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Isolation of Quercetin and Kaempferol From Azadirachta Indica Leaves and Its Synergistic Action Against Clinically Isolated Multi Drug Resistant Strains MRSA

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Review Article

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ABSTRACT

The aim of this study was to isolate the two major flavonoids Quercetin and kaempferol by P-HPLC from *Azadirachta Indica* leaves and its activity against clinical isolated MRSA strains. The flavonoids were separated and identified by analytical column, while isolated by preparative column Intersil ODS-3 C-18 and its composition as in analytical procedure. Antibiotics (Cefixime, Ceftriaxone, Erythromycin, Ciprofloxacin, Cephradine, Methicillin, Ampicillin, Levoflaxacin and Amoxicillin) and flavonoids (quercetin, kaempferol and combination of quercetin + kaempferol) were evaluated against clinically isolated MRSA. The results showed that the Cefixime, Ceftriaxone, Ciprofloxacin, Cephradine, Methicillin, Ampicillin and Amoxicillin were found inactive against clinical isolated MRSA when tested alone, but when combine with quercetin, kaempferol and Q+K showed valuable anti-MRSA activity, while tested flavonoids showed were found to be no impact on Erythromycin and causing reduction in Levoflaxacin and Ciprofloxacint.

INTRODUCTION

Phenolic compounds are important secondary plant metabolites reported for diverse and significant biological and pharmacological activities, like anti-bacterial, anti-fungal, anti-viral, anti-ulcer, anti-allergenic and anti-inflammatory [1-4]. These compounds also appear to be effective in certain kind of cancerous process and some cardiovascular diseases [4,5]. Phenolic compounds are also used as natural antioxidant or as food additive to preserve food for organoleptic and nutritional quantities [6,7]. Flavonoids is one of the important and largest class of phenolic compounds, generally known to be present in plant and plant base product, associated with a broad spectrum of health-promoting effects [8]. The presence and concentration of these compounds varies even in same plant at different ripening stages and organs of the same plant. Some plants are found to be an excellent source of phenolic compounds and suggested to use for preservation of food as well as to get health benefits [9]. Quercetin and kaempferol are the basic flavonoids possess antioxidant, antimicrobial, antiviral, antimutagenic and antiinflammatory properties [10].

Azadirachta Indica (A. indica) is known by Urdu name Neem, is a member of mahogany family Meliaceae [11,12]. Different parts (roots, stem, bark, leaves, flower, fruits, and seeds) of the *A. indica* have been used traditionally for the treatment of infections, inflammation, fever, dental disorders and skin diseases [13-15]. More than 140 active compounds have been isolated from *A. indica*, including phenolic compounds, alkaloids, triterpenoids, flavonoids, carotenoids, ketones and steroids [16-19].

MATERIAL AND METHODS

Collection of Bacterial Cultures

Staphylococcus aureus (n=100) were collected from the microbiology laboratory of tertiary care hospitals Khyber Teaching Hospital, Lady Reading Hospital and Hyathabad Medical complex Peshawar KPK Pakistan. The collected MRSA strains were carried out to Microbiology microbiological laboratory (ISO-17025) PCSIR labs complex Peshawar and cultured on the same day within 2 hours of collection. The resistant strains were isolated and stand for further analysis.

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Collection of Plant

The *A. indica* was collected from botanical garden of Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories Complex Peshawar, KPK, Pakistan. The fresh fully green leaves were selected at the initial stage of maturity during April- July 2012. All the sample leaves were washed with plenty of water, dried in vacuum oven at 40°C for 48hours. The dry leaves were crashed with commercial grinder and sieved. The powder sample was stored in poly ethylene bags in desiccating cabinet for further analysis.

Preparation of Extract

20 g of dry leaves were macerated with 200 ml distilled water. The aqueous extract was fractionated with 50 ml petroleum ether to remove fatty material. The aqueous extract was then extracted with 50ml ethyl acetate. The ethyl acetate extract was dried over anhydrous H_2SO_4 and evaporated to dryness under N_2 stream at 40°C.

Characterization of Quercetin and Kaempferol by HPLC

The two flavonoids were separated by analytical column Intersil ODS-3 C-18 (5 µm, 4.6 × 250 mm) using mobile phase acetonitrile and aqueous solution of 0.2% formic acid composition 70:30 at a flow rate of 1 ml/min in total run time of 20 minutes by using UV-Vis detector.

Isolation of Quercetin and Kaempferol by P-HPLC

The scaled preparative chromatographic procedure was similar to the analytical procedure, having the ability to collect the peak of interest. The quercetin and kaempferol were isolated and achieved by semi preparative column Intersil ODS-3 C-18 (5 µm, 6.0 × 250 mm), using mobile phases and its composition as in analytical procedure. The acceptable preparative separation achieved using 0.10 ml injection. The flow rate was set of 10 ml/minute.

RESULTS

Collection of Quercetin and Kaempferol

The quercetin and kaempferol was identified by external standard analytical HPLC procedure. Fraction collection was setup through software and the peaks collected in window from 7.0 to 8.0 for quercetin and 11.0 to 12.0 for kaempferol, while the other peaks outside of the window were not collected. The purity of collected compounds showed UV area% purity of 86 and 89% for quercetin and kaempferol.

Quercetin and Kaempferol Against MRSA

Quercetin, kaempferol and quercetin + kaempferol (Q+K) were evaluated against clinical isolated MRSA. Quercetin and kaempferol are found to be more active then combination. The zone of inhibition of quercetin (500 µg/ml) was 13.42 mm and kaempferol (500 µg/ml) was 18.30 mm, while zone of inhibition of Q+K (250+250 µg/ml) was 10.30 mm of the combination against MRSA. The MIC value of these compounds were determined by dilution method and given in **Table 1**. The MIC of quercetin was found 410 µg/ml and kaempferol 370 µg/ml.

Antibiotic Sensitivity Assays

Antibiotics and flavonoids were evaluated against MRSA strains. Most of the antibiotic were found inactive when tested alone, potentiate the activity when combine with quercetin, kaempferol and Q+K. 500 µg/ml of quercetin showed highest activity 18.0 mm on Cephradine disc and lowest 4.5 mm with ciprofloxacin. The kaempferol showed highest activity 22 mm against MRSA when combine with Ceftriaxone, while lowest 7.0 mm when combine with ciprofloxacin. The combination of Q+K comparatively showed lowest activities when combine with antibiotics. Quercetin and kaempferol were found to more active than Q+K combination.

Table 1. Average Zone of Inhibitions in (mm ± Standard deviation) of antibiotic alone and with flavonoids against Clinical Isolated MRSA strains.

S. No	Antibiotic	Alone	Quercetin	Kaempferol	Q+K
			With Antibiotic		
1	Cefixime	00.0	13.6 ± 0.48	18.2 ± 0.91	11.12 ± 0.51
2	Ceftriaxone	00.0	17.5 ± 0.57	22.5 ±1.20	12.3 ± 0.73
3	Erythromycin	21.5 ± 1.42	21.0 ± 0.83	21.0 ± 0.89	21.0 ± 0.94
4	Ciprofloxacin	10.0± 1.09	04.5 ± 0.14	07.0 ± 0.26	04.2 ± 0.19
5	Cephradine	00.0	18.0 ± 0.59	13.0 ± 0.48	07.0 ± 0.13
6	Methicillin	00.0	16.0 ± 0.43	18.5 ± 0.61	08.4 ± 0.53
7	Ampicillin	00.0	17.0 ± 0.57	16.0 ± 0.57	13.7 ± 0.51
8	Levoflaxacin	16.2 ± 1.24	11.23 ± 1.15	08.3 ± 1.08	04.1 ± 0.41
9	Amoxicillin	00.0	16.5 ± 0.51	20.0 ± 0.94	10.0 ± 0.34

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MIC of Flavonoids and Flavonoids-Antibiotics by Incremental Increase Approach

MIC of quercetin, kaempferol, Q+K alone and combination with antibiotics against MRSA were determined by half dilution method is given in **Table 2.**

Table 2. MIC of Antibiotic with flavonoids.

S. No	Antibiotic	Quercetin	kaempferol	Q+K
			With Antibiotic	
1	Cefixime	462 ± 2.0	478 ± 3.0	500 ± 3.0
2	Ceftriaxone	460 ± 4.0	360 ± 1.0	480 ± 2.0
3	Cephradine	446 ± 3.0	480 ± 4.0	586 ± 2.0
4	Methicillin	464 ± 2.0	438 ± 2.0	584 ± 4.0
5	Ampicillin	462 ± 5.0	462 ± 2.0	468 ± 3.0
6	Amoxicillin	460 ± 4.0	384 ± 2.0	488 ± 2.0

The MIC of Quercetin, Kaempferol and Q+K was determined to test the susceptibility and resistance of microbial strains. The results showed the MIC value of quercetin was found 460 µg/ml against MRSA when combined with resistive drugs i.e. Cefixime, Ceftriaxone, Methicillin, Ampicillin and Amoxicillin, while 440 µg/ml when combine with Cephradine.

The MIC value of kaempferol was found in range 360 to 480 µg/ml against MRSA in combination with different drugs. Its MIC value was 360 µg/ml when combine with Ceftriaxone, 380 µg/ml with Amoxicillin and 440 µg/ml with Methicillin, 460 µg/ml with Ampicillin and 480 µg/ml with Cephradine and Cefixime. Catechins showed activity in range of 480 to 500 µg/ml against MRSA. The MIC value of catechins was 500 µg/ml against MRSA when combine with Cefixime, Cephradine, Methicillin and Amoxicillin, while 480 µg/ml when combine with Ampicillin and Ceftriaxone. The gallic acid was found resistant against MRSA below 500 µg/ml when combine with Cephradine, Methicillin, Ampicillin and Amoxicillin, while inactive below 480 µg/ml when combine with Ceftriaxone and Cefixime.

DISCUSSION

In vitro assessment the antimicrobial susceptibility tests are used to find out the resistance of microbial strains against an antimicrobial agent. These agents produce naturally by microbes or synthetically in laboratory. Antibiotic, antiviral, antifungal and other plant bioactive compounds such as phenolic used as antimicrobial agents.

In current study different antibiotics (Cefixime, Ceftriaxone, Erythromycin, Ciprofloxacin, Cephradine, Methicillin, Ampicillin, Levoflaxacin and Amoxicillin), and flavonoids (quercetin, kaempferol and Q+K) were evaluated against *MRSA*. Many researchers isolated the flavonoids from plants sources that possessed antibacterial activity, Such as apigenin by Basile, et al [20], galangin by Cushnie and Lamb [21], Naringenin by Rauha, et al. [22], Luteolin by Sato, et al. [23], quercetin by Cushnie and Lamb [21], rutin by Hendra, et al [24] and kaempferol by Cushnie and Lamb [21]. Dhayanithi, et al. [25] studied the leaves extract of different plants, showed the activity against *MRSA* strains, which was resistant against Methicillin, vancomycin and ciprofloxacin. Liu, et al. [26] studied the effect of kaempferol on antibiotic resistant bacteria MRSA they found that combination of kaempferol greatly affected fluoroquinolones. The antibiotic Cefixime, Ceftriaxone, Ciprofloxacin, Cephradine, Methicillin, Ampicillin and Amoxicillin were found inactive when tested alone, potentiate the activity when combine with quercetin, kaempferol and Q+K. while tested flavonoids have were found to be no impact on Erythromycin and causing reduction in Levoflaxacin and Ciprofloxacin002E **(Figures 1 and 2).**

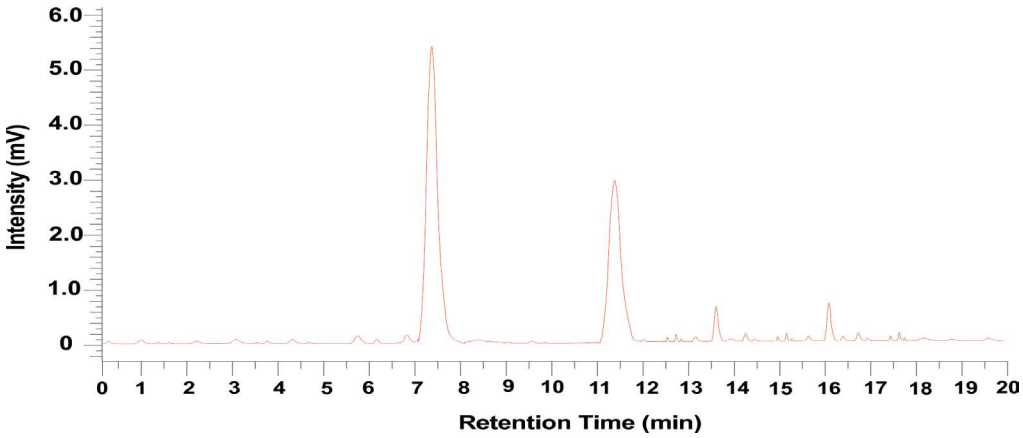

Figure 1. HPLC Chromatogram of Identification Quercetin and Kaempferol.

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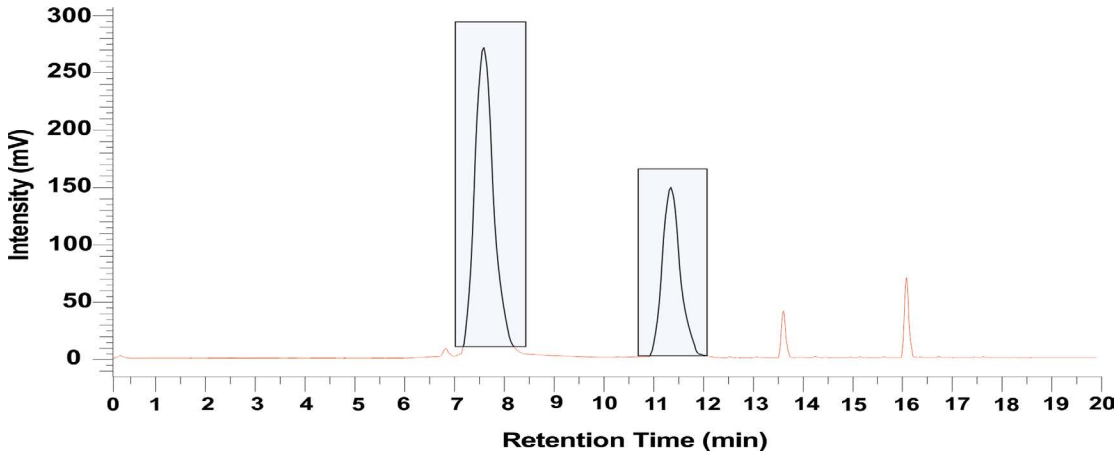

Figure 2. HPLC Chromatogram of Isolation of Quercetin and Kaempferol.

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