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PHARMACOGNOSTICAL EVALUATION AND CHEMICAL SCREENING OF *MICROCOSMUS EXASPERATUS*

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ABSTRACT

Marine organisms are a rich source of structurally novel and biologically active metabolites. Ascidians are marine sedentary animals. *Microcosmus exasperatus* is a simple ascidian belonging to the family Pyuridae. It is found in plenty throughout the year in the Thoothukudi harbour area. Microscopic examination of the test, mantle, branchial tentacles, dorsal tubercle, dorsal lamina, branchial folds, stigmata, liver lobes and gonads have been carried out to confirm identification of the species. Physico chemical parameters and preliminary chemical screening of petroleum ether, benzene, methylene chloride, chloroform, ethanol, methanol, and water extracts were performed. A characteristic yellow fluorescence was noticed in petroleum ether (40 ^{0}-60 ^{0}C), green in benzene, dark green in methylene chloride, yellowish green in chloroform, greenish yellow in ethanol, methanol and aqueous extracts under UV light (365 nm). Percentage of total ash was 47.91 with acid insoluble ash 4.19 and water soluble ash 2.23. The residue on ignition was nil. The percentage of extractive value was minimum in petroleum ether (1.05), maximum in ethanol (13.95), methanol (12.12) and water (11.94). Alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, quinones, anthraquinones, proteins, carbohydrates and lipids were observed in this species. TLC analysis showed the presence of single spot in petroleum ether, benzene; two in methylene chloride, chloroform, ethanol and three in methanol extracts. An analysis of these parameters in *Microcosmus exasperatus* is the first attempt of standardisation of marine drug powder.

Keywords: Ascidian, *Microcosmus exasperatus*, Pharmacognosy, Chemical screening.

INTRODUCTION

The marine environment is an exceptional reservoir of bioactive compounds, which exhibit structural features not found in terrestrial natural products. The numbers of pharmacologically potent substances isolated from marine organisms are increasing rapidly and have resulted in the isolation of more or less 10,000 metabolites [1]. A large proportion of these have been extracted from marine animals, especially sponges, bryozoans, molluscs, ascidians and some of them are currently in clinical trials [2]. Ascidians or sea squirts are marine sedentary animals with cosmopolitan distribution. They are ciliary filter feeders found occurring from the littoral zone to the deep sea. It has been widely demonstrated that the ascidians are rich in bioactive substances [3-8]. A chemical screening strategy can benefit from the use of standards or target-molecules which can be rapidly characterized by diagnostic parameters. Pharmacognostical evaluation, qualitative and quantitative estimation of the chemical constituents will be very much useful in the standardisation of drugs. A review of literature shows that studies on the GC- MS analysis, vitamins by HPLC, phenolic compounds, flavonoids by HPTLC, antidiabetic, antimicrobial, hepatoprotective, CNS depressant, antitumor and immunomodulatory activity [9-16] of *Microcosmus exasperatus* are available. In the present study, an attempt has been made to assess the macroscopic, microscopic characters, physicochemical parameters and to evaluate the chemical compounds.

MATERIALS AND METHODS

Animal material

Samples of *Microcosmus exasperatus* were collected from Thoothukudi harbour area and identified using key to identification of Indian ascidians [17]. The colour and external appearance of the animals were noted. A voucher specimen AS 2240 has been deposited in the museum, Department of Zoology, A.P.C. Mahalaxmi College for Women, Thoothukudi - 628002.

Preparation of the animal powder

Epibionts adhering to the surface of the test of *Microcosmus exasperatus* were carefully removed. The specimen was washed several times with sterile sea water.

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It was dried under shade, homogenized to get a coarse powder which was stored in an air-tight container and used for all further investigations.

Preparation of extract

100 gm powder was extracted with different solvents such as petroleum ether, benzene, methylene chloride, chloroform, ethanol, methanol and water using soxhlet apparatus, cooled to room temperature and evaporated in a rotary evaporator to get a residue.

Macro and microscopical characterization

Macroscopical studies were done by naked eye, internal and external characters observed using binocular microscope. Colour photographs were taken by canon photomicrography unit.

Fluorescence analysis

The animal powder and their extracts in various solvents were examined under ordinary light and UV light (365 nm). The powder was also treated with 1N NaOH (aqueous), 1N NaOH (ethanolic), 1N HCl, 1:1 H_2SO_4 and 1:1 HNO_3 and changes in colour were recorded [18].

Physicochemical parameters

The percentage of loss of weight on drying, total ash, acid-insoluble ash, water soluble ash and residue on ignition were obtained by employing standard method of analysis [19].

Preliminary chemical screening

The presence of alkaloids, terpenoids, steroids, coumarins, tannins, saponins, flavonoids, quinones, anthraquinones, phenols, aromatic acids, catechins, proteins, amino acids, carbohydrate, glycosides, starch, free sugar and lipids were tested by standard procedures [20-23].

Chromatographic studies

Thin layer chromatography

Thin layer chromatographic studies have been performed for petroleum ether (40 - 60 °C), benzene, methylene chloride, chloroform, ethanol, methanol and water extracts using Silica gel plates. The Silica gel-G for TLC was poured as thin layers on glass plates by preparing semi-solid slurry with distilled water. The plates were dried until they are free from moisture and activated in an air-oven at about 110 °C for about 3 hours. The solvent systems selected were 100% chloroform and chloroform: ethanol (8.5:1.5). Different solvent systems were found to be effective to get the maximum number of spots for the various extracts. The developed TLC plates have been first viewed through ultraviolet fluorescence viewing cabinet (365 nm) before keeping in an Iodine chamber and the R_f values of the fluorescing spots and the spots appeared after keeping in Iodine chamber were measured.

RESULTS AND DISCUSSION

Macro and microscopic characters (Plate 1)

Individuals are oval measuring about 1.5 cm in height, Test is hard and free from sand and other particles. Surface of the test is much corrugated. The branchial and atrial siphons are clearly visible directed opposite to each other. Fresh specimen is orange red in colour. Thick musculature on the mantle is arranged regularly to form neat cross stripes on the dorsal half**.** The branchial tentacles are branched with small primary, secondary and tertiary out growth.

Dorsal tubercle is well developed. The opening of the dorsal tubercle is U shaped with two horns curved inside. Branchial sac is with ten folds, inner longitudinal vessels arranged as follows; D3(12)3(11)3(11)2(10)3(11)3 (10)2(7)2(9)2(7)0E. There are 5-6 stigmata in each mesh. Gut loop is parallel to each other, dark brownish in colour with three or four liver lobes. The compact liver lobes are formed by groups of parallel, shallow elongate pouches which project out from the gut wall in the pyloric region. Anus is without lobes. Gonads are present inside as well as outside the gut loop.

The specimen of *Microcosmus exasperatus* collected from the undersurface of barges of Thoothukudi harbour area by SCUBA diving was analyzed macroscopically and microscopically. The characters studied revealed that they belong to the family Pyuridae and in all respects the samples were identical to those that have already been reported from India [24].

Fluorescence Characters

Many drugs give fluorescence when the cut surface or the powder is exposed to UV radiation. The dry powder of *Microcosmus exasperatus* was treated with various reagents and the change in colour was recorded (Table 1). The powder as such and with aqueous, ethanolic alkali showed greenish yellow fluorescence; with acids dark yellow, lemon yellow and green under UV light. A characteristic yellow fluorescence was noticed in petroleum ether (40 ^{0}-60 ^{0}C), green in benzene, dark green in methylene chloride, yellowish green in chloroform and greenish yellow in ethanol, methanol and aqueous extracts under UV light (365 nm). This characteristic fluorescence can be used as a diagnostic tool for the correct identification of *Microcosmus exasperatus* and to test adulteration (if any) in the species.

Physico-chemical characters

The determination of ash value is useful for detecting low grade products, exhausted drugs and excess of sandy or earthy matter. The percentage of total ash, acid insoluble ash, water soluble ash, residue on ignition and extractive values are presented in Table 2. There was 89.27% loss of weight on drying. Percentage of total ash was 47.91 with acid insoluble ash 4.19 and water soluble ash 2.23. The residue on ignition was nil. The percentage of extractive value was minimum in petroleum ether (1.05), maximum in ethanol (13.95), methanol (12.12) and water (11.94).

Physico-chemical characters such as ash, extractive values are usually employed to detect the presence of adulterants in herbal medicinal preparation. An

analysis of these parameters in *Microcosmus exasperatus* is the first attempt of standardisation of marine drug powder.

Preliminary chemical screening

Presence of alkaloids, terpenoids, steroids, coumarins, tannins, saponins, flavonoids, quinones, anthraquinones, phenols, aromatic acids, catechins, proteins, amino acids, carbohydrate, glycosides, starch, free sugar and lipids were tested qualitatively using the crude extracts of petroleum ether (40 - 60 °C), benzene, methylene chloride, chloroform, ethanol, methanol and water. The results are presented in Table 3. Alkaloids, terpenoids, steroids, quinones, phenols, proteins, carbohydrate, glycosides and lipids have been detected in all the extracts whereas coumarins and catechins were not observed in any of the extracts. Tannins were present in methylene chloride, ethanol, methanol and saponins were found in all extracts except ethanol and water. Flavonoids were observed in methylene chloride, chloroform and methanol. Anthraquinones were present in petroleum ether, chloroform, ethanol and water while aromatic acids where detected in methylene chloride only. Amino acids were found in all extracts except petroleum ether and benzene whereas starch was detected in petroleum ether, benzene and chloroform extract. Free sugars were present in petroleum ether, methylene chloride, chloroform and water.Marine derived alkaloids show powerful anti-proliferative, antimicrobial, anti HIV and cytotoxic activity [25]. They also function as strong anesthetics, pain relievers, narcotics etc. More than 200 aromatic alkaloids isolated from marine tunicates are grouped into structural types and discussed in terms of their reported pharmacological activity [26]. Alkaloids are reported to have cardio vascular effects [27]. In plants, terpenoids provide chemical defense against environmental stress and repair mechanism for wounds and injuries [28]. Total sterols were isolated from *Halocynthia aurantium* by column chromatography [29]. Dietary intake of plant sterols help to reduce the blood cholesterol levels [30]. Tannins are natural phenolic compounds reported to have very good anti-diarrhoeal, anti-septic antiviral, antibacterial, antiparasitic effects, anti-inflammatory, antiulcer and antioxidant property for possible therapeutic applications [31-33]. They are also employed as antidotes in poisoning [34].

Saponins have antimicrobial, anti-inflammatory, anti-feedent, and hemolytic effects [35-36]. A member of the class of cyclic organic compounds, quinones occur as pigment in bacteria, fungi and certain higher plants. Animals containing quinones obtain them from plants they eat. They are used in manufacturing dyes and fungicides [37]. Flavonoids, the major group of phenolic compounds are known for their antimicrobial, antiviral, spasmolytic activity, reduce the risk of various cancers and prevent menopausal symptoms [38]. Phenolic compounds, saponins and flavonoids may be linked or suggested to be involved with antibacterial, antiviral and antidiarrhoeal activity in plants [39]. Phenolic phytochemicals have antioxidant, antidiabetic, anticarcinogenic, antimicrobial, antiallergic, antimutagenic and anti-inflammatory properties [40,41]. Anthraquinones are the largest group of naturally occurring quinones. Alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, quinones and anthraquinones observed in the different extract gives proof for the occurrence of bioactive principles with pharmacological applications. The presence of proteins, carbohydrates and lipids in *Microcosmus exasperatus* indicates their nutritive value [42,43].

Chromatographic studies

Thin layer chromatography

Crude extracts of petroleum ether (40 - 60 °C), benzene, methylene chloride, chloroform, ethanol and methanol of *Microcosmus exasperatus* have been subjected to thin layer chromatographic study. The results are presented in Table 4. TLC analysis showed the presence of single spot in petroleum ether, benzene under ultraviolet fluorescence and Iodine chamber with R_f values of 0.67, 0.91 respectively. Methylene chloride, chloroform, ethanol extracts exhibited two spots with R_f values of 0.96, 0.15; 0.17, 0.91; 0.09, 0.86 and methanol extract indicated three spots with R_f values of 0.96, 0.79 and 0.18.

TLC studies of different extracts and fractions help to confirm the presence of phytoconstituents [44-46]. This in addition to giving knowledge about adulteration informs us the number of components available in the different extracts. In the present study, three spots have been detected in the methanol extract indicating the presence of three compounds.

Table 1. Fluorescence characters of *Microcosmus exasperatus* and their extracts in various solvents

S. No	Treatments	Under ordinary light	Under UV light (365 nm)
1	Powder as such	Grey	Greenish Yellow
2	Powder +1N NaoH (aqueous)	Brownish black	Greenish Yellow
3	Powder +1N NaoH (ethanolic)	Yellow	Greenish Yellow
4	Powder +1 N HCL	Yellow	Dark Yellow
5	Powder +1: 1 H_2SO_4	Orange	Lemon Yellow
6	Powder +1:1 HNO_3	Orange	Green
	Extracts:		
	a. Petroleum ether	White	Yellow
	b. Benzene	Yellow	Green
	c. Methylene chloride	Yellow	Dark Green
	d. Chloroform	Yellowish brown	Yellowish Green
	e. Ethanol	Yellow	Greenish Yellow
	f. Methanol	Yellow	Greenish Yellow
	g. Water	Brown	Greenish Yellow

Table 2. Physico - Chemical characters of *Microcosmus exasperatus*

S. No	Particulars	Percentage
1	Loss of Weight on drying	89.27
2	Total ash	47.91
3	Acid insoluble ash	4.19
4	Water soluble ash	2.23
5	Residue on ignition	NIL
	Extracts:	
	a. Petroleum ether	1.05
	b. Benzene	1.28
	c. Methylene chloride	1.50
	d. Chloroform	5.92
	e. Ethanol	13.95
	f. Methanol	12.12
	g. Water	11.94

Table 3. Chemical screening of *Microcosmus exasperatus*

S. No.	Test	Petroleum Ether	Benzene	Methylene Chloride	Chloroform	Ethanol	Methanol	Water
1	Alkaloids	+	+	+	+	+	+	+
2	Terpenoids	+	+	+	+	+	+	+
3	Steroids	+	+	+	+	+	+	+
4	Coumarins	-	-	-	-	-	-	-
5	Tannins	-	-	+	-	+	+	-
6	Saponins	+	+	+	+	-	+	-
7	Flavonoids	-	-	+	+	-	+	-
8	Quinones	+	+	+	+	+	+	+
9	Anthraquinones	+	-	-	+	+	-	+
10	Phenols	+	+	+	+	+	+	+
11	Aromatic acids	-	-	+	-	-	-	-
12	Catechins	-	-	-	-	-	-	-
13	Proteins: Biuret	+	+	+	+	+	+	+
14	Aminoacid: Ninhydrin	-	-	+	+	+	+	+
15	Carbohydrate: Molisch	+	+	+	+	+	+	+
16	Glycosides: Anthrone	+	+	+	+	+	+	+
17	Starch: Iodine	+	+	-	+	-	-	-
18	Free sugar: Benedict	+	-	+	+	-	-	+
19	Lipids: Bragdon	+	+	+	+	+	+	+

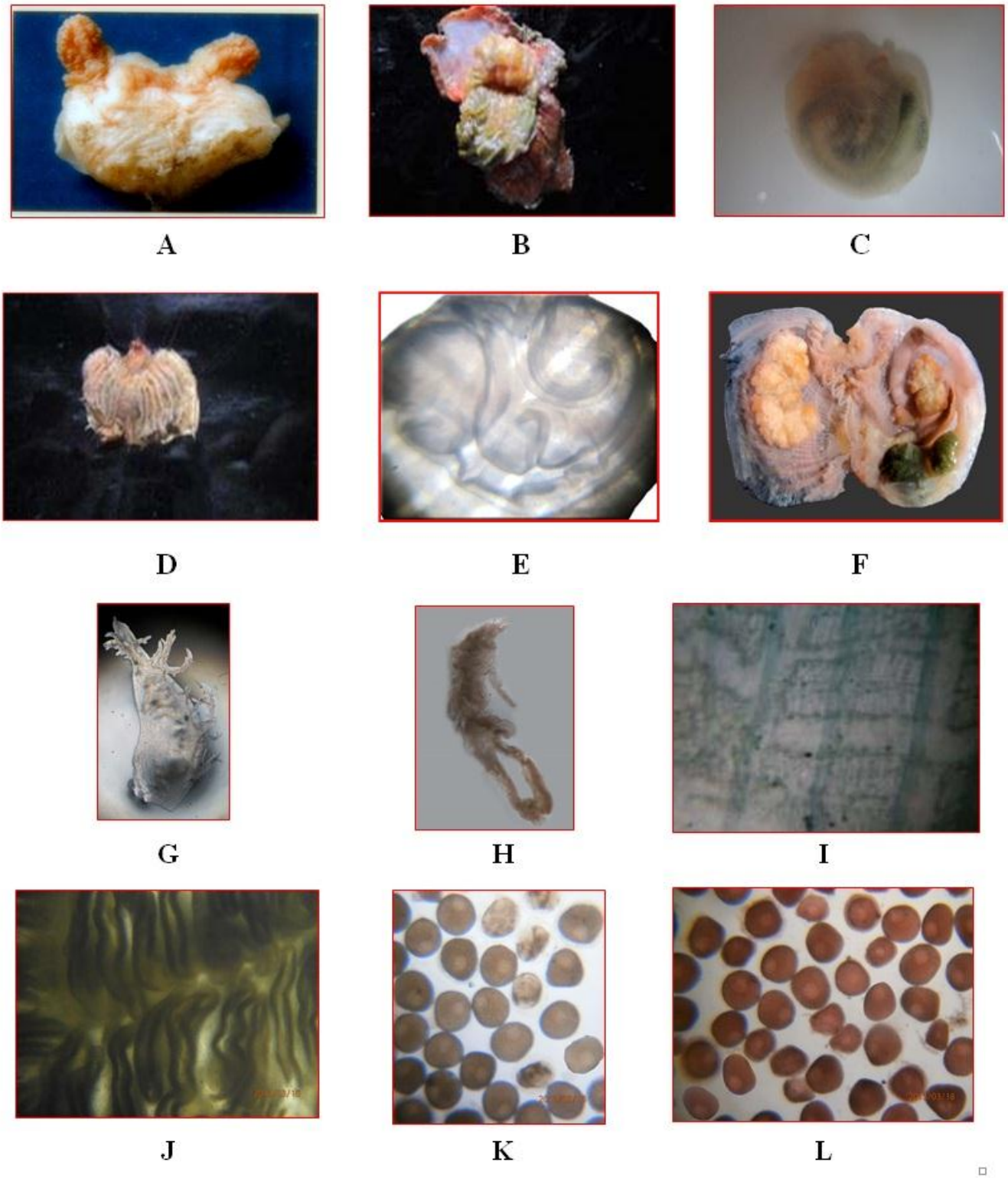
+Present; -Absent

Table 4. R_f Values of the spots of the various extracts of *Microcosmus exasperatus*

S. No	Extracts	Solvent system used	Rf values of the spots	
			Under UV light (365 nm)	In Iodine Chamber
1.	Petroleum ether	100% Chloroform	0.67 (Yellowish - Brown)	0.67♦
2.	Benzene	Chloroform: Ethanol (9.5:0.5)	0.91 (Yellowish - Brown)	0.91♦
3.	Methylene chloride	Chloroform: Ethanol (8.5:1.5)	(Yellowish - Brown)	0.96♦, 0.15○
4.	Chloroform	Chloroform: Ethanol (8.5:1.5)	0.91 (Yellowish - Brown)	0.17○, 0.91♦
5.	Ethanol	Chloroform: Ethanol (8.5:1.5)	(Yellowish - Brown)	0.09○, 0.86♦
6.	Methanol	Chloroform: Ethanol (8.5:1.5)	(Yellowish - Brown)	0.96♦, 0.79□, 0.18○

♦ - thick spot; □ - moderate spot; ○ - mild spot

Plate 1. *Microcosmus exasperatus* – External and Internal characters



A. *Microcosmus exasperatus* external appearance, **B**. open branchial sac, **C**. mantle musculature and gut loop, **D**. branchial sac with folds, **E**. opening of the dorsal tubercle, **F**. gut loop with gonads, **G**. branched tentacle, **H**. C.S of endostyle, **I**. gill slits,**J**. section of liver **K**. fertilized eggs showing different stages of development, **L**. male follicles.

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