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Antimicrobial Potential of 2',4'- Dihydroxy-4-Prenyloxychalcone Combined with Ciprofloxacin and Fluconazole

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ABSTRACT

Infectious diseases caused by bacteria and fungi affects millions of people worldwide. Antibiotics have been effective in treating infectious diseases; increased use of these drugs has led to an increased resistance by the microorganisms. This study examined the antibacterial and antifungal activities of 2',4'-dihydroxy-4-prenyloxychalcone isolated from the aerial parts of *Indigofera pulchra* (willd) and its synergistic potential when combined with ciprofloxacin and fluconazole. The aerial parts of the plant were subjected to cold maceration using ethanol to afford the extract which was further fractionated using hexane, chloroform, ethyl acetate and butanol. Extensive phytochemical investigation of chloroform fraction using silica gel column chromatography followed by preparative thin layer chromatography led to the isolation of 2',4'-dihydroxy-4-prenyloxychalcone. The structure was elucidated using IR, ^{1}H-NMR, ^{13}C-NMR and DEPT techniques. Results of the antimicrobial study indicated that the isolated compound inhibited the growth of the test microorganisms (*MRSA, S. aureus, E. coli, P. mirabilis, C. albicans and C. tropicalis*) with zone of inhibition range of 29 – 37 mm, and was found to be greater than that observed for ciprofloxacin and fluconazole (25 – 33 mm). Synergistic effect of the isolated compound was observed when combined with ciprofloxacin and fluconazole resulting in an increase in zones of inhibition of the drugs to a range of 25 – 40 mm. The result of this study had justified the use of *Indigofera pulchra* in ethno-medicine for the treatment of bacterial and fungal infections and the isolated compound might be a potential lead with antibacterial and antifungal activities.

Keywords: Isolation, Antimicrobial, *Indigofera pulchra*, Synergy, Zone of inhibition.

Introduction

Natural products are purified organic compounds isolated from natural sources that are produced by the pathways of primary or secondary metabolism.[1] A large amount of archaeological evidence exists which indicates that humans were using medicinal plants during the Paleolithic period, approximately 60,000 years ago. Furthermore, animals such as non-human primates, monarch butterflies and sheep are also known to ingest medicinal plants to treat illness.[2]

Indigofera pulchra (Willd) is an erect stiff, grey-pubescent softly wooded under-shrub that grows up to 1–1.5 m high. It is widely distributed from Senegal to Nigeria and over Eastern and central Africa from Ethiopia to Angola.[3] In ethno medicine, the leaves are used to treat infected wound, itching skin and as snake antidote;[3] as prophylactic against snake bite[4] and for the treatment of malaria and dysentery.[5,6]

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In the treatment of drug-resistant infections, combinations of antibiotics have often been used as this takes advantage of different mechanisms of action. The use of antimicrobial agents displaying synergy is one of the well-established indications for combination antimicrobial therapy.[7] Antimicrobial synergism occurs when two or more antibiotics, in combination exert an inhibitory effect that is greater than the additive effects of the individual antibiotics. Combinations of antimicrobials that demonstrate an *in vitro* synergism against infecting strains are more likely to result in a successful therapeutic outcome. Thus, evidence of *in vitro* synergism could be useful in selecting optimal combinations of antimicrobials for the empirical therapy of serious bacterial infections.[8]

Plant extracts and plant-derived compounds have long been established to possess antimicrobial activity. However, plant-derived compounds have been seen to lack the broad spectrum and potent antimicrobial activity often displayed by bacterial or fungal produced antibiotics.

It had been hypothesized that, in addition to the production of intrinsic antimicrobial compounds, plants also produce multi-drug resistance (MDR) inhibitors which enhance the activity of the antimicrobial compounds.[9] This hypothesis was tested and showed that the activity of putative plant antimicrobials against gram positive and gram negative organisms was significantly enhanced by synthetic MDR inhibitors of MDR efflux proteins.[10] Those findings provided a basis to believe that plants can be potential sources of natural MDR inhibitors that can potentially improve the performance of antibiotics against resistant strains.

The screening of crude plant extracts for synergistic interaction with antibiotics is expected to provide leads for the isolation of MDR

inhibitors. The ability of crude extracts of plants to potentiate the activity of antibiotics had been observed by some researchers and it is anticipated to form the basis for the bioassay-directed fractionation of potential resistance modulators from plants. In a study of some Jordanian plants, results showed that the efficacy of the antibiotics, gentamycin and chloramphenicol against *S. aureus* were reportedly improved by the use of plant materials.[11] It was also observed, the synergistic interactions between extracts of Brazilian medicinal plants and eight antibiotics on *S. aureus*.[12]

Previous studies reported the isolation of 2',4'-dihydroxy-4-prenyloxychalcone[13] and recent pharmacological studies of *Indigofera pulchra* (Willd) reported the antibacterial activity the methanol leaves extract of *Indigofera pulchra*.[14] Other pharmacological studies reported include: the analgesic and anti-inflammatory activities of the methanol leaves extract of *Indigofera pulchra* (Willd)[15] and anti-*Plasmodium berghei* activities of the methanol leave extract and its n-butanol soluble fraction.[16] There is probably no documented report on the antimicrobial activity of 2',4'-dihydroxy-4-prenyloxychalcone or its possible synergistic antimicrobial potential combining ciprofloxacin or fluconazole. The present study was therefore carried out to evaluate the antimicrobial potential of 2',4'-dihydroxy-4-prenyloxychalcone and its synergistic activity with ciprofloxacin and with fluconazole.

Materials and Methods

General Experimental Procedure

Infrared (IR) absorption spectra were recorded using an infrared spectrophotometer Shimadzu 8400S and NMR spectra were obtained on a Bruker AVANCE (400 MHz for ^{1}H and ^{13}C) spectrometer. Dimethylsulfoxide (DMSO) was used as a solvent. Chemical shift values (δ) were recorded in parts per million (ppm) relative to TMS internal standard.

Plant Material

The plant *Indigofera pulchra* (Willd) was collected from Ahmadu Bello University, Zaria, Samaru Campus and was authenticated at the Herbarium Unit, Department of Biological Sciences, Ahmadu Bello University Zaria by comparing with an existing specimen voucher no. 410. The fresh aerial parts of the plant material werecarefully cut, air-dried at room temperature, made into powder using mortar and pestle and subsequently referred to as powdered plant material of *Indigofera pulchra* (Willd).

Extraction

Measured 1.5 kg of the plant material of *Indigofera pulchra* (Willd) was extracted with 7.5 litres of ethanol using cold maceration method for ten days. The solvent was evaporated at reduced pressure and a dark green gummy material (89.25 g) was obtained and referred to as the ethanol extract of *Indigofera pulchra* (Willd). The extract was treated successively with hexane, chloroform, ethylacetate and n-butanol to afford 10.26 g, 25.5 g, 7.45 g and 4.67 g of the fractions, respectively.

Chromatographic Analysis of Choloroform Fraction

Chloroform fraction (25 g) was chromatographed over silica gel (60 – 120 mesh size) packed column of dimension (100 x 4 cm). The column was eluted with 100% n-hexane then followed by hexane:ethyl-acetate in the ratio of 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40 to 30:70. Ninety three fractions (100 mL each) were collected and were pooled together based on their TLC profiles to give 15 pooled fractions (A1-A15). Sub-fraction A6 consisting of one major spot and two minor spots was subjected to purification using preparative thin layer chromatography (PTLC) with hexane-ethyl-acetate (3:1) to afford a pure yellow crystalline solid compound coded L1.

Antimicrobial Synergy Study

Antimicrobial activity of isolated compound (L1) and its synergistic potential on the activities of ciprofloxacin and fluconazole was determined using some pathogenic microbes.[17]

The test organisms used for the study were clinical isolates which include two Gram-positive bacteria (Methicillin Resistant *Staphylococcus aureus* and *Staphylococcus aureus*), two Gram-negative bacteria (*Proteus mirabilis* and *Escherichia coli*) and two yeasts (*Candida albicans* and *Candida tropicalis*) obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital Zaria.

Agar well-diffusion method was used for the screening of L1 and the standard antibiotics while Mueller-Hinton agar was used as the growth medium and was prepared according to the manufacturer's instruction, sterilized at 121ºC for 15 minutes, poured into a sterile petri dish and allowed to cool and solidify. The sterilized medium was seeded with 0.1ml inoculums of the test microbe, spread evenly over the surface of the medium using a sterile cotton swab and a 6 mm well was cut at the centre of each inoculated medium using cork borer of 6 mm in diameters.

compound L_1 (1 mg) was dissolved in 10 mL of DMSO to obtain a concentration of 100 µg/mL and 1mg each of the drugs (ciprofloxacin and fluconazole) were dissolved separately in 10 mL each of DMSO to obtain a concentration similar to L1. Solution of L1, ciprofloxacin and fluconazole (0.2 mL, 20µg) each were separately introduced into the wells of the inoculated media, incubated (24 and 48 hours, respectively) and the zones of inhibition were observed.[17]

Results and Discussion

Isolation of L1

Compound L1 was found to be a yellow crystalline powder and completely soluble in ethyl acetate with a melting point range of $137^{o}C – 141^{o}C$.

Thin layer chromatographic analysis of L1

The one-dimensional thin layer chromatography of compound L1 showed a yellow colour single spot with R_f value 0.59 using hexane: ethyl acetate in the ratio of 2:1 as the solvent system (mobile phase) and 10% sulphuric acid as spraying agent.

Infrared analysis of L1

The IR spectrum of compound L1 showed absorption bands around 3310 cm^{-1} for O-H stretching vibration for a hydroxyl group, at 3146 cm^{-1} indicating aromatic C-H stretching and at 2865.88, 2916.74 and 2970.30 cm^{-1} showing presence of methyl protons. Strong absorption at 1737.39 cm^{-1} signifies a carbonyl vibration of a ketonic functional group, Ar-OH at 1200 cm^{-1}, 1143.92 cm^{-1} for aromatic C-O- and Ar-H bending vibrations around 835.46 cm^{-1}.[18]

NMR analysis of L1

The ^{1}H-NMR spectrum of compound L1 showed resonance at δ 6.21, δ 6.32, δ 6.35, δ 6.92, δ 7.75 and δ 8.08. It also revealed the presence of proton resonances at δ 7.78, δ 6.95 and at δ 4.53, δ 5.39, δ 1.64, and δ 1.9. It exhibited signals for meta coupled (J = 2.0 Hz) aromatic protons (δ 6.21, 1H; 6.32, 1H), ortho-coupled (J = 9.0 Hz) protons (δ 7.78 1H; 6.36, 1H), due to 1, 2, 4, trisubstituted benzene and an AA'BB' aromatic splitting pattern (6.95, 2H, t, J = 9 Hz; 7.28, 2H, dd, J = 9.2, 2.0 Hz) indicating a 1, 4, disubstituted benzene. It also revealed the presence of two protons J = 15.0 Hz (δ 7.75, 1H; 7.25, 1H), this is typical of olefinic protons in trans arrangement. Other resonances at δ 4.53, 2H, J = 7.0 Hz due to oxymethylene, 5.39, 1H, due to methine proton,1.64, 3H, s and 1.67, 3H, s representing two methyl groups are indicative of the presence of a prenyl moiety as a side chain of the molecule.[19, 20]

The ^{13}C-NMR spectrum of compound L1 revealed a total of 18 carbon resonances of which 10 correspond to carbon atoms in the aromatic region (C2 and C6 resonating within the same chemical environment as C3 and C5), 1 for the carbonyl carbon and 7 for aliphatic carbon resonance. The ^{13}C – DEPT - NMR spectrum of compound L1

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revealed the presence of one methylene at δ64.63 ppm, two methyl groups at δ18.08 and δ25.48 ppm and eight methine resonances.
The 13C and DEPT NMR spectra of L1 revealed signals at δ 64.63, 119.61, 137.68, 18.08 and 25.48, these carbon signals are typical of prenyloxy side chain.[19] Also observed is a signal at 191.58 which is due to carbonyl carbon, other resonances observed were for nine aromatic methine and five quaternary aromatic carbons, these resonances suggest the presence of chalcone nucleus. The assignment of carbons and the placement of the prenyloxy side chain were achieved by comparison with NMR spectra from an existing literature.

Antimicrobial Studies
The zones of inhibition of L1 measured in millimetre (mm) showed that compound L1 had greater antibacterial activity than ciprofloxacin against *P. mirabilis* and *E. coli* and also greater than fluconazole against *C. albicans and C. tropicalis as seen* from their observed zones of inhibition. The antimicrobial action of L1 and its effect on the antibacterial activity of ciprofloxacin and the antifungal activity of fluconazole are shown in Table 3. The results of this study have shown that compound L1 isolated from the chloroform fraction of the ethyl alcohol extract of *Indigofera pulchra* (Willd) aerial parts possess a wide range of antimicrobial activity against both Gram-positive and Gram-negative bacteria and against yeast (*Candida*). This therefore, showed that the antimicrobial effect of *Indigofera pulchra* (Willd) on some isolates is believed to be due to the presence of constituents like flavonoids, saponins and tannins which have been shown to possess antimicrobial properties.[21]
The isolated compound L1 show appreciable antimicrobial activity against all the test microorganisms used and was found to enhance the antibacterial activity of ciprofloxacin against all the test microorganisms but with L1 alone showing better activity against *E. coli* and *P. mirabilis* than ciprofloxacin. Synergistic effect of compound L1 on the antifungal activity of fluconazole was also observed against both *C. albicans* and *C. tropicalis* with L1 showing better activity than fluconazole. This can therefore be a basis to attribute the antimicrobial activity of *Indigofera pulchra* (Willd) to the presence of the isolated compound L1.

Table 1: NMR data of L1

Position	δ_c of compound L1	δ_c of reference compound (D5)	DEPT
1	127.19	129.0	C
2,6	131.00	131.5	CH
3,5	115.12	116.2	CH
4	160.79	162.7	C
1’	113.07	114.8	C
2’	165.12	166.5	C
3’	102.65	103.6	CH
4’	165.87	167.5	C
5’	108.24	108.9	CH
6’	133.00	133.4	CH
1’’	64.63	66.1	CH_2
2’’	119.61	120.8	CH
3’’	137.68	139.2	C
4’’	18.08	17.5	CH_3
5’’	25.48	25.5	CH_3
1’’’	191.58	193.5	C=O
2’’’	118.49	119.3	CH
3’’’	143.87	145.2	CH

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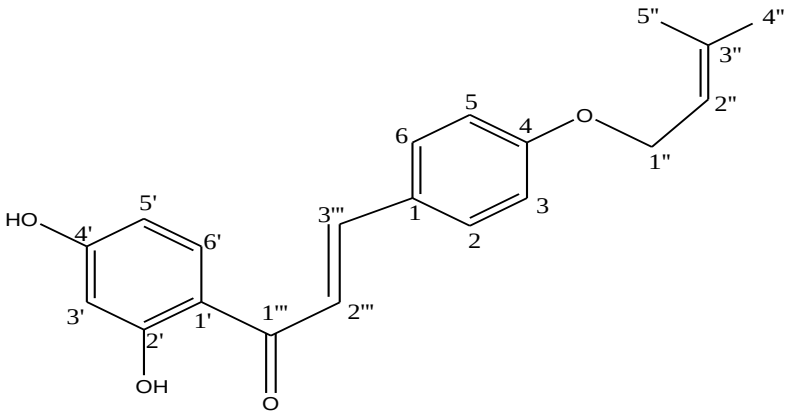

Figure 1: Structure of 2',4'-dihydroxy-4-prenyloxychalcone.

Table 2: Results of the susceptibility test of the isolated compounds and the standard antibiotics against selected organisms

Test Organisms	L1 (20 µg)	Cp(20 µg)	Fz(20 µg)
MRSA	29 mm	29 mm	-
S. aureus	30 mm	33 mm	-
E. coli	31 mm	28 mm	-
P. mirabilis	35 mm	27 mm	-
C. albicans	32 mm	-	26 mm
C. tropicalis	30 mm	-	25 mm

Key: Cp = Ciprofloxacin, Fz = Fluconazole

Table 3: Effect of L1 on the antimicrobial activity of the standard antibiotics

Test Organisms	Cp(20µg)	Fz(20µg)	L1 (10µg) + Cp (10µg)	L1 (10µg) + Fz (10µg)
MRSA	29 mm	-	30 mm	-
S. aureus	33 mm	-	26 mm	-
E. coli	28 mm	-	27 mm	-
P. mirabilis	27 mm	-	25 mm	-
C. albicans	-	26 mm	-	29 mm
C.tropicalis	-	25 mm	-	27 mm

Key: Cp = Ciprofloxacin, Fz = Fluconazole

Conclusion

The present investigation indicates that compound L1 isolated from the aerial parts of *Indigofera pulchra* (Willd) have promising antimicrobial activity against *MRSA, S. aureus, E. coli, P. mirabilis, C. albicans and C. tropicalis.* Combinations of the isolated compound with ciprofloxacin slightly increased the activity of the drug against *MRSA* and with fluconazole increased the antimicrobial activity against *C. albicans* and *C. tropicalis.* The isolated compound however, appeared to produce better antimicrobial activity against the Gram-negative bacteria and the fungi than ciprofloxacin and fluconazole.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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