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Full Length Research Paper

A novel filarial topoisomerase II inhibitor produced by native isolate *Micrococcus luteus* B1252

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A novel inhibitor of filarial topoisomerase II was isolated from the culture filtrate of native isolate *Micrococcus luteus B1252*. The methanolic extract of the cell pellet was partially purified by silica column chromatography and preparative high performance liquid chromatography (HPLC). The active fraction obtained from HPLC was subjected to gas chromatography-mass spectroscopy (GC-MS) analysis which revealed the presence of 11 compounds. The fatty acid ester derivatives (Z) 15-tetracosenoic acid, methyl ester present in the partially purified extract of *M. luteus B1252* is suggested to be the active principle which inhibits topo II.

Key words: Filariasis, topoisomerase II, *Micrococcus luteus B1252,* fatty acids, (Z) 15-tetracosenoic acid, methyl ester.

INTRODUCTION

Lymphatic filariasis is a major public health problem which affects 120 million people throughout the tropics (Mariappan, 2007). The disease was therefore targeted by the World Health Organization (WHO) for elimination by the year 2020 (Bebehani, 1998; Ottesen, 2000; Oqueka et al., 2005). India accounts for 45.2% of the global prevalence (Omkumar, 2008). Even though the disease is not fatal, the disabling occurring in chronic state is a cause of social stigma. Filariasis is a vector borne disease caused by the nematode *Wuchereria bancrofti* (90% cases) and *Brugia malayi* (10% cases). These endo- parasites complete their life cycle in two hosts, mosquitoes and human; human harbors the adult worms and allows reproduction in the body. At present no vaccines are available against filarial infections and the drugs available target both adult worms (macrofilaricidal) and microfilaria (Microfilaricidal). Several drugs have been designed from plant extracts (Mishra et al., 2005) and marine products (Hwang et al., 1999) that targets mostly the enzymes and arrest the metabolic activity of the parasite. The drugs currently used are Suramin, also known as antryol, diethylcarbenzamide, (such as hete-razan, banocide and notezine); targets mostly microfilaria but also has macrofilaricidal activity (Langham and

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Abbreviations: **HPLC,** High performance liquid chromatography; **GC-MS,** gas chromatography-mass spectroscopy; **NIB,** nuclei isolation buffer; **EDTA,** ethylenediaminetetraacetic acid; **EGTA**, ethylene glycol tetraacetic acid; **SDS,** sodium dodecyl sulfate; **DTT,** dithiothreito; **TAE,** tris/borate/ethylenediaminetetraacetic acid; **RI,** retention index; **IC,** inhibition concentration; **TIC,** total ion chromatogram; **PMSF,** phenylmethylsulfonyl fluoride; **DMSO,** dimethyl sulfoxide; **NIST,** national Institute of standards and technology; **ATP,** adenosine triphosphate.

Kramer, 1980) and levamisole which interferes with the production of succinate dehydrogenase leads to muscular paralysis of the parasite (Lewis et al., 1980). New strategies affordable and without undesirable side effects, are desperately needed. Novel drug targets are under trials along the drug development pathway.

The identification of deoxyribonucleic acid (DNA) topoisomerase II (topo II) as drug target for various diseases such as cancer, bacterial infections etc, also promised a new lead candidates for the development of antifilarial drugs . Studies show that the parasite's topoisomerase is sufficiently distinct from their human counterparts so as to allow differential chemical targeting and will therefore make good cellular targets. Among the various target enzymes reported for drug development against parasitic diseases, DNA topoisomerases II (topo II) have been chosen as a novel target due to their role in replication, transcription and recombination of DNA. Topo II play pivotal roles in the organization of DNA within the cell nucleus as well as in its structure and function (Katiyar et al., 2005). This enzyme acts in the relaxation of DNA supercoils that accumulate during gene transcription along with replication fork. Moreover, only topo II can carry out the decatenation of replicated circular double-stranded DNA, and it is obligatorily involved in the remodelling of chromatin during mitosis (Nitiss, 1998).

Topo II inhibitors are classified into two groups: topo II catalytic inhibitors and topo II poisons. Topo II catalytic inhibitors inhibit the catalytic activity of topo II without generating DNA breaks, thus depriving cells of topo II activity leading to cell death (Andoh and Ishida,1998). Topo II poisons stabilize the cleavable complex leading to cytotoxic DNA break accumulation.

So far, Topo II poisons find applications as antitumor agents whereas topo II catalytic inhibitors have wide applications as antineoplastic agents, cardioprotectors or modulators that increase the efficacy of other agents (Larsen et al., 2003). Several microorganisms have been screened for topoisomerase inhibitors (Suzuki et al., 2003). The coumarin antibiotics novobiocin, clorobiocin, and coumermycin A1 isolated from several S*treptomyces* strains bind topo II and exhibit potent activity against Gram-positive bacteria (Donnelly and Blagg, 2008). Five different types of inhibitors designated as 2280 – DTI, 2890 – DTI (Suzuki et al., 1998a), macrostatin (Suzuki et al., 1998b), topostatin (Suzuki et al., 1998 c,d,e) and isoaurostatin (Suzuki et al., 2001) have been identified from various micro organisms which belong to the cleavable complex-nonforming type. A novel inhibitor of topo II designated as 2070 – DTI was isolated from a culture filtrate of *Streptomyces* species strain 2070 (Suzuki et al., 2003). Though number of natural inhibitorshas been isolated from microbial source against topo II and they are reported as anti filarial or anti cancer drugs, still the emergence of such type of drug from the natural source is much warranted due to its bio availability and less toxicity. In this context, this study was focused on the analysis of the topo II inhibitory activities of *M. luteus B1252* isolated from the Western Ghat Hills, Tamil Nadu, India.

MATERIALS AND METHODS

Isolation and partial purification of topoisomerase II from *Setaria cervi*

Enzyme topo II was partially purified from the parasite *S. cervi* isolated from the goat intestine (Pandaya et al., 1999). Filarial parasites were homogenized in Nuclei Isolation Buffer (NIB) (2.5 mM potassium phosphate buffer, pH 7.0; 2 mM $MgCl_2$; 0.1 mM ethylenediaminetetraacetic acid (EDTA) 1 mM ethylene glycol tetraacetic acid (EGTA); 1 mM dithiothreito (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Aldrich) and 3000×g. The pellet was washed with NIB and resuspended in NIB containing 4 mM EDTA; 0.35% (v/v) Triton X-100 and 0.375 M NaCl (Himedia Mumbai). The suspension was gently agitated for 15 min on ice and polyethylene glycol (9%, w/v) was added. The mixture was kept on ice for 1 h with occasional shaking and centrifuged at 10,000 x *g* for 30 min. The enzyme was partially purified using G-50 CM Sephadex chromatography (Reddy et al., 2009). The protein content was determined (Lowry et al., 1951).

Topoisomerase relaxation assay

Topo II activity was measured by monitoring the relaxation of supercoiled pBR322 DNA (Pandya et al., 1999). Assay mixture (20 µl) contained 50 mM Tris–HCl, (pH 7.5); 50 mM KCl; 1mM $MgCl_2$; 1mM adenosine triphosphate (ATP); 0.1 mM EDTA; 0.5 mM DTT; 30 µg/ml BSA; 0.25 µg pBR322 DNA and enzyme protein. The reaction mixture was incubated at 37°C for 30 min and 5 µl stop buffer [0.25%, bromophenol blue, 1M sucrose, 1mM EDTA, 0.5% sodium dodecyl sulfate (SDS)] was added. The entire reaction mixture was loaded on 1.2% agarose gel and electrophoresed in tris/borate/ethylenediaminetetraacetic acid (TAE) (40 mM Tris–acetate buffer, pH 8.3, 1mM EDTA) at 25 V for 18 h. The gel was stained with ethidium bromide (0.5 µg/ml) and photographed in Alphadigidoc Pro gel documentation system (Alpha Innotech CA). The effect of inhibitor on the enzyme activity was measured by incubating enzyme protein with the inhibitor for 15 min at 37°C and starting the reaction by adding pBR322. Heat inactivated enzyme blank was also maintained.

Extraction of topo II inhibitor from *Micrococcus luteus* BI252

The strain *M. luteus BI252* isolated from Western Ghat Hills (Accession number: EU.176162) was used in this study. *M. luteus BI252* was cultivated for two days at 37°C on a rotary shaker (120

rpm) in a medium composed of 0.5% peptone, 0.2% yeast extract, 0.3% beef extract and 0.3% NaCl (Himedia Mumbai) and the medium was adjust to pH 7.0. After cultivation, cellular residues of the culture broth were removed by centrifugation at 7000 x *g* at 4°C for 10 min. The cell pellet was homogenized with 2 volumes of methanol and the extract obtained was concentrated *in vacuo*. The crude methanolic extract obtained was checked for the topo II inhibition by relaxation assay as mentioned above.

Partial purification of topo II inhibiting compound

The methanol extract obtained as described previously was fractionated on a silica (60 to 120 mesh) column by gradient elution with a mixture of solvent methanol: chloroform in the order of increasing proportion (5% increment) from 5 to 100% of MeOH in $CHCl_3$. Five fractions each 2.0 ml were collected in each proportion of mobile phase. The fraction dried *in vacuo* were dissolved in dimethyl sulfoxide (DMSO) and tested for anti topo II activity. The subfractions 50% MeOH and 50% $CHCl_3$ showed a high inhibitory effect on the topo II activity and the fractions were pooled together. The extract was further purified using preparatory HPLC (Shimadzu, LC 10 A phenomenex C_{18} column, 20 x 250 mm, 25 µm pore size analysed through photo diode array detector UV Max of 200 to 800 nm, Shimadzu Corporation, Japan). About 5.0 ml of concentrated fraction was injected. The mobile phase methanol: water (60:40) was pumped isocraticaly at a flow rate of 25 ml/min (all the above mentioned solvents were HPLC grade purchased from Merck India, Mumbai). The obtained eluants dried *in vacuo* were dissolved with DMSO and tested for topo II inhibition at various concentrations of 5, 10,15,20,25 and 30 µg/ml by relaxation assay.

GC-MS analysis

The active fraction obtained from preparatory HPLC was subjected to GC-MS analysis. GC-MS analyses were performed (Clarus 500 Perkin Elmer Elite-1 gas chromatograph interfaced to Turbo mass gold-Perkin Elmer Mass detector, USA) and a vaporization injector operating at 250°C in the split mode (1:100) was used. 100% dimethyl poly siloxane column (Perkin Elmer USA Elite-M1column 30 m x 0.53 mm ID x 1.5 µm df) was used. The oven temperature was programmed to 110°C 2 min hold up to 280°C at the rate of 5°C/min-9 min hold. High purity helium was used as carrier gas at 1ml/min. Electron ionization mass spectra in the range of 45 to 450 Da were recorded at 70 eV electron energy for 46 min. The identity of each compound was determined by comparison of its retention index (RI) as well as of its total ion chromatogram with the NIST mass spectral library version 08.

RESULTS

Isolation and partial purification of topoisomerase II from *Setaria cervi* Topo II enzyme was extracted from *Setaria cervi* and to screen topo II inhibition, visible shift of the pBR322 super coiled DNA in comparison with the control was achieved using various concentration of partially purified enzyme from *Setaria cervi* (Figure 1*)*. The maximum visible shift of the band was seen with 2.4 µg (1U) of enzyme preparation and this concentration was used for further inhibition analysis.

Partial purification of topoisomerase II inhibiting compound

Methanolic extract of *M. luteus BI252* was evaluated for *in vitro* topo II inhibitory activity against filarial topo II from *Setaria cervi* (Figure 2). About 50 µg of the crude methanol extract showed 80% of inhibition. The methanol extract was fractionated on the silica gel using MeOH: $CHCl_3$ solvent system in the order of increasing proportion (from 5 to 100% of MeOH in $CHCl_3$). About hundred fractions were collected and all the fractions were screened for inhibition against topo II. However, the fraction obtained with 50:50 MeOH-$CHCl_3$ showed the highest inhibition. The active fraction was further fractionated by preparative HPLC with photo diode array detector. As shown in Figure 3, seven peaks were obtained, each peak was collected separately and screened for topo II inhibition activity. A major fraction with retention time of 8.0 min and UV maximum at 206 nm showed the highest topo II inhibition activity. The active fraction showed dose dependent inhibition with IC_{50} value at 11.2 µg/ml (Figure 4a and b). Specific inhibitor of topo II, nalidixic acid (5 mg/ml) was used as the positive control throughout the experiments. Nalidixic acid showed dose dependent inhibition with 50% inhibition concentration (IC_{50}) at 40 µg/ml.

GC-MS analysis

GC-MS data, and total ion chromatogram (TIC) showed around 11 peaks (Figure 5). The compound profile of each active fraction and structures are illustrated based on NIST library matching factor with more than 85% spectral similarity (Table 1and Figure 6). The fatty acid n-hexadecanoic acid, (Z)-15-tetracosenoic acid, methyl ester and methyl tetradecanoate was dominant with a percentage of 61.4%. The representatives of aliphatic alcohols were 1, 15-pentadecanediol and 4-tridecanol. Furthermore, ester compounds found were butyl cyclo-butyl ester and cyclobutyl heptyl ester. In addition, other compounds such as 2-methyl-2-nitro- propane, 4,6-di (1,1-dimethylethyl)-2-methyl- phenol, sulfurous acid, cyclohexylmethyl isobutyl ester and 5-methyl-2-trimethylsilyloxy-acetophenone were also found.

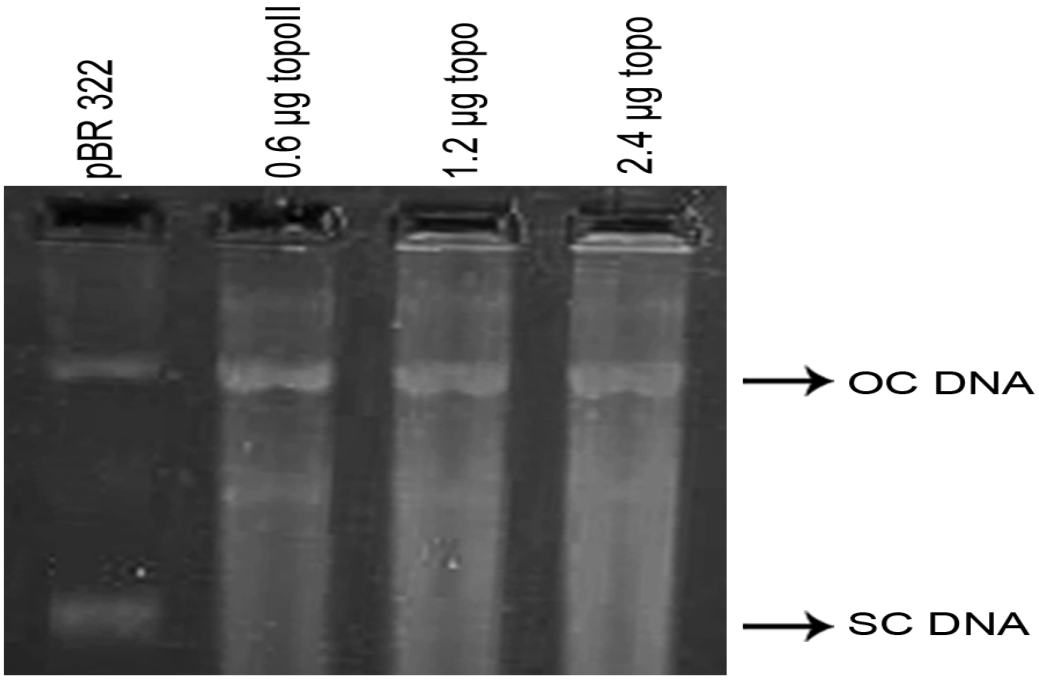


Figure 1. Topoisomerase II activity of the partially purified *Setaria cervi* enzyme. The activity of the extract was assayed at various concentration of the protein (0.6, 1.2 and 2.4 µg).

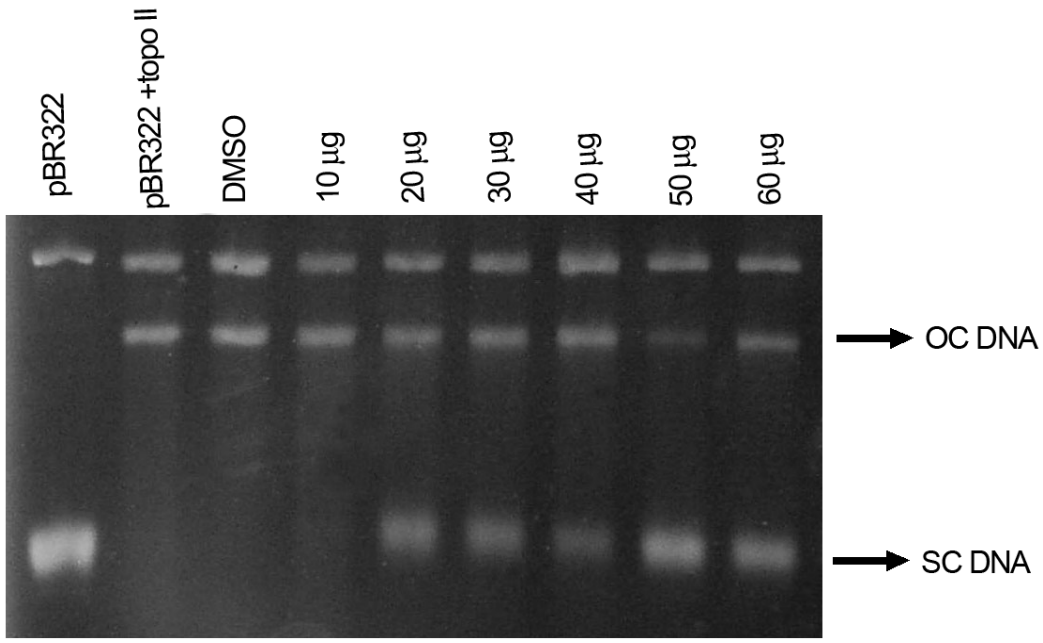


Figure 2. Inhibitory effect of crude methanolic extract of *Micrococcus luteus BI252* and inhibitory effect of crude methanolic extract of various concentrations (10 to 60 µg) against *Setaria cervi* topoisomerase II activity. The inhibition was determined using relaxation assay. OC DNA strands for open circular DNA and SC DNA for supercoild DNA.

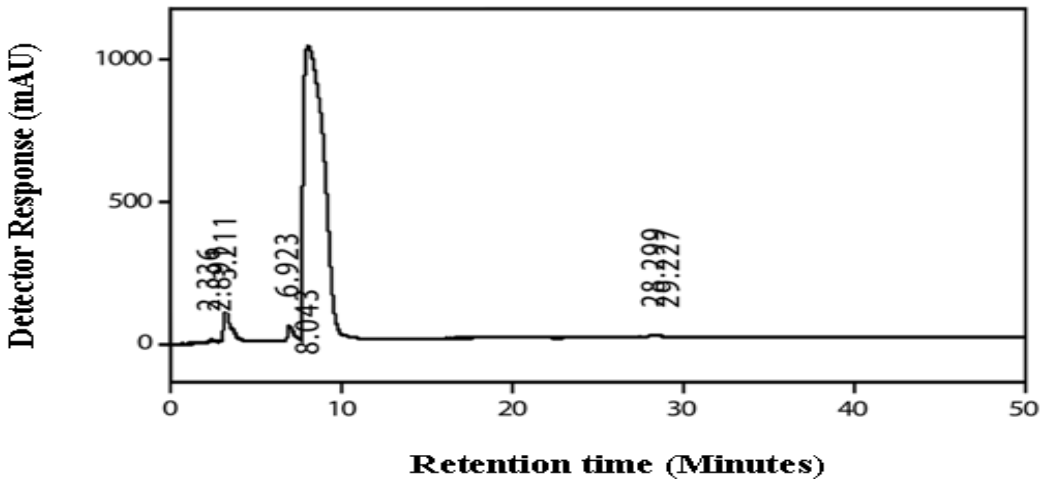


Figure 3. HPLC chromatogram of the partially purified methanol extract, *Micrococcus luteus BI252* using silica column. Extract was subjected to preparative HPLC Simadzu, LC 10 A phenomenex C_{18} column, (20 x 250 mm) and the mobile phase of methanol: water (60:40). The compounds of the peak with retention time 8.043 min show area percentage of 94.24% active anti topo II activity with area percentage of 94.24%.

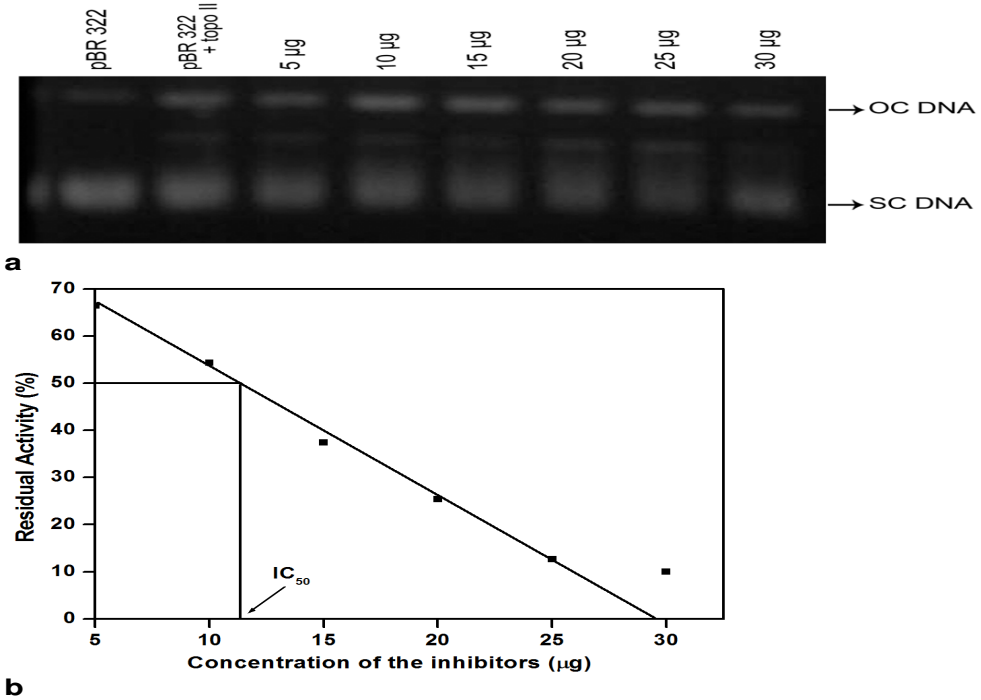


Figure 4. a, Effect of *Micrococcus luteus* B1252 partially purified HPLC extract on *Setaria cervi* topo II. Lane 1, pBR322 DNA alone; lane 2, *Setaria cervi* enzyme activity; lane 3 to 8 HPLC fractions 5,10,15,20,25 and 30 µg/ml concentration. OC DNA strands for open circular DNA and SC DNA for supercoild DNA. **b,** The inhibitory effect of HPLC purified extract on topo II. The relative percentage of inhibition in terms of residual activity remaining was plotted against various concentration of the extract.

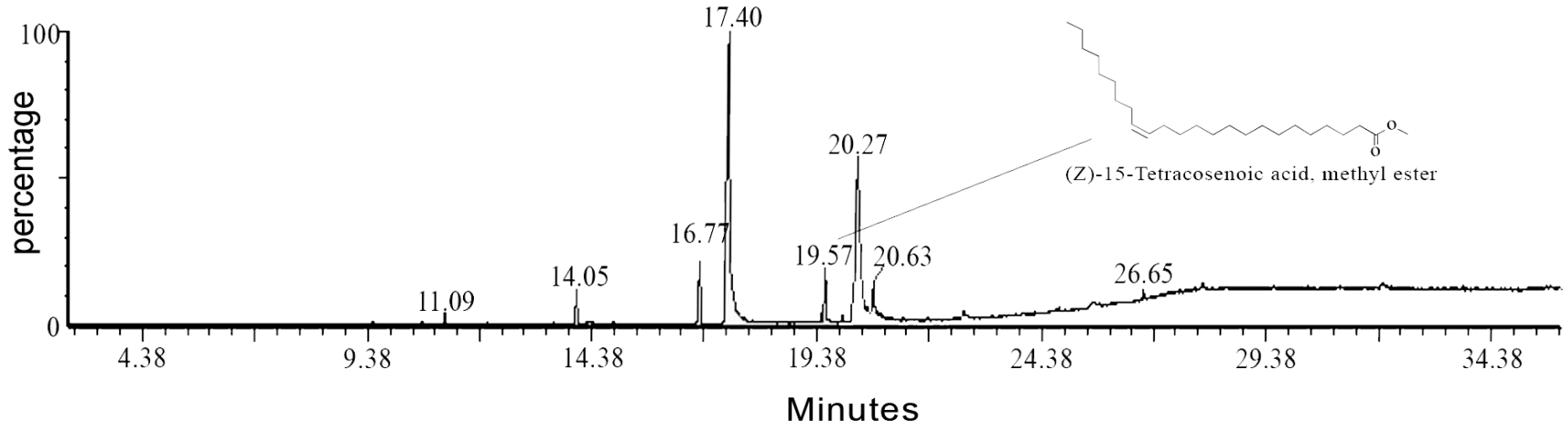
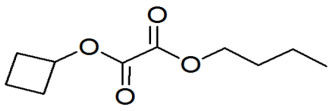


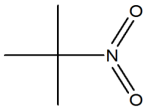
Figure 5. GC-MS profile of active fraction of *Micrococcus luteus BI252* with topo II inhibitory. Eleven peaks were observed at various mass levels and further, the compounds were identified by NIST library matching factor with more than 85% spectral similarity.

Table 1. Compound profile of partially purified extract of *Micrococcus luteus* B1252 by GC-MS analysis.

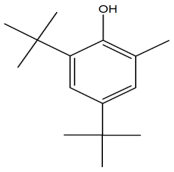
S/N	Retention time	Name of the compound	Molecular formula	Molecular weight	% Peak area	Compound nature
1	9.52	Oxalic acid, butyl cyclobutyl ester	$C_{10}H_{16}O_4$	200	0.08	Ester compound
2	10.59	2-methyl-2-nitro- Propane	$C_4H_9NO_2$	103	0.14	Nitrogen compound
3	11.09	4,6-di(1,1-dimethylethyl)-2-methyl- Phenol	$C_{15}H_{24}O$	220	0.64	Phenolic compound
4	12.06	Oxalic acid, cyclobutyl heptyl ester	$C_{13}H_{22}O_4$	242	0.19	Ester compound
5	14.05	Sulfurous acid, cyclohexylmethyl isobutyl ester	$C_{11}H_{22}O_3S$	234	2.27	Acidic compound
6	16.77	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242	5.61	Myristic acid ester
7	17.40	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	51.50	Palmitic acid
8	19.57	(Z)-15-Tetracosenoic acid, methyl ester	$C_{25}H_{48}O_2$	380	4.29	Fatty acid ester
9	20.27	1,15-Pentadecanediol	$C_{15}H_{32}O_2$	244	30.15	Aliphatic alcohol
10	20.63	4-Tridecanol	$C_{13}H_{28}O$	200	3.96	Aliphatic alcohol
11	26.65	5-Methyl-2-trimethylsilyloxy-acetophenone	$C_{12}H_{18}O_2Si$	222	1.18	Ketone compound



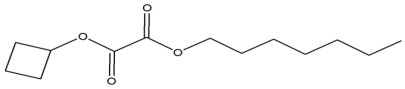
Oxalic acid,butyl cyclobutyl ester



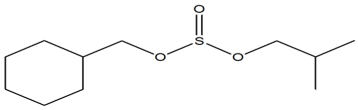
2-methyl-2-nitro Propane



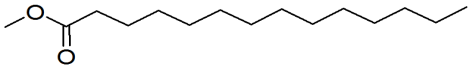
4,6-di(1,1-dimethylethyl)-2-methyl-Phenol



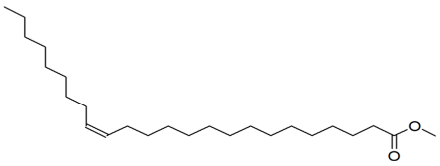
Oxalic acid Cyclobutyl heptyl ester



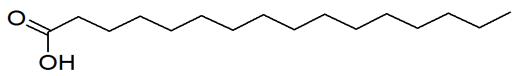
Sulfuruous acid,cyclohexylmethyl isobutyl ester



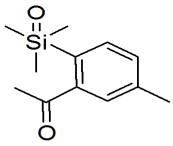
Methyl tetradecanoate



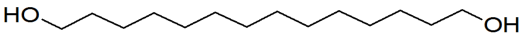
(Z)-15-Tetracosenoic acid,methyl ester



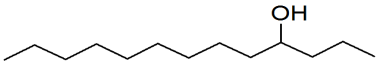
n-Hexadecanoic acid



5-methyl-2-trimethylsilyloxy-acetophenone



1,15-Pentadecanediol



4-Tridecanol

Figure 6. Structure of the compounds identified by GC-MS analysis.

DISCUSSION

Novel therapeutic targets from the myriad parasitic enzymes and metabolic pathways are being utilized in drug development against parasitic diseases. Despite the significant advances made in the chemotherapy of parasitic diseases, the treatment and eventual eradication of filariasis still remains a major uphill task. A suitable, safe and effective filaricide would allow more impact to be made on controlling filarial diseases. Filarial parasite DNA topo II is a major target for the development of antifilarial agents (Katiyar et al., 2003; Misra-Bhattacharya et al., 2004; Bajpai et al., 2005; Prabhakaran et al., 2011). This investigation deals with the anti topo II activity of the microorganism *Micrococcus luteus BI252* to target filarial infection. The methanolic crude extract was found effective against the topo II activity *in vitro*. The extract was purified using silica column chromatography and HPLC. The active fraction subjected to GC-MS analysis revealed the presence of major group of fatty acids, fatty acid ester, fatty alcohol along with nitrogen, silicon and sulfur derivatives; n-hexadecanoic acid, (Z)-15-tetracosenoic acid, methyl ester, methyl tetradecanoate with total percentage of 61.4%. Unusual fatty acids such as n-tetradecanoic (n-C_{14}), n-hexadecanoic (n-C_{16}) and n-octadecanoic (n-C_{18}) acids were previously reported as major constituents of cell membranes and waxes in a number of organisms, including terrestrial and aquatic plants, phytoplankton, and microbes (Farrington and Quinn 1973; Cranwell 1982; Meyers and Ishiwatari 1993; Chikaraishi and Naraoka, 2005). Fatty acid having a chain length of C_{18} or higher are proven to inhibit topo II activity (Mizushino et al., 2000). According to the above said report, in our study, the saturated compound viz n-hexadecanoic acid and methyl tetradecanoate (chain length less than C_{18}) in the extract are less possible to inhibit the topo II activity.

The inhibitory potency of fatty acid is dependent on the carbon chain length and the position of the double bond in the fatty acid molecule (Suzuki et al., 2000). Cis unsaturated fatty acids (C_{18} or Higher) are known to inhibit human topoisomerase II (Mizushino et al., 2000). Mizushino and his coworkers revealed that the amino acid Thr, His, Leu and lys form a pocket in the enzyme where the fatty acid molecule binds to inhibit topo II. In the observation, the saturated fatty acid compounds and fatty alcohols, 4-tetracanol and 1, 15-pentadecanedol in the extract have very less carbon chain length and in addition to the absence of cis unsaturation position may lead to lack of topo II inhibition activity. A recent report showed three 4'-ester derivatives of GL-331 inhibited topo II activity (Zhiyan et al., 2004). When they compared the inhibition potential of esterified compounds, the results suggested that the electron withdrawing substituents on the β-carbon of ester moiety might enhance the topo II inhibition (Han et al., 2004). Based on our results and the above said facts, the probable active compound responsible for topo II inhibition is (Z) 15-tetracosenoic acid, methyl ester. The data indicate that the active compound can be utilized as lead molecules for antifilarial chemotherapy after purification and after extensive investigations in the pharmacological point of view.

Conclusions

Our study suggests that the fatty acid ester derivatives (Z) 15-tetracosenoic acid, methyl ester fatty acid is an alternative target for development of anti-virulence drug lead compounds. Future work of screening novel fatty acid ester derivative inhibitors could be done by molecular docking based virtual screening followed by molecular dynamics simulations.

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