American Journal of Ethnomedicine, 2014, Vol. 1, No. 3, 174-185
Available online at http://www.ajethno.com

UV, FTIR, HPLC Confirmation of Camptothecin an Anticancer Metabolite from Bark Extract of *Nothapodytes nimmoniana* (J. Graham)

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ABSTRACT

Nothapodytes nimmoniana belonging to family *Icacinaceae* is an endangered medicinal plant from Western Ghats of Maharashtra. This plant possesses a potent antineoplastic agent, mainly camptothecin and 9-methoxy camptothecin. In present paper, phytochemical evaluation, identification, isolation, purification and extraction procedure of camptothecin by TLC has been described in detail. *Nothapodytes nimmoniana* tannin free extract was used for purification by HPLC. Here, the CPT was extracted from dried bark of *Mappia* by soxhlet extractor and TLC fingerprinting carried out by comparing with standard CPT using the solvent system Ethyl acetate: Methanol (3:1). UV spectroscopical λ_{max} values were observed at 256, 290 and 354nm approximately. FTIR spectropical data suggest the unique functional group present in the compound. Purification of CPT by mobile phase acetonitrile / H_2O (25:75) using HPLC with PDA detector and DAD signal at 365nm was done. To finalize a relation of anticancer ability with antioxidant and antibacterial potential of the plant, TLC bioautography techniques were used to support it as a best and multifaceted chemo preventative agent.

Keywords- *N. nimmoniana* Graham, Camptothecin, Phytochemical analysis and TLC bioautography.

INTRODUCTION

Nothapodytes nimmoniana Graham previously known as *Mappia foetida* Miers. This plant is known as ghanera in Maharashtra and durvasanemara in Karnataka state of India. *N. nimmoniana* is highly popular as a source of anticancer drugs curing sarcomas such as lungs, breast and uterine cervical cancers. There are numerous reports stating Camptothecin (CPT) to be present in several species (Khan

et al., 2012). However, *N. nimmoniana* reports for higher concentration (CPT) with potent antitumour isoquinoline alkaloid originally isolated from *Camptotheca acuminata* from China (Wall and Wani, 1966) and there are few small trees geographically distributed in the Western Ghats of India (Rajasekharan and Kareem, 2011). As this plant is an only source that produces an anticancer compound in large quantity, has attracted scientist and researchers to divert their focus for isolation of CPT in pure and huge quantity (Fulzele et al., 2005).

Camptothecin antitumour activity has been confirmed against a variety of tumor models. The real mechanism of Camptothecin against tumor is by inhibiting topoisomerase- I enzyme (Redinbo, 1998) essential for the DNA replication in the dividing eukaryotic cells. That results in the formation of putative covalent reaction intermediate, a reversible Topo, I CPT-DNA ternary complex. Among many other cellular responses, the interaction between the DNA replication machinery and the ternary complex causes cell death (Kjeldesen, 1992). There are derivitized CPT based drugs are available in the market that has been approved by FDA of the USA and National health agencies. At present, four semisynthetic water-soluble derivatives of camptothecin- topotecan, irinotecan, 9-aminocamptothecin and 9-nitro camptothecin are being used widely for the management of various malignancies.

Thus in this study the CPT was extracted from dried bark of Mappia by soxhlet extractor, preliminary characterized by using TLC along with marker CPT. Further purified from TLC scrapping, analysed by FTIR and purified by HPLC using an acetonitrile / H_2O (25:75) solvent system with PDA detector with at 365nm. In final, the antioxidant and antimicrobial potential of the total extract was determined by TLC bioautography to establish the relation of anticancer ability.

MATERIALS AND METHODS

Plant Collection and Identification

The plant saplings and dried plant parts were collected from Chiplun part of Western Ghats, Maharashtra. Few saplings and some of its parts like bark and seeds were also procured from Salem, South India. These saplings were then maintained in green house. The plant was authenticated using standard flora and cross-checked and stored in the herbarium records at Department of Biotechnology, Sant Gadge Baba Amravati Unversity, Amravati, India as ***Nothapodytes nimmoniana* (J. Graham)** with an accession number-BTSGBAU-07.

Preparation of extract

The plant bark was sun dried and crushed to mesh size of 0.1 to 0.5 mm. About 10 gm of powdered material were kept in a thimble into solvent extractor and extracted it with 95% methanol for 9 hrs. Further 200ml of extract was allowed to evaporate at room temperature, it takes approximately 7-8 days. The extract obtained was somewhat greenish brown gummy solid, which was used for phytochemical screening to hunt for the presence of alkaloids along with other metabolites such as Saponins, Tannins, Cardiac Glycoside, Steroid, coumarin, carbohydrates and flavonoids.

Preliminary phytochemical screening

The phytochemical screening was carried out by using standard procedures described by Kokate (1986), Harborne (1998), Patil et al. (2012). The results were shown in Table no 1.

TLC separation

The presence of active metabolites in *N. nimmoniana* extract was evaluated by TLC fingerprinting. The preparative normal phase TLC was performed on 15x10 cm glass plates coated with 1 mm layer of silica gel, dried in room temperature for 30 minutes and activated in the oven at 110˚ C for next 30 minutes. The solvent system used was Ethyl acetate: Methanol (3:1). About 10 µl of extract was spotted on the silica plates. The experimental plates were developed in a chromatographic chamber, saturated by 100 ml of the solvent system. Plate development required about 15 to 20 minutes; it was then visualized at 365 nm under UV transilluminator (Cleaver). Rf values of bands were recorded by using formula and were compared with standard CPT (1mg/ml). The results were shown in Figure no. 1.

$$Rf = \frac{\text{Distance traveled by sample.}}{\text{Distance traveled by Solvent.}}$$

Purification and characterization of camptothecin

The camptothecin compound was processed for preparative TLC, and corresponding band was scrapped from the TLC plates along with the silica. Resuspended in the solvent (methanol) and shaken vigoursly for 15 min and afterwards centrifuged at 4000 rpm for another 25min. Further, the above supernatant was next collected in separate beaker and evaporated to dryness in the vacuum. The residual compound remaining are the purified camptothecin weighing about 0.36 g.

UV- Spectroscopic analysis

The Camptothecin compound was scraped from TLC plate under UV transilluminator at 365nm. Both of compounds were dissolved in methanol solvent. The diluted compound samples were set for UV analysis (using UV spectrophotometer 1800 Shimadzu) under range of 1100-190nm. Methanol was used for correction of baseline as blank. The results were shown in Figure no 2.

Fourier-Transform Infrared Spectroscopy (FTIR) Analysis

The purified scraped compound was used for detection of unique functional group using FTIR spectroscopy (The IRAffinity-1S FTIR spectrophotometer). The functional group detections were recorded in the wave number range of 400–4000 cm−1 at a resolution of 4 cm−1. Sample powder was dried at 80 °C for 24 h, then, mixed with KBr powder at 1%, pressed to obtain self-made sample disks. The results were presented shown in Table no 2 and Figure no 3 shows FT-IR spectra of CPT purified from TLC.

HPLC purification of CPT

The tannin-free extracts prepared as per the method describe by Wall et al. (1996). Tannins are plant polyphenols commonly found in plant extracts, which bind and precipitate proteins and various other organic compounds, including amino acids and alkaloids, thus interferes the drug efficiency and efficacy (Sarkar et al., 2005). Thus, tannin-free extract was used for determination of CPT content; the concentrate was transferred into polypropylene micro centrifuge tubes, mixed with HPLC grade MeOH (1ml) vortexed for 20 second followed by centrifugation at 4000rpm for 15min. All chemicals used were of HPLC grade. The clear supernatants were directly applied on HPLC. The column used was ZORBAX Eclipse Plus C18 (4.6 × 100 mm, 3.5). Acetonitrile: H_2O (25:75) solvent system with flow rates 1ml/min was used (Mingzhang et al., 2011). Detection was done at 365nm; elution gradient was isocratic elution with 10µl of sample. The

flow rate kept was 1ml/min. DAD signal at 365 nm was kept for detection. Reference compound was commercially available camptothecin of Sigma Aldrich with concentration 1mg/ml. Figure no 4 showed HPLC chromatogram of *N. nimmoniana,* bark peak is overlapping with standard CPT at 7 min.

DPPH free radical scavenging (antioxidant) property of extracts

The solvent system used is Ethyl acetate: Methanol (3:1), it produces the significant results showing 5-6 fluorescent bands at 365nm in UV transilluminator. DPPH (2,2-Diphenyl-1-picrylhydrazyl, $C_{18}H_{12}N_5O_6$) was used in this assay to assess the free-radical scavenging (antioxidant) property of extracts. DPPH (4 mg) is dissolved in Methanol (50 mL) to obtain a concentration of 2mg/ml in methanol. It is allowed to develop for 30 min. The white spots against a violet-yellow background indicate the antioxidant activity. The results were shown in Figure no 5, confirming the presence of antioxidant metabolites in total bark extract of *N. nimmoniana.*

TLC Bioautography of crude extract

DNA topoisomerases are the targets of important anticancer and antibacterial drugs. The anticancer compound obtained if possess antibacterial property that would be beneficially used against DNA topoisomerases (Pommier et al., 2011). Thus TLC Bioautography assay confirm antibacterial activity of compound (Camptothecin). In order to find out the bioactive fraction possessing bactericidal property from total extract TLC Bio-autography by agar overlay method was performed (Hostettmann et al., 1990). In N.A medium, a suspension of test pathogen *S. aureus* and *E. coli* was added aseptically in the molten agar at about 50°C, further spread on the TLC plate. Allow to solidify and then plates were kept in moist chambers and incubated at 37°C for overnight. Next day, the solution of tetrazolium salts 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 2mg/ml) was sprayed on the plate. The results were shown in Figure no 6 and Figure no 7, confirming the fact that camptothecin do posseses the antibacterial potential against *S. aureus* and *E. coli*.

RESULTS AND DISCUSSION

Extraction of Material

The initial weight of bark powder before extraction was 10 gm, after extraction the pure crude extract was of 0.76 gm. The extraction scheme resulted in efficient method as it removed the tannins, saponins and other interfering compounds.

Phytochemical Analysis

Characteristic's phytochemical tests showed the presence of alkaloids, carbohydrates, phenols and tannins, Glycosides, flavonoids, Steroids, Anthraquinones and Terpenoids, while proteins and amino acids, saponins, Fats and oils, gum and mucilage showed absence from bark extract. The results were shown in Table no 1.

TLC characterization

The amount of TLC purified camptothecin is 0.36 g respectively. The TLC analysis was done under UV transilluminator at 365 nm, and TLC plates show two lanes. Lane 1 shows standard CPT band in the identical range as that of the test sample bands. Lane 2 and 3 are same sample in duplicate representing two protruding bands. Top green color band is of 9-methoxy CPT with the Rf value 0.92 and lower band a prominent blue color of camptothecin at Rf 0.88 in first lane.

UV Spectroscopic analysis

The Camptothecin extract were scanned in range of 1100-190nm and characteristic peak were noted for Camptothecin. Prominent peaks were observed at 256, 290 and 354nm with absorption at 0.3, 0.9 and 0.5 respectively. Figure no 2 shows UV spectroscopic analysis of CPT extracted and purified from TLC plates.

FTIR analysis

The use of FTIR technique allows, pointing out the implication of the different functional groups of guest and host molecules by analyzing the significant changes in the size and position of the absorbance bands. The principal absorption peak of standard Camptothecin showed a –OH stretching at 3,425.7 cm^{-1}, ester stretching 1,736 cm^{-1}, C=O stretching at 1,648 cm^{-1}, pyridone, C=C and C–N stretching at 1,597, 1,572, and 1,436 cm^{-1}, and C–C(=O)–O stretching at 1,148 cm^{-1}, respectively. Peak at 767 cm^{-1} appears to be a contribution of four adjacent hydrogen bonds on the hetero-aromatic nucleus. The CPT extracted from dried bark, purified by TLC scrapping showed a – OH stretching at 3,412.08 cm−1, ester stretching 1,740.62 cm−1, C=O stretching at 1,645.28 cm−1, pyridone, C=C and C–N stretching at 1,590, 1,401.02, and C–C(=O)–O stretching at 1,026 cm−1 respectively. Peak at 491.26 cm−1 appears to be a contribution of four adjacent hydrogen bonds on the hetero-aromatic nucleus. The results were shown in Table no 2 respectively.

HPLC Analysis

The bark extracts of *N.nimmoniana* analyzed by HPLC showed chromatographically homogenous peaks with baseline separation and same retention time at 7.05 min parallel to standard CPT. Test sample and standard CPT have exact retention time and overlapping peaks, which confirm the presence of anticancer compound as camptothecin.

Antioxidant assay of extracts

DPPH (2,2-Diphenyl-1-picrylhydrazyl, $C_{18}H_{12}N_5O_6$) was used to assess the free-radical scavenging (antioxidant) property of extracts. TLC analysis under UV shows 5-6 blue color fluroscent bands. The blue color (A) band just below the solvent front (SF) has been confirmed as Camptothecin by standard on TLC, however, this band is not showing antioxidant activity. The blue color band (B) below Camptothecin band confirms antioxidant potential presented in Figure no 5. Thus in future, identification, separation of the band (B) may yield potent compound, which needs to be carried out in further work.

TLC bioautography

The inhibitory zones (colorless) against a blue-violet background indicate the antibacterial activity. The results clearly indicate bands as a colourless against violet background confirming inhibition of bacterium and also antibacterial potential of camptothecin. Figure no 6 (a) against *S.aureus* and Figure no 7 against *E. coli*.

CONCLUSION

The present study concludes that selected solvent system for TLC gives prominent bands of CPT. Further characterize by FTIR and confirmed by HPLC. The good amount of alkaloid content has been established. It was thought that CPT shows antioxidant activity however DPPH assay confirms other metabolite for antioxidant activity that needs to clarify in further research work. TLC Bioautography confirms antimicrobial activity of methanolic extract of bark of *N. nimmoniana* againts *S. aureus* and *E.coli.*

ACKNOWLEDGEMENT

We express our sincere thanks to UGC for providing financial assistance under Major Research Project to Dr Anita Patil and Dr. Surendra Patil (F.No.42-212/2013 (SR). We are also appreciative of Shri Vasant Pusalkar, Aruna Planta Medica for authentication of plant and Bipin Lade for editing of manuscript and Department of Biotechnology, Sant Gadge Baba Amravati University, Amravati (M.S) for providing the research facilities.

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Table 1. Phytochemical screening of bark extracts (methanolic) of *Nothapodytes Nimmoniana.* with details of plant constituents and tests used.

S. No.	NAME OF TEST	TYPE OF TEST	Test Result
1.	ALKALOIDS	WAGNER'S TEST	+
		MAYER'S TEST	+
		HAGER'S TEST	+
		DRAGENDORFF'S TEST	+
2.	CARBOHYDRATES	MOLISCH'S TEST	+
		BENEDICT'S TEST	+
		FEHLING'S TEST	+
3.	PHENOLS & TANNINS	FERRIC CHLRIDE TEST	+
		GELATIN TEST	+
		LEAD ACETATE TEST	+
4.	GLYCOSIDES	BORNTRAGERS TEST	+
	CARDIAC GLYCOSIDES (CARDENOLIDES)	KELLER KILLIANI TEST	+
5.	PROTEINS & AMINO ACIDS	BIURET TEST	-
		NINHYDRIN TEST	-
6.	SAPONINS	FOAM TEST	-
7.	FLAVONOIDS	SODIUM HYDROXIDE TEST	+
8.	COUMERINS	BONTRAGER'S TEST	-
9.	FATS & OILS	SPOT TEST	-
10.	GUM & MUCILAGE		-
11.	STEROIDS		+
12.	ANTHRAQUINONES	BORNTRAGERS TEST	+
13.	TERPENOIDS	SALKOWSKI TEST	+

Table 2. FTIR spectroscopy data of Camptothecin

S. No.	Functional groups	Standard CPT (cm-1)	CPT (cm-1)	CPT extracted from bark
1.	OH	3600-3400	3432.47	3412.08
2.	Ester	1740-1730	1740.62	1740.00
3.	C=O	1655-1645	1652.57	1645.28
4.	C=C	1610-1550	1580.30	1590.00
5.	C=N	1450-1350	1438.42	1401.02
6.	C–O	1320-1000	1157.27	1026.13
7.	H	----	767.00	491.26

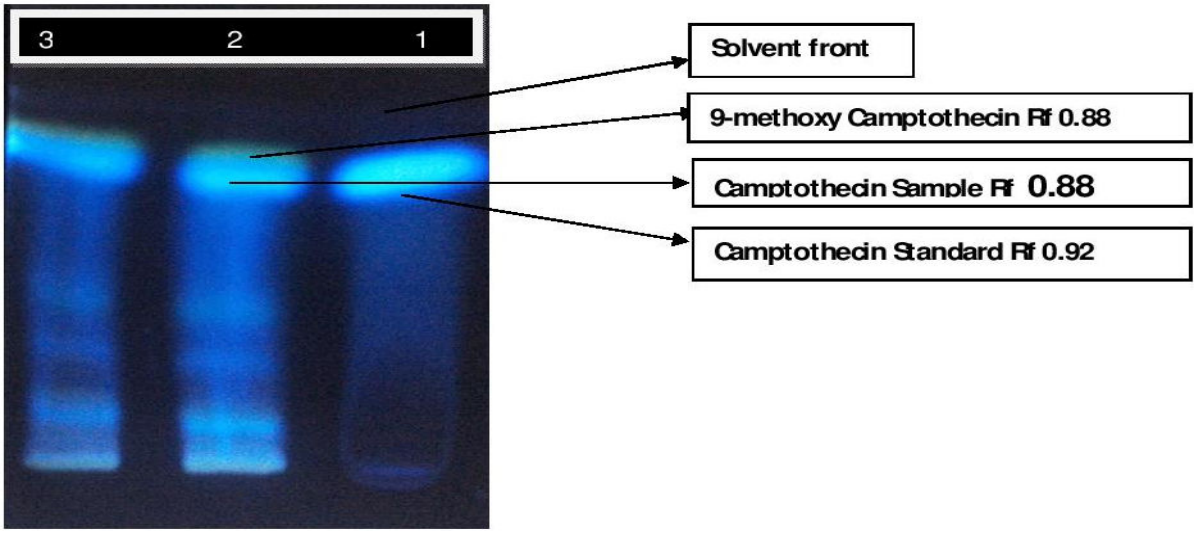

Fig. 1. TLC of Bark Sample of *N. Nimmoniana* observed under 365nm

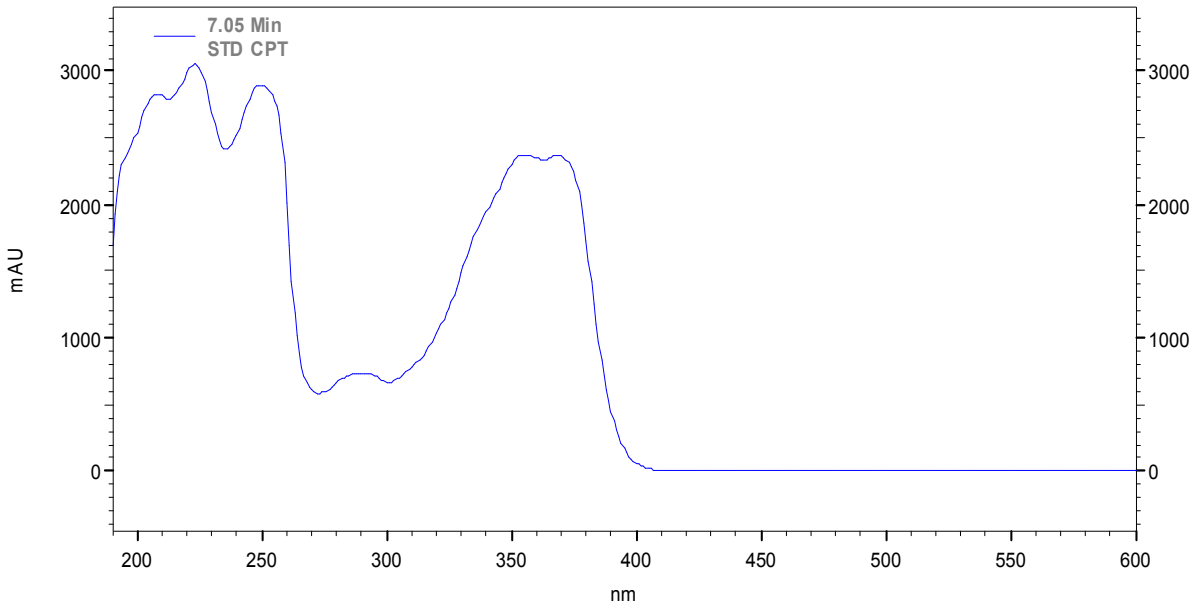

Fig. 2. UV – visible absorption spectra of CPT

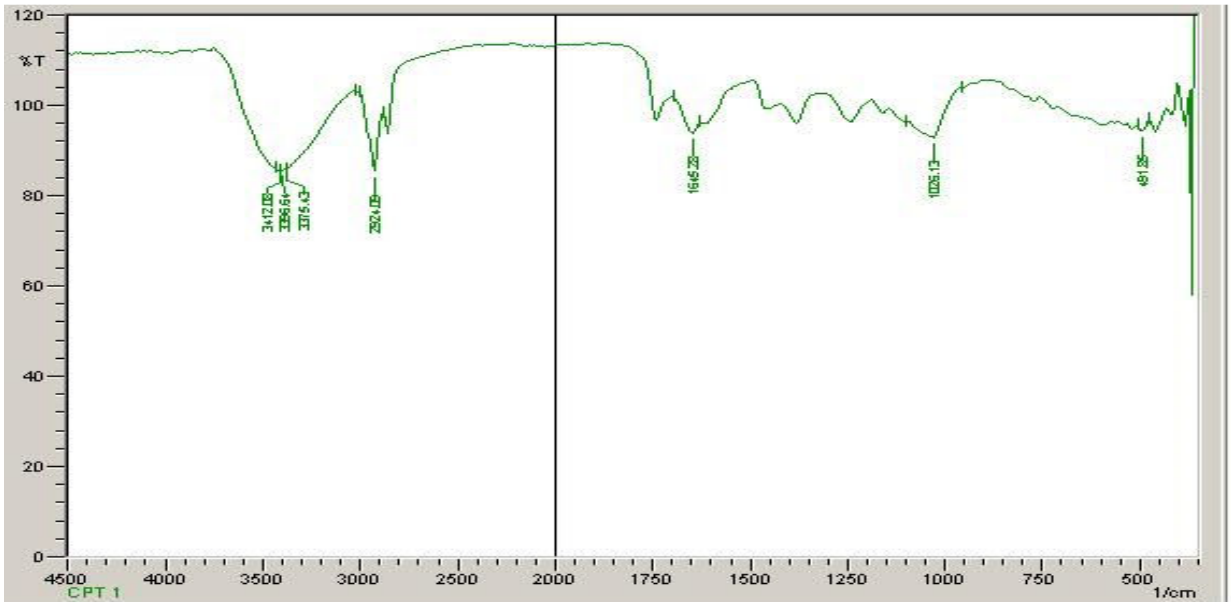

Fig. 3. FT-IR spectra of CPT purified from TLC plates

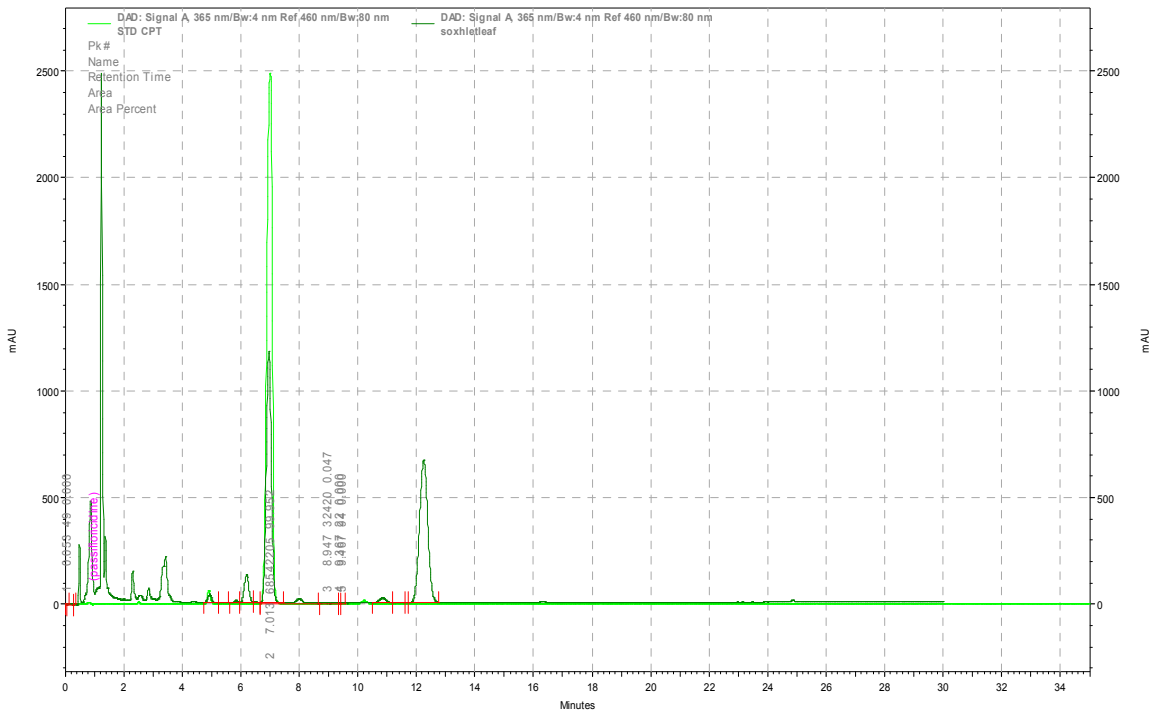

Fig. 4. HPLC chromatogram of *N.nimmoniana,* bark peak is overlaping with standard CPT at 7.05 min

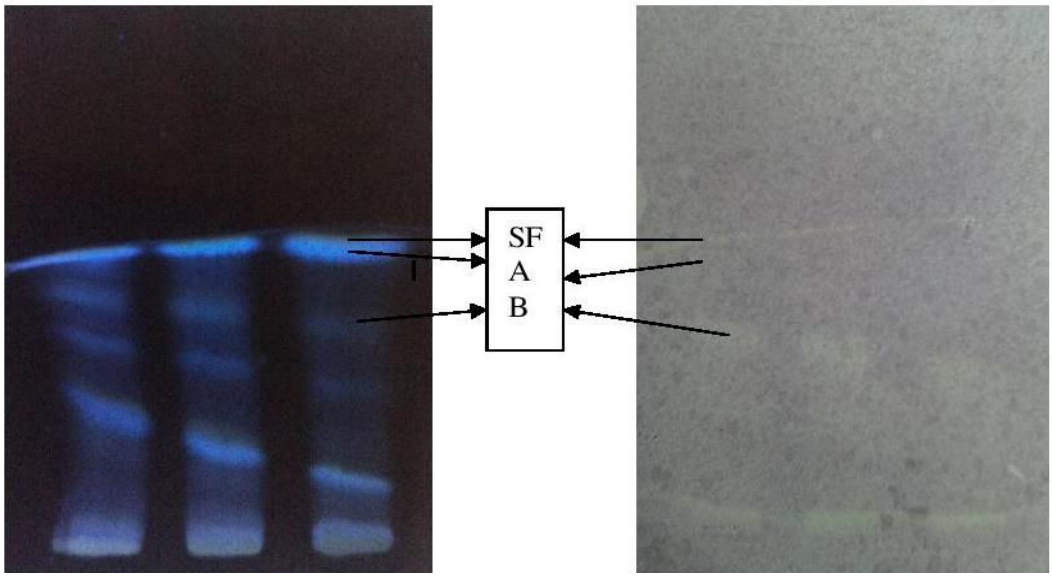

Fig. 5. a. TLC separation of methanolic extract b. DPPH spray on TLC for antioxidant assay Antioxidant activity of band (B) metabolites of methanolic extract of *N. nimmoniana* show potential activity and band (A) CPT is not showing any Antioxidant activity. SF: solvent front, A: CPT band, B: Unknown compound (antioxidant).

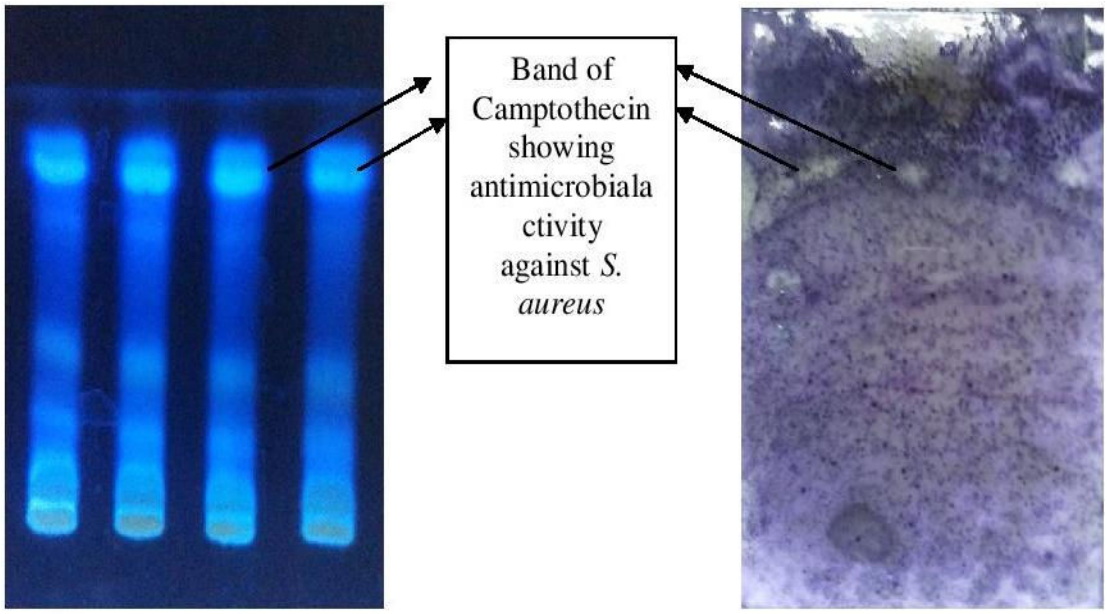

Fig. 6. TLC Bioautography of methonolic extract of bark of *N. nimmoniana* againts *S. aureus*

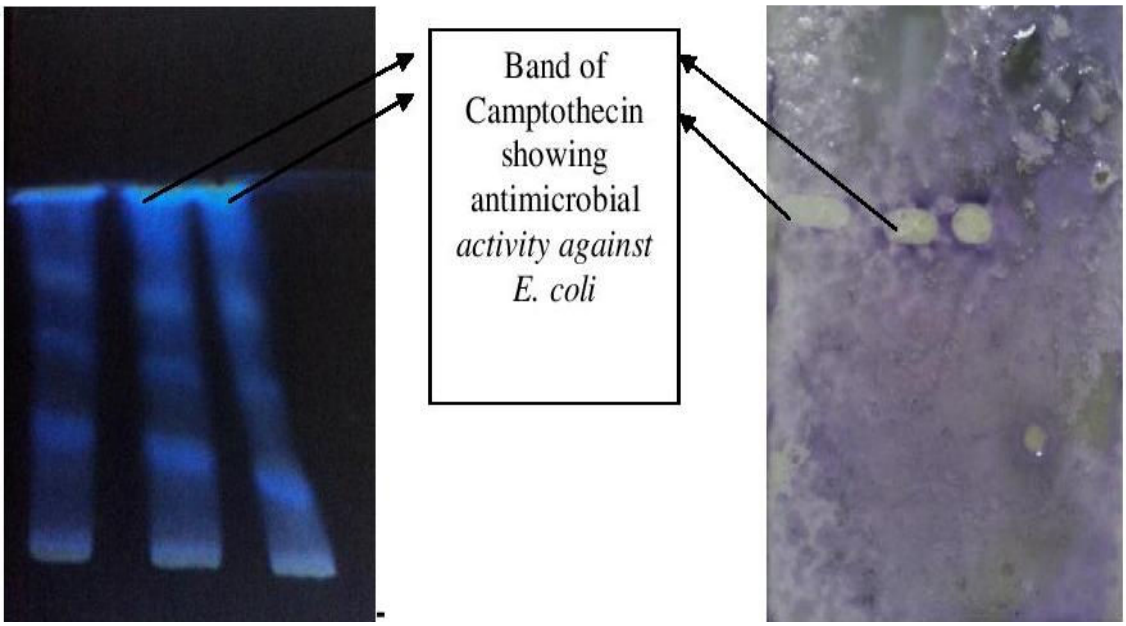

Fig. 7. TLC Bioautography of methonolic extract of bark of *N. nimmoniana* againts *E.coli*