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ANTIMICROBIAL ACTIVITY OF CRUDE EXTRACT OF CITRUS *HYSTRIX* AND *CITRUS MAXIMA*

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ABSTRACT

Since plants are used as therapeutic agents, the present study was conducted to evaluate the antibacterial activities of methonolic extracts of different components of *Citrus hystrix* and *Citrus maxima* (Red and White) fruit. Studies on the antibacterial activity of methanolic extracts of leaf, peel, and pulp of *Citrus hystrix* and *Citrus maxima* (Red and White) fruit was conducted using agar disc diffusion method. The microorganisms used include *Staphylococcus aureus* (MTCC 3160), *Salmonella typhi* (MTCC 3215), *Escherichia coli* (MTCC 40), *Pseudomonas aeruginosa* (MTCC 424), *Klebsiella pneumoniae* (MTCC 3384). The maximum activity was observed against all organisms except *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The minimum inhibitory concentration ranged between 12.5mg/mL and 200mg/mL depending on microorganism and various extract. *Citrus hystrix* and *Citrus maxima* (Red and White) fruit were observed to have antibacterial activity and can be used for medicinal purposes.

INTRODUCTION: *Citrus hystrix* DC. (Tamil name- kolumichi; Family-Rutaceae) is a small and bushy tree, about 3-5 m tall, which grows all over India and South East regions of Asia [1]. It has folkloric reputation to be used in flu, fever, hypertension, abdominal pains and diarrhoea in infants [2]. The fruits are used as pickle as well as in cooking. The fruit juice is rubbed onto the skin to soften or mixed with bath water to control body odor [3]. It is known as medicinal lime for killing land leeches as an anti-leech rub [4].

The fruits are also used in shampoo as an insecticide for washing the head as a hair shampoo. The stem bark of *C. hystrix* showed mild to moderate antimicrobial activity [5] while the methanolic extract of leaves is known to inhibit the herpes virus [2] and also used as mosquito repellent [6]. The fruit oil contained citronellal, geranial and d-limonene [7].

Citrus maxima, (Tamil name- pamblimasu; Family-Rutaceae) is a medium sized tree; its leaves have the smell and winged petioles. The flowers are bisexual and sweet smell [8]. The fruit is always round shape, big size, a native plant of Asia and commercially grown in India. Two types viz, the white-fleshed and red or pink-fleshed are available in India and named accordingly. In traditional medicine, the fruit peel has been used for cough, swelling, and epilepsy, because of the effectiveness of the volatile [9]. The root bark contains β-sitosterol and several acridone alkaloids, and coumarins which shows antimicrobial activity.

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Citrus flavonoids have a large spectrum of biological activity including antibacterial, antifungal, antidiabetic, anticancer and antiviral activities [10, 11]. Flavonoids can function as direct antioxidants and free radical scavengers, and have the capacity to modulate enzymatic activities and inhibit cell proliferation [12]. In plants, they appear to play a defensive role against invading pathogens, including bacteria, fungi and viruses [13].

Flavonoids are generally present in glycosylated forms in plants, and the sugar moiety is an important factor determining their bioavailability. Preparation from peel, flowers and leaves of bitter orange (*Citrus aurantium* L.) are popularly used in order to minimize central nervous system disorders [14]. In addition the fiber of citrus fruit also contains bioactive compounds, such as polyphenols, the most important being vitamin C (or ascorbic acid), and they certainly prevent and cure vitamin C deficiency-the cause of scurvy [15]. The peel of *Citrus* fruits is a rich source of flavonoid glycosides, coumarins, β and γ-sitosterol, glycosides and volatile oils [16]. Many polymethoxylated flavones have several important bioactivities, which are very rare in other plants [17].

An antimicrobial is a substance that kills or inhibits the growth of microorganism such as bacteria, fungi or protozoans. Antimicrobial drugs either kill microbes (microbicidal) or prevent the growth of microbes (microbistatic). Disinfectants are antimicrobial substances used on nonliving objects. The aim of the present study was to determine the antibacterial activity of various extracts of *Citrus hystrix* and *Citrus maxima* (Red and White) fruit and leaf samples. Antibacterial sensitivity test was conducted against standard antibiotics such as Gentamycin, Ciprofloxacin, Amikacin, Tetracycline and Streptomycin.

The antimicrobial activity of the methonolic extract of citrus fruits at different concentration viz, 1000µg and 2000µg were determined by disc diffusion assay [(18)]. The results obtained were expressed as the zone of inhibition (mm).

In this study, the anti-microbial effects of these three plant extracts were tested against some human bacterial pathogens. This is an attempt to provide scientific basis for the use of these extracts in traditional medicinal practice and the possibility of using this knowledge to produce new antimicrobial agents that may be effective against some of the emerging and re-emerging bacterial pathogens.

MATERIALS AND METHODS:

Plant material: The plant of *Citrus hystrix & Citrus maxima* (Red and White) fruit and leaf samples were collected from Mayiladuthurai, Nagai district, Tamil Nadu. Fresh fruit and leaves sample were collected during the month of April 2010. The fresh fruit samples were separated into peel, pulp and juice. Juices were collected in a separate container and stored under -20°C for further analysis. Thus obtained fruit peel and pulp and leaves were dried at room temperature for about ten days and ground into fine powder using electric blender. The powdered fruit and leaf samples were stored in a separate container until further analysis.

Preparation of Extract:

1. **Extract from Dried Powder:** Each 25 g of *Citrus hystrix & Citrus maxima* (Red and White) dried leaf, peel, pulp powder were taken in a separate container, to this 175 mL of methanol was added and kept for 48 h in a shaker at room temperature. Filtered through Whatmann No-4 filter paper and extract was collected, the extraction process was repeated twice. Thus the collected extracts were pooled. The various extracts of *Citrus hystrix & Citrus maxima* fruit were concentrated using vacuum evaporator and dried at 60°C. The dried extract powder was used for the study and screened against selected bacterial strains.

Antibacterial Assay:

1. **Microorganisms Tested:** The following microorganisms were used for the Study. Standard strain of *Staphylococcus aureus* (MTCC 3160), *Klebsiella pneumoniae* (MTCC 3384), *Pseudomonas aeruginosa,* (MTCC 424), *Salmonella typhi* (MTCC 3215), *Escherichia coli* (MTCC 40). The microorganisms were purchased from the MTCC; imTECH, Chandigarh, India. They were maintained on agar slants at 4^{o}C in the refrigerator.

2. ***In vitro* determination of Antibacterial Activity:** Stock cultures were maintained at 4°C on slants of nutrient agar. Active cultures for experiments were prepared by transferring a loopful of colonies from the stock culture to peptone water and incubated for 4h at 37°C. Antibacterial activity was determined by agar disc diffusion method [18]. Standard suspension of bacteria was inoculated on the surface of Muller-Hinton (Himedia) agar plates. Dimethyl Sulphoxide and Methanol (1:1) mixture was used to dissolve the plant extract. Sterilized filter paper discs (5mm) containing 20µL of each extract (100mg/mL) were arranged on the surface of the inoculated plates and incubated at 37^{o}C for 18-24h. Along with this, Gentamycin, Ciprofloxacin, Amikacin, Tetracycline, Streptomycin (10mg/disc (Himedia standard) was used as positive control and paper disc treated with DMSO was used as negative control. Each extract was analyzed in triplicate was studied for antimicrobial activity.

3. **Minimum Inhibitory Concentration (MIC):** The minimum inhibitory concentration values were determined by broth dilution assay. Varying concentrations of the extracts (200mg/mL, 150mg/mL, 100mg/mL, 50mg/mL, 25mg/mL and 12.5mg/mL) were prepared. 0.1mL of each concentration was added to each 9mL of nutrient broth containing 0.1mL of standardized test organism of bacterial cells. The tubes were incubated at 37°C for 24h. Positive controls were equally set up by using solvents and test organisms without extracts. The tube with least concentration of extract without growth after incubation was taken and recorded as the minimum inhibitory concentration [19].

RESULT AND DISCUSSION: The present study carried out on the plant extract revealed the possession of medicinal activities. *In vitro* antibacterial activity of methanol extracts of leaves, peel and pulp of *Citrus hystrix & Citrus maxima* (Red and White) fruit were observed (**Table 1**). The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments [20]. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances e.g. the phenolic compounds which are part of the essential oils, as well as tannin [21]. According to Draughon [22] the presence of flavonoids, tannins and alkaloids may be the responsible for activity against the pathogenic bacteria.

The extracts were found to have moderate activity against all organisms. Methonol extracts of leaves and pulp were found to have maximum activity compared to peel extracts against all microorganisms. Leaf extract of *Citrus hystrix* have been shown to have moderate activity (11.5-9.5 mm) against all five microorganisms followed by the *Citrus maxima* (Red and White) leaf extracts. Peel extract of *Citrus hystrix* have been shown to have moderate activity (10.5-8.5 mm) against all five microorganisms followed by the *Citrus maxima* (White) leaves extracts. Whereas, Peel extract of *Citrus maxima* (*red*) has been shown to have the lowest activity (8-9mm) against all five microorganisms. Pulp extract of *Citrus maxima* (red) has registered moderate activity (12-8 mm) against all five microorganisms followed by the *Citrus maxima* (White) and *Citrus hystrix* pulp extracts.

TABLE 1. *IN VITRO* ANTIBACTERIAL ACTIVITY OF METHANOL EXTRACTS OF LEAVES, PEEL AND PULP OF *C. HYSTRIX & C. MAXIMA* (RED AND WHITE) FRUIT. (VALUES ARE MEAN OF THREE REPLICATES)

STRAIN (MTCC code no)	INHIBITION ZONES diameter (mm) INDUCED													
	CHL	CMRL	CMWL	CHP	CMRP	CMWP	CHPU	CMRPU	CMWPU	GEN	CIP	TET	AMI	STR
***S. aureus* (3160)**	10	11.5	11	8.5	8	10	10	11	11	18	15	20	18	16
***K. pneumoniae* (3384)**	11	9.5	9.5	9	9	8	7	8	6.5	16	18	15	25	20
***P.aereuginosa* (424)**	11	11	10	13	8	9	11	12	11.5	20	25	18	22	17
***S.typhi* (3215)**	11.5	11	10.5	10.5	9	10	9	9	10	16	21	22	20	20
***E.coli* (40)**	10.5	10	11.5	10	8	9	9	10	9.5	14	15	14	13	12

(CHL- *Citrus hystrix* leaf; CMRL- *Citrus maxima* Red leaf; CMWL- *Citrus maxima* white leaf. CHP- *Citrus hystrix* peel; CMRP- *Citrus maxima* Red peel; CMWP- *Citrus maxima* white peel. CHPU- *Citrus hystrix* pulp; CMRPU- *Citrus maxima* Red pulp; CMWPU- *Citrus maxima* white pulp; GEN-Gentamycin; CIP-Ciprofloxacin; TET- Tetracycline; AMI-Amikacin; STR-Streptomycin).

The minimum inhibitory concentration ranged between 12.5 mg/mL and 200mg/mL depending on microorganism and various extracts are shown in **Table 2**. The result of minimum inhibitory concentration suggests that methonolic extract of *Citrus hystrix & Citrus maxima* (Red and White) fruit could possibly act as a bactericidal agent to these microorganisms. Highest minimum inhibitory concentration were shown to the leaves, peel, and pulp extract of *Citrus hystrix & Citrus maxima* (Red and White) against *S. aureus* and *P.aereuginosa* followed by *S. typhi*, and the lowest minimum inhibitory concentration were shown to the leaves, peel, and pulp extract of *Citrus hystrix & Citrus maxima* (Red and White) against *E. coli* followed by *K. pneumonia*. The present investigation confirmed the antimicrobial activity of leaf, peel and pulp extract of *Citrus hystrix & Citrus maxima* (Red and White).

Table 2. Minimum inhibitory concentration of methanol extracts of leaves, peel and pulp of *C. hystrix & C. maxima* (Red and White) fruit. (Values are mean of three replicates).

MICROORGANISMS	MINIMUM INHIBITORY CONCENTRATION(MIC) SAMPLES(mg/ml)								
	CHL	CMRL	CMWL	CHP	CMRP	CMWP	CHPU	CMRPU	CMWPU
***S. aureus* (3160)**	50	12.5	25	75	75	25	25	12.5	12.5
***K. pneumoniae* (3384)**	25	75	50	50	50	75	75	75	100
***P. aereuginosa* (424)**	25	50	50	12.5	50	50	25	25	12.5
***S. typhi* (3215)**	25	50	25	25	50	50	50	75	75
***E. coli* (40)**	50	75	12.5	25	75	50	75	50	50

(CHL- *Citrus hystrix* leaf; CMRL- *Citrus maxima* Red leaf; CMWL- *Citrus maxima* white leaf. CHP- *Citrus hystrix* peel; CMRP- *Citrus maxima* Red peel; CMWP- *Citrus maxima* white peel. CHPU- *Citrus hystrix* pulp; CMRPU- *Citrus maxima* Red pulp; CMWPU- *Citrus maxima* white pulp; GEN- Gentamycin; CIP- Ciprofloxacin; TET- Tetracycline; AMI- Amikacin; STR-Streptomycin).

Ontengco *et al*., [23] reported that the essential oil of the pomelo shows *in vitro* activity against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922, and were found to have significant potential as a broad-spectrum antibacterial raw material for galenic preparations. Rodrigues et al., [24] confirmed that the experiments performed in Guinea-Bissau, limes were shown to prevent or reduce food-borne transmission of *Vibrio cholerae*. They may therefore be considered an effective protectant against cholera when added to food.

Cano *et al*., [25] reported the higher antimicrobial activity of *Citrus paradise* due to the presence of essential oils, vitamin C and flavonoids. Dhanavade *et al*., [26] reported the presence of essential oils, protopine and corydaline alkaloids, lactons, polyacetylene, acyclic sesquiterpenes, hypericin and pseudohypericin compounds from lemon peel are effective toward inhibition of various bacteria. The extract in solvent ethanol found to have higher antimicrobial activity against tested microorganisms in comparison with the extracts of lemon peel in other solvents like methanol and acetone. The study reveals that the peel of lemon is not only an astringent but also is a good antimicrobial agent.

Mathur *et al*., [27] reported that the aqueous extract of citrus fruits peel and pulp contained the significant antimicrobial activity. Mokbel and suganuma [28] reported the antimicrobial activity of methanol extract albedo of pomelo fruit agaist the several bacteria. Okwu *et al*., [29] confirmed the antifungal activity of citrus plants extract on Okra plant against the *Fusarium oxysporum* fungi and they suggested the antifungal compound from citrus plants which may used as alternative to synthetic fungicides. This antibacterial activity may be of the presence of metabolic toxins or broad spectrum antibiotic compounds.

CONCLUSION: Plants produce chemicals known as secondary metabolites that are not directly involved in the process of growth but acts as deterrents to insects and microbial attack. These plant secondary metabolites include several classes such as terpenoids, flavonoids and Phenolic compounds in the citrus fruits having diverse chemical structures and biological activities. Antimicrobial activity of the leaf, peel and pulp extract is directly concerned with the bioactive compounds that they contain. This antibacterial activity may be indicative of the presence of metabolic toxins or broad spectrum antibiotic compounds.

The present study exhibited the antibacterial effect of various extracts of *Citrus hystrix & Citrus maxima*. The inhibitory effect of the extract justified the medicinal use of *Citrus hystrix & Citrus maxima* and further study is required to find out the active component of medicinal value. Thus it can be said that leaves, peel and pulp of citrus fruits are useful for consumption and are beneficial for health. This study may lead to the formulation of a nutraceuticals.

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