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# PHYTOCHEMICAL INVESTIGATION AND ANTI-INFLAMMATORY ACTIVITY OF AN ISOLATED FRACTION OF STEROLS FROM THE FRUIT EXTRACT OF *CARISSA CARANDUS*

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## ABSTRACT

The sterols fraction from methanolic extract of the fruits of *carissa carandus* was investigated for anti-inflammatory activity in animal models.The powder of *carissa carandus* fruits was subjected to extraction with methanol in soxhlet extractor. The methanolic extract after preliminary phytochemical investigation showed the presence of sterols, triterpenoids, phenolic compounds and flavonoids. Results of the study revealed that sterols possesses significant anti-inflammatory activity. The anti-inflammatory activity was studied using acetic acid induced vascular permeability and croton oil induced ear edema at a two different doses (200 and 400 mg/kg) of methanolic extract. The methanolic extract of *carissa carandus* fruit was exhibited significant anti-inflammatory activity at the dose of 400 mg/kg in both models when compared with control group. Indomethacin (10 mg/kg) was also shown significant anti-inflammatory activity in both models.

**KEYWORDS:** *Carissa carandus*, Phytochemical investigation, Anti-inflammatory activity, Sterols, Methanolic extracts

**INTRODUCTION-** *Carissa carandus* is a large dichotomously branched evergreen shrub with short stem and strong thorns in pairs, belonging to the family apocynaceae. The whole plant possesses medicinal properties useful in the treatment of skin diseases, inflammatory diseases, rheumatism and jaundice. Various cardiac glycosides, a triterpenoidal constituents carissone and β- sitosterol were reported from the root extract of the plant (hedge et al),

where as tannin, triterpenes and carissic acid were reported from the leaves extracts. The fruit extract of *carissa* were reported carissol and isoamyl alcohol. (singh rastogi and pal). Phytochemical investigation of *carissa* reported various phytoconstituents such flavanoids, tannin, phenols, sterols etc (Vaghasiya et al). Steroids work by decreasing inflammation and reducing the activity of the immune system. Inflammation is a process in which the body's white blood cells and chemicals can protect against infection and foreign substances such as bacteria and viruses. High content of sterols were present in fruits extracts of *carissa carandus* (Pal et al). A number of scientific study reported sterols possesses anti-inflammatory activity. The fruit in clusters of 3-10, is oblong, broad-avoid or round, ½ to 1 inch long, has fairly thin but tough, purplish-red or nearly black when ripe (Devmurari).

However so far no systematic study on phytochemical and anti-inflammatory activity has been reported in literature. The present study is focused to evaluate the anti-inflammatory activity of fruit extract of *carissa carandus*..

## MATERIAL AND METHOD

**PLANT MATERIAL -** The fresh fruits of plant *Carissa carandus* were collected in the month of October from local market, Bhopal (M.P.)

The plant material was authenticated by Dr. Ziaul Hassan, Professor, Department of Botany Saifia college of Science, affiliated to Barkatulla university peer gate, Bhopal, Madhya Pradesh, bears authentication number 211/ BOT/ SAIFIA/ 2011 and the specimen is deposited in the herbarium.

**EXTRACTION-** The fresh fruits of *Carissa carandus* were cut into pieces, shade dried and powdered in a mixer grinder. These powdered drugs were used for extraction.

### Preparation of petroleum ether extract

The powdered material (1 kg) was defatted exhaustively with petroleum ether (60-80 ºC) in a soxhlet extractor for about 48 hours. The pet. ether soluble fraction was collected and dried in rotary vaccum evaporator.

### Preparation of hydroalcoholic extract

After petroleum ether extraction the air dried marc was extracted with methanol: water (70:30) to get hydroalcoholic extract. The filtrate was concentrated using a rotary evaporator at low temperature 390º C. The weight of the crude extract was 50 gm.

These crude extract (10gm) was triturated with 90% methanol and then subjected to solvent-solvent partitioning designed by Kupchan and Tsou (1973). The prepared solution was then fractionated successively using solvents of increasing polarity, such as *n*-hexane, carbon tetrachloride and chloroform. All the fractions were evaporated to dryness by using rotary evaporator at low temperature of 390º C and kept in air tight containers for further analysis.

**Saponification process**

Concentrated extract was treated with 250 ml hydro-alcoholic solution of ethanol and 50 ml of potassium hydroxide (150gm/100 ml), dried in rotary vaccum evaporator. Extracts obtained were then weighed and the percentage yields were calculated in terms of air dried weight of the plant material. Dried extracts were stored in refrigerator until further studies. These saponification process was used to remove fatty material and to obtained unsaponified matter.

**ISOLATION AND PURIFICATION OF STEROL**

After the completion of saponification process the fatty component separates and separated unsaponified fraction dissolved in pet. ether was fractionated with ethylene chloride in separating funnel. The separated component was treated with 10% hydro alcoholic solution of ethanol. Afterwards anhydrous sodium sulphate was added and filtered. Extract was concentrated in rotary vacuum evaporator. Concentrated component was dissolved in ethylene chloride. Mixture was kept for overnight and separated in centrifuge at controlled temperature. Obtained crystals were redissolved in *n*-hexane and distilled water was added and mixture was kept overnight for crystallization. After centrifugation in cooling microfuge, collected component was kept in air tight container for further analysis (Kamboj and Saluja).

The column chromatography was used for purification of the sterols. The column was run using hexane, chloroform and methanol by gradient elution technique. Total 100 ml volume was collected in the mentioned solvent system. Then the column was eluted with pet. ether, ethyl acetate and methanol mixtures of increasing polarities to provide 31 fractions (50 ml each). Evaporation of the solvents, to obtained colorless needle shaped crystals from the fraction no. 24-27. The yield of isolated crystal is 4.3 gm. Again the qualitative chemical test and TLC was performed in order to check the presence of sterols. (Muhit et al).

These crystals were further subjected to IR, NMR and mass spectroscopy to ascertain the chemical structure.

**PRELIMINARY PHYTOCHEMICAL ANALYSIS –** The result of preliminary phytochemical investigation of extract shown in **Table-1**

**IDENTIFICATION OF ACTIVE PRINCIPLE BY TLC-** The chloroform, ethyl acetate, formic acid, toluene and distilled water were subjected to thin layer chromatography.

The details of TLC were as follows (For sterols)

**Adsorbent:** Silica gel 60-F.

**Visualization:** Iodine chamber and spraying reagents.

**Detecting reagent used:** U.V at 254 nm and 366 nm.

**Mobile phase:**

**Solvent system I-** Chloroform: Ethyl acetate: Formic acid (7.5:6:0.5)

**Solvent system II-** Toluene: Acetic acid (7:3)

**Solvent system III-** Toluene: Methanol: Acetone: Acetic acid (14:4:1:1)

The spot visualized as pink colour (Bhawani et al).

The Rf value of each shown in **Table-2.**

**ANIMALS -** Adult swiss albino mice of either sex weighing between 30-35 g were selected for anti-inflammatory activity. For both methods the animals were randomly distributed into groups and housed into polyacrylatic cages (3 per cage) and maintained under standard laboratory condition. All animal were feed with standard pellet diet and water ad libidum. The project was cleared by institutional Animal Ethical Committee (IAEC) and care of animals were taken according to CPCSEA guidelines. Institutional Animal Ethical Committee experiment protocols no. PBRI/IAEC/12/PN-237.

**ACUTE TOXICITY STUDY**

Acute oral toxicity was performed as per OECD 423 guidelines. Four dose levels were selected for acute oral toxicity. 5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg were used as dose range. As per annexure II A of OECD 423 guidelines following methodology was used. Dose was selected as $1/10^{th}$ and $1/5^{th}$ of observed NOAEL (i.e. 2000 mg/kg) as 200 mg/kg and 400 mg/kg. Acute oral toxicity of both, methanolic extract and isolated component was performed.

**ACETIC ACID INDUCED VASCULAR PERMEABILITY**

Animals were divided into three groups (N=6). The control group received vehicle orally, while other groups received test drug and standard drug respectively followed by the

injection of 0.25 ml of 0.6% solution of acetic acid intraperitoneally. Immediately after administration, 10 mg/kg of 10% (w/v) Evans blue is injected intravenously through the tail vain. Thirty minutes after Evans blue injection the animals are hold by a flap of abdominal wall and the viscera irrigated with distilled water over a petri dish. The exudate is then filtered and makes the volume up to 10 ml. The dyes leaking out into the peritoneal cavity measured spectophotometrically using visible spectra at 10 nm and compared with the control group. The concentration of Evans blue in the peritoneal cavity was measured by the absorbance at 630 nm in a spectrophotometer. The vascular permeability was represented in terms of the absorbance (A= 630) which leaked into the cavity. Experiments were performed in triplicate (Prabhu et al, 2011).

$$\text{Vascular permeability \% inhibition} = \frac{\text{Abs. Control} - \text{Abs. Test}}{\text{Abs. Control}} \times 100$$

**CROTON OIL INDUCED EAR EDEMA**

**Procedure –** Croton oil was dissolved in 5% acetone solution (v/v) and 10µl were applied with an automatic pipette to both anterior and posterior surfaces of the right ear. The left ear received the vehicle. One hour after the oral administration of vehicle. The indomethacin, methanolic extract and isolated component applied topically at a dose of 10 µl on both sides of the right ears. The left ear was kept untreated to serve as control. Four hours after the treatment, the mice were killed by cervical dislocation and a plug (6 mm in diameter) was taken from both the treated and untreated ears with a punch. The punches were weighed immediately after decapitation and the difference in weight was use to assess the inflammatory response. (Okoli et al ).

$$\text{Ear edema \% inhibition} = \frac{\text{Abs. Control} - \text{Abs. Test}}{\text{Abs. Control}} \times 100$$

**STATISTICAL ANALYSIS**

All data were presented in Mean ± SD (N=6). Data were analyzed by one way ANOVA, followed by Dunnet's test. $P<0.05$ was considered as level of significance. Data were analyzed by primer software.

## RESULTS AND DISCUSSION

**Phytochemical Screening-** Preliminary Phytochemical screening of various extracts from fruit of *carissa carandus* showed positive results for steroids, flavanoids, tannin, alkaloids and glycosides etc.

**Table 1: Physical examination of fruit extracts of *C.carandus***

| Name of extract | Yield (%w/v) | Colour and odour | Appearance |
|---|---|---|---|
| Petroleum ether extract | 4.44 | Yellow and aromatic | Thick and oily |
| Methanolic extract | 50.90 | Dark brown | Thick, sticky and resineous |

**Table 2: Inferences of qualitative phytochemical examination of pet. ether and methanolic extracts of *carissa carandus* fruits extract.**

| S. No. | Constituents | Petether fraction | Methanolic extracts |
|---|---|---|---|
| 1. | Carbohydrate | - | + |
| 2. | Saponins | - | - |
| 3. | Flavanoids | - | + |
| 4. | Alkaloids | - | + |
| 5. | Steroids | + | + |
| 6. | Tannins & Phenolics | + | + |
| 7. | Glycosides | - | + |
| 8. | Proteins | - | - |

**(+): Present, (-): Absent**

**Table 3: Thin layer chromatography**

| Sr. No. | Solvent | No. of spots | $R_f$ value |
|---|---|---|---|
| 1. | Chloroform: Ethyl acetate: Formic acid (7.5 : 6 : 0.5) | 3 | 0.92<br>0.85<br>0.69 |
| 2. | Toluene : Acetic acid ( 7 : 3 ) | 4 | 0.94<br>0.84<br>0.58<br>0.29 |
| 3. | Toluene : Methanol : Acetone : Acetic acid (14 :4 : 1 : 1) | 5 | 0.95<br>0.86<br>0.43<br>0.31<br>0.26 |

**Table 4: Effect of *C. carandus* on acetic acid induced vascular permeability**

| Group No. | Treatment | Evans Blue leaked* | % Inhibition |
|---|---|---|---|
| 1. | Vehicle | 0.97±0.13 | -- |
| 2. | Extract 200 mg/kg | 0.67±0.07** | 31 |
| 3. | Extract 400 mg/kg | 0.65±0.04** | 33 |
| 4. | Isolated component 200 mg/kg | 0.56±0.03** | 42 |
| 5. | Isolated component 400 mg/kg | 0.40±0.03** | 58 |
| 6. | Indomethacin | 0.39±0.03** | 59 |

*All Data are presented in Mean ± SD (N=6)

** Significant difference as compared to vehicle treated group (P<0.05)

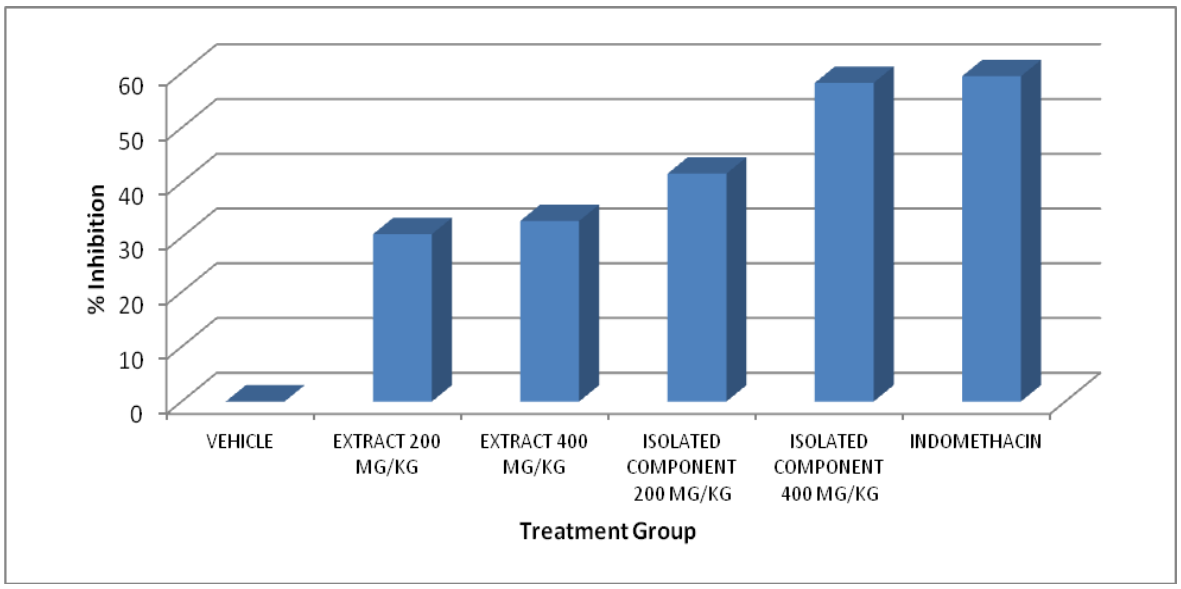


**Figure 1: Effect of *C. carandus* on acetic acid induced vascular permeability**

**Table 5: Effect of *C. carandus* on croton oil induced ear edema**

| Group No. | Treatment | Ear weight* | % Inhibition |
|---|---|---|---|
| 1. | Vehicle | 4.0±0.63 | -- |
| 2. | Extract 200 mg/kg | 2.5±1.38** | 0.37 |
| 3. | Extract 400 mg/kg | 2.0±0.89** | 0.50 |
| 4. | Isolated component 200 mg/kg | 1.7±0.52** | 0.58 |
| 5. | Isolated component 400 mg/kg | 1.5±0.55** | 0.625 |
| 6. | Indomethacin | 1.5±0.84** | 0.625 |

*All Data are presented in Mean ± SD (N=6)

** Significant difference as compared to vehicle treated group (P<0.05)

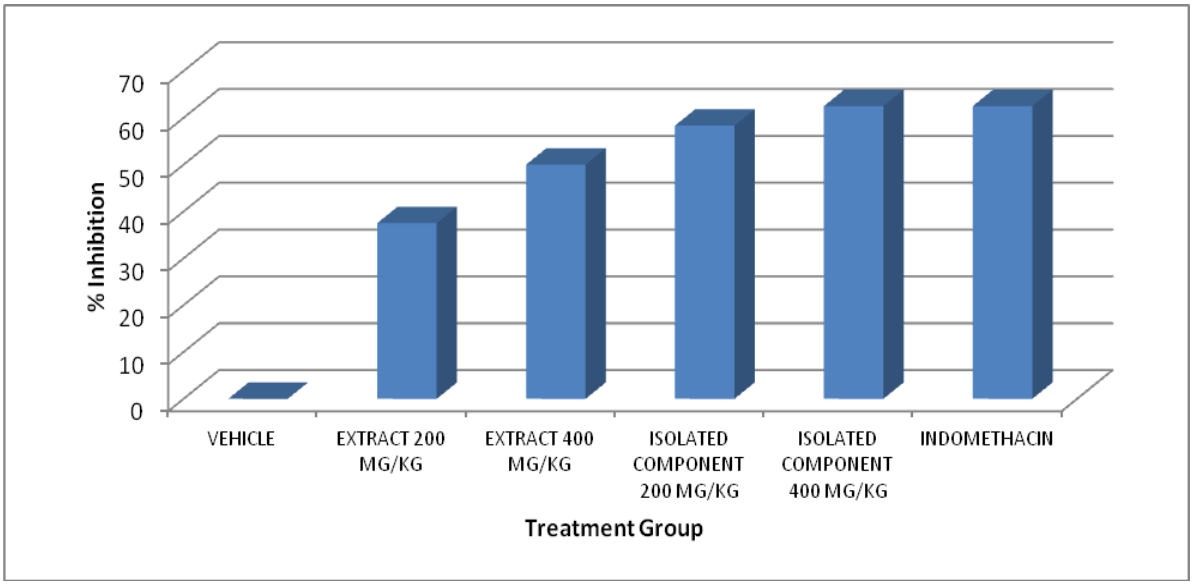


**Figure 2: Table - Effect of *C. carandus* on croton oil induced ear edema**

In present study acetic acid was used as inflammatory agent and protein binding dye was used to assess anti-inflammatory activity. It was observed that both extract and isolated component significantly decreased vascular permeability at 200 mg/kg and 400 mg/kg doses (Table 4 and Figure 1). Even percentage inhibition in isolated component at 400 mg/kg was found to be 58 % which was not significantly less as compared to that use standard anti-inflammatory agent indomethacin which showed % inhibition of 59 %.

Similarly in croton oil induced ear edema significantly decreased in extract and isolated component treated group at 200 mg/kg and 400 mg/kg (Table 5 and Figure 2). Both of these models confirmed anti-inflammatory activity of extract and isolated component in acute model of inflammation.

## CONCLUSION

Thus from investigation it was confirmed that methanolic extract of ***C. carandus*** and isolated component which was found to be sterol in nature possess significant anti-inflammatory activity. Although further detailed study is required for assessment of mechanism of action for said activity and assessment of sub chronic toxicity and chronic toxicity is also an important requirement.

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