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Augmentation Effects of Novel Naringenin Analogues and Ciprofloxacin as Inhibitors for Nora Efflux Pump (EPIs) and Pyruvate Kinase (PK) Against MRSA

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Abstract: New naringenin derivatives bearing halogen moiety (1-6) were designed and synthesized. The structure of the newly synthesized compounds was elucidated by elemental analyses and spectral data. The in-vitro antimicrobial activity of the obtained compounds has been evaluated. All new analogues tested show inhibitory activity against microorganisms under investigation, the most potent compounds are 1-4. The rest of compounds show nearly same antimicrobial activity as ciprofloxacin reference drug used in this study. The new analogues showed also augmentation effect when used together with ciprofloxacin.

Key words: Naringenin analogues, pyruvate kinase, NorA efflux pump, MRSA

INTRODUCTION

The emergence of MRSA strains resistant to methicillin and other antimicrobial agents has become major concern because of the higher mortality due to systemic MRSA infections (Tarai and Kumar, 2013). Kingdom of Saudi Arabia is considered potential spot for the collection of Methicillin-Resistant Staphy lococcus aureus (MRSA) because up to 6 million of their populations are expatriates from many countries. In addition, KSA hosts about four million Muslim pilgrims from all over the world (Yousef *et al*., 2013).

Many efflux pumps are encoded chromosomally and their presence enhances resistance mediated by microorganisms as MRSA. The *S. aureus* chromosome codes for homologues of NorA efflux pump. A single pump can provide bacteria with resistance to a wide array of chemically and structurally diverse chemicals which constitutes a great challenge for development of novel antibiotics or resistance-modifying agents (Hermenean *et al*., 2013).

Recently, MRSA Pyruvate Kinase (PK), an enzyme essential for *S. aureus* growth and survival was suggested to be as a potential *S. aureus* drug target (Zoraghi *et al*., 2011).

Naringenin is a bioactive flavanone comprising many activities. It is recently reported to have antibacterial activity against both G+ve and G-ve bacteria, moreover it inhibits growth of *Klebsiella pneumonia* strain which is resistant to Ciprofloxacin broad-spectrum antibiotic. Moreover, it was reported for some naringenin analogues and synthetic derivatives to possess antimicrobial activity against MRSA through inhibition of NorA efflux pump activity (Rai and Kon, 2013).

To modulate the pump action in order to inhibit the efflux of drugs, the newly designed inhibitors should exhibit superior pharmacophore fit of flavonolignan due to its high activity as NorA inhibitors. Naringenin analogues were also reported to inhibit the enzymatic activity of MRSA pyruvate kinase which is a key enzyme for *S. aureus* growth and survival.

So, as a continuation for our previously published work in development of new antimicrobials, herein we designed, synthesised, elucidated the structure of newly synthesised naringenin derivatives. Then pharmacological investigation against MRSA was carried out to be tested as NorA efflux pump modulators and inhibitors of PK activity.

Bacterial multidrug efflux pumps are the major contributors of microbial resistance to several classes of antibiotics (Weinstein and Hooper, 2005; Webber and Piddock, 2003). Different pumps can efflux specifically a drug or group of drugs such as the NorA system that transports different antibiotics and large variety of molecules (Poole, 2000, 2005; Levy, 2002; Neyfakh *et al*., 1993). The problem of antibiotic efflux can be overcomed by addressing any of the following four strategies:

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- Inhibiting drug binding to the cytoplasmic membrane pumps
- Inhibiting interaction of different components of a multicomponent pump
- Targeting energy sources of a pump
- Targeting the regulatory network that controls the expression of efflux pumps (Kumar and Schweizer, 2005; Kumar *et al*., 2012)

Combination of an antibiotic with an Efflux Pump Inhibitor (EPI) would be expected to restore the effectiveness of antibiotics that at present cannot be used any longer. Combination therapy might even synergistically increase the susceptibility of the bacteria (Holler *et al*., 2012; Tegos *et al*., 2011).

MRSA Pyruvate Kinase (PK), an enzyme essential for *Staphylococcus aureus* growth and survival was suggested to be as a potential *Staphylococcus aureus* drug target (Zoraghi *et al*., 2010). MRSA PK was chosen since this protein was found to be essential for network integrity and therefore, represents an unexploited and attractive target for the development of novel classes of antimicrobials (Zoraghi *et al*., 2011).

Flavanones inhibited MRSA PK enzymatic activity (NorA+PK) in addition flavanones reported also to inhibit NorA efflux pumps (Bremner, 2007; Sasaki *et al*., 2012). So, if flavanone analogues underlie both mechanisms, they can act as a poly-anti-MRSA agent to the ordnance of current and past antibiotic therapies.

Naringenin is one of the flavanones present in grapefruits and tomatoes and it has a wide range of pharmacological properties, including anti-oxidant, anti-fibration, anti-cancer, anti-atherogenic and anti-proliferative activities (Yoon *et al*., 2013; Raza *et al*., 2013).

Naringenin could be a potential agent in the treatment of *S. aureus* infections (Tsuchiya and Iinuma, 2000). Anti-virulence drugs are recommended to be used in combination with conventional antimicrobials to extend the useful lifespan of the antibiotics (Zhang *et al*., 2013). It could potentially be used synergistically with α-lactam antibiotics. The limitations in using naringenin on wide bases are; its scarcity and its solubility problem in aqueous solution, so more derivatives have to be designed for obtaining more bioavailable and potent agents. So, it can be used as starting material for the synthesis of other novel antimicrobials with enhanced activity through different mechanisms of action.

While a number of synthetic and natural product inhibitors of the NorA efflux pump are known, there is still a need to develop new and more potent inhibitors (Bambeke *et al*., 2000; Walmsley *et al*., 2003; Pages *et al*., 2005; Kaatz, 2005; Poole, 2005). As the 3D structure of this protein is still not available, a ligand based approach wasfunctionalized using CATALYST program. The advantage of ligand based approach is that the only requirement is a collection of ligands that interact similarly with the target (Kurogi and Guner, 2001). CATALYST (Accelrys) is a computer program designed to assist the generation of ligand based pharmacophores.

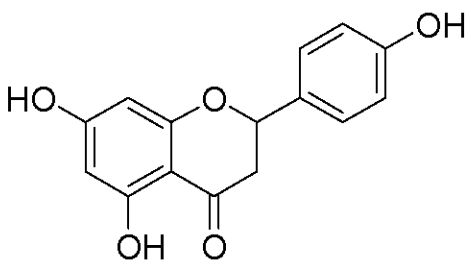


Fig. 1: Naringening

Due to the high activity and superior pharmacophore fit of flavanone class of molecules (Morel *et al*., 2003), naringenin, one of these structures wasused as the starting point for the de novo design as NorA EPIs. And also the new derivatives was tested for PK activity inhibition so as to act as poly-anti-MRSA agents (Fig. 1). From all the above findings, the aim of this research work was:

- To use a pharmacophore to design a range of novel active naringenin analogues using CATALYST program
- To synthesise these compounds via conventional synthetic procedures besides microwave assisted synthesis
- Elucidation of the newly synthesised compounds's structures via microanalytical methods and spectroscopic ones as IR, Mass, 1H-NMR, 13C-NMR
- Pharmacological investigation of new compounds for NorA efflux pump inhibition alone and in combination with ciprofloxacin on clinical MRSA local strains isolated from Kingdom of Saudi Arabia to evaluate the possible augmentation effects
- MIC for ciprofloxacin alone and naringenin derivatives each alone then combination of ciprofloxacin and each of the newly synthesised compound
- Pharmacological testing of the new compounds for enzymatic inhibition of Pyruvate Kinase in the selected MRSA strain.
- Structure activity relationship study

MATERIALS AND METHODS

Design of NorA inhibitors based upon naringenin structure and pharmacophore generation.y overlaying naringenin structure on the pharmacophore and removing unnecessary features, a backbone compound was identified. Then naringenin structure was optimised via different substitutions to maximize the interaction with the pharmacophore features (Sutter *et al*., 2000). In addition to the maximal receptor interaction, ease of synthetic accessibility was also considered in the design of these compounds.

Pharmacophore generation settings: Hypo Gen® and Hypo Refine® from CATALYST® 4.9: hypotheses 10, Spacing 300, Min points 4, Min Subset Points 4, Superpsition Error 1. Misses 1, Feature Misses 1, Complete Misses 0, Tolerance Factor 1, Check Superposition 1, Weight Variation 0.3, Mapping Coeff 0, Memm 60, Ideal HB ond Geom Only 0, Variabble Weight 1, Variable Tolerance1 (Li *et al*., 2000).

The CATALYST ® program will run on Silicon Graphics Fuel workstation, consisting of a 600 MHz MIPs R14000 (IP35) CPU, SGI Origin 3000 system architecture and running under the IRIX64 6.5.23m operating system.

Chemistry

Chemical synthesis: Synthetic pathways (Bovicelli *et al*., 2007) was followed to obtain different analogues and derivatives for naringenin

Naringenin: Regioselective glycosylation of naringenin by Koenigs-Knorr method (Oyamaa and Kondo, 2004). Different mono and di-substitutions at position 6 and 8 was carried according to reported procedures. General procedure for the halogenation of naringenin (Bovicelli *et al*., 2007).

To a 0.01 M solution of naringenin and NaX (1 equivalent for every halogen atom to be introduced in the substrate) in a 1:1 acetone/water mixture, the time for the reaction was monitored by Thin-Layer Chromatography (TLC) (hexane/ethyl acetate 1:1). The mixture was then extracted with ethylacetate. The organic extracts were washed with NaCl solution, dried over anhydrous sodium sulfate and the solvent evaporated under vacuum. The compound obtained was purified by flash column chromatography after the workup.

Reaction for disubstituted naringenin hologen formation: Same previous reaction was held to achieve direct addition of 2 moles of halogen to the 6, 8-positions of the aromatic ring of naringenin. Heating the mixture in presence of a catalyst to achieve iso-1,3-dione derivatives (Abulrob *et al*., 2004). Same products were also obtained at room temperature but with extended time.

Attempted microwave -induced synthesis: Naringenin was added together with different halogens in the specified solvents to microwave flask. The reaction then was programmed to the specified heat, time and pressure. Target compounds were obtained in 15-20 min.

Chemicals and reagents: Naringenin and the different reagents used for the chemical reactions, together with the suitable solvents as ethanol and methanol was purchased from Sigma-Aldrich. The designed compounds that was prepared in this study was synthesized by standard synthetic procedures.

Verification of structures of the newly synthesised compounds: All the newly synthesised naringenin derivatives were subjected to structure elucidation via microanalytical analysis and spectral as: IR, Mass, 1H-NMR, 13C-NMR. The flash points were measured. The IR spectra of the compounds was recorded by FT-IR spectrometer with KBr pellets. 1H and 13C NMR spectra was recorded using a Bruker 300 NMR spectrometer operating at 400.13 and 100.77 MHz, respectively. Microanalyses was obtained with an Elemental analyses system (element analyzer). The purity of the compounds was checked by TLC on pre-coated SiO_2 gel (200 mesh) aluminium plates and visualized in UV chamber.

Pharmacological and microbiological investigations

Bacterial strains: The bacterial strains consisted of epidemic *S. aureus* which is antibiotic-sensitive. MRSA clinical isolates strains wasobtained from appropriate sources from Saudi Arabia hospitals. Their antibiotic susceptibility and resistance was described

MIC determination (Lechner *et al*., 2008)

Antibacterial activity test by Minimal Inhibitory Concentration (MIC): The MICs of naringenin analogues and ciprofloxacin were determined in a microdilution assay utilizing an inoculum of 100 μL MRSA-KSA-ICU strain suspended in saline solution at 0.5 of the McFarland scale was added in Brain Heart Infusion (BHI) in Eppendorfs. Then, each sample was transferred to 96-well microtiter plates and serial dilutions of each substance were performed with concentrations ranging from 0.5-512 μg mL^{-1} The plates were incubated at 37°C during 24 h and bacterial growth was determined using the sodium Resazurincolorimetric method. The MIC was defined as the lowest concentration in which no bacterial growth was observed, according to CLSI. The

antibacterial assays were performed in triplicate and the results were expressed as average of the replicates.

Evaluation of efflux pumps inhibition by reduction of MIC: To analyze whether naringenin analogues and ciprofloxacin might affect the efflux pump activity, we evaluated the potential of these substances to decrease the MIC of ciprofloxacin. The inhibition of the efflux pump was evaluated using sub-inhibitory concentrations of naringenin derivatives and a solution containing inoculums, suspended in saline solution at 0.5 of the McFarland scale was added in Brain Heart Infusion (BHI) in Eppendorfs. Then, each sample was transferred to 96-well microtiter plates and serial dilutions of each substance were performed with concentrations ranging from 0.5-512 μg mL^{-1} The plates were incubated at 37°C during 24 h and bacterial growth was determined using the sodium Resazurin colorimetric method. Ciprofloxacin MICs were used as controls. The antibacterial assays were performed in triplicate and the results were expressed as average of the replicates.

Statistical analysis: All experiments were made in triplicate. The data were analyzed using two-way ANOVA and the Tukey's post test using GraphPad Prism Software 5.0 (GraphPad, San Diego, CA). The values are expressed as geometric means and the differences with $p<0.05$ were considered significant.

According to Abdel-Nasser A. briefly MIC determinations wasundertaken for naringenin alone, ciprofloxacin alone and a combination of the two to determine intrinsic antibacterial activity. The MIC for the combination of ciprofloxacin and each of the naringenin analogues was determined and compared with the combination of m-chlorophenylhydrazone CCCP (0.75 mg L^{-1}) and ethidium bromide. The range of ethidium bromide concentration was used at 0-100 mg L^{-1} plates was incubated at 37°C for 24 h and the MIC was taken as the lowest concentration that inhibited growth.

Modulation assay for efflux pump activity: Coldham *et al*. (2010). The new compounds were further screened for their synergistic effects with EtBr prior to the efflux assays, according to modification of a method described by Lechner and co-workeres. The concentration of the compounds was kept the same throughout the experiment whereas the EtBr was serially diluted for MIC determination with and without new compounds. A Modulation Factor (MF) was used to express the Modulating Effects of compounds on the MIC (EtBr), where MF5MIC (EtBr)/MIC (EtBr+compound). EtBr accumulation assay by the fluorometric method (Coldham *et al*., 2010; Rodrigues *et al*., 2008). Then efflux assay was carried out as follow.

EtBr efflux assay by the fluorometric method: The effect of the new agents on EtBr efflux activity was measured according to a modification of recently reported fluorescence techniques (Coldham *et al*., 2010; Rodrigues *et al*., 2008). The following condition steps was used:

- Accumulation
- Centrifugation
- Replication
- Monitoring the EtBr efflux from the cells

Assay of pyruvate kinase activity inhibition: Naringenin and its novel synthesised analogues was assayed for their ability to inhibit enzymatic activities of MRSA Pks. PK activity was determined using a continuous assay coupled to lactate dehydrogenase in which the change in absorbance at 340 nm because of oxidation of NADH, measurement using a Benchmark Plus microplate spectrophotometer.

Initiation of reactions: Expression of PK activity as specific activity (μmol/min/mg) which is defined as the amount of PK that catalyzes the formation of 1 μmol of either product per minute. Application of Naringenin and its novel synthesised analogues. IC50 values was calculated by curve fitting on a four-parameter dose response model with variable slope using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA). All values determined represent three measurements, each in triplicate.

Statistical analysis: Data was expressed as mean± standard deviation. Statistical analyses and significance, was measured by the Student's t-test for paired samples was performed using Prism software version 4.0 (GraphPad Software, CA, USA). In all comparisons, $p<0.05$ was considered statistically significant.

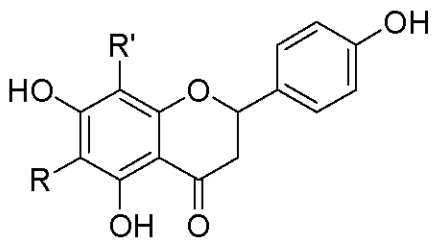
RESULTS AND DISCUSSION

Chemistry: The following novel mono and disubstituted narengenin analogues were obtained. Products were obtained in deep orange-brown coloured thick oil. All target compounds were structurally confirmed and all spectral and micro analytical data come into accordance with postulated following structures:

- Fluoro-2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)chromen-4-one (1)
- 6-Chloro-2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)chromen-4-one (2)
- 6-Iodo-2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)chromen-4-one (3)

Table 1: *In vitro* activities of new naringenin analogues alone against MRSA-KSA-ICU strain and/OR in combination with ciprofloxacin

Compounds	MIC (μg mL^{-1})	MIC (μg mL^{-1}) in combination with ciprofloxacin
Naringenin	0.6	0.30
1	0.2	0.05
2	0.4	0.20
3	0.5	0.30
4	0.1	0.01
5	0.4	0.20
6	0.5	0.30



1, R= F, R' = H
2, R= Cl, R' =H
3, R= I, R' = H
4, R=R'=F
5, R=R'=Cl
6, R=R'=I

Fig. 2: Chemistry chromen

- 6,8-Difluoro-2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl) chromen-4-one (4)
- 6,8-Dichloro-2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl) chromen-4-one (5)
- 6,8-Diiodo-2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl) chromen-4-one (6)

Target compounds were newly designed and synthesised in order to assess their antiviral activities and their activities in combination with ciprofloxacin, the reference antibiotic used in the current study. All compounds revealed extra bands in IR spectra attributed to the introduced halogen atoms, confirming their incorporation. ^{1}H-NMR spectra displayed shift in signals attributed to OH group due to adjacent halogen substitution. In addition, signals attributed to protons at positions 6 and 8 disappeared, confirming halogen substitution at these positions (Fig. 2 and Table 1).

CONCLUSION

Microbiological investigation: Antibacterial activity test by Minimal Inhibitory Concentration (MIC). From the results obtained in the previous table, it is clear that naringenin and its new analogues exert antibacterial activities against MRSA-KSA-ICU. This activity is increased upon combination with ciprofloxacin. Fluoro analogues 1 and 4 displayed the most potent activities alone and in combination with ciprofloxacin.

Naringenin and its new analogues presented a MIC 0.1-0.6 μg mL^{-1} and as such they do not exhibit clinically relevant antibacterial activity. However, when associated with ciprofloxacin (MIC = 0.5 μg mL^{-1}) against MRSA-KSA-ICU strain carrying efflux pumps, naringenin and its new analogues reduced the MIC values of ciprofloxacin. Substances that reverse bacterial resistance when associated with antibiotics by reducing their MIC are defined as "modifiers of the antibiotic activity" which can alter the bacterial susceptibility to antibiotics by inhibiting microbial efflux pumps (Costa *et al*., 2008, 2013; Kim *et al*., 2013). Thus, if a substance causes a reduction in the MIC = 3 dilutions when combined with the inhibitor, this is an indicative that this substance affects the bacterial resistance by inhibiting the efflux pump activity (Davies and Wright, 1997).

This comes into accordance with previous work by Prorokova *et al*. (2015), who proved in their research the improvement of antibacterial activity through direct fluorination. In conclusion, naringenin derivatives exert synergestic activity with ciprofloxacin against MRSA- KSA-ICU strain. Fluioro derivatives1 & 4 were the most potent.

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