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3,5-DIMETHOXY-4'-O-(2",3"-DIHYDROXY-3"-METHYLBUTYL)-DIHYDROSTILBENE ISOLATED FROM THE AERIAL PARTS OF *Indigofera pulchra* POTENTIATES THE ANTIMICROBIAL ACTIVITIES OF CIPROFLOXACIN AND FLUCONAZOLE

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ABSTRACT
Bacterial and fungal infections continue to present a major threat to human health leading to serious injuries. Microorganisms' resistance to currently existing drugs is an increasingly problematic issue that leads to millions of deaths in the world every year. This study examined the microbial inhibitory potential of 3,5-dimethoxy-4'-O-(2",3"-dihydroxy-3"-methylbutyl)-dihydrostillbene isolated from the aerial parts of Indigofera pulchra (Willd) and its ability to enhance the activities of Ciprofloxacin and Fluconazole against bacteria and fungi respectively. The aerial parts were extracted by cold maceration using ethyl alcohol to obtain the crude extract which was further separated by liquid-liquid fractionation to afford the hexane, chloroform, ethylacetate and butanol fractions. Extensive phytochemical investigation of chloroform fraction using column and thin-layer chromatographic techniques led to the isolation of 3,5-dimethoxy-4'-O-(2",3"-dihydroxy-3"-methylbutyl)-dihydrostillbene. The structure was elucidated using IR, 1H-NMR, 13C-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 maximum zone of inhibition of 37 mm, and was found to be greater than that observed for ciprofloxacin (33 mm) and fluconazole (26 mm). The isolated compound was observed to enhance the antimicrobial activities of ciprofloxacin and fluconazole when combined from a range of 25 – 33 mm to a range of 25 – 40 mm. The result of this study had justified the use of Indigofera pulchra in traditional medicine for the treatment of bacterial and fungal infections and the isolated compound can be used for clinical trials to develop a new drug, more potent and effective for the treatment of microbial infections.
Key words: Indigofera pulchra, isolation, antimicrobials, resistance, synergy.

INTRODUCTION

A natural product is a chemical compound or substance produced by a living organism that is, found in nature (NCCIH, 2013). Within the field of organic chemistry, the definition of natural products is usually restricted to mean purified organic compounds isolated from natural sources that are produced by the pathways of primary or secondary metabolism (Hanson, 2003). Plants are a major source of complex and highly structurally diverse chemical compounds (phytochemicals), this structural diversity attributed in part to the natural selection of organisms producing potent compounds to deter herbivores (Dang and van Damme, 2005). Animals also represent a source of bioactive natural products. In particular, venomous animals such as snakes, spiders, scorpions, caterpillars, bees, wasps, centipedes, ants, toads, and frogs have attracted much attention. This is because venom constituents such as peptides, enzymes, nucleotides, lipids, biogenic amines etc. often have very specific interactions with a macromolecular target in the body e.g. α-bungarotoxin from cobras (Dossey, 2010).

The World Health Organization (WHO) estimates that 80 percent of the populations of some Asian and African countries presently use herbal medicine for some aspects of primary health care. This may be due to the fact that pharmaceuticals are prohibitively expensive for most of the world's population, half of which lives on less than $2 U.S. per day (Edgar *et al.*, 2002).

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 (Sule *et al.*, 2003). In ethno medicine, the leaves are used to treat infected wound, itching skin and as snake antidote; as prophylactic against snake bite (Sule *et al.*, 2003) and for the treatment of malaria and dysentery (Hanson, 2003).

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.

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 (Fortin *et al.*, 2001). 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 infection (Stermitz *et al.*, 2000).

The WHO defines antimicrobial resistance as a microorganism's resistance to an antimicrobial drug that was once able to treat an infection by that microorganism which can occur by natural resistance in certain types of bacteria, by genetic mutation, or by one specie acquiring resistance from another (WHO, 2014). Resistance can happen spontaneously owing to random mutations, to a build-up of resistance over time or to misuse of antibiotics or antimicrobials. Resistant microbes become increasingly difficult to treat, requiring alternative medications or higher doses, both of which may be more costly or more toxic. According to Centre for Disease Control and Prevention (2015), microbes which are resistant to multiple antimicrobials are called multidrug resistant (MDR); these organisms are often referred to as superbugs.

Microorganisms exhibit resistance to antimicrobials through drug inactivation or modification, alteration of target site, alteration of metabolic pathway, reduced drug accumulation, horizontal gene transfer and unlinked point mutations in the pathogen genome at a rate of about 1 in 10^8 per chromosomal replication. Mutations are rare but the fact that bacteria reproduce at such a high rate allows for the effect to be significant (Chan *et al.*, 2011).

Synergy describes the fact that a system (the combination and interaction of two or more agents or forces) is such that the combined effect is greater than the sum of their individual effects. This definition implies that there are three possible ways of such an "interaction of agents or forces", these forces could simply add up, not affecting each other (no interaction; A+B = A+A or B+B), their combination could produce a greater than expected result (synergy; A+B > A+A or B+B), or the combination could lead to a result that is less than the sum of the individual effects; antagonism: A+B < A+A or B+B (Hewitt and Vincent, 2003).

Recent studies reported the antibacterial activity (Gibbons *et al.*, 2008), analgesic and anti-inflammatory activities, and anti-*Plasmodium berghei* activities of the methanol leaves extract of *Indigofera pulchra* (Willd) and its n-butanol soluble fraction (Ibrahim *et al.*, 2011).

A 2',4'-dihydroxy-4-prenyloxychalcone was isolated from the aerial parts of *Indigofera pulchra* (Willd) and reported to have inhibited the growth of some clinical isolates as well as potentiating the anti-bacterial and anti-fungal activities of ciprofloxacin and fluconazole when combined respectively (Dauda *et al.*, 2019). A mixture of two new dihydrostilbenes, 3-methoxy-4,5-methylenedioxy-4'-*O*-(2,3-dihydroxy-3-methylbutyl)-dihydrostilbene and 3,5-dimethoxy-4'-*O*-(2,3-dihydroxy-3-methylbutyl)-dihydrostilbene), was isolated from *Indigofera pulchra* as an inseparable mixture. Several isolation strategies, including multiple preparative TLC, SPE and finally HPLC were undertaken in an attempt to separate these metabolites, but to no avail (Gibbons *et al.*, 2008).

There is probably no documented report on isolation of 3,5-dimethoxy-4'-*O*-(2,3-dihydroxy-3-methylbutyl)-dihydrostilbene in its pure form from *Indigofera pulchra* (Willd) talk less of either its antimicrobial activity or synergistic antimicrobial potential when combined with

ciprofloxacin and fluconazole. Therefore, this study was carried out to investigate ability of 3,5-dimethoxy-4'-*O*-(2,3-dihydroxy-3-methylbutyl)-dihydrostilbene to synergistically potentiate the antimicrobial activities of ciprofloxacin and fluconazole when combined.

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 1H and 13C) 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 were carefully 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). Fraction A11 (1.06g) was packed for repeated silica gel column chromatography using hexane: chloroform of varying the polarity (5%) to chloroform 100%. Then with chloroform: ethylacetate mixture in ratio order of 99.5:0.5, 99:1, 98.5:1.5, 98:2, 97.5:2.5, 97:3, 96.5:3.5, 96:4 and 91:9 where one hundred and twenty two sub-fractions were collected. Two major spots with R_f values 0.33 and 0.31 were observed on the TLC profile with one minor spot from pooled sub-fractions C17 and C18; purified using preparatory thin layer chromatography (PTLC) to afford a white amorphous solid compound coded L2.

Antimicrobial Synergy Study

Antimicrobial activity of isolated compound (L2) and its synergistic potential on the activities of ciprofloxacin and fluconazole was determined using some pathogenic microbes (Mukesh *et al.*, 2008). 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 L2 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 L2 (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 L2. Solution of L2, 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.

Isolation of L2

Compound L2 was a white crystalline solid showing a red coloured single spot with R_f value 0.33 on TLC using hexane: ethylacetate in the ratio of 2:1 as the mobile phase and 10% sulphuric acid as spraying reagent. L2 was found to have a melting point of 144-147^{O}C and soluble in ethylacetate while insoluble in hexane, chloroform, butanol and methanol.

Infrared analysis of L2

The IR spectrum of L2 shows absorptions at 3568.50cm^{-1} and 3389.02cm^{-1} for O-H stretching. Strong absorption at 1148.44cm^{-1} signifies presence aromatic C-O- (ether) and Ar-H bending vibrations around 873.97cm^{-1} (Kalsi, 2004).

NMR analysis of L2

Similar to that of flavonoids, ^{1}H-NMR spectra of compound L2 shows signals within a range of 6-8ppm. For ring A, signal at δ6.31ppm (2H, d) shows absorption for C-2 and C-6 protons which appear at lower field than C-4 proton at δ6.24ppm (1H, d) due to shielding effect of C-3 and C-5 methoxyl groups. Ring B protons appears downfield to A-ring protons; B-ring proton signals ranges within 6.65-7.1ppm depending on the nature of oxygen attachment to the ring on the ring (Mabry *et al.,* 1970). Therefore, C-3′ and C-5′ protons shows absorption at δ6.7-6.8ppm (2H, dd) upfield to where C-2′ and C-6′ protons absorb δ6.9-7.1ppm (2H, dd) due to shielding effect of oxygen at C-4′. Other protons include the C-4″ and C-5″ methyl protons that appeared around δ1.0-δ1.2ppm, the C-1‴ and C-2‴ at δ2.3-2.8ppm, oxymethylene C-1″ at δ4.88, δ4.9ppm doublet and hydroxyl protons at a range of δ4.0-4.4ppm (Kalsi, 2004).

13C-NMR of L2 signals at δ24.31 and 27.35 shows Sp^3 C-4″ and C-5″ methyl carbons, δ35.96 and δ37.64 for C-1‴ & C-2‴ ethylene carbons and δ55.05 for methoxyl groups at C-3 and C-5. Signals at δ69.72 signals for oxy-methylene carbon C-1″, δ75.86 for the methine carbon. These coclusions were confirmed by comparison of the spectra of compound 2 (Gibbons *et al.,* 2008).

The ^{13}C - DEPT-NMR spectrum of compound L2 revealed the presence of three methylene (for O-CH_2) at δ35.97, δ37.64 and δ69.76ppm, seven methine (CH) at δ24.31, δ27.37, δ75.86, δ97.74, δ106.47, δ114.30, δ129.29ppm, two methyl carbons (CH_3) at δ24.31 and δ27.37, and two methoxy carbons (O-CH_3) at δ55.05ppm and δ25.5ppm. The assignment of all carbons and protons were achieved by comparison with NMR spectra from an existing literature.

Antimicrobial studies

The zones of inhibition of compound L2 measured in millimetre showed that L2 was able to enhance the antibacterial activity of ciprofloxacin against MRSA and *E. coli* with L2 showing better activity against *E. coli* and *P. mirabilis* than ciprofloxacin. Synergistic effect of compound L2 on the antifungal activity of fluconazole was observed against both *C. albicans* and *C. tropicalis* with L2 showing better activity than fluconazole. This can therefore be a basis to attribute the antimicrobial activity of *Indigofera pulchra* to the presence of L2.

Table 1: NMR data of L2

Position	δ_c of compound L2	δ_c of reference compound (2)	DEPT
1	143.49	145.0	C
2	106.46	115.5	CH
3	160.35	162.3	C
4	97.73	99.1	CH
5	160.35	162.3	C
6	106.46	115.5	CH
1′	133.24	135.3	C
2′,6′	129.28	130.5	CH
3′,5′	114.29	115.6	CH
4′	157.15	158.1	C
1″	69.76	70.2	CH_2
2″	75.86	76.7	CH
3″	70.87	71.9	C
1‴	37.64	39.4	CH_2
2‴	35.96	38.2	CH_2
4″	24.31	26.0	CH_3
5″	27.35	27.1	CH_3
3-OMe	55.05	55.5	CH_3
5-OMe	55.05	55.5	CH_3

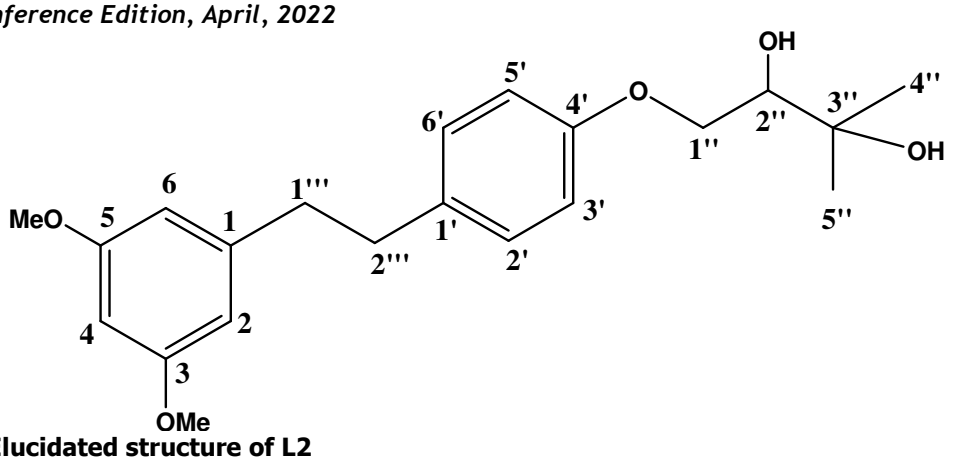

Figure 1: Elucidated structure of L2

Table 2: Results of the susceptibility test of L2 and the standard antibiotics against selected organisms.

Organisms	L2 **(20µg)**	Cp **(20µg)**	Fz **(20µg)**
MRSA	0 mm	29 mm	-
S. aureus	31 mm	33 mm	-
E. coli	33 mm	28 mm	-
P. mirabilis	34 mm	27 mm	-
C. albicans	37 mm	-	26 mm
C. tropicalis	32 mm	-	25 mm

Keys: Cp=Ciprofloxacin, Fz=Fluconazole

Table 3: Effect of L2 on antimicrobial activity of the standard antibiotics.

Test Organisms	Cp **(20µg)**	Fz **(20µg)**	L2 **(10µg)** + Cp **(10µg)**	L2 **(10µg)** + Fz **(10µg)**
MRSA	29 mm	-	33 mm	-
S. aureus	33 mm	-	31 mm	-
E. coli	28 mm	-	29 mm	-
P. mirabilis	27 mm	-	25 mm	-
C. albicans	-	26 mm	-	40 mm
C.tropicalis	-	25 mm	-	39 mm

Keys: Cp=Ciprofloxacin, Fz=Fluconazole

CONCLUSION

Based on the findings of this study, it can be concluded that 3,5-dimethoxy-4'-*O*-(2,3-dihydroxy-3-methylbutyl)-dihydrostilbene is a strong contributing factor that might probably justify the scientific basis for the use of *Indigofera pulchra* (Willd) aerial parts in the treatment of infections caused by bacteria and fungi. Students, researchers, pharmaceutical industries and the government will indeed find this study highly relevant with a view to curtailing the problems of severe deaths, injuries and threats caused by antimicrobial resistance to currently available antibiotics.

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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