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Research Article

Eradication effects of citrus *hystrix* (kaffir lime) against methicillin resistant *staphylococcus* aureus – an ethnopharmacology approach

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Abstract

Citrus hytrix (Rutaceae) is well known as Kaffir lime was found to be beneficial as essential oil – perfumery. Ethanolic extracts of *Citrus hystrix* leaves revealed the presence of phytochemical constituents *viz*., Saponins, Tannins, Lipids, Flavonoids and Terpenoids. The objective of the present study is to determine the efficacy of ethanolic extracts of *Citrus hystrix* for bactericidal effects against MHSA and also analyzed for antioxidant potentials. Ethanolic extract of *Citrus hystrix* has been used for estimation of bactericidal effects against Methicillin Resistance *Staphylococcus aureus* at various eluted fractionated compounds of *Citrus hystrix*. The peptides possess the high activity subjected for HPTLC fingerprinting analysis. DPPH analysis were also performed to assess the free radical scavenging activity. Antioxidant activity displayed superior activity with an IC_{50} of 2.8µg/ml respectively. Fractions (EF13, EF14, EF15) showed high bactericidal activity against MRSA. Furthermore, HPTLC of the fractionated compound indicate the presence of biological active products - Quercetin, Rutin and Gallic acid with high area. This study suggests that, ethanolic extracts of *Citrus hystrix* leaves exhibits antibacterial activity against Methicillin resistance *Staphylococcus aureus*. Phytomedicines are now an important and beneficial zone of the recommended treatment.

Keywords: *Citrus hystrix*, Methicillin resistant *Staphylococcus aureus*, Antioxidant activity, HPTLC fingerprinting, DPPH.

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is considered as one of the major causes for nosocomial infections and ranked third among the pathogens causing infection. It is a biotype of *Staphylococcus aureus* that has adverse the resistance to β-lactams which enclosed penicillin (methicillin, dicloxacillin, natcillin, oxacillin, etc.), cephalosporins, and carbapenems. The development of such resistance does not make the organism to be more intrinsically virulent than other *S. aureus* strains that are susceptible to these drugs, except that the resistance makes treatment with standard antibiotics more complicate and thus more critical (Chongtrakool et al., 2006). The prevalence of health-care associated MRSA (HA-MRSA) is comparatively higher than health-care associated methicillin-susceptible *S. aureus* in several countries. While, MRSA infections are associated with increased fatality and costs for the healthcare systems in developed countries, the impact can be poor in the developing countries. The spread of HA-MRSA in resource-limited settings can have desolating consequences because of the insufficiency of microbiology laboratories with outbreak investigation capability and the facilities are inadequacy for the bacterial

identification and antimicrobial susceptibility testing as well as the high cost of antibiotic drugs required to treat severe HA-MRSA infections (Khan et al., 2018) leads to high morbidity and mortality and is known as "*superbug*" being resistant to most of the antibiotics (Abirami et al., 2013). In the recent years, many researchers have reported the importance of phytomedicines for management of MRSA. This strategy could significantly reduce the MRSA mediated morbidities. Hence, this study focused to spot the potent compound act against the MRSA through phytochemical approach.

Citrus hystrix DC (Kaffir lime), is a small, thorny bush with aromatic leaves have irregular bumpy surface with dark green fruits found everywhere in Southeast Asia. It is a key ingredient in many countries like Thai, Cambodian, Indonesian, Laotian, Malaysian, and Philippine cuisines (Srisukha et al., 2012). It has folkloric reputation to be used in flu, fever, hypertension, abdominal pains and diarrhea in infants. Many kinds of organic compounds have been detected in kaffir lime leaves extracts - Flavonoids, tannin, saponin, glycoside, coumarine, bergamottin and pinene (Fortin et al., 2002). A number of volatile compounds are reported to have bioactive properties that may be useful in promoting health and fighting disease (Putri Christy et al., 2017). Kaffir lime has huge effective in research and merchandising for aromatherapy and spa practices, solution for insect repellent, production of shampoo, antioxidants compound and cosmetic product (Kasuan et al., 2013). They are effective sources of novel drugs particularly against bacterial pathogens (Srisukh et al., 2012). Kaffir lime oil has effective bactericidal effect such as *Propionibacterium* acnes, 20 serotypes of *Salmonella* as well as other bacteria such as *Bacillus subtilis, Staphylococcus epidermis, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* (Doreen et al., 2011).

Furthermore, the extract of kaffir lime leaf has cytotoxic effect against HL60 (promyelocytic leukemia), K562 (chronic myelocytic leukemia), Molt4 (lymphoblastic leukemia) and U937 (monocytic leukemia) 5 cells (Tunjunga et al., 2015) and the compounds help in the development of new mosquito control agent by using the WHO prescribed methodology (Mya et al., 2015). The individual organic compounds from kaffir lime leaves might have different biological activities. Fractionation is needed to separate and purify those compounds, in order to identify each compound's biological activity and to determine which of these compounds appear to be more biologically active for specific health purposes. Therefore, the objective of this study is to separate and identify the volatile organic compounds of kaffir lime leaves by ethanolic extracts and their fractions. Furthermore, this study will also determine each extract potential acts against the MRSA pathogens.

MATERIALS AND METHODS

Plant collection

The plant of *Citrus hystrix* leaf samples were collected from Thondaputhur, Coimbatore district, Tamil Nadu, India. The leaves were allowed to shade dried at room temperature for about two weeks.

Preparation of plant extract

Shade dried leaves of *Citrus hystrix* was powdered in a blender. 25 g of dried leaf powder were taken in a separate container, to this 175 mL of ethanol was added and kept for 3 days in a shaker at room temperature. The extract was obtained by filtration through Whatmann No-1 filter paper and this extraction process was repeated twice. Thus, the collected extracts were pooled, and dried using a vacuum evaporator before further experiments.

Phytochemical screening

The different solvent (Chloroform, Acetone, Ethanol and Petroleum ether) extracts of *Citrus hystrix* leaves were subjected to preliminary phytochemical tests. Tests were performed to detect phenolic compounds, tannins, saponins, terpenoids, phytosteroids, flavonoids and lipids in the extract as per standard procedure (Kokate, 2004).

Column chromatography of ethanolic extract

The column was packed by wet packing using silica gel adsorbent 60 F254 (Cascioferro et al., 2021) and separation was started by eluting with solvent gradually with increasing order of polarity using different solvents. The entire fraction was collected at regular intervals separately and pooled. The collected fractions were used to detect the antimicrobial activity against MRSA. The fraction that showed activity against the MRSA was used for further study.

Thin layer chromatography

The fractions containing compound were checked by TLC for purity. TLC was performed by using various solvent systems. The identified spots were scrapped from the plate and dissolved in concerned solvent mixture and centrifuged in cooling centrifuge for 15 min. This fraction contains the purified phytochemical were tested against MRSA pathogens.

HPTLC analysis

In HPTLC fingerprinting analysis, 2 µL of the ethanolic extract solution and 2 µL of standards (Rutin, Gallic acid and Quercetin) were loaded as 5 mm band length in Silica gel 60F254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapour) with respective mobile phase (toluene: ethyl acetate: formic acid: methanol (3:6:1.6:0.4) and the plate was stable in the respective mobile phase upto 90 mm. The developed plate was allowed to dried by hot air to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAG 3) and captured the images at white light, UV 254 nm and UV 366 nm (Yamunadevi et al., 2011).

Column chromatography

An appropriate size long cylindrical glass column (based on the amount of the sample) should be stand firm on a column-chromatography stand. Completely dried plant extract sample were mixed with silica gel to make a fine powdered form for easy delivery of sample in already packed silica gel column. Sample powdered mass must be placed on the top of the pre-packed silica column and sample should be covered with a layer of cotton. Then solvents of different polarities were pass through the column at uniform rate under gravity to fractionate the sample extract. Each fraction was collected separately in a test tube and numbered consecutively for further analysis on thin layer chromatography (Bajpai et al., 2016).

Bactericidal activity

Agar diffusion method was carried out for the assessment of antibacterial activity of *Citrus hystrix* leaf samples. Methicillin resistant *S. aureus* strain were previously isolated and identified in our early work. Standard suspension of bacteria was then inoculated on the surface of Muller-Hinton (Himedia) agar plates. The plates have been kept to dry and a sterile cork borer (5 mm in diameter) was used to punch wells in the agar medium at different sites on the plates. Subsequently, wells were filled with fractions of ethanolic extract which was dissolved in Dimethyl Sulphoxide (DMSO) and gently allowed to diffuse at room temperature for 2hrs and incubated at 37°C for 18-24h. Along with this, vancomycin (10mg/disc (HiMedia standard) were used as positive control. All experiments were performed in triplicate and values were expressed as mean ± S.D (Bakkali et al., 2008).

DPPH radical scavenging activity

DPPH was dissolved in methanol to make 0.025 g/L. The plant extract was diluted with dimethyl sulfoxide (DMSO) to get sample solution. 5 µL of the sample solution in a 96-well plate and 195 µL DPPH working solution was added to each well. After a 20 min reaction at room temperature, the absorbance of the solution was measured at 515 nm. The free radical scavenging activity of each fraction was determined by comparing its absorbance with that of a blank solution (Molyneux, 2004). The ability to scavenge the DPPH radical was expressed in percentage inhibition and calculated using the following equation:

ABTS scavenging activity (%) = $(A_0 - A_1)/A_0 \times 100$

where A_0 - absorbance of the control, and A_1 - absorbance of the sample.

RESULTS AND DISCUSSION

Phytochemical investigation suggested the ethanolic extracts showed the presence of phenolic compounds, tannins, saponins, terpenoids, phytosteroids, flavonoids and lipids than other solvents (Figure 1 & Table 1).

Preliminary antibacterial activity

Four different types of solvents were used to collect the plant leaves extracts. Ethanol plants extracts possess good activity against MRSA. Chloroform shows moderate activity against MRSA. The Acetone and Petroleum ether shows no activity against MRSA. Hence, ethanol extracts were used for further studies (Figure 2).

Antimicrobial activity of collected fractions

There are fifteen fractions obtained from column chromatography using different solvent mixtures. The entire fifteen fractions were used to detect the bactericidal activity against MRSA. The F13, F14 and F15 fractions showed good activity against MRSA. Maximum zone of inhibition of 20 ±0.45 mm was obtained for F14 and F15 (Figure 3). Hence, the fractions 13, 14 and 15 were used for further characterization process. Effect of limonene, terpinen-4-ol, and α-terpineol showed inhibitory effect on MRSA (Samraj and Rajamurugan, 2017) which highly correlated with this study.

MRSA bactericidal activity

The F13, F14, F15 fractions were treated with seven different solvent mixtures which were used as mobile phase in TLC plates. Nine spots were observed and scrapped from the plates and used for detecting the antimicrobial activity against MRSA. The S2EF13 (29 mm) spot showed high activity against MRSA. This S2EF13 was identified as quercetin while comparing with the Rf value of standard quercetin (Figure 4).

HPTLC fingerprinting analysis

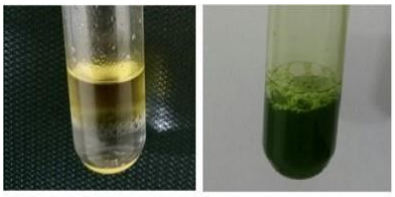
At desired resolution, Rutin, Gallic acid and Quercetin with reproducible peaks were succeeded using toluene: ethyl acetate: formic acid: methanol (3:6:1.6:0.4) as the mobile phase. Figure 5 shows the HPTLC Chromatogram of ethanolic leaf extract of *Citrus Hystrix* with standards of the Rf value of 0.74, 0.18 and 0.84 respectively.

Antioxidant activity

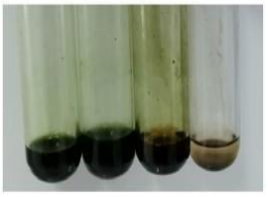
The free radical scavenging activity of the extract was estimated by comparing the percent inhibition of *Citrus hystrix* leaf extract with standard ascorbic acid. The IC_{50} value of extract and ascorbic acid was found to be 2.8 µg/ml and 2.5 µg/ml respectively. This antioxidant activity may be attributed by the flavonoids present in the extract (Butryee et al., 2009). The highest DPPH inhibition was exerted by CHP, with an IC_{50} value of 6.43 µL/mL (Warsito et al., 2018), which was recorded maximum comparatively.

CONCLUSION

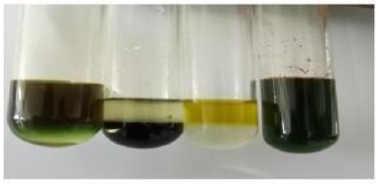
MRSA is formidable, versatile and unpredictable. Its capacity for genetic adaptation and the serial emergence of successful epidemic strains causes it to remain a major



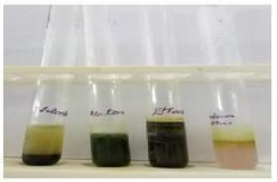
Presence of Saponins



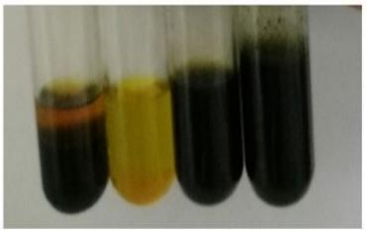
Presence of Terpenoids



Presence of Phytosterols



Presence of Oils and Fats



Presence of Phenolic Compounds and Tannins

Figure 1. Phytochemicals tests of *Citrus hystrix.*

Table 1: Phytochemical analysis of *Citrus hystrix***.**

Test name	Ethanol extract
Carbohydrate	-
Phenolic Compounds and Tannins	+
Amino acid	-
Saponins	+
proteins	-
Glycosides	-
Terpenoids	+
Phytosterols	+
Flavanoids	+
Test for lipids	+

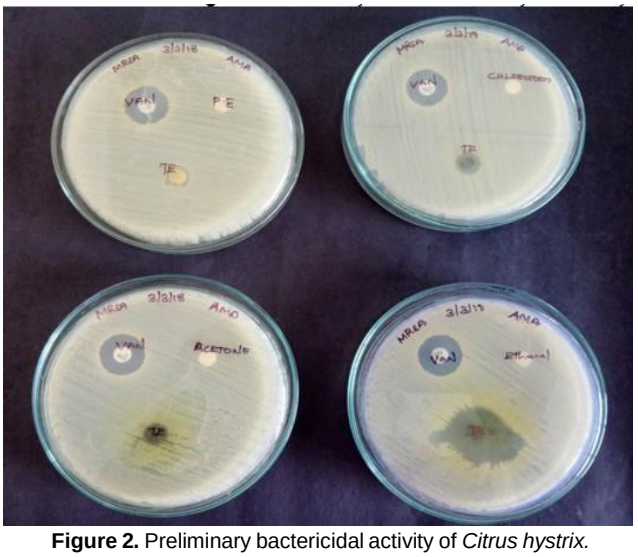


Figure 2. Preliminary bactericidal activity of *Citrus hystrix.*

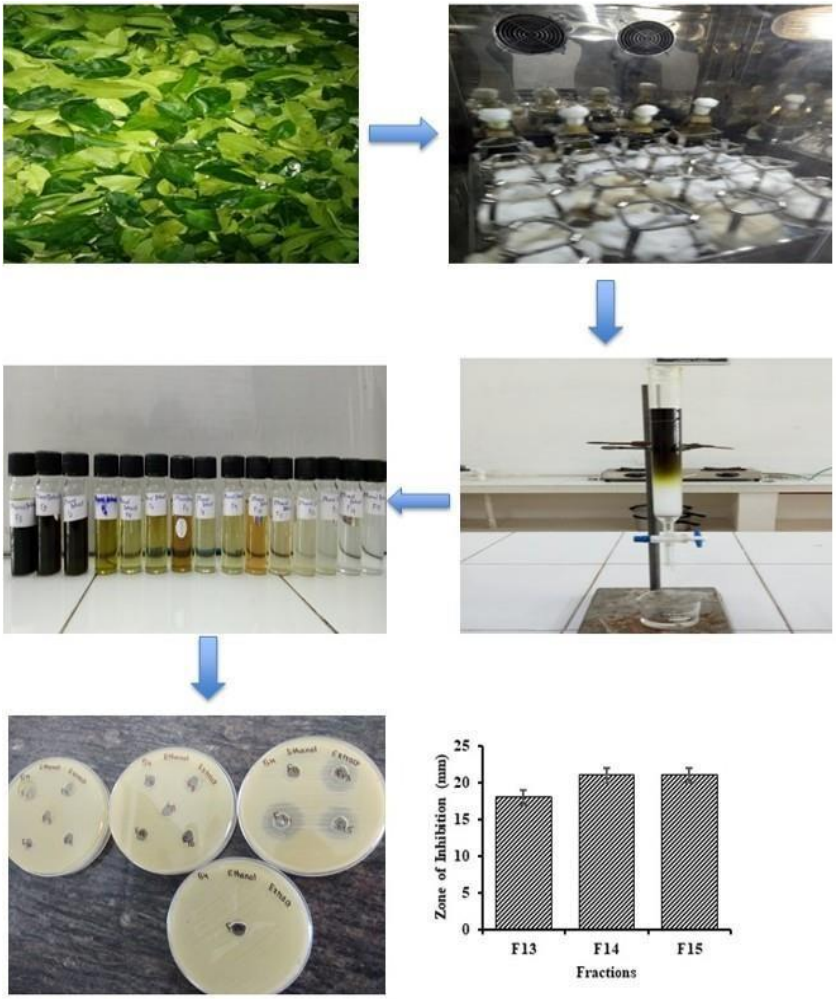


Figure 3. Extraction and Fractionation of *Citrus hystrix*.

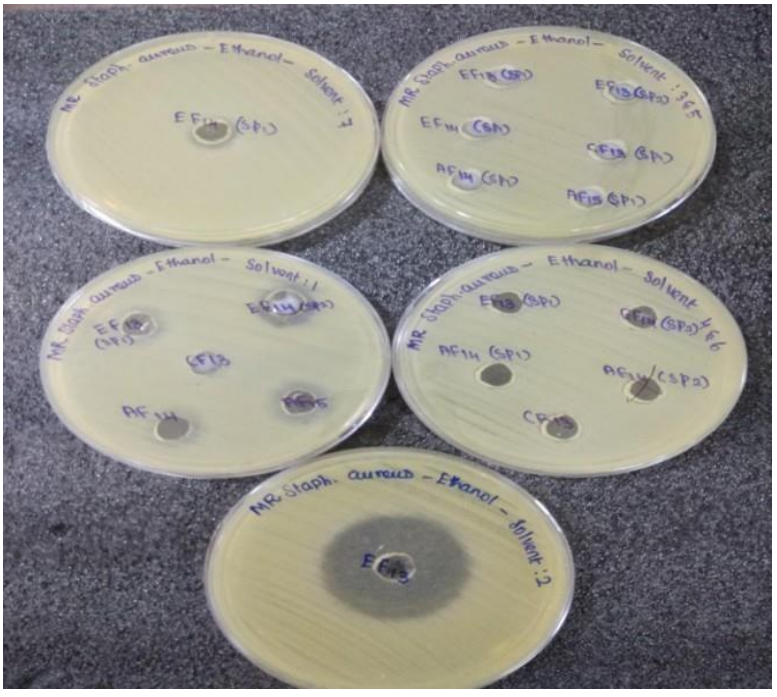


Figure 4. MRSA bactericidal activity.

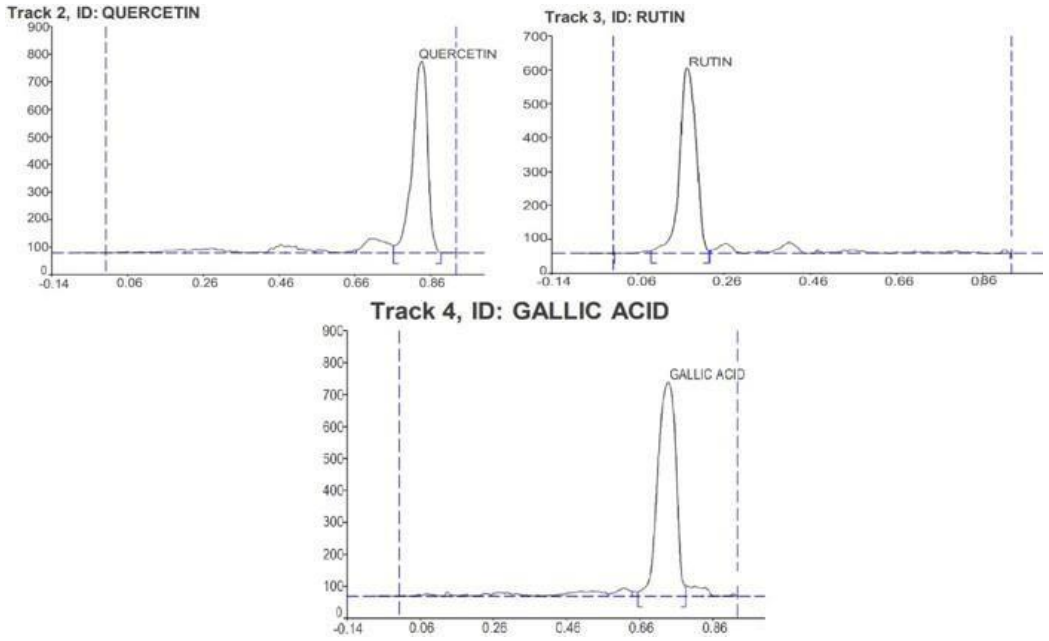


Figure 5. HPTLC fingerprinting analysis

threat to human health. The persistent of high mortality associated with invasive MRSA infection despite the fact that multiple antibiotics with effectiveness against MRSA have been approved by the FDA since 2014 - highlights the need for high-quality trials to determine optimal management for these patients. Phytochemical approaches without side effects can be the future remedy, one such is *Citrus hystrix*. The compound Quercetin, Rutin can may be consider for the betterment of the management of MRSA infections.

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