Anti-inflammatory Activity of *Sclerotium stipitatum* Berk. et Curr. an Ethnomedicinal Fungus, in Chronic and Acute Animal Models of Inflammation

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Authors’ contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

*Sclerotium stipitatum* Berk. et Curr., locally known as ‘nilamanga’ is a rare macro fungus, traditionally used to treat numerous diseases like arthritis, earache, jaundice etc. The present study aims to evaluate the anti-inflammatory activity of ethanol extract of *S. stipitatum* and identify the bioactive compounds present in them. Phytochemical screening of extracts obtained using different solvents like petroleum ether, chloroform, ethanol and water were done. The best extract was chosen for the acute carrageenan-induced and chronic formalin-induced anti-inflammatory studies. Diclofenac was used as the standard drug. Ethanol extract showed significant inhibition of inflammation induced by carrageenan and formalin-induced paw edema models compared to the control. GC-MS analysis shows certain bioactive compounds. The significant inhibitory effect on paw edema proves that *S. stipitatum* possesses remarkable anti-inflammatory activity, and isolation and identification of bioactive compounds can be used for new drug formulations.

Keywords: *Sclerotium stipitatum*; anti-inflammatory; paw edema; carrageenan; formalin; ethanol extract.

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1. INTRODUCTION

Inflammation is the immune system's response against harmful stimuli such as damaged cells, pathogens, irradiation or toxic compounds and acts by eliminating the injurious stimuli and starting the healing process [1]. So, it is a vital defense mechanism to health [2]. The cellular and molecular events and interaction can efficiently reduce the impending infection or injury during acute inflammatory responses. Thus it resolves the acute inflammation and restores the tissue homeostasis [3]. However, acute inflammation may become severe and contribute to chronic inflammatory diseases when it becomes uncontrollable [4]. NSAIDs are the most common medications used for inflammation and related disorders worldwide. They are carboxylic acid-containing drugs with salicylic derivatives [5]. However, even though they have a high potential against inflammation, severe side effects like gastrointestinal (GI) ulceration, obstruction, perforation, and bleeding have limited NSAIDs' therapeutic usage [6]. In this case, natural medicines are the best alternatives [7]. Researchers have now found many alternative medicines of plant origin that can cope with the side effects of non-steroidal anti-inflammatory drugs (NSAIDs) against inflammation.

The various phytochemicals like alkaloids, flavonoids, terpenoids, and saponins seem to contribute to plants' anti-inflammatory activity [8]. Not only plants but also many fungi have anti-inflammatory properties. The compounds like Tsugaric acid in Ganoderma lucidum, Fumigaclavine C in Aspergillus fumigatus, Rutilin in Hypoxylonrutilum are responsible for the anti-inflammatory activities in those respective fungi [9].

S. stipitatum is found by Berkeley in 1860 from the white ants' nest in South India [10]. From ancient times itself, they were widely used for many medications by the tribes. They used to preserve this rare fungus whenever they got it [11]. Due to their restricted habitat, they have not been exploited for many studies. Only a few studies have been done on this species. It has got excellent medicinal use in Ayurveda and ‘Parambharyavydyam.' An ethnobotanical study reveals that it effectively treats several ailments, such as earache, arthritis, stomach pain, dehydration, jaundice and even stomach cancer [12]. So here we are, trying to explore the anti-inflammatory activity and bioactive compounds present in the fungus.

2. MATERIALS AND METHODS

2.1 Fungal Material

S. stipitatum was collected from the tribal colonies and forests in Palakkad district, Kerala, India. It is a hypogeous fungus usually associated with termite nests under the soil. “The mass consists of very irregular swollen and sometimes constricted more or less anastomosing and more or less densely compacted threads,” says the Rev. M. J. Berkeley [10]. The outer covering of S. stipitatum is black, and the inner portion is white. On drying, it becomes rigid, and the inner portion is spongy and opaque [13]. Its identity is confirmed at the National Fungal Culture Collection of India (NFCCI) and the specimen is deposited at Ajrekar Mycological Herbarium (AMH) with accession number: AMH-10322 (Fig. 1).

Fig. 1. Depicts the fresh specimen of S. stipitatum

2.1.1 Preliminary qualitative analysis

The material was cut into small pieces and dried by keeping in a hot air oven for 48hours at 60°C. Then it is powdered and extraction is done by the hot soxhlet method. The solvents are removed by distillation over water bath. Preliminary
phytochemical screening of extracts was done using the standard phytochemical tests [14]. Finally, the extract with the maximum number of compounds are chosen for further study.

GC-MS analysis was carried out on a Thermo GC-Trace Ultra Ver: 5.0, Thermo MS DSQ II and gas chromatograph connected to a mass spectrometer (GC-MS) instrument under the following conditions:- column DB 5- MS Capillary standard non- polar column (Sample ID: EM - 473), helium (HE) was used as carrier gas at a static flow of 1.0 ML/MIN, dimension was used as 30 MTS, ID:0.25mm Film: 0.25µM. The oven temperature was programmed from 70°C raised to 260°C at 6 C/MIN. The injection volume taken was 1 Microliter.

2.2 Animal Study

2.2.1 Acute toxicity Study and drug preparation

Before moving on to the in-vivo animal experiments toxicity study was done to understand the drug's toxicity. For that 6 Swiss albino mice were used. 3 males and 3 females. An acute toxicity study was conducted. Ie, a higher concentration of the drug was given as a single dose. The concentration used was 2gm/kg body weight (b. wt). The drug was prepared by dissolving ethanol extract in water. After administering the drug orally, the mortality rate of the mice was noted. The dosage was determined based on the acute toxicity investigation performed following OECD guidelines 423 [15], which demonstrated that the ethanol extract did not cause death at doses up to 2 gm/kg b.wt. Furthermore, no toxicity symptoms were seen in the dose group up to 2 g/kg b.wt during the research period.

The concentration of drug thus chosen for anti-inflammatory study was 200mg/kg (milligram/kilogram) b.wt (body weight) and 50 mg/kg b. wt. 1/10th of the dosage used for the acute toxicity study was used as higher dose and a lower dose was also chosen. Diclofenac was used as the standard drug. The concentration of the standard was 10mg/kg b. wt.

Animal experiments were carried out at Amala Cancer Research Centre, Thrissur. Female Swiss albino mice (20-25g/b. wt) were used for the anti-inflammatory studies as they were readily available. They were brought from Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India. They were maintained in polypropylene cages with regular standard rat feed and water ad libitum.

2.2.2 Acute Carrageenan induced model

Acute anti-inflammatory activity was evaluated by the method of Winter et al. [16]. Animals were grouped into four with 6 animals each. First two groups were pretreated orally with drugs of higher dose 200 mg/kg and lower dose 50mg/kg for 6 days. On the 6th day, diclofenac was also injected intraperitoneally to the 3rd group. 4th group was kept as control. After 1-hour, carrageenan was induced by subplantar carrageenan injection in 0.1% carboxymethylcellulose (CMC) in the right hind paw of every mouse. Using a vernier caliper, the paw thickness was measured 1 hour before and every hour up to the 6th consecutive hour after carrageenan administration. The percentage inhibition of paw thickness was calculated by:

%Inhibition of thickness = [(tCn-tC0) – (tTn- tT0) / tCn – tC0] x 100

where tCn- paw thickness at the particular time period of control animal
T0- paw thickness before induction of control animal
Tn- paw thickness at the particular time period of treated animal
T0- paw thickness before induction of treated animal

2.2.3 Chronic formalin-induced model

As described in the carrageenan-induced model, the animals were grouped into four, with six animals each and pretreated with drugs for 6 consecutive days. Moreover, after the sixth day of administration, 2% freshly prepared formalin was injected into the right hind paw. Then the paw thickness was calculated using vernier caliper before and after the formalin administration. Next, the edema was measured every day for up to 7 days [17]. Then the percentage of inhibition was calculated using the above formula.

2.3 Statistical Analysis

Results were expressed as mean±SD. Statistical analysis was carried out using a one-way analysis of variance (ANOVA). P<0.05 was considered statistically significant.
3. RESULTS

3.1 Preliminary Qualitative Analysis

Preliminary qualitative analysis revealed that petroleum ether extract of *S. stipitatum* contained phytosterol. The chloroform extract had phytosterols, glycosides and lactones. In acetone extract were detected tannins and lactones. The ethanol extract showed the presence of alkaloids, flavonoids, phenol, saponin and aleurone grains. The aqueous extract contained flavonoids, phenols and naphthoquinones. Since ethanol extract extracted the most compounds, it was selected for the further studies (Table 1).

3.1.2 GC-MS analysis of ethanol extract

The ethanol extract of *Sclerotium stipitatum* has the following composition: 2-Propanone,1-(dimethylamino)-, 2-Pyrrolidinone, N-Trimethylsilyl-2-pyrrolidinone, 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester, Hexanediamide,N,N'-di-benzoyloxy-, 2,5-Methylene-d,l-rhamnitol, 2,5-Methylene-d,l-rhamnitol, 2,5-Methylene-d,l-rhamnitol, Ribitol, 1,2-Benzenecarboxylic acid, diisooctyl ester. The retention time of above compounds were 4.16, 9.18, 9.80, 11.30, 13.05,13.97, 14.73, 21.20, 25.90, and 30.41 respectively.

The compounds ribitol and 2,5-Methylene-d,l-rhamnitol are the most abundant compounds present in the extract. All other compounds are present in only a minute quantity compared to this (Figs. 2; 3).

3.2 Animal Study

3.2.1 Acute toxicity

The acute toxicity study revealed that the administration of ethanol extract was safe up to 2gm/kg b.wt, with no toxicity or mortality.

3.2.2 Carrageenan induced inflammation

The ethanol extract of *Sclerotium stipitatum* significantly inhibits the acute inflammation induced by carrageenan. The extracts at concentrations 200 and 50 mg/kg reduced the paw thickness 83.33 and 66%, respectively, compared to control. The activity of standard reference drug Diclofenac at 10mg/kg b.wt showed 88.88%(Fig. 4) (Table 1).

3.2.3 Formalin induced inflammation

The ethanol extract of *Sclerotium stipitatum* significantly inhibits the chronic inflammation induced by formalin. The extracts at concentrations 200 and 50 mg/kg reduced the paw thickness 94 and 80%, respectively, compared to control. The activity of standard reference drug Diclofenac at 10mg/kg b.wt showed 98%(Fig. 5) (Table 2).

<table>
<thead>
<tr>
<th>Chemical constituents</th>
<th>Petroleum ether</th>
<th>Chloroform</th>
<th>Acetone</th>
<th>Ethanol</th>
<th>Distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytosterol</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Triterpenoids</td>
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<td>Saponin</td>
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<td>+</td>
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<td>Alkaloids</td>
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<td>+</td>
<td>-</td>
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<tr>
<td>Flavonoids</td>
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<td>Lactones</td>
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<td>Tannins</td>
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<tr>
<td>Aleurone grains</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Naphthoquinones</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Fig. 2. Depicts the result of GCMS analysis of ethanol extract of *S. stipitatum*.

- **Fig. 3.** Depicts the structure of compounds present in the ethanol extract of *S. stipitatum*.
Table 2. Effect of *Sclerotium stipitatum* ethanol extract on carrageenan induced paw edema

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial paw thickness (cm)</th>
<th>Final paw thickness (cm)</th>
<th>Percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.190 ± .010</td>
<td>0.280 ± .010</td>
<td>-</td>
</tr>
<tr>
<td>Diclofenac (10mg/kg)</td>
<td>0.190 ± .015</td>
<td>0.200 ± .005**</td>
<td>88.88</td>
</tr>
<tr>
<td>SS Extract (200mg/kg)</td>
<td>0.190 ± .010</td>
<td>0.205 ± .010**</td>
<td>83.33</td>
</tr>
<tr>
<td>SS Extract (50mg/kg)</td>
<td>0.180 ± .012</td>
<td>0.210 ± .012**</td>
<td>66.00</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± Standard deviation (SD), n = 6, **p<0.01 compared to control considered as significant.

Fig. 4. Depicts the change in paw thickness at each hour in carrageenan induced model

Fig. 5. Depicts the change in paw thickness at each day in formalin induced model
4. DISCUSSION

Secondary metabolites like alkaloids, flavonoids, tannins, saponins, coumarins etc., act as the sources of anti-inflammatory agents [18]. The GC-MS analysis shows the presence of some bioactive compounds present in the extract. For example, 2-Pyrrolidinone has significant antioxidant and anti-cancer activity [19]. Many derivatives of 2-Pyrrolidinone have proved to possess anti-inflammatory activity; in addition, the template 2-Pyrrolidinone also contributes to the anti-inflammatory activity of new compounds [20]. The most prevalent chemicals found in the extract are ribitol and 2,5-Methylene-d,l-rhamnitol. In comparison to this, all other chemicals are present in only minute amounts, especially 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester, and 1,2-Benzenedicarboxylic acid, diisooctyl ester. The primary bioactive compound ribitol has been shown to possess excellent anti-inflammatory activity [21]. So, the secondary metabolites and the major bioactive compounds obtained through GC-MS may have contributed to the anti-inflammatory activity of the extract.

In order to reveal the medicinal potential of this fungus, we chose to perform the anti-inflammatory activity considering the period, availability of chemicals and other facilities required for the experiments. Also, chronic inflammation can predispose a person to cancer, and inflammation can also be due to infection. So, treating inflammation can act as a remedy for these causes. Also, it further clarifies the need to study the anti-tumor and antioxidant properties and thus to evaluate the overall anti-cancer potential of the extract in future.

Carrageenan-induced inflammation is the most suitable procedure for screening acute anti-inflammatory agents [22]. The current investigation on ethanol extract of *S. stipitatum* reveals its ability to reduce paw edema in a dose-dependent manner remarkably. In carrageenan, the inhibitory effect of extract at high dose (200 mg/kg) was 83.33%, the low dose was 66% and that of standard diclofenac was 88.88% (Table 1). In all groups, there is an initial increase in paw thickness for the first 2 hours after the induction of carrageenan. After that, however, it starts to reduce, and the drug-treated groups show a drastic reduction compared to the control. Reduction in the paw edema in the control group is minor and will not reduce beyond a specific limit. In the standard group, it almost gets reduced to the normal level. Moreover, the drug-treated ones also show a good result (Fig. 4).

The formalin-induced paw edema model is the best method for screening chronic anti-inflammatory agents closely related to human arthritis [22]. In the formalin-induced model, the inhibitory effect of extract at high dose (200mg/kg) was 94%, the low dose was 80%, and standard diclofenac was 98%. Here also, all groups show an increase in paw edema initially, but it is comparatively less in drug-treated groups. Then from the third day onwards, edema starts to decrease, and ethanol extract treated ones and standard treated ones almost come to the normal paw thickness. Thus, the *S. stipitatum* ethanol extract at high and low doses gives a promising result comparable to the standard drug (Fig. 5).

| Table 3. Effect of *Sclerotium stipitatum* ethanol extract on formalin induced paw edema |
|---------------------------------|---------------------------------|---------------------------------|
| Initial paw thickness (cm)     | Final paw thickness (cm)        | Percentage inhibition          |
| Control                        | 0.190 ± .010                    | 0.290 ± .010                   | -                              |
| Diclofenac (10mg/kg)           | 0.190 ± .020                    | 0.192 ± .020****              | 98.00                          |
| SS Extract (200mg/kg)          | 0.200 ± .010                    | 0.196 ± .010**                | 94.00                          |
| SS Extract (50mg/kg)           | 0.190 ± .010                    | 0.210 ± .010**                | 80.00                          |
| **Values are expressed as Mean ± SD, n = 6, **** p< 0.0001, ** p< 0.01 compared to control considered as significant.**

Inflammation is mediated by the activation of prostaglandins, Platelet Activating Factor (PAF), and other mediators of inflammation like TNF-α, interleukin, NO etc. [23]. Furthermore, it is attributed to releasing histamines, kinins, serotonin, etc. [24]. Here the anti-inflammatory activity of ethanol extract in both models is comparable with that of the diclofenac, the conventional anti-inflammatory drug. Moreover, the anti-edematous effect maybe because of the inhibition of histamine release or the inhibitory effect of extract at high dose (200 mg/kg) was 83.33%, the low dose was 66% and that of standard diclofenac was 88.88% (Table 1). In all groups, there is an initial increase in paw thickness for the first 2 hours after the induction of carrageenan. After that, however, it starts to reduce, and the drug-treated groups show a drastic reduction compared to the control. Reduction in the paw edema in the control group is minor and will not reduce beyond a specific limit. In the standard group, it almost gets reduced to the normal level. Moreover, the drug-treated ones also show a good result (Fig. 4).

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inhibition of carrageenan-induced inflammation. Thus it might be downregulating the prostaglandin synthesis and the Coxygenase-2, which promotes the prostaglandin synthesis [25]. Then the proliferative phase of inflammation is represented by the formalin-induced paw edema [26]. So the drug also seems to act by inhibiting the proliferative phase of inflammation.

5. CONCLUSION

The current study demonstrated the anti-inflammatory activity and the various biologically active compounds present in the ethanol extract of S. stipitatum. Thus, it supports the traditional usage of S. stipitatum and formulation of new drugs can be done. Moreover, fungi are an unexplored group when compared to plants. So, this anti-inflammatory work is highly significant and reveals the importance of further research to investigate fungi’s magical properties. The study also emphasizes the usefulness of “Ayurveda,” an old Indian system still practiced in some regions of the country. This old notion should be carefully assessed considering current medical knowledge, and if proven to be acceptable, it can be utilized partially.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All the animal experiments were carried out with prior approval from Institutional Animal Ethical Committee with approval number-ACRC/IAEC/18(2) P-2, following the internationally accepted laboratory animal use and care guidelines and rules of CPCSEA (Approval no. of institution – 149/PO/Rc/S/99/CPCSEA).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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