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Phytochemical Characterization of Topoisomerase-II Inhibitor from Freshwater Algae *Chlorosarcinopsis eremi (Chantanachat, S. and Bold, H.C)*

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Abstract
The freshwater algae C*hlorosarcinopsis eremi,* belong to the family Chlorophyceae. The methanol extract of the algae of the present study were assayed for inhibition of relaxation activity of topoisomerase II. The methanol extract was subjected to thin layer chromatography and column chromatography to separate the phytochemical constituent present in the methanol extract. The obtained fractions were again assayed for inhibition of relaxation activity of topoisomerase II. The inhibition of relaxation activity of topoisomerase II enzyme was found maximum in Fraction II compared to other fractions of methanol extract. From the Gas Chromatography - Mass Spectrometery analyses of methanol fraction II, the present study concluded 2-Allyl-t-Butylhydroquinone may be the active metabolite responsible for Topoisomerase II enzyme inhibition.

Keywords
Chlorosarcinopsis eremi; Topoisomerase II inhibition; Thin layer chromatography; Column chromatography; GCMS; 2-Allyl-t-Butylhydroquinone.

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INTRODUCTION
Chlorosarcinopsis eremi belongs to the order Chlorococcales of the family Chlorophyceae. *Chlorosarcinopsis eremi* is coccoid, which occur in freshwater habitats. The species shows antimicrobial activity against *Escherichia coli*, *Klebsiella* sp, *Pseudomonas* sp, *Salmonella* sp, *Staphylococcus aureus*, *Proteus* sp, Methicilin resistant *Staphylococcus aureus*, *Serriata* sp and *Bacillus* sp. It was also found to contain phytochemicals including Alkaloids, Anthraquinones, Cardiac glycosides, Flavonoids, Reducing sugars, Saponins and Terpenoids [1]. With this earlier report the present study is focused on further exploration of the phytochemical constituent of the algae as topoisomerase II inhibitor. In the present study the compounds present in method extract of the algae were separated using thin layer chromatography and column chromatography. The compounds of the fraction were also quantitatively and qualitatively analysed using Gas chromatography and Mass spectroscopy. The fractions were further assayed to

analyse the inhibition of Topoisomerase II enzyme using Relaxation assay.

1. MATERIALS AND METHODS

1.1 Extraction of algal sample

The algal sample of the present study *Chlorosarcinopsis eremi* were collected and dried under shade. The shade dried algal powders were dissolved in methanol solvent and extracted using pestle and mortor. The extract was placed under room temperature for evaporation of excess solvents. The residue of the extract was taken for further analysis [2].

1.2 Thin layer chromatography (TLC)

Standardization of solvent system for the maximum separation of active constituents of methanol extract was identified by performing thin layer chromatography. The mobile phase was standardized using combination of solvents in different ratio (chloroform, methanol and water). Analytical TLC plates were prepared by pouring the absorbent silica gel G slurry on the glass plates. The thickness of the absorbent layer was 150µm. Prepared chromo plates were dried for 30 minutes in air and then in oven at 110°C for 30 minutes. The origin line was marked at 2cm from the bottom of the plate. A volume of 5µl of sample was applied at the Centre of the origin line using micropipette. The chromatogram was developed by the ascending technique by immersing the plate in the standardized mobile phase (chloroform: methanol: water) to a depth of 0.5cm. Development is allowed to proceed until the solvent front has travelled the required distance. The plate was then removed and placed under iodine chamber and the solvent front immediately marked with a pointed object. The qualitative evaluation of the chromatogram was done by determining the migration behavior of the separated substance in the form of R_f value (relative front or retardation factor).

R_f =Distance travelled by the compound **/** Distance travelled by the solvent.

1.3 Column chromatography (CC)

The standardized mobile phase (chloroform: methanol: water) was used in Column chromatography for the separation of individual compounds from methanol extract. The classical preparative chromatography column was used. The column prepared using wet method. Chloroform was used as stationary phase. The methanol extract was loaded on top of the stationary phase. The Eluent is slowly passed through the column to advance the separation of the organic material. The individual components were retained by the stationary phase differently and separated from each other while they were running at different speeds through the column with the eluent. The eluent was collected in a series of fractions. Separated fractions were taken for the further analyses.

1.4 Gas chromatography-MS analysis (GC-MS)

The compounds present in the fraction were quantitatively and qualitatively analysed using Trace GC Ultra and DSQII model MS from Thermo Scientific Co. The compounds were identified based on comparison of their retention indices with those of NIST4 and WILEY9. Only those compounds with spectral fit values equal to or greater than 700 were considered positive identification.

1.5 Extraction of Topoisomerase II enzyme

Crude Topo II enzyme extracts were prepared from MCF-7 cell line by a modified method of Deffie *et al*. [3]. Exponentially growing human Breast cancer cell line, MCF-7 was collected by centrifugation and washed in ice cold phosphate buffered saline (PBS). The washed cells were resuspended in PBS, mixed well and centrifuged at 1800 rpm for 10 min. The cell pellet was resuspended in lysis buffer (Lysis buffer consist of 10Mm Tris HCl (pH 7.5), 1mM $MgCl_2$, 1mM EGTA, 0.5% (v/v) CHAPS, 10% (v/v) glycerol, 5mM β-mercaptoethanol, 1mM PMSF) and incubated in ice for 25 min with intermittent vortexing. Cells were sonicated for 1 min. DNA and nuclear debris was pelleted by centrifugation at 4°C, 1800 rpm for 20 min and the supernatant were collected. Protein concentration in the extract was determined by Folin-Ciocaltaeu method [4] using BSA as standard.

1.6 Topoisomerase II inhibition - Relaxation assay

The separated fractions were assayed for the Inhibition of Relaxation activity of DNA topoisomerases on supercoiled plasmid pBR 322 DNA (Fermentas, USA) [5]. The reaction was performed in 20µl reaction mixtures containing 50mM Tris HCL buffer (pH 8.0), 100mM KCl, 10mM $MgCl_2$, 0.5mM dithiothreitol, 0.5mM EDTA, 1mM ATP, 30µg/ml BSA, pBR 322 plasmid DNA(125ng), 4µg of topoisomerase II enzyme (or 1U of enzyme) and the inhibitors (fractions I –V). The reaction mixtures were incubated at 37°C for 30 min and terminated by adding 3µL of loading buffer consisting of 40% sucrose, 0.25% bromophenol blue, 0.1mM EDTA. The mixtures were subjected to 1% agarose gel electrophoresis in TBE (Tris- Borate- EDTA) running buffer. The agarose gels were stained with ethidium bromide and DNA was visualized on UV trans illuminator. Gel statin, Gel doc LamIlage ID L320 (Medicare Scientific, India) was used for densitometric quantitative analysis of the supercoiled DNA.

2. RESULTS AND DISCUSSION

2.1 Thin layer chromatography

Colorless components were detected by using visualizing agent, iodine vapors. The chromatogram of methanol extract gave the maximum separation in eight spots. The refractive-index of the spots was calculated to be 0.3, 0.49, 0.6, 0.7, 0.82, 0.86 and 0.9cm (Fig 1).

Fig 1 Thin layer chromatogram

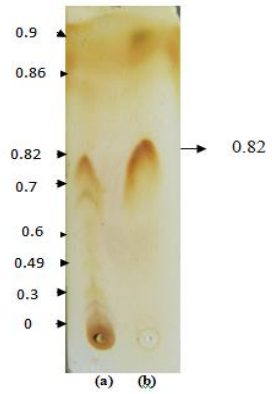


Fig (a) Chromatogram of Methanol extract (b) Chromatogram of methanol fraction II.

2.2 Column chromatography

The classical preparative chromatography column with a diameter of 5mm and a height of 1m with a tap at the bottom is used for elution. Different fractions were formed. The fractions were further analyzed for their respective compounds using thin layer chromatography. The respective spots were visualized under iodine chamber. Fraction I was observed to show a single spot, fraction II showed single spot, fraction III showed 5 spots, fraction IV showed three spots, fractions V showed eight spots and fraction VI showed two spots. Fraction V and VI were clapped as fraction V for convenience (Table 1) These methanol fractions were further subjected to Topoisomerase II inhibition assay.

Fig 2 GC-MS of fraction II of methanol extract of *Chlorosarcinopsis eremi*

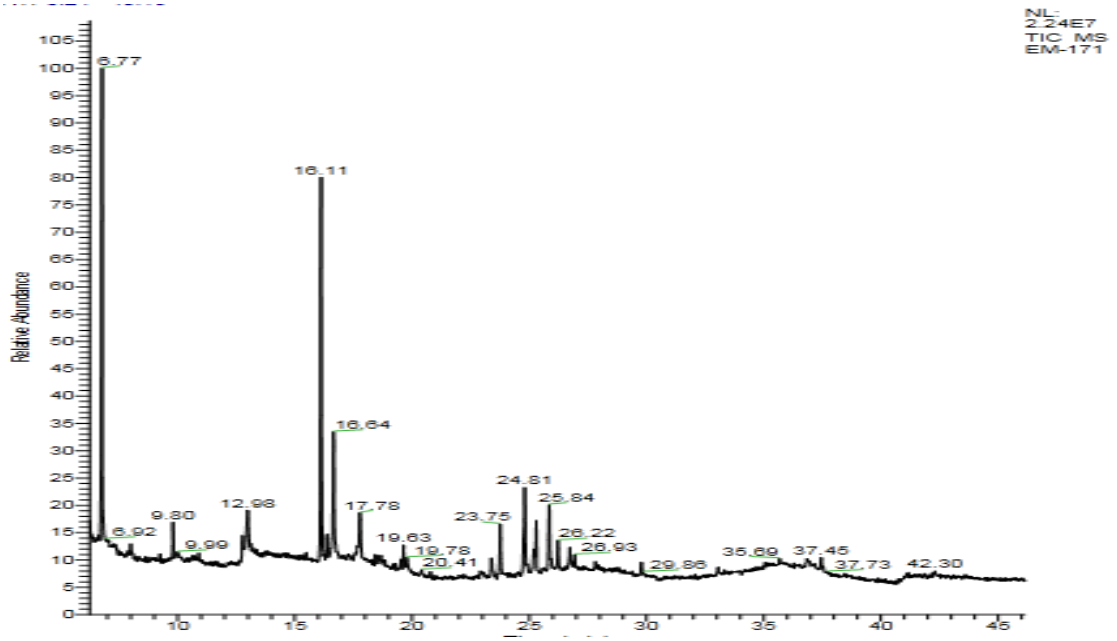


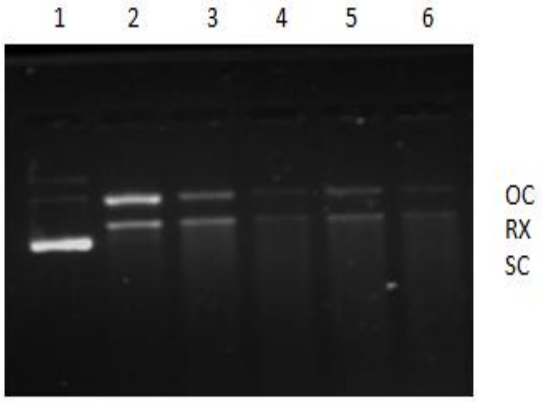
Table 1 Column chromatography of methanol extract of *Chlorosarcinopsis eremi*

Fraction	Eluting agent Chloroform: ethanol: water (v:v)	Elution volume	Order of fractions	No. of bands observed in TLC
I	100:0	25ml	1	1
II	95:5	25ml	2	1
III	90:10	25ml	3	5
IV	85:15	25ml	4	3
V	80:20	25ml	5	8
VI	80:20:7	25ml	6	2

2.3 Extraction of Topoisomerase II enzyme

The concentration of protein obtained from human breast cancer cell line (MCF-7) was 10µg /µl. The Complete relaxation of 125ng of pBR322 was achieved by the enzyme at 4µg concentration [Fig 3]. One unit of the enzyme was calculated as the amount of enzyme capable of relaxing 0.125µg DNA in 30 min at 37°C.

Fig 3 Relaxation of supercoiled pBR322 by topoisomerase II enzyme

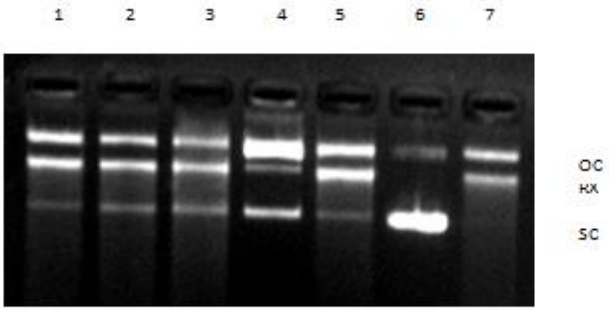


DNA topoisomerase II activity assay was performed by incubating pBR322 DNA (125 ng) with topoisomerase II enzyme extracted from MCF-7 cell lines. Lane 1. pBR322 DNA as control: Lane 2-6. pBR322 DNA with 5-1µg of enzyme extract. OC. Open circular DNA; RX. Relaxed; SC. Supercoiled.

2.4 Topoisomerase II inhibition – relaxation assay

The methanol fractions (I-V) were tested for the inhibition of relaxation of supercoiled DNA by inhibiting topoisomerase II enzyme activity. Among the fractions assayed, methanol fraction II showed significant inhibition on the relaxation of supercoiled DNA (Fig 4) The present work is more or less comparable with the few earlier reports. Triterpenoid fomitellic acids (FAs) extracted from *Fomitella fraxinea* (Basidiomycetes) showed inhibition of DNA topoisomerase II enzyme [6]. He also showed the inhibition of Topoisomerase II enzyme by Ursolic acid (UA) extracted from *Tabebuia caraiba* and *Campsis radicans*. Two coumarins, (-)-Epicatechin and 9S, 12S, 13S-Trihydroxy-10E-octadecenoic acid extracted from the bark of *Tilia amurensis* showing inhibitory activity against Topoisomerase II enzyme [7].

Fig 4 Effect of methanol fractions on relaxation of supercoiled pBR323 plasmid DNA in inhibiting topoisomerase II enzyme



The influence of methanol fractions on relaxation of plasmid DNA in inhibiting topoisomerase enzyme. Lane 6, pBR322 DNA as control; Lane 7, pBR322 DNA with 1U of topoisomerase; Lane 1-5, 10µg of fraction V-I of methanol extract (inhibitors). OC, Open circular DNA; RX, Relaxed; SC, Supercoiled.

Table 2 GC-MS of methanol fraction II of *Chlorosarcinopsis eremi*

S.No	SI	RSI	Compound Name	Probability	Molecular Formula	Molecular Weight	Area %
1	925	929	2-Allyl-5-t-butylhydroquinone	7.12	C13H18O2	206	6.73
2	822	902	4-O-Methylconhypo-protocetraric acid	24.11	C19H18O8	374	1.51
3	838	951	3-[4'-(t-Butyl)phenyl]furan-2,5-dione	40.98	C14H14O3	230	1.13
4	893	906	9-(Methylthio)-8H-acenaphtho[1,2-c]pyrrole-7-carboxylic Acid	29.37	C16H11NO2S	281	0.57

2.5 GC- MS

The GC –MS analysis of methanol fraction II of *C. eremi* exhibited the presence of compounds at four different peaks. Among the peaks 2-Allyl-5-t-butylhydroquinone showed a highest peak at 16.11 retention time with 6.73% of area (Table 2) (Fig 2).

CONCLUSION

The present study of phytochemical characterization of methanol extract of fresh water algae *Chlorosarcinopsis eremi* revealed the presence of phenolic compound 2-Allyl-t-Butylhydroquinone. From the topoisomerase ii enzyme inhibition assay result the present study also concluded that 2-Allyl-t-Butylhydroquinone may be the active metabolite responsible for the inhibition of topoisomerase ii enzyme. In future the compound 2-Allyl-t-Butylhydroquinone will be analysed for the inhibition of Topoisomerase II enzyme at different stages of its catalytic cycle to understand the mechanism of action.

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