<th>Saponins</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-hexane</td>
<td>M+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>R+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CHCl3</td>
<td>M+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>R+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acetone</td>
<td>M+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>R+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Hydroalcohol</td>
<td>M-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>R-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>
Anti-Inflammatory Activity  The anti-inflammatory activity was evaluated by using 2 models like carrageenan induced paw edema in Wistar rats and cotton pellets induced granuloma model for chronic inflammation [23,24].

Carrageenan induced rat paw edema
The rats weighing 150–250 gm were used for the study. The animals were fed on standard diet and water provided with ad-libitum. The animals were kept on fasting over night before the experimentation.

The rats were divided into 5 groups (n = 6) such as Group I (Control) – Normal; Group II (Standard) - Diclofenac sodium (10 mg/kg) in normal saline; Group III (Test) - AEIF 200 mg/kg; Group IV (Test) - ACMF 200 mg/kg; Group V (Test) - AERF 200 mg/kg. The standard and test drugs were given orally to the animal 1 h prior to carrageenan injection. Acute paw edema was induced by injecting 0.1 ml of 1 % (w/v) carrageenan solution, prepared in normal saline in sub-plantar region of the left hind paw of the rat. The perimeter of paw was measured by using digital vernier caliper. Measurements were taken at 0, 1, 2, 3 and at 5 h after the administration of the carrageenan. The % inhibition of Edema was calculated by following formula

\[
\text{% Inhibition} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100
\]

Cotton pellet-induced granuloma model
This model is used for chronic inflammation and based on the foreign body granuloma which is produced in rats by subcutaneous implantation of pellets of compressed cotton [24–26]. The rats were divided into 5 groups (n = 6). After shaving the fur, the rats were anaesthetized under light ether and 20 mg of sterile cotton pellets were inserted, one in each axilla of the rats. Control vehicle, indomethacin (10 mg/kg), AEIF (100 mg), ACMF (100 mg), AERF (100 mg) were administered orally for 7 consecutive days from the day of cotton pellet implantation. The animals were anaesthetized on the eighth day and cotton was removed surgically and made free from extraneous tissue. The pellets were dried at constant weight at 600 C. The increment in the dry weight of the pellet was taken as a measure of the granuloma formation [27].

Statistical analysis
All the data were expressed as mean ± SEM (n = 06). The statistical analysis was performed by using one way and 2 way ANOVA followed by Bonferroni post test. The level of statistical significance was set at P < 0.05.
Result and Discussion

According to survey and other research carried out in different countries scientific (or standard) medicine in developing be countries serve only in minority (estimated at 30 to 50% of total population) while the rest of population attends to its health needs by the use of what are called as traditional medicine or folk medicines. These medicines are based specially on the use of low cost medicinal plants that are easily accessible to the entire population. As the synthetic drugs have very potent pharmacodynamic effects; as they are active many also have strong and possibly dangerous and harmful side effect. A large number of Indian medicinal plants are attributed with various pharmacological activities because they contain a diversified class of phytochemicals. It is believed that current analgesia inducing drugs such as opioids and NSAIDs are not useful in all cases, because of their side effects and potency. The plants like Azadirachta indica, Cassia tora, Acacia nelotica, Zingiber officinalis, Ocimum sanctum etc. are used traditionally for inflammation. Carissa is genus of shrub and small tree distributed in the warmer part of world. About 12 species are found in India almost all of them bearing edible fruits. The roots of Carissa carandas have been used to treat inflammation and pain and to reduce the fever by the folklore people of Salem, Dharmapuri and Coimbatore district, Tamilnadu state, India. Carissa edulis and some other species of Carissa also exhibit anti-inflammatory activity [4]. As per our knowledge the fruits of Carissa carandas have not been explored for anti-inflammatory activity till date.

The n-hexane, chloroform and acetone extract of fruits of C. carandas at all stages showed positive tests for steroids and triterpenoids except for the acetone extract of immature fruits. In addition the acetone and hydroalcoholic extracts of immature, mature and ripe fruits exhibited the presence of alkaloids, tannins, glycoside and saponins (▶ Table 1). Quantitative estimation of steroids for n-hexane and chloroform extract was carried out and the result showed the carbohydrate content was more in acetone and hydroalcoholic extracts of ripe fruits whereas the total phenolic and flavanoid content was more in the acetone and hydroalcoholic extracts of mature fruits. It was also observed that the carbohydrate, phenolic and flavanoid contents were more in acetone extract than the hydroalcoholic extracts. (▶ Table 2). The thin layer chromatography has been performed for all the extracts and it was found that acetone extract showed 7 spots in the mobile phase consisting of benzene: methanol: formic acid (8:1:1) after spraying with anisaldehyde reagent. Since the acetone extract of mature fruits showed more number of bands on TLC plate, this extract was subjected to column chromatography (▶ Table 3 and ▶ Fig. 1).

The column chromatography study of acetone extract, the fractions which have same Rf value were pooled and collected. No single spot has been observed and isolated by column chromatography. The TLC pattern and UV spectra of fraction 578–607 were similar and hence elutes were concentrated and purified by fraction with n–hexane and toluene. Preliminary phytochemical test of fractions showed positive response to Libermann-Bruchard reagent. Elutes of chloroform:methanol (98:2) were collected and further purified with n–hexane and petroleum ether. It gives positive response to Liebermann-burchard test. The TLC pattern of these elutes showed 2 distinct spots and UV spectra revealed the presence of 2 peaks at wavelength 279.40 nm and 243.60 nm having absorbance at 0.4244 and 0.3323 respectively (▶ Fig. 2).

The present study establishes the significant acute anti-inflammatory activity of C. carandas fruit extracts in experimentally induced acute inflammation in Wistar rats. The inflammatory response can be readily produced in the form paw oedema with the help of irritants or phlogistic agents. Such agents like carrageenan, formalin, bradykinin, histamine, serotonin etc when injected into the dorsum of the foot of the rats they produce acute paw oedema within a few minutes of injection. Carrageenan induced rat paw oedema has been most commonly used as an ideal experimental animal model for acute inflammation. [28, 29].

Carrageenan-induced acute inflammatory oedema is generally believed to be a biphasic response. The early phase (1–2 h) of the carrageenan model is mainly mediated by histamine and serotonin (5-HT). The late phase (2–4 h) is mediated by bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages [30]. In the present study, both the test extracts produced significant inhibition of carrageenan induced rat paw oedema after a period of 3 h (▶ Table 4 and ▶ Fig. 3). The anti inflammatory activity was studied on Wistar rats using carrageenan induced paw edema model and cotton pellets induced granuloma. There was a consistent increase in % inhibition at concentrations of 100 and 200 mg/kg up to 3 h. The mature fruits showed highest activity at 3 h with 200 mg/kg dose. In carrageenan induced rat paw edema

Table 4  Data of Anti-Inflammatory Activity.

<table>
<thead>
<tr>
<th>Groups</th>
<th>0hr</th>
<th>1hr</th>
<th>2hr</th>
<th>3hr</th>
<th>4hr</th>
<th>5hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.14 ± 0.02</td>
<td>6.5 ± 0.01</td>
<td>7.06 ± 0.02</td>
<td>8.24 ± 0.02</td>
<td>7.6 ± 0.01</td>
<td>6.9 ± 0.02</td>
</tr>
<tr>
<td>Standard</td>
<td>4.470 ± 0.016</td>
<td>6.2 ± 0.01</td>
<td>6.6 ± 0.02</td>
<td>6.9 ± 0.03</td>
<td>6.7 ± 0.01</td>
<td>7 ± 0.02</td>
</tr>
<tr>
<td>Immature</td>
<td>4.8 ± 0.01</td>
<td>6.880 ± 0.0081</td>
<td>7.020 ± 0.16</td>
<td>7.003 ± 0.02</td>
<td>7.080 ± 0.016</td>
<td>7.040 ± 0.010</td>
</tr>
<tr>
<td>Mature</td>
<td>5.5 ± 0.02</td>
<td>6.44 ± 0.02</td>
<td>6.95 ± 0.02</td>
<td>7.27 ± 0.02</td>
<td>7 ± 0.01</td>
<td>7.1 ± 0.02</td>
</tr>
<tr>
<td>Ripe</td>
<td>5.190 ± 0.01</td>
<td>7.190 ± 0.0081</td>
<td>7.68 ± 0.0081</td>
<td>7.81 ± 0.1022</td>
<td>7.600 ± 0.012</td>
<td>6.12 ± 0.016</td>
</tr>
</tbody>
</table>
the % inhibition at concentration of 100 mg was found to be more with immature fruits (68 %) as compared to the % inhibition observed with mature and ripe fruits after 3 h. After 5 h, the increase in % inhibition was observed with the extract of ripe fruits and a decline in % inhibition was observed with the extract of immature fruits (▶ Table 5 and ▶ Fig. 4). In cotton pellets method inhibition of granuloma was found to be less than 50 % of standard (45 %) with the extract of immature and ripe fruits whereas it was 24.91 % with the extract of mature fruit (▶ Table 6 and ▶ Fig. 5).

**Conclusion**

Based on the results obtained from the present preliminary study and pharmacological studies, it can be concluded that mature fruits of Carissa carandas L. possessed comparably effective acute anti-inflammatory actions in Wistar rats. Further studies are presently necessary to confirm the identity of the bioactive principles responsible for these actions.

**Table 5** % inhibition for anti-inflammatory activity.

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of animal</th>
<th>3 hr</th>
<th>5 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>6</td>
<td>78</td>
<td>85</td>
</tr>
<tr>
<td>Immature</td>
<td>6</td>
<td>66</td>
<td>20</td>
</tr>
<tr>
<td>Mature</td>
<td>6</td>
<td>58</td>
<td>57</td>
</tr>
<tr>
<td>Ripe</td>
<td>6</td>
<td>37</td>
<td>66</td>
</tr>
</tbody>
</table>

**Table 6** Effect of stages of fruits on cotton pellets granuloma in rats.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Group specification</th>
<th>Wet weight, of cotton pellets in mg</th>
<th>Dry weight of cotton pellets in mg</th>
<th>% inhibition of granuloma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>120.17 ± 1.21</td>
<td>60.21 ± 1.92</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Diclofenac sodium</td>
<td>74.03 ± 0.92</td>
<td>33.32 ± 0.82</td>
<td>44.66</td>
</tr>
<tr>
<td>3</td>
<td>Immature</td>
<td>80.10 ± 1.02</td>
<td>47.01 ± 1.14</td>
<td>21.93</td>
</tr>
<tr>
<td>4</td>
<td>Mature</td>
<td>78.42 ± 0.96</td>
<td>45.21 ± 0.97</td>
<td>24.91</td>
</tr>
<tr>
<td>5</td>
<td>Ripe</td>
<td>81.21 ± 1.10</td>
<td>48.08 ± 1.18</td>
<td>20.14</td>
</tr>
</tbody>
</table>
Conflict of Interest

Declared None.

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[16] Meda A, Lamien CE. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. J of Food Chemistry 2005; 571–577