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# TLC-Bioautography guided screening for compounds inhibitory to Klebsiella pneumoniae from Justicia wynaadensis (Nees) T. Anders

KEYWORDS: Justicia wynaadensis, TLC, Bioautography, Kodagu

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**ABSTRACT** *The increasing occurrence of opportunistic infections as well as infections caused by multi-drug resistant organisms has led to new efforts in the search for novel antimicrobial compounds. Justicia wynaadensis is a scandent herb found growing in the forest and estate regions of Kodagu District. The present investigation deals with the TLC-Bioautography of the methanolic extract of J. wynaadensis, followed by GC-MS analysis of active fractions. TLC-Bioautography resulted in four clearly visible active spots showing large inhibition zones. The antibacterial activity of the methanolic extract of J. wynaadensis against Klebsiella pneumoniae MTCC 3384 could be attributed to volatile components such as phytol and to the fatty acids such as myristic acid, palmitic acid, linoleic acid and stearic acid.*

## INTRODUCTION

The increasing occurrence of opportunistic infections as well as infections caused by multi-drug resistant organisms has led to new efforts in the search for novel antimicrobial compounds. Higher plants are a rich source of molecules with pharmaceutical properties, thus offering themselves as sources of lead compounds for the development of new drugs. The metabolites, secondary metabolites and secretory products contribute to the pharmacological activities of the plant such as analgesic, antitumor, antimicrobial, antihyperglycemic, antipyretic, anti-inflammatory, antiarthritic, antioxidant and immunomodulatory properties (Fabry (1996); Abreu (1999); Woodman (2005).

New plant based drugs can be found by subjecting the plant extracts to chemical screening and investigating their biological activity. A very convenient and simple way of testing plant extracts and pure substances for their effects on both human and plant pathogens is bioautography; TLC-Bioautography allows the screening of plant material and subsequent bioassay-guided fractionation and isolation (Hostettmann and Marston, 1994).

Since the GC-MS chromatogram of the methanolic extract of Justicia wynaadensis, indicated the presence of 24 phytocomponents (Ponnamma and Manjunath, 2012), several of them being antimicrobial, the present investigation deals with the TLC-Bioautography of the methanolic extract of J. wynaadensis tested against the bacterium Klebsiella pneumoniae MTCC 3384, followed by GC-MS analysis of the active fractions.

Justicia wynaadensis is a scandent herb found growing in the forest and estate regions of Kodagu District. Traditionally, the aqueous extracts of the leaves and stem of the plant is consumed by the local population during peak monsoon, when the plant is believed to have acquired maximum medicinal properties. The extract, deep-violet in colour and with a unique flavor, is incorporated in the preparation of dessert, the consumption of which, is said to keep them healthy throughout the year. The plant is said to possess catalase and peroxidase activity; also polyphenols and flavonoids have been identified and estimated (Medapa et al., 2011).

## MATERIALS AND METHODS

Plant Material: The aerial parts of the plant, Justicia wynaadensis (Nees) T.Anders was collected from the estate regions of Kodagu district, Karnataka, India on the 18$^{th}$ day of the locally called 'Kakkada' month of monsoon.

Preparation of the extract and standards: The leaves and stem of Justicia wynaadensis was shade dried, crushed by hand and ground into coarse powder using an electric grinder. To 1 gram of the powdered plant material, 10 ml of methanol was added and boiled for 5-10 minutes over a water bath and filtered. The extract was concentrated to a volume of 2ml.

Inoculum: Colonies of Klebsiella pneumoniae MTCC 3384 from Mueller Hinton agar plates were suspended in sterilized 0.85% sodium chloride solution and added to sterile, cooled (45°C) Mueller Hinton agar medium, to give a concentration of $10^8$ cfu/ml.

Indicator solution for the determination of bacterial growth: 2,3,5- Triphenyl Tetrazolium Chloride (TTC) (6mg/ml); TTC a redox indicator, is reduced to insoluble formazan by actively metabolizing cells, resulting in the formation of pink to red colonies; zones of bacterial growth inhibition will remain colourless.

Positive control: Ampicillin disc (10mcg) was placed on the test plate to check for the inhibitory effect of the antibiotic on the test organism Klebsiella pneumoniae MTCC 3384 and to test the effect of the chromogen, TTC.

***TLC-Bioautography agar overlay process:***

0.1ml of the extract was loaded onto the TLC silica gel $60F_{254}$ Merck chromatographic plate; a reference plate was similarly prepared. Both plates were developed to a distance of 10 cm in the same tank using the pre-determined mobile phase, petroleum ether : ethyl acetate (7:3). The mobile phase was removed from the plates by drying with a stream of cool air and observed under visible light. The $hR_f$ values were recorded.

About 22 ml of the freshly prepared inoculum described above was uniformly spread on the TLC chromatogram using a sterile Pasteur pipette. After solidification, the plates

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were placed in a petridish lined at the bottom with moist cotton wool. The plates were incubated at 35°C for 24 hours, following which they were sprayed with an aqueous solution of TTC (6mg/ml) and incubated further for 4-6 hours to observe for colorless inhibition zones against a red background that would indicate the antimicrobial effect of the chemical in the separated fraction. $hR_f$ of spots showing inhibition were noted. The results are presented in Figure 1.

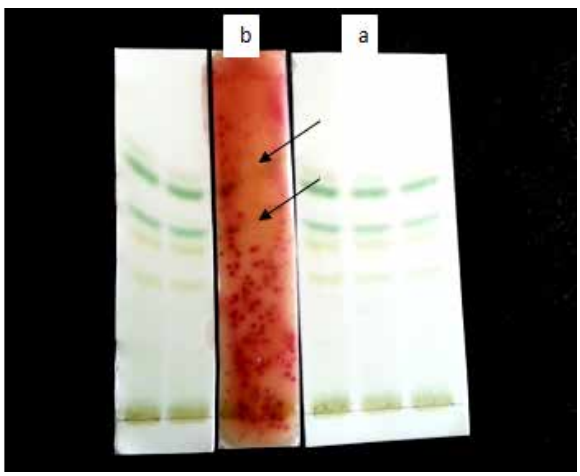


**Fig. 1 Bioautography agar overlay method**
(a) Thin layer chromatogram of the methanolic extract. (b) Bioautogram of the methanolic extract of J. *wynaadensis* (*arrows indicate zones of antimicrobial activity*)

***GC-MS analysis of the active fractions:***
Bands on TLC corresponding to $hR_f$ 88.0 and 83.0 (together as Fraction 1) and bands corresponding to $hR_f$ 69.0 and 64.0 (together as Fraction 2) were recovered by scraping the adsorbent from the reference plate. The bands within these fractions exhibited a single spot of antibacterial activity; hence these fractions were scraped off separately, collected into an Eppendorf vial, eluted with ethyl acetate and subjected to GC-MS analysis.

**RESULT**
TLC-Bioautography resulted in four clearly visible active spots showing large inhibition zones ≥ 10 mm. Active spots were visible around bands of hRf 93.8, 90.0, 88.0, 83.0, 69.0, 64.0. Fraction 1 produced five major peaks corresponding to compounds Neophytadiene, Phytol, Palmitic acid, and Stearic acid, the major component being Palmitic acid followed by that of Phytol. Fraction 2 produced five major peaks corresponding to compounds Myristic acid, Phytol, Palmitic acid, Linoleic acid, Methyl linoleate and Stearic acid the major component beings Palmitic acid followed by that of Linoleic acid and its methyl ester. These compounds have attributed to the antimicrobial activity against Klebsiella pneumoniae MTCC 3384 as has been observed by Bioautography.

Thus, both saturated (Myristic acid, Palmitic acid, Stearic acid) and unsaturated fatty acids (Linoleic acid) have been identified in the methanolic extract of J. wynaadensis.

**DISCUSSION**
Antibacterial activity of diterpene phytol has been demonstrated against S. aureus (Inone et al., 2005). Antimicrobial activity of terpenoids, neophytadiene and phytol, has been studied by Ragasa et al. (2009) and Plaza et al. (2010). Antibacterial activity of palmitic acid, myristic acid, linoleic acid and stearic acid has been demonstrated by Yff et al. (2002), Huang et al. (2011) and Lograda et al. (2012). Desbois and Smith (2010) have reasoned that the mechanisms of antibacterial activity of free fatty acids is primarily targeted on the various essential processes within and at the cell membrane which could be: solubilization of the membrane by the detergent effect of the fatty acids leading to the loss of the vital components of the electron transport chain, cell lysis due to leakage resulting in the insertion of unsaturated fatty acids into the bacterial membrane, impairment of nutrient uptake and inhibition of enzyme activity in the membrane or cytosol.

Suleimana et al. (2010) screened the hexane, acetone, dichloromethane and methanol extracts of seven medicinal plants of South Africa for their antimicrobial activity by the bioautographic procedure. All the extracts exhibited antimicrobial activity against at least one of the test microorganisms; S. aureus and C. neoformans were susceptible to most of the medicinal plants tested.

The results from this study form the basis for further studies to individually isolate the compounds responsible for the observed growth inhibitory effects against Klebsiella pneumoniae MTCC 3384.

**CONCLUSION**
Thus, from our studies, it could be concluded that the antibacterial activity of the methanolic extract of J. wynaadensis against Klebsiella pneumoniae MTCC 3384 could be attributed to the volatile components such as phytol and to the fatty acids such as myristic acid, palmitic acid, linoleic acid and stearic acid. The fractions 1 and 2 have to be further investigated to determine and isolate the antimicrobial component. Also, the non-volatile antimicrobial components need to be investigated.

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