Pyrazole, Isoxazoline and Bpyrimidine Derivatives from Polygonum senegalense and Psiadia punctulata Flavonoids and their Anti-Microbial Activities

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Original Article

ABSTRACT
Currently, researchers have given special attention to the synthesis of heterocyclic compounds with nitrogen and oxygen five or six-membered ring systems. This is mainly due to their wide range of biological activities. Hence, in this study, isoxazoline, pyrazole and bipyrimidine derivatives were synthesized from flavonoids, previously isolated from Polygonum senegalense and Psiadia punctulata, and assessed for their anti-fungal activity. A flavone was reacted with hydrazine hydrate to afford a pyrazole analogue 5-methoxy-2-(5,2,3,4,5-tetramethoxyphenyl)-1H-pyrazol-3-yl-benzene-1,3-diol (1). Two isoxazoline derivatives namely, 2,4(5)-dihydro-5-phenylisoxazol-3-yl)-5-methoxybenzene-1,3-diol (2) and 2(4,5)-dihydro-5-phenylisoxazol-3-yl)-3,5-dimethoxyphenol (3) were successfully synthesized by the reaction of chalcones with hydroxylamine hydrochloride. An oxime derivative (4) was also generated from a similar procedure. A reaction between a chalcone and thiourea gave a bipyrimidine derivative, 4,5-dihydro-6(2,4-dihydroxy-3,6-dimethoxyphenyl)-4-phenylpyrimidine-2(1H)-thione (5). The products were then assessed for their anti-bacterial and anti-fungal activity.

All compounds showed no significant activity except compound 2 that demonstrated activity against standard anti-bacterial agent, Strep tokoccus aureus with IC50 value of 7.56 and anti-fungal Candida neoformans, Candida krusei and Candida glabrata strains with IC50 values 8.01, 8.11 and 13.74 μg/mL respectively. We, therefore, recommend synthetic optimization of compound 2 as a potential anti-microbial agent.

Key words: Bipyrimidine, Isoxazolines, Oxime, Pyrazole, Chalcones

INTRODUCTION
Pyrazoles fused with different heterocycles are known to contribute to various chemotherapeutic effects and have emerged as anti-microbial, anti-fungal and anti-viral agents. 1-3 Similarly, a large number of pyrimidine derivatives have been reported to exhibit anti-microbial1,2 and anti-fungal3 activities.

The isoxazolines possess a variety of significant and diverse pharmacological activities including anti-fungal, anti-bacterial, anti-muscarinic, anti-inflammatory, anticancer and anti-depressant activities.1 Consequently, there have been extensive studies on these compounds as promising disease-controlling agents.1 In addition, different pyrimidine derivatives have remarkable pharmaceutical importance because of their biological activity as anti-HIV, anti-tubercul and anti-diabetic compounds.2,3 In view of the above mentioned facts, it was thought of interest to synthesize some new heterocyclic compounds containing isoxazoline, pyrazole and pyrimidine rings and examine their anti-microbial properties. The substrates were flavonoids of Polygonum senegalense,10 and Psiadia punctulata flavonoids.11

Polygonum senegalense has been found to be rich in flavonoids, especially chalcones.5,10 This class of compounds has been extensively employed as intermediates in the synthesis of heterocyclic and carbocyclic systems.2,12 This is due the presence of reactive keto-ethylenic group in their general skeleton. A natural flavone, 5,9-dihydroxy-2′,3′,4′,5′-tetramethoxy-flavone from Psiadia punctulata,11 was reacted with hydrazine hydrate to produce the semi-synthetic pyrazole product 1 (Equation 1). The protocol followed was a modification of the procedures of Hassan et al. (2010).3 Two chalcones were successively converted to isoxazoline derivatives, 2 and 3 (scheme 1). An effort to convert a chalcone to a substituted oxazole by reacting it with hydroxylamine hypochloride and sodium acetate in ethanol, in accordance with a modified procedures of Ragini et al., 2010,12 yielded an oxime (4) analogue instead (scheme 1). A substituted bipyrimidine derivative (5) was also obtained by reacting a chalcone, 3,6-dihydroxy-2,4-dimethoxychalcone, with thiourea (scheme 1).
Pyrazole, isoxazoline and bipyrimidine analogues

MATERIALS AND METHODS

Procedure for preparation of pyrazole derivative
A sample of 100 mg of flavone 8-hydroxy-6-methoxy-3-(2,3,4,5-tetramethoxyphenyl)naphthalen-1-(4H)-one was dissolved in 10 ml of ethanol and excess of hydrazine was added, according to modified procedure of Essam et al (2012). The mixture was poured into a quick-fit flask fitted with a thermometer and a refluxing condenser and refluxed for 3 hours on a magnetic stirrer. The mixture was allowed to stand overnight. Shiny pale yellow crystals from ethanol were filtered and washed with excess ethanol.

General procedure for preparation of isoxazoline analogues
A sample of 100 mg of a chalcone was mixed with 10 ml of hydroxyamine hydrochloride in 15 ml pyridine. The mixture, in a quick-fit flask fixed with a thermometer and a refluxing condenser and on a magnetic stirrer, was heated in a reflux for 3 hours (the reaction was monitored by Thin Layer Chromatography, TLC). The reaction mixture was allowed to cool and then poured over crushed ice in a 250 ml beaker. A few drops of concentrated HCl were added. A precipitate formed that was filtered and washed with a lot of water. A TLC analysis was done and product (isoxazoline derivative) was crystallized out of the mixture from ethanol.

Procedure for preparation of oxime derivative
Anhydrous sodium acetate (0.02 mol) was dissolved to 10 ml of acetic acid. Hydroxylamine hydrochloride (0.01 mol) in ethanol (10 ml) was added to the solution of 2'-4'-dihydroxy-6'-methoxychalcone (50 mg) in ethanol. The solution of sodium acetate in acetic acid was transferred to this reaction mixture in a quick-fit flask fitted with a thermometer and refluxing condenser on a magnetic stirrer and refluxed for 8 hr at temperature range of 40-60°C. The progress of the reaction was monitored by TLC. The completion of the reaction was poured into ice cold water. A brown precipitate that formed was washed with excess water and spotted on a TLC. The precipitate gave a single spot with Rf 0.53 in 90 % CH₂Cl₂ in n-hexane.

Procedure for preparation of bipyrimidine derivative
An amount of 100 mg of a,b-unsaturated chalcone, (E)-1-(2,4-dihydroxy-3,6-dimethoxynaphthalen-1-(4H)-3-phenylprop-2-en-1-one, was mixed with 100 mg of thiourea and dissolved in 20 ml of ethanol. A catalytic amount of KOH was added. The mixture, in a quick-fit flask fitted with a thermometer and refluxing condenser and on a magnetic stirrer, was heated on reflux for 12 hours. It was then transferred to a 250 ml beaker into which crushed ice was poured. A few drops of HCl were added. A precipitate formed, which almost immediately was filtered out and washed with excess water to afford product 5. It registered an Rf value of 0.46 in 40% EtOAc (ethyl acetate) in n-hexane and a yield of 59%. The spectral data is recorded in table 1.

In vitro anti-microbial activity assay
The anti-microbial susceptibility assays were done using Clinical and Laboratory Standard Laboratory Institute (CLSI) method. The positive controls were ciprofloxacin (≥ 98% purity assessed by HPLC at ICN Biomedicals, Ohio) for bacteria and amphotericin B (≈ 80% purity assessed by HPLC at ICN Biomedicals, Ohio). The test organisms, C. albicans (ATCC 90028), C. glabrata (ATCC 90030), C. krusei (TCC 6258), A. fumigatus (ATCC 90906), C. neoformans (ATCC 9011), S. aureus (ATCC 29213), Methicillin-resistant S. aureus (ATCC 33591), E. coli (ATCC 35218), P. aeruginosa (ATCC 27853) and M. intracellulare (ATCC 23068) were obtained from the American Type Culture Collection, ATCC (Manassas, Virginia).

RESULTS AND DISCUSSION

structure elucidation
5-Methoxy-2-(5-(2,3,4,5-tetramethoxyphenyl)-1H-pyrazol-3-yl) benzene-1,3-diol (1)

It was isolated as a light yellow solid with Rf value of 0.63 in 2% MeOH in CH₂Cl₂ and a yield of 74% w/w. Electron Spray Ionization High Resolution Mass Spectrometry (ESI-HRMS) spectrum of this product gave molecular ion peak at m/z 402 (1), which was consistent with its molecular formula, C₂₀H₁₉N₂O₂. In ¹H-NMR spectrum, characteristic imine and amine C-5 and C-3 peaks appeared at δ 147.7 and 149.9 respectively. They appeared downfield due to aromaticity of pyrazole ring and deshielding anisotropic effects in unsaturated systems. The NH proton was also downward shifted to δ 11.50 because of the deshielding effects of the heteroatomic nitrogen. A summary of both ¹H- and ¹³C-NMR chemical shift assignments is given in Table 1. Scheme 2 below outlines the proposed mechanism for this conversion.

An attempt to convert another flavone, 5,7-dihydroxy-3,4’-dimethoxyflavone, in the same way was not successful. This could be attributed to the bulky methoxy group at C-3 which hinders nucleophilic attack on C-2 (see Scheme 3).

2-(4,5-dihydro-5-phenylisoxazol-3-yl)-5-methoxy

Scheme 2: Proposed mechanism for the conversion of flavone to pyrazoles
Pyrazole, isoxazoline and bypyrimidine analogues

**Scheme 3**: Conversion of 3-methoxysubstituted flavones to pyrazoles

**Scheme 4**: Proposed mechanism for conversion of chalcones to isoxazoline analogues

with the axial and geminal CH₃ protons and formed doublet of doublets in the range δH 5.18-5.22 (Jeq=12.0, Jen=4.0). The methine proton was downfield of CH₂ because the former experiences more deshielding anisotropic and electron withdrawing effect of the benzene ring and the heteroatomic oxygen respectively; otherwise a proton on a sp³ carbon in a five-membered cyclic compound appears below δH 3.00.

Another characteristic peak is that of quaternary aromatic C-6 of the monosubstituted aryl ring typically appearing at δH 140.0. The two pairs of chemically equivalent carbons, C-7/11 and 8/10 produced intense signals at δH 128.9 and δH 126.9 respectively. C-9 at the para position resonated at δH 128.8. The corresponding protons of these ArCs were observed as multiplets in the region of δH 7.33 to 7.49.

In 'H-NMR, the signal at 11.42 was due to phenolic proton of C-2' which undergoes strong chelation by the imine group. A summary of spectral and 13C- and 'H-NMR assignments are recorded in Table 1.

**Scheme 5**: Reaction of 2',4'-dihydroxy-6'-methoxychalcone with ammonium hydroxide

**Scheme 6**: Stepwise synthesis of oxime analogues from a,b-unsaturated chalcones

**Oxime derivative (4)**

A reaction between 1',4'-dihydroxy-6'-methoxychalcone and hydroxylamine hydrochloride, adopted for synthesis of an oxazole, gave an oxime derivative as revealed by NMR analyses.

It was obtained as an amorphous brown solid with Rf 0.53 in 90% CH₂Cl₂ in n-hexane and a yield of 78%. Both 13C-NMR and DEPT spectra of the compound showed the chemical shifts of the two methylene carbons at δC 28.2 and 33.7 in the upfield region of 13C-NMR spectrum. The triplets at δC 3.04 (Jen=8.0) and δC 3.15 ('H=8.0) were due to protons bonded to these carbons. The imine carbon was typical at δC 153.5.

The 'H-NMR signal at δH 10.80 was due to proton of the phenolic hydroxyl group on C-2'. This proton experiences great deshielding caused by chelation by the imine moiety. However, the imine group proton was highfield shifted and appeared as a broad signal at δH 3.25 because of the immense shielding from oxygen to which it is directly bonded. A summary of spectral data is given in Table 1.

The formation of the oxime derivative followed 1, 2-addition reaction where there was nucleophilic attack on the chalcone carbonyl carbon (see Scheme 6).

**4,5-dihydro-6-(2,4-dihydroxy-3,6-dimethoxyphenyl)-4-pyrimidinylthione (5)**

This a brown solid with Rf of 0.48 in 40% EtOAc in n-hexane and a yield of 57%. HRMS showed its molecular ion at m/z 346.1921 which was in agreement with the formula C₁₇H₁₆O₅N₂S. Both 'H- and 13C-NMR signals characteristic were observed. The thiocarbonyl carbon (C=S) of

**Table 1**: Chemical shift assignments of product 3 are given in Table 1.
Table 1: $^1$H- and $^{13}$C-NMR spectral data of compounds 1-5

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Table 2: Anti-microbial activity

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<th>C. krusei (IC$_{50}$)</th>
<th>A. fumigatus (IC$_{50}$)</th>
<th>C. neoformans (IC$_{50}$)</th>
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KEY: NA – not active; ND - not determined
bpyrimidine ring was typical at $\delta_7$ 188.7 in $^{13}$C-NMR. The imine carbon appeared at $\delta_6$ 164.6. The deshielded methine carbon, due to its close proximity to phenyl ring was downfield shifted to $\delta_5$ 78.8. The methine peak is characteristic of methine carbons attached to amino group in six-membered heterocyclic ring that normally appear in the region of $\delta_4$ 77-110.

From DEPT and $^{13}$C-NMR analyses, the methylene carbon of the pyrimidine ring appeared at $\delta_5$ 45.0. Both methylene and methine protons appeared at $\delta_6$ 2.73 ($dd, J=13.2$) and 2.98 ($dd, J=10.4, 2.4$) and the latter at $\delta_7$ 5.50 ($J=8.8$). The broad $^1H$-NMR signal at $\delta_7$ 7.36 was due to the NH proton. A complete spectral assignment is given in Table 1.

**Anti-microbial activity**

The compounds were tested for anti-microbial activities. All derivatives showed no significant activity ($\geq 40 \mu g/mL$) against standard strains of the diseases. However, compound 2, an isoxazoline, was reported for moderate anti-microbial activity against *Streptococcus aureus*, *Candida neoformans*, *Candida krusei* and *Candida glabrata* strains with inhibitory concentration to 50% microorganism (IC$_{50}$) values of 7.56, 8.01, 8.11 and 13.74µg/ml respectively (see table 2).

**CONCLUSION**

The main focus of this study was to synthesize semi-synthetic pyrazole, isoxazoline and bpyrimidine analogues and evaluate them for their bioactive effects. Five new compounds were synthesized. A reaction of chalcones with hydroxylamine hydrochloride in pyridine yielded isoxazoline heterocyclic systems. Earlier literature indicated that when chalcones are reacted with hydroxylamine hydrochloride in acetic acid, oxazole analogues are found. However, the NMR spectral analysis showed the formation of an oxime. The study also revealed that it is possible to convert $\alpha,\beta$-unsaturated ketones to bpyrimidine ring systems (scheme 1).

Besides, flavones and other related compounds can be modified to pyrazole derivatives (equation 1).

All compounds were tested for anti-fungal activity against *Streptococcus aureus*, *Candida neoformans*, *Candida krusei* and *Candida glabrata* strains of fungal diseases. Only compound 2 showed moderate activity with inhibitory concentration to 50% microorganism (IC$_{50}$) values ranging from7.56 to 13.74 µg/mL. Hence, we recommend further synthetic optimization of this compound and other isoxazoline analogues for the better-expected anti-fungal activity.

**ACKNOWLEDGEMENT**

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**REFERENCES**


Pyrazole, isoxazoline and bipyrimidine analogues

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