Anti-inflammatory activity of *Sclerotium stipitatum* Berk.*et.*Curr. an ethnomedicinal fungus, in chronic and acute animal models of inflammation

Abstract

Sclerotium stipitatum Berk. *et*. Curr., locally known as 'nilamanga' is a rare macro fungus, traditionally used for the treatment of numerous diseases like arthritis, ear ache, jaundice etc.The purpose of the present study is to evaluate the anti-inflammatory activity of ethanolextract of *S. stipitatum*and to 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 when 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.

Key words: *Sclerotium stipitatum,* Anti-inflammatory, Acute, Chronic, Ethanolextract

1. Introduction

Inflammation is the response of immune system against harmful stimuli such as, damaged cells, pathogens, irradiation or toxic compounds and acts by eliminating the injurious stimuli and starting the healing process (Medzhitov, 2010). So it is a vital defense mechanism to health(Gonçalves et al., 2011). 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 (Zhou, et al., 2016). However acute inflammation may become severe and can contribute to chronic inflammatory diseases when it becomes uncontrollable (Chen et al., 2017).NSAIDs are the most common medications used for inflammation and related disorders worldwide. They are carboxylic acid containing drugs with salicylic derivatives (Rao et al., 2010). Even though they have high potential against inflammation their severe side effects like gastrointestinal (GI) ulceration, obstruction, perforation and bleeding has limited the therapeutic usage of NSAIDs (Wograkpanich et al., 2017). In this case, natural medicines are the best alternatives (Ghasemian et al., 2016). Researchers have now found many alternative medicines of plant origin which can cope up with the side effects of non-steroidal anti-inflammatory drugs (NSAIDs) against inflammation.

The various phytochemicals like alkaloids, flavonoids, terpenoids and saponins seems to contribute towards the anti-inflammatory activity of plants (Shaikh et al., 2016). Not only plants but also there are many fungi that 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 fungus(Deshmukh et al., 2012).

S. stipitatum is found by Berkeley in 1860 from the white ants nest in South India (Berkeley, 1860). From ancient time itself they were widely used for many medications by the

tribes. They used to preserve this rare fungus whenever they get it. Due to its restricted habitatthey have not been exploited for much studies. Only few studies have done in this species. It has got excellent medicinal use in Ayurveda and 'Parambharyavydyam'. An ethnobotanical study reveals that it is effective in curing a number of ailments, such as earache, arthritis, stomach pain, dehydration, jaundice and even stomach cancer (Balakrishnan and Anil, 2001). So here we are trying to explore theanti-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.Itis a hypogeal fungus usually associated with termite nest under the soil. "The mass consist of very irregular swollen and sometimes constricted more or less anastomosing and more or less densely compacted threads" says the Rev. M. J. Berkeley (Berkeley, 1860). The outer covering of *S. stipitatum* is black and the inner portion is white in color. On drying it becomes very hard and the inner portion is spongy and opaque in nature. (Anto et al., 2015) Its identity is confirmed at National Fungal Culture Collection of India (NFCCI) and the specimen is deposited at Ajrekar Mycological Herbarium (AMH) with accession number: AMH-10322. (Figure 1)

2.1.1 Preliminary qualitative analysis

The material were cut into small pieces and dried by keeping in a hot air oven for 48hours at 60℃. Then it is powdered and extraction is done by hot soxhlet method. The solvents are removed by distillation over water bath.Preliminary phytochemical screening of extracts were done using the standard phytochemical tests (Kokate et al., 1995). The extract with maximum number of compounds are chosen for further study.

GC-MS analysis was carried out on a Thermo GC-Trace Ultra Ver: 5.O, 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. Injection volume taken was 1 Micro liter.

2.2 Animal study

The ethanol extract was dissolved in water. The concentration of drug was 50 milligram/kilogram (mg/kg) body weight (b. wt) and 200mg/kg b.wt. 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. They were brought from Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India. They were maintained in polypropylene cages with normal standard rat feed and water ad libitum. Experiments were carried out with prior approval from Institutional Animal Ethical Committee(Approval No: ACRC/IAEC/18(2) P2) as per the CPCSEA guidelines.

2.2.1 Acute Carrageenan induced model

Acute anti-inflammatory activity was evaluated by the method of Winter et al. (1962). Animals were grouped into four with 6 animals each. First two groups were pretreated orally with drugs of higher dose 200mg/kg and lower dose 50mg/kg for 6 days. On the 6^{th} day diclofenac was also injected intraperitonially to the 3^{rd} group. 4^{th} group was kept as control. After 1 hour carrageenan was induced by subplantar injection of carrageenan 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 for every hour upto 6^{th} consecutive hour after carrageenan administration. The percentage inhibition of paw thickness was calculated by:

%inhibition of thickness = $[(tC_n\text{-}tC_0) – (tT_n\text{-}tT_0) / tC_n – tC_0] \times 100$

where, tC_n- paw thickness at particular time period of control animal

tC_0- paw thickness before induction of control animal

tT_n- paw thickness at particular time period of treated animal

tT_0- paw thickness before induction of treated animal

2.2.2 Chronic formalin induced model

As described in carrageenan induced model, the animals were grouped into four, with six animals each. Pretreated with drugs for 6 consecutive days. And after 6^{th} day of administration, 2% freshly prepared formalin was injected in the right hind paw. Then the paw thickness was calculated using vernier calipers before and after the formalin administration. The edema was measured everyday up to 7 days (Chau, 1989). 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 one way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

3 Results

3.1 Preliminary qualitative analysis

The petroleum ether extract of *S.stipitatum* shows positive result for phytosterol. The chloroform extract had shown positive results for phytosterols, glycosides and lactones. The acetone extract shows positive result towards tannins and lactones. The ethanol extract showed the presence of alkaloids, flavonoids, phenols, saponin and aleurone grains. The aqueous extract showed the presence of flavonoids, phenols and naphthoquinones. Since ethanol contains most number of compounds, it is chosen for the further studies.

3.1.2 GC-MS analysis of ethanol extract

The ethanol extract of *Sclerotium stipitatum* has the following major composition: 2-Propanone,1-(dimethylamino)-, 2-Pyrrolidinone, N-Trimethylsilyl-2-pyrrolidinone, Hexanediamide,N,N'-di-benzoyloxy-,2,5-Methylene-d,l-rhamnitol, 2,5-Methylene-d,l-rhamnitol, 2,5-Methylene-d,l-rhamnitol, 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester, 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 (Figure 2; 3).

3.2 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 as compared to control. The activity of standard reference drug Diclofenac at 10mg/kg b.wt showed 88.88%(Figure 4) (Table 1).

3.2.1 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 as compared to control. The activity of standard reference drug Diclofenac at 10mg/kg b.wt showed 98%(Figure 5) (Table 2).

4 Discussion

Secondary metabolites like alkaloids, flavonoids, tannins, saponins, coumarins etc. acts as the sources of anti-inflammatory agents (Mohammed et al., 2014). The GC-MS analysis shows the presence of some bioactive compounds present in the extract. 2-Pyrrolidinone have significant anti-oxidant and anti-cancer activity (Thangam et al., 2013). 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 (Moutevelis-Minakakis et al., 2011). Hexanediamide,N,N'-di-benzoyloxy- have potential anti-tumor activity (Prabhu et al., 2020). 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl)ester and1,2-Benzenedicarboxylic acid, diisooctyl ester have shown promising antimicrobial activity (Arora & Meena, 2018; Kalaivani et al., 2012). So the secondary metabolites as well as these compounds may have contributed to the anti-inflammatory activity of the extract.

Carrageenan induced inflammation is the most suitable procedure for the screening of acute anti-inflammatory agents (Greenwald, 1991). The current investigation on ethanolextract of *S. stipitatum*reveals its ability to remarkably reduce the paw edema in a dose dependent manner. In carrageenan the inhibitory effect of extract at high dose (200mg/kg) was 83.33%, low dose was 66% and that of standard diclofenac was 88.88% (Table 1). In all groups there is an initial increase in the paw thickness for first 2 hours after the induction of carrageenan. But then it starts to reduce and the drug treated groupsshow a drastic reduction when compared to the control. Reduction in the paw edema in control group is very less and it will not reduce beyond a certain limit. In the standard group it almost gets reduced to the normal level. And the drug treated ones also show a good result (Figure 4).

Formalin induced paw edema model acts as the best method for the screening of chronic anti-inflammatory agents which are closely related to human arthritis (Greenwald, 1991).In formalin induced model the inhibitory effect of extract at high dose (200mg/kg) was 94%, low dose was 80% and that of 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 3rd 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 dose and low dose gives a promising result almost comparable to the standard drug (Figure 5).

Inflammation is mediated by the activation of prostaglandins, Platelet Activating Factor (PAF), and other mediators of inflammation like TNF-α, interleukin, NO etc. (Hwang et al., 1986). And also, this attributes to the release of histamines, kinins, serotonins etc. (Larsen and Henson, 1983). Here the anti-inflammatory activity of ethanolextract in both models aremore or less comparable with that of the diclofenac, the established anti-inflammatory drug. And the

anti-edematous effect may be because of the inhibition of histamine release or inhibition of cyclooxygenase enzymes which are responsible for the formation of prostaglandins. Cycloxygenase inhibition proves to be more effective for the inhibition of carrageenan induced inflammation. Thus it might be down regulating the prostaglandin synthesis as well as the Coxygenase-2, which promotes the prostaglandin synthesis (Giuliano and Warner, 2002). Then the proliferative phase of inflammation is represented by the formalin induced paw edema (Ahmed and Ramabhimaiah, 2012). 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 thevarious biologically active compounds present in the ethanol extract of*S. stipitatum*.Thus, it supports the traditional usage of *S. stipitatum* andformulation of new drugs can be done.Moreover, fungi are an unexplored group when compared to the plants.So, this anti-inflammatory work is highly significant and reveals the importance of further researchin orderto investigate the magical properties of fungi.

NOTE:

The study highlights the efficacy of " Ayurveda and Parambharyavydyam" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

6 COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. 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. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Tables

Table 1: Depicts the result of phytochemical screening

Chemical constituents	Petroleum ether	Chloroform	Acetone	Ethanol	Distilled water
Phytosterol	+	+	-	-	-
Triterpenoids	-	-	-	-	-
Saponin	-	-	-	+	-
Alkaloids	-	-	-	+	-
Flavonoids	-	-	-	+	+
Lactones	-	+	+	-	-
Tannins	-	-	+	-	-
Sterols	-	-	-	-	-
Resins	-	-	-	-	-
Glycosides	-	+	-	-	-
Volatile oils	-	-	-	-	-
Phenol	-	-	-	+	+
Aleurone grains	-	-	-	+	-
Naphthoquinones	-	-	-	-	+

Table 2: Effect of *Sclerotium stipitatum* ethanol extract on carrageenan induced paw edema

	Initial paw thickness (cm)	**Final paw thickness (cm)**	**Percentage inhibition**
Control	0.19 ± .01	0.28± .01	-
Diclofenac (10mg/kg)	0.19 ± .015	0.20 ± .005**	88.88
SS Extract (200mg/kg)	0.19 ± .01	0.205± .01**	83.33
SS Extract (50mg/kg)	0.18 ± .012	0.21 ± .012**	66

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

Table 3: Effect of *Sclerotium stipitatum* ethanol extract on formalin induced paw edema

	Initial paw thickness (cm)	**Final paw thickness (cm)**	**Percentage inhibition**
Control	0.19 ± .01	0.29± .01	-
Diclofenac (10mg/kg)	0.19 ± .02	0.192± .02****	98
SS Extract (200mg/kg)	0.20 ± .01	0.196± .01**	94
SS Extract (50mg/kg)	0.19 ± .01	0.210± .01**	80

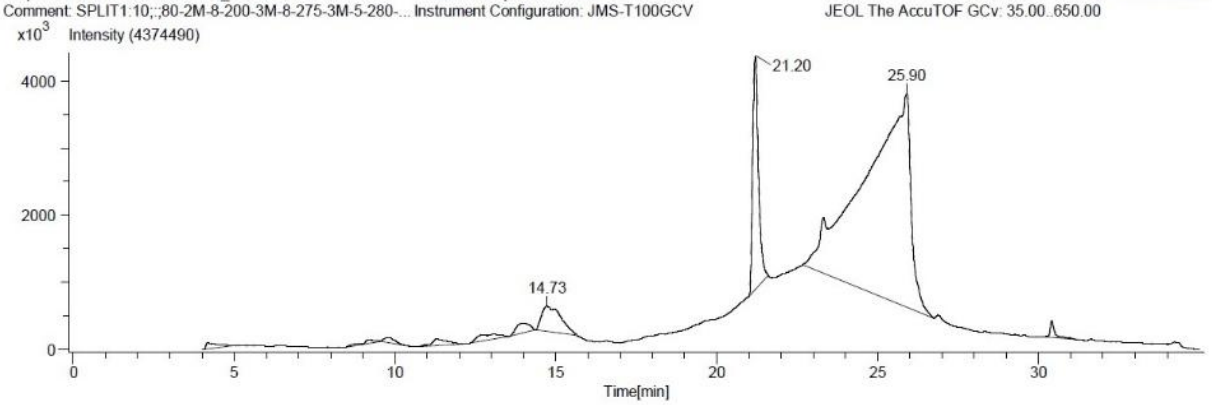
Values are expressed as Mean ± SD, n = 6, **** p< 0.0001, ** p< 0.01 compared to control considered as significant.

Figures

Figure 1: Depicts the fresh specimen of *S. stipitatum*

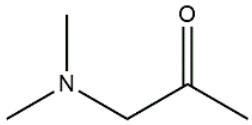
Figure 2: Depicts the result of GCMS analysis of ethanol extract of *S.stipitatum*

Ionizing Volt: 70[V]
Acq. Data Name: e152st thomas_ssee1 External Sample Id: SSee1 Experiment Date/Time: 12/23/2019 4:45:56 PM
Comment: SPLIT1:10;;;80-2M-8-200-3M-8-275-3M-5-280-... Instrument Configuration: JMS-T100GCV Ionization Mode: EI+
JEOL The AccuTOF GCv: 35.00..650.00

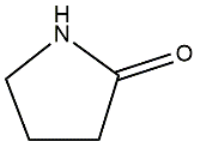


Peak Number	Time [min]	Type	Peak Width(FWH [min]	Area [Intens. * sec]	Height	Description	Start Point		End Point	
							Time[min]	Height	Time[min]	Height
1	4.16	BB	0.9141	2343435.76	93027.72		4.08	348	5.03	58452
2	9.18	BB	0.4188	1349041.27	53854.07		8.50	36202	9.52	118512
3	9.80	BB	0.3969	1779566.47	73279.37		9.54	120015	10.44	49870
4	11.30	BB	0.5489	3150143.03	97710.57		10.75	43413	12.13	82538
5	13.05	BB	1.0027	3868192.87	72597.01		12.34	93584	13.57	195494
6	13.97	BB	0.5241	4280565.76	149085.98		13.57	195494	14.35	289733
7	14.73	BB	0.6711	16019591.27	377000.41		14.37	288970	15.65	212437
8	21.20	BB	0.1915	43971058.94	3477945.37		21.00	789691	21.60	1103353
9	25.90	BB	1.8572	311300866.46	3178619.18		22.67	1264878	26.77	461983
10	30.41	BB	0.1109	2690236.09	259402.16		30.10	188196	31.36	140512

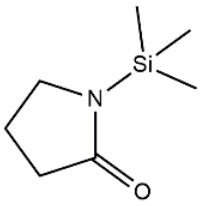
Figure 3: Depicts the structure of major compounds present in the ethanol extract of *S.stipitatum*



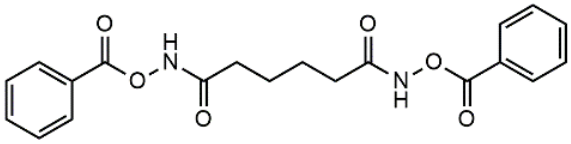
2-Propanone,1-(dimethylamino)-



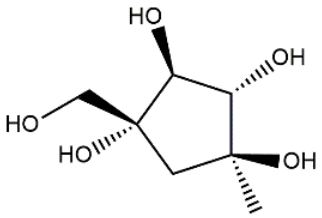
2-Pyrrolidinone



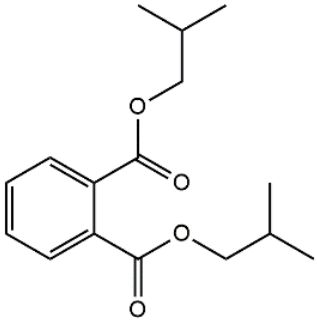
N-Trimethylsilyl-2-pyrrolidinone



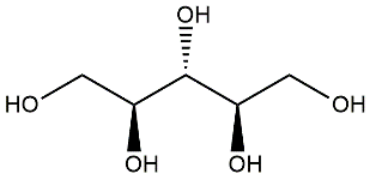
Hexanediamide,N,N'-di-benzoyloxy-



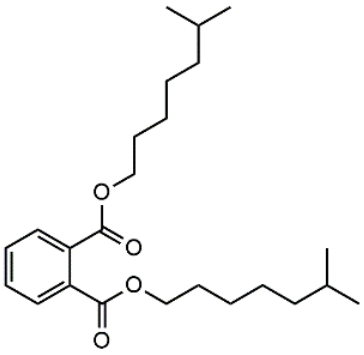
2,5-Methylene-dl-rhamnitol



1,2-Benzenedicarboxylic acid, bis(2-methylpropyl)ester



Ribitol



1,2-Benzenedicarboxylic acid, diisooctyl ester

Figure 4: Depicts the change in paw thickness at each hour in carrageenan induced model

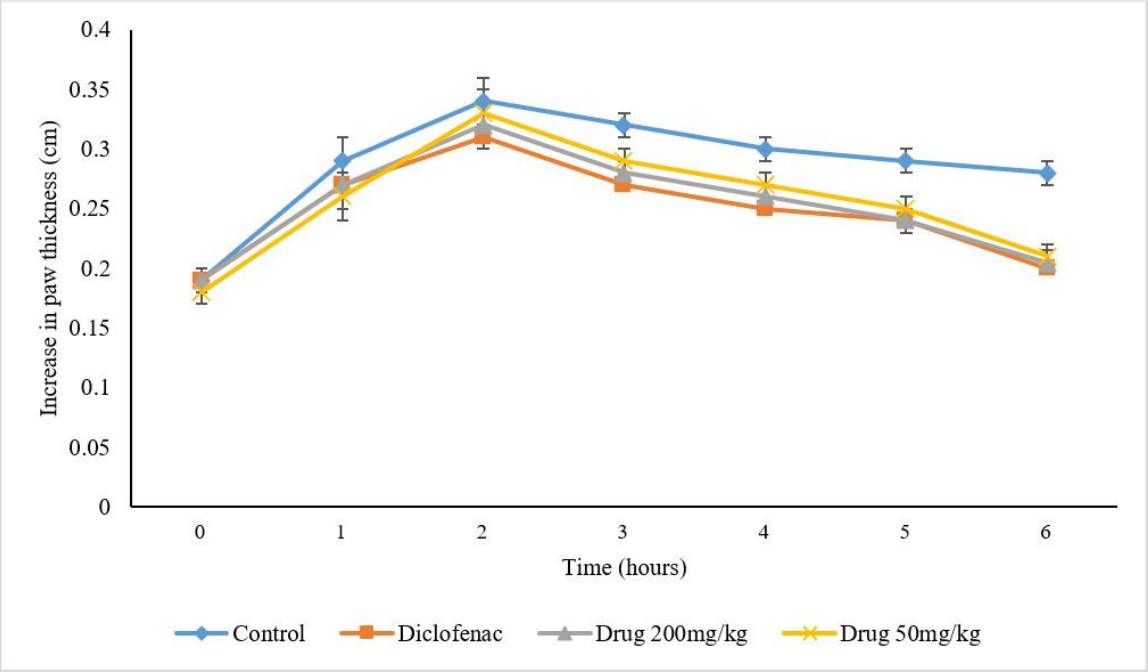


Figure 5: Depicts the change in paw thickness at each day in formalin induced model