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Research Article

NOSOCOMIAL INFECTIONS: ANTI-MRSA ACTIVITY OF CANNABIS SATIVA AND THUJA ORIENTALIS ACTIVE COMPONENTS OBTAINED BY TLC

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ABSTRACT

Staphylococcus aureus infections in human as this bacterium is resistant to methicillin. Natural products from higher plants may give a new source of antimicrobial agents. In this study, Cannabis sativa and Thuja orientalis have been chosen and findings of their individual contents' inhibitory role on MRSA is studied and compared.

The 3 MRSA bacterial cultures were isolated, as MG1, IR0 and IR2, first two were found to be resistant to Methicillin antibiotics while, IR0 showing the highest zone of inhibition with C. sativa and T. orientalis plant extracts. Respectively, 9-THC, CBDA, CBD and CBN components were separated from the Cannabis leaves and LEA^1, LEA^2, LEA^3 and LEA^4 various unknown components were separated from Thuja leaves using thin layer chromatography. However, a higher zone of inhibition was observed in 9-THC component of Cannabis against MG1 and LEA4 component of Thuja against IR2 isolates.

9-THC and CBD components of Cannabis proved best. These natural components can be formulated for topical use to cure infections caused by MRSA because of their promising anti-MRSA activity.

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INTRODUCTION

Staphylococcus aureus is also known as the "superbug" gram-positive bacteria (Timothy, 2004). *Methicillin-Resistant Staphylococcus aureus* (MRSA) is responsible for several difficult-to-treat infections for humans (Singh et al., 2018). MRSA is any strain of MRSA that has developed through horizontal gene transfer and natural selection of multiple drugs resistant to β-lactam antibiotics (Wilder., 2013). *Staphylococcus aureus* is nonspore forming, catalase and coagulase positive, aerobic as well as facultative anaerobic bacteria. The initial presentation of MRSA is small red bumps that resemble pimples and spider bites and may be accompanied by fever and rashes (Carr., 2017). *Staphylococcus aureus* infection can lead to serious bloodstream infections, skin infections and pneumonia as well as a greater risk of nosocomial community-acquired infections (Thomas and Suzanne., 2002). The term HA-MRSA (healthcare-associated or hospital-acquired MRSA or nosocomial associated), CA-MRSA (community-associated MRSA) reflects this, methicillin is a β-lactam antibiotic of the Penicillin class. It is formerly used in the treatment of bacterial infections caused by organisms of the *Staphylococcus spp.* Basically, Methicillin is a semi-synthetic derivative form of penicillin antibiotics (Eun Ju Choo., 2017). MRSA is different from other types of *Staphylococcus* because it cannot be treated with certain antibiotics and now it has developed resistance to methicillin as well. Resistance is usually conferred by the acquisition of a non-native gene encoding a penicillin-binding protein (PBP2a), with significantly lower affinity for β-lactams (Kristoffer et al., 2014).

Cannabis, known as marijuana among various identified names; is a psychoactive drug from the Cannabis plant intended for medical or recreational use. That core psychoactive part of cannabis is tetrahydrocannabinol (THC) and cannabidiol (CBD). Cannabis sativa, the hemp plant, is native to central Asia subcontinent. Throughout history, hemp was extensively used for a broad range of purposes, from fabrication of textile fibers to relief of pain. Cannabis resin has been in disrepute because of its intoxicating effect while some countries allow cannabis for the treatment of various diseases (Antonio Waldo Zuardi., 2006). The effect is based on the cannabinoids, of which Cannabidiol (CBD), Δ9-tetrahydrocannabinol (THC), cannabinol (CBN) are studied best and show such antimicrobial activity**.** Thuja orientalis, which is currently known as Platycladus orientalis, is a distinct genus of evergreen coniferous trees in the Cypress family. It is

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native to many countries active compounds are terpenoids, flavonoids, tannins, etc. which show many applications in medical prospect.

Recently, multiple drug resistance has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of diseases making it a globally growing problem. Natural products of higher plants may give a new source of antimicrobial agents. Thuja orientalis is an evergreen species widely cultivated as a common ornamental plant. The stem of this plant is used in the treatment of coughs, colds, dysentery, etc. The root and bark can be used for the treatment of burns and scalds. Fresh leaves of the plant are used as an anti-inflammatory drug. The most prominent components of the plant are thujone, isothujone, fenchone, etc. α-pinene and α-cedrol are also other major constituents of T. orientalis (Singh and Singh., 2019).

Thin layer chromatography (TLC) is a technique used for the separation of different type of biomolecules in an assortment of components. It is performed on a sheet of glass or plastic or aluminum foil which is coated with a thin layer of adsorbent material, usually silica gel. This layer of adsorbent is known as the stationary phase. It works on the principle that different compounds have different solubilities and adsorption to the new phases between which they are to be partitioned. TLC is a solid-liquid technique in which the two phases are a solid (stationary phase) and a liquid (moving phase), various types of solvent system is used for the separating of different compounds. The distance traveled by the solute is divided by the distance traveled by the solvent. This ratio is called the retardation factor (Rf).

Separation of compounds is based on the competition of the solute and the mobile phase for binding sites on the stationary phase. It is commonly said that "strong" solvents push the analyzed compounds up the plate whereas "weak" solvents barely move them. The order of strength/weakness depends on the coating (stationary phase) of the TLC plate. For silica gel coated TLC plates, the solvent strength increases in the following order perfluoroalkane (weakest), hexane, pentane, carbon tetrachloride, benzene/toluene, dichloromethane, diethyl ether, ethyl acetate, acetonitrile, acetone, water, methanol, acetic acid and formic acid (strongest). Based on these, we choose an appropriate solvent system and run the chromatography process (Goutam et al., 2015).

METHODOLOGY

Collection of sample*:* The samples were collected from patients of pyogenic ailments from Department of Microbiology, GSVM Medical College, Kanpur. Samples were collected using a sterile swab and spread on Mannitol Salt agar plates (Singh et al., 2018)

Media Preparation*:* The culture was obtained to grow on MSA Media (Mannitol Salt Agar). Mannitol Salt Agar is commonly used for the selective and differential medium for the growth of *Staphylococcus spp*. It encourages the growth of a certain group of bacteria while inhibiting the growth of others. It contains a high concentration of salt (NaCl), making it selective for gram-positive bacteria since this level of salt is inhibitory to most other bacteria (Smyth and Kahlmeter., 2005). This is also a differential medium for mannitol-fermenting *staphylococci*, containing carbohydrate mannitol and the indicator phenol red, a pH indicator for detecting acid produced by mannitol-fermenting *Staphylococci. S. aureus* produces yellow colonies with yellow zones. If an organism can ferment mannitol, an acidic byproduct is formed that causes the phenol red in the agar to turn yellow. It is used for the selective isolation of presumptive pathogenic *Staphylococcus species*. The single colony picked with the inoculation loop from the spreading plate were streaked on the plate having MSA media and it is left for overnight incubation at 37ºC for 24 hours, the plate shows the yellow colonies that are *Staphylococcus spp.* (Habib et al., 2015). The biochemical confirmatory test catalase, coagulase, citrate, oxidase and urease were work done through the Wladimir et al., 2000 followed method. Gram staining also was done by (a Combined compendium of food additive specification book., 2005) standardized book method.

Antibiotic Susceptibility Test: It was done as per according to National Collaboration for Clinical and Laboratory Standards (NCCLS) protocols followed by Jan Hudzicki., 2009. Disc diffusion method is used here as an antibiotic sensitivity is the sensitivity of bacteria to antibiotics. It is usually done to determine which antibiotic will be most successful in treating bacterial infection. Small antibiotic discs are placed onto a plate upon which bacteria are growing. If the bacteria are sensitive to the antibiotic, a clear ring or zone is observed around the disc indicating poor growth. Mueller-Hinton Agar with saline solution (MHA) is most frequently used in this antibiotic susceptibility test.

Preparation of Plant Extracts: Leaf extracts of *Cannabis sativa* and *Thuja orientalis* were prepared in an ethanol solvent and later used for checking its antimicrobial activity against MRSA (Singh et al., 2018).

TLC (Thin layer Chromatography) for the separation of Cannabinoids and Thuja Components

The plates were coated with silica gel mixed with water. This mixture was spread as a thin layer on the TLC plate. The plate is dried and activated by heating in an oven for 20-30 minutes at 110°C and thickness of the adsorbent layer is around 0.1-0.25 mm for analytical purpose and around 0.5-2.00 mm for preparative TLC.

For Cannabis components, the solvent systems used were Chloroform: Ethanol (9:1 ratio) 150 ml total solvent was prepared in a 9:1 ratio in a glass jar. It is kept covered and left for 1hour for saturation of the air in the chamber. Using a capillary tube, 1 µl of the *cannabis* and thuja leaf extracts were spotted in different glass sheet at a distance of 1.5 cm from one end of TLC plate. Then allowed for air dry for 10 minutes and again spot the extract for a more concentrated spot (around 4-5 spots). A different number of spots can be spotted at some distance away from each other each of which was moved in its own adjacent lane from its own starting point. The dried TLC plate (10x 15cm) is then placed in the chambers so that one edge dips in the solvent system but the spotted area remains out of the solvent. The lid is then closed. The TLC is allowed to run for about 30-45 mins till the solvent reaches 5cm below the end of the slide. The solvent moves up by capillary action. The slide is removed and the distance traveled by the solvent is

marked. This is the solvent front. Cannabis and thuja extracts plates were analyzed under UV or iodine chamber. Then calculate the Rf value using this formula:-

$$Rf = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by the solvent}}$$

The Rf value obtained is compared with a standard table and the component is identified as per the given standard (**Goutam et al., 2015)**.

For Thuja components, The above steps are applied for Thuja also with a difference in the solvent system. Thuja components need a different ratio of the solvents to get separated. Here Chloroform and methanol in the ratio 8:2, 9:1, 7:3, 6:4 were used.The different spots obtained after TLC were scraped off and stored in sterile vials or tubes and labeled. The anti-microbial activity of these individual spots was then done after elution in 200µl of sterile water or absolute ethanol. The eluents were first centrifuged in 200-500µl of the solvent and supernatant discarded.

Inhibitory Effect of The Different TLC Purified Components: A lawn of overnight grown culture of the isolated MRSA was spread on MHA medium plate, sterile whatman filter paper No 44 discs of 5mm diameter incorporated with 5µl of the different eluates, were air dried under laminar flow and placed gently on the bacterial lawn. The plates were incubated at 37ºC for 24 hours after which the zone of inhibition was recorded. It was compared with those obtained using standard antibiotic discs (Methicillin, vancomycin, Penicillin and Ampicillin).

RESULTS

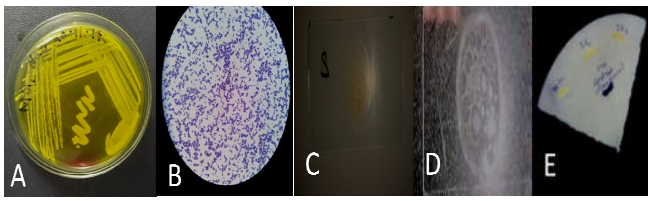
The 3 bacteria isolated were named as MG_1, IR_0 and IR_2.

Isolation of MRSA: Isolate *Staphylococcus aureus* bacteria from clinical and non-clinical samples, It was identified via culture medium and biochemical method after subculturing every week through the streaking method on the Mannitol Salt Agar plates. The colonies on MSA plate appeared yellow colour with a halo around after overnight incubation at 37ºC. A single colony was selected from each plate and purified and maintenance on a fresh MSA slants.

Biochemical Identification: The isolated culture were shown Gram staining-positive, Catalase and Coagulase positive. They are also positive for urease and citrate test, negative for oxidase test done by the book standardized method (a Combined compendium of food additive specification book., 2005).

Table 1 Biochemical test for isolate culture.

Culture name	Catalase	Coagulase	Urease	Citrate	Oxidase
IR_2	+ve	+ve	+ve	+ve	-ve
MG_1	+ve	+ve	+ve	+ve	-ve
IR_0	+ve	+ve	+ve	+ve	-ve



The pictures were observed in the Isolates of *S. aureus* on MSA plates (A), biochemical test (B), Gram staining (C), Catalase test (D) Coagulase test (E) and Oxidase Test

Table 2 Zone of inhibition formed by Antibiotics Sensitivity Test (AST) of known antibiotics.

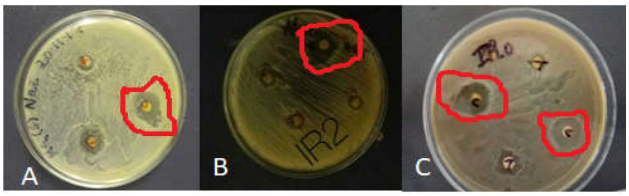
Isolate Name	Zones of inhibition by AST of known antibiotics in mm			
	Methicillin	Vancomycin	Penicillin	Ampicillin
IR_2	26	8	0	0
MG_1	1	7	0	1
IR_0	0	1	0	0

Sample MG_1 and IR_0 were found to be resistant to Methicillin antibiotics. This is Methicillin-Resistant *Staphylococcus aureus* (MRSA).

Pictures were observed in AST of known antibiotics (Vancomycin, Methicillin, Ampicillin and Penicillin). (A) IR_0 culture shows resistance to all four antibiotics were observed, (B) IR_2 culture were observed shows resistance to penicillin and Ampicillin and the clear zone was observed around methicillin and Vancomycin and (C) MG_1 shows resistance to all three whereas a small zone around Vancomycin is seen.

Table 3 Zones of inhibition (mm) formed by plant extracts

Isolate Name	Cannabis sativa	Thuja orientalis
IR_2	14	9
MG_1	17	8
IR_0	19	10



Pictures were analyzed through the zones observed against *C. sativa* and *T. orientalis* extracts are an appearance on red circle MG_1 Culture (A), IR_2 culture (B) and IR_O culture (C). *Cannabis sativa* extract shows more sensitivity than *Thuja orientalis* extracts.

Identified Component of Cannabis and Thuja plant Leaf

Table 4 using the TLC, analysis of psychoactive plant *Cannabis* sativa L components standardized by Goutam et al., 2015.

TLC for separation of Cannabis components:			Standard Value
S. No.	Rf value of the spot (%)	Identified Component	Rf value of spot (%)
1	17	9-THC	0
2	73	CBDA	77
3	86	CBD	88
4	90	CBN	90

Table 5 According to Ojeswi et al., 2010, the standard table referred for the identification of Thuja compounds are as follows.

TLC for separation of Thuja components			Standard Value
S. No.	Rf value of the spot (%)	Component	Rf value of spot (%)
1	11	LEA^1	4
2	66	LEA^2	66
3	81	LEA^3	81
4	89	LEA^4	85

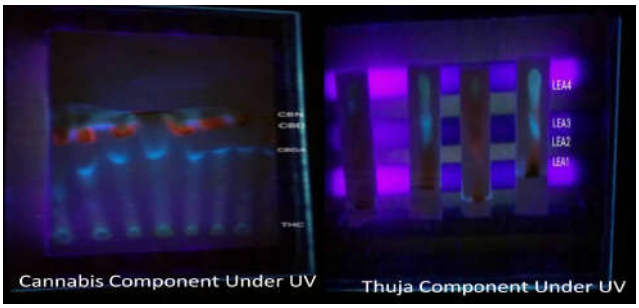
Table 6 Zones of inhibition formed by the Cannabis and Thuja components against MRSA isolate.

Cannabis sativa

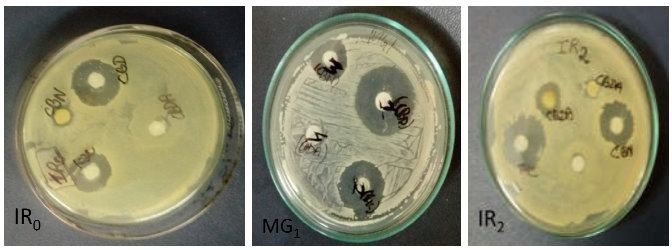
S. No.	Rf value	Component	Zones in mm		
			IR_0	MG_1	IR_2
1	17	9-THC	12	21	16
2	73	CBDA	5	7	5
3	86	CBD	13	20	12
4	90	CBN	7	13	12

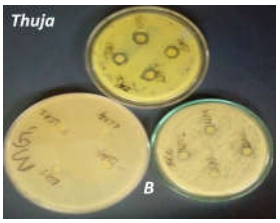
Thuja orientalis

S. No.	Rf value	Component	Zones in mm		
			IR_0	MG_1	IR_2
1	11	LEA^1	6	8	9
2	66	LEA^2	6	8	12
3	81	LEA^3	5	10	11
4	89	LEA^4	9	5	13



Pictures were analysis by TLC for separation of Cannabis and Thuja components observed under UV light.





Pictures were shown on zones of inhibition observed in different components of *C. sativa* and (B) *Thuja orientalis* against MRSA on MG_1, IR_2 and IR_O culture.

DISCUSSION

In this paper study, the various plant extracts show anti-microbial activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA). There are ever increasing reports about the anti-microbial activity of plant extracts. In this work, my aim to find the active principles of plant extracts that were acting against the MRSA have investigated the active components present in two common plants; Cannabis sativa and Thuja orientalis with respect to their anti-MRSA activity. The MRSA is known to be resistant to methicillin and other beta-lactam antibiotics and so they do not respond well to the beta-lactam antibiotics like penicillin, methicillin etc. Basically, we use used the two plants, *Cannabis sativa* and *Thuja orientalis* and have been found to be responding well in inhibition to the growth of MRSA. The extract of ***C. sativa*** leaves showed a more inhibitory effect on the MRSA strain in comparison to ***T.orientalis*** although ***T.orientalis*** also shows some activity. In this work, the components of the two plants were extracted and the anti-microbial activity exhibited by them was observed. On separation, we have seen that each of the components alone responded very well and the growth of MRSA was inhibited by the effect of these components. Compared with Goutam et al., 2015 the components separated from *C. sativa* were CBD, THC, CBN and CBDA out of which CBD, THC and CBN showed the higher inhibitory property. The unknown components were separated from *T. orientalis* extract LEA^1, LEA^2, LEA^3 and LEA^4 for further study (Ojesvi BK et al., 2010). All the four components responded but the components of *C. sativa* showed more better and satisfactory results.

In the future, there is a need to exploit the biologically active metabolites of these plants to combat the resistance of these strains to antibiotics to help to respond to the current health care situation.

CONCLUSION

Most of the fractions obtained after TLC separation are biologically active and show anti-MRSA activity (Table 3). Components separated from Cannabis sativa extracted by Thin Layer Chromatography in Chloroform-Ethanol solvent are CBD, THC, CBN and CBDA. They showed good anti-MRSA activity and components separated from Thuja orientalis extracts by Thin Layer Chromatography in Chloroform-Methanol solvent were LEA^1, LEA^2, LEA^3 and LEA^4 and they also responded to some extent (Table 4 and 5). The plant components responded better than the antibiotics (Table 6: A and B). Hence, these components can be studied further and can be used in the formulation for topical use to cure infections caused by MRSA because of their promising anti-MRSA activity.

Note: The medium was mixed in distilled water and it was autoclaved at 15 lbs at 121ºC for 20 min, after autoclaving the media was allowed to cool then add 18-20 ml/plate poured under the laminar air flow.

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