J. Chem Soc. Nigeria, Vol. 44, No. 7, pp 1313 -1319 [2019]

In-vitro anti-typhoid efficacy of an isoquinolone derivative from *Bligha sapida* Koenig seeds

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Received 28 June 2019; accepted 08 August 2019, published online 31 October 2019

Abstract
Qualitative and quantitative screening of the 70% ethanol extract of *Bligha sapida* seeds (labeled B) revealed a strong presence of alkaloids. Separation and extraction of the crude alkaloidal portion (labeled Ba) of extract B using chromatographic techniques led to the isolation and purification of a compound (labeled Ba3i). Structural elucidation of compound Ba3i using physical, chemical and spectral revealed it as 3, 4-dihydro-6,7-dimethoxy-2*H*-isoquinolin-1-one in comparison with literature data. *In-vitro* anti-typhoid assay of B, Ba and Ba3i against typhoid and paratyphoid strains using the agar dilution method revealed that all test compounds showed no activity against *S. typhi* in comparison with Ciprofloxacin. The extract B, at 1000 μg/cm^3 was active against *Salmonella paratyphi* A, B and C, while, the alkaloidal portion, Ba and isolated compound, Ba3i, at 500 μg/cm^3 and 100 μg/cm^3 respectively were active against *S. paratyph*i B and C only. The findings from this study have provided some evidence for the ethnomedicinal use of the seeds of *B. sapida* as an antibacterial agent and in treatment of fevers, especially those caused by paratyphoid bacilli.

Keywords: Alkaloids, anti-typhoid, *Bligha sapida*, isolation, isoquinolone, seeds

Introduction

Enteric fever, which includes typhoid and paratyphoid fevers are caused by the invasive *Salmonella* genus. The causal organism of typhoid fever is *Salmonella typhi*, while paratyphoid which is milder than typhoid fever is caused by *Salmonella paratyphi* A, B and C. Both diseases are endemic in developing nations due to lack of provision of clean water, poor sewage disposal, unhygienic handling of food by food vendors and generally, low standard of sanitation and personal hygiene, all constituting a huge health problem. These fevers, amongst others, have become endemic, coupled with the emergence of multi-resistant strains with decreased susceptibility to conventional antibiotics, such as, Ciprofloxacin and Chloramphenicol [1] has led to the search for newer and alternative antimicrobial agents from natural products, such as; *Bligha sapida* K. D. Koenig (family Sapindaceae).

B. sapida commonly known as Ackee-apple/breadfruit (English), *Gwanjakusa* (Hausa), *Okpu* (Igbo) and *Ishin* (Yoruba) is a tropical and sub-tropical woody perennial large tree crop widely distributed in the Caribbean islands, Central and South America and most African countries, especially, West Africa. Its leaves are alternate with shiny leaflets; bark is smooth gray black, while, branches are thick and bilateral. Flowers are small, greenish and staminate; fruits are pear-shaped, red with yellow or orange capsules which splits open when fruits are matured revealing cream-colored arils attached to smooth, shiny black oblong seeds as shown in plate 1 [2]. Ethnomedicinally, various parts of the plant are useful in the treatment of fever, malaria, yellow fever, epilepsy, dysentery, constipation, conjunctivitis, oedema, migraine, headache, diabetes, abortifacient, dental decay, internal haemorrhage and whitlow [2 - 5].The potentials of the seeds in both food and non-food industries have been reported [5]. Experimentally, the plant has been shown to possess anti-diabetic [6] and repellant properties [7]

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Plate 1: Matured fruits of the plant showing the black seeds attached to the arils

Preliminary phytochemical screening of the plant revealed a rich presence of saponins, sterols, fatty acids, amino acids, alkaloids, flavonoids and tannins [8]. From the arils and seeds of the unripe fruits, two diastereoisomers; hypoglycin A and hypoglycin B have been isolated [9, 10]. Reportedly, the unripe fruit contains hypoglycin A at concentrations 100 times higher than those of the ripe fruits, making the ripe fruits more edible [9]. Other compounds isolated include; blighnone [11] and (2s, 1's, 2's)-2-(2'-carboxycyclopropyl) glycine [12]. Characterization of the seed oil of the plant revealed a high content of mono-unsaturated fatty acids, eicosenoic acid and arachidic acid. Other compounds, includes; stigmasterol, β-sitosterol, α- and γ- tocopherol [13]. Oleic acid was identified as the major fatty acid in the aril, pod and raphe of the fruit, while gondoic acid was identified in the seed of the plant [14].

Extensive literature search revealed not much information about extraction, isolation and characterization of some of the alkaloids of the seeds of the plant, as well as investigation of the anti-typhoid potentials of such alkaloids against typhoid and paratyphoid bacilli. This study, therefore, presents the *in-vitro* anti-typhoid activity alkaloid(s), the crude (total) alkaloidal portion and 70% crude ethanol extract of *B. sapida* seeds in comparison with Ciprofloxacin

Materials and Methods

Extraction of plant material

Fresh ripe fruits of *Bligha sapida* bearing seeds were collected in the month of April 2018 from New-Bussa town, Borgu Local Government Area of Niger State, Nigeria. Seeds with reference number NIPRD/H/6740 were identified and authenticated by Mr. Akeem Lateef of the herbarium section of National Institute for Pharmaceutical Research and Development, (NIPRD), Idu, Abuja. The seeds were separated from the fruits, air-dried and pulverized. 500 gram of the pulverized plant sample was extracted exhaustively by hot maceration with 70 % ethanol for ten (10) days. Resulting mixture was filtered, concentrated *in-vacuo* and evaporated to dryness over a water bath to yield 'crude ethanol extract of seeds of *B. sapida*' (coded B, dark brown gummy mass, 10.8 % yield).

Qualitative screening for alkaloids

A small portion of the extract, B, was hydrolyzed with 1% aqueous HCl over a steam bath for about 5 minutes and the mixture filtered. The resulting filtrate (1 cm^3 each) was treated separately with 2 drops each of Dragendorff's (potassium bismuth iodide solution) and Wagner's (solution of iodine in potassium iodide) reagents. Formation of a reddish brown- and brown-colored precipitate of each reagent by the extract in each tube respectively, indicates the presence of alkaloids [15].

Quantitative screening for alkaloids

This was determined using the method of Harbone [16]. A portion of the extract, B (2 g) was solubilized in a solution mixture of 10% acetic acid in ethanol in a 250 cm^3 beaker and allowed to stand for 4 hours. The resulting extract was concentrated *in-vacuo* to one-quarter of the original volume followed by addition of several drops of conc. NH_4OH until complete precipitation was achieved immediately after filtration. After 3 hours of mixture sedimentation, the supernatant was discarded and the precipitate washed with 20 cm^3 of 0.1 M of NH_4OH and then filtered. The residue was dried in an oven and the % alkaloid expressed as:

$$\frac{weight\ of\ alkaloid}{weight\ of\ extract\ used}\ x\ 100$$

Extraction of crude (total) alkaloids

The crude (total) alkaloids present in the crude 70 % ethanol extract of *B. sapida* seeds (B) was extracted using the method of Wang *et al*. [17]. The extract was suspended in a 5% dilute acidic medium (H_2O and HCl) and then partitioned with hexane. The aqueous acidic portion was then basified (pH 9) with aqueous ammonia and further extracted with $CHCl_3$ till the extractant was colorless. The combined $CHCl_3$ portion was concentrated *in-vacuo* to

yield 'alkaloidal portion/total crude alkaloids of ethanol extract of *B. sapida*' (coded Ba, golden brown gummy mass, 11.5% yield). Trace of water was removed using anhydrous Na_2SO_4. Precipitation of Dragendorff's (orange-red) and Wagner's (reddish-brown) reagents when separately added to the portion, confirmed they were alkaloids.

Isolation, purification and characterization of an alkaloid

Crude alkaloidal portion, Ba (3 g) was applied to a column (100 g of alumina as stationary phase), gradient elution commenced with $CHCl_3$ (100%) and gradually increased from $CHCl_3$: MeOH 100:0 to 0:100. Eluents were monitored on TLC using $CHCl_3$: MeOH: NH_3 (9: 1: 1 v/v/v), as mobile phase and chromatograms sprayed with Dragendorff's reagent. Similar fractions were pooled to yield a total of 4 major fractions, coded Ba1 – Ba4. Fraction Ba3 (eluents 91-157; 120 mg; 2 spots on TLC, $CHCl_3$: MeOH: NH_3, 19:1:0.5 v/v/v) was further applied to a flash column (30 g alumina, elution with $CHCl_3$: MeOH, 1:1) to afford a compound, coded Ba3i.

Characterization of compound Ba3i

Melting point of compound was uncorrected using a Gallenkamp melting point apparatus. IR and UV spectra were recorded in MeOH using FT-IR 8400 spectrometer and T60 UV-Visible spectrophotometer respectively. ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectra were recorded in $CDCl_3$ on Bruker spectrophotometer operating at 400 MHz, while, GC and MS was recorded on Agilent technologies 7890A and Agilent technologies 5975C respectively.

Anti-typhoid assay of test compounds

Collection and identification of test organisms

The test organisms, *Salmonella typhi*, *Salmonella paratyphi* A, B and C, were collected in a lyophilized form from the Vaccine Laboratory, Department of Microbiology, Federal University of Technology, Minna, Niger State, Nigeria. They were reactivated by sub-culturing them on freshly prepared nutrient agar slants and maintained until required for assay. Test organisms were verified using the reference method (Microbact^{TM} gram negative 12qeA (12E) kit) as described by Mugg [18].

Susceptibility testing of test compounds (B, Ba and Ba3i)

The viability test for each organism was carried out by resuscitating each organism on nutrient agar medium and incubating at 37^{o}C for 24 h. 0.2 cm^3 of each 24 h culture of typhoid bacilli was dispensed into 20 cm^3 of Mueller Hinton broth (MHB) and incubated for 5 h at 37^{o}C to obtain 1x10^6 cfu/cm^3 of each organism. A loopful of the standardized culture was used for assay using the agar dilution method. 5 mg of crude extract, B, 2.5 mg of alkaloidal portion, Ba and 0.5 mg of isolated compound, Ba3i were separately reconstituted in 5 cm^3 of sterile distilled water each to afford concentrations of 1000, 500 and 100 μg/cm^3 respectively. 1 cm^3 of each reconstituted test compound was then dispensed in 19 cm^3 of Mueller Hinton agar (MHA) in sterile Petri dishes. Agar was allowed to solidify for each dish at room temperature and a loopful of each standardized test organism was streaked unto each solidified agar plate. Plates were prepared in duplicates. Control plates, including standard control (Ciprofloxacin; 0.05 mg; 10 μg/cm^3), extract sterility control (ESC), organism viability control (OVC) and medium sterility control (MSC) were also prepared. All plates were incubated aerobically at 37^{o}C for 24 h and checked for activity in terms of growth/no growth.

Results and Discussion

Characterization of compound Ba3i

Physical parameters

White needles (15 mg) re-crystallized from a mixture of Me_2CO: MeOH; melting point, 170-172^{o}C [lit. 173-175^{o}C] [19]. TLC, $CHCl_3$: MeOH: NH_3 (9: 1: 0.1, R_f 0.77), CH_2Cl_2: MeOH: NH_3 (19:1:0.1, R_f 0.69), spot was colorless (sunlight), brown (254 and 366 nm), golden brown (I_2 vapour) and orange-red (Dragendorff solution).Compound was soluble in $CHCl_3$, Et_2O and Me_2CO, slightly soluble in MeOH, EtOH and H_2O, insoluble in hexane and petroleum ether. GC-MS revealed its molecular weight and molecular formula to be 207 gmol^{-1} and $C_{11}H_{13}NO_3$ respectively. An odd mass number indicates odd number of nitrogen atom (s) [20].

Table 1: ^{1}H-NMR, ^{13}C- NMR and DEPT-135^{o} spectral data of compound Ba3i in comparison with literature values*

Position	δH (ppm)	δH (ppm)*	δ C (ppm)	δ C (ppm)*	DEPT (ppm)	DEPT (ppm)*
1	-	-	163.8	164.7	- (nulled)	-
2	8.58 (s)	8.62 (s)	-	-	-	-
3	3.50 (m)	3.53 (m)	38.9	39.7	38.9 (inverted)	39.7
4	3.20 (m)	3.16 (m)	27.0	27.5	27.0 (inverted)	27.5
5	7.12 (s)	7.07 (s)	112.8	111.6	112.8 (normal)	111.6
6	-	-	155.0	153.1	- (nulled)	-
7	-	-	145.3	147.1	- (nulled)	-
8	7.50 (s)	7.49 (s)	111.5	110.0	- (normal)	110.0
9	3.82 (s)	3.85 (s)	57.2	56.1	57.2 (normal)	56.1
10	3.74 (s)	3.75 (s)	57.2	56.1	57.2 (normal)	56.1
a	-	-	130.8	131.4	- (nulled)	-
b	-	-	121.8	121.4	- (nulled)	-

Keys: s = singlet, m = multiplet, - = no peak/signal observed *****[23]

Color reactions

Compound gave an orange-red and yellow ppt. with Dragendorff's and Wagner's reagents respectively, confirming it is a nitrogenous base [15]. It also formed an orange precipitate with 2, 4-dinitrophenylhydrazine (DNPH) indicating the presence of a carbonyl moiety on the molecule, while it gave no color change with Schiff's reagent, confirming it is a ketone [21].

Spectral data

IR (ν cm^{-1}): 3410.7 (N-H stretch of CONH), 3050.8 (=C-H stretch of aromatics), 1736.5 (C=O stretch of CONH), 1620.1 (N-H bending of CONH) and 1580.4 (aromatic C-C stretch).

UV (λmax, nm): 310 (tetrahydroisoquinoline moeity), 261 (C=O of CONH), 227 (Ar-OMe).

^{1}H-NMR (δ ppm): The obtained peaks in comparison with literature (Table 1) revealed a highly de-shielded singlet broad peak at δ8.58 which is attributed to electronegative nitrogen atom attached to the proton at position 2 and thereby causing de-shielding of such a proton. The singlet peaks each at δ7.12 and δ7.50 are attributable to de-shielded aromatic protons (Ar-H), while the sharp singlet peak at δ3.74 was assigned to the methyl protons at position 9, an indication that they are in a chemically identical environment, hence, same chemical shift. This also applies to the three protons at position 10, though slightly more de-shielded than those at position 9 [22].

^{13}C-NMR (δ ppm): The obtained peaks in comparison with literature revealed a total of ten proton-decoupled peaks (Table 1) of which a downfield peak at 163.8 ppm was assigned to the carbonyl carbon of amide group at position 1, while, aromatic carbons at positions 6 and 7 both bearing an electronegative oxygen atom were assigned 155.0 and 145.3 ppm respectively. The de-shielded methyl carbon atoms attached to the electron withdrawing oxygen atoms at positions 9 and 10 both occur in the same chemical environment and hence, same chemical shift and therefore, a bold, sharp peak at 57.2 ppm.

DEPT-135 (δ ppm): Amongst the 10 carbon resonances obtained from carbon-13 NMR, DEPT-135 in comparison with literature (Table 1) revealed that five carbon atoms were quaternary (nulled/disappeared in the spectrum) at positions 1, 6, 7, a and b; two were methine of benzene (normal/above in the spectrum) at positions 5 and 8; two were methylene of dihydroisoquinolone (inverted/below in the spectrum) at positions 3 and 4; while, two (superimposed on each other, same chemical shift) were methyl (normal/above in the spectrum) at positions 9 and 10.

GC-MS (m/z; % relative intensity): The GC-MS fragmentation pattern of the compound were typical of those of an aromatic amide, whose major fragment ions were produced from the cleavage of the acyl moiety, bonds next to nitrogen atom and ring opening [20] as shown in Fig. 1.

From the molecular ion peak (M^+) **(I)**, there is likely a partial ring opening around the nitrogen moiety to form a resonance-stabilized benzoyl cation derivative **(II)**, which subsequently loses the carbonyl ion to give a benzyl cation derivative **(III)**. In another fragmentation pattern, the M^+ could lose the entire isoquinolone ring to form a phenyl cation derivative **(IV)**.Other fragment ions generated by the molecule were of very low abundance (< 20%).

A comparative study of the obtained physical, chemical and spectroscopic data of compound **Ba3i** with those published in literature, the compound was identified as anisoquinoline/isoquinolone derivative, named 3,4-dihydro-6,7-dimethoxy-2*H* -isoquinolin-1-one/1,2,3,4-Tetrahydro-6,7-dimethoxy-2*H*-isoquinolone (Fig. 2). The compound also called 'Corydaldine' had earlier been isolated from other plants [23, 24].

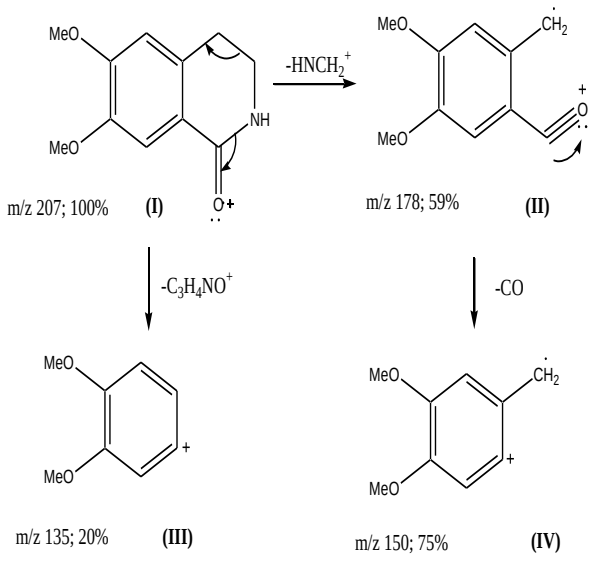

Figure 1: Some proposed fragmentation patterns of compound Ba3i

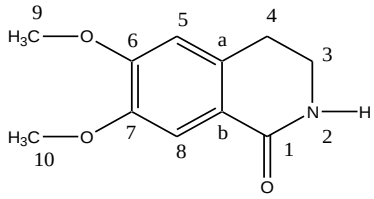

Fig 2: Structure of compound Ba3i (3, 4-Dihydro-6,7-dimethoxy-2*H*-isoquinolin-1-one/1, 2, 3, 4-tetrahydro-6,7-dimethoxy-2*H*-isoquinolone)

Anti-typhoid Assay

The crude ethanol extract of *Bligha sapida* seeds, B, its alkaloidal portion, Ba and isolated compound, Ba3i at 1000, 500 and 100 μg/cm^3 inhibited the growth of *S. paratyphi* B and C respectively in comparison with Ciprofloxacin at 10 μg/cm^3 (Table 3).

Table 3: *In-vitro* anti-typhoid activity of extract of *B. sapida* (B), alkaloidal portion (Ba) and isolated compound (Ba3i) in comparison with Ciprofloxacin (C) against test organisms

Test organisms	Activity of test compounds (μg/cm^3) against test organisms			
	B (1000)	Ba (500)	Ba3i (100)	C (10)
S. typhi	-	-	-	+
S. paratyphi A	+	-	-	+
S. paratyphi B	+	+	+	+
S. paratyphi C	+	+	+	+

+ = activity, - = no activity

Both the alkaloidal portion, Ba and isolated compound, Ba3i did not inhibit the growth of *S. typhi* and *S. paratyphi* A at 500 and 100 μg/cm^3 respectively, an activity that could probably be improved at higher concentration (dose dependent) or maybe activity could increase in the presence of other bioactives (synergism) or an indication that they might not be useful in the treatment of typhoid and paratyphoid fever illnesses caused by *S. typhi* and *S. paratyphi* A respectively. Although, generally, the organisms tested are reportedly more susceptible *in-vitro* than *in-vivo* to a variety of antibiotics [25], some strains of *S. typhi* and *S. paratyphi* A exhibit resistance against some common antibiotics, such as chloramphenicol and fluoroisoquinolone (a derivative of compound Ba3i) [26].The isolated compound has earlier been reported to

possess efficacy against some pathogens [27]. Isoquinoline alkaloids and their derivatives which are known to form one of the largest groups of plant alkaloids are produced by a large range of plant families and are known to display significant antibacterial potentials against both Gram-positive and Gram-negative pathogens [27].

Conclusion

In-vitro anti-typhoid investigation of the alkaloidal portion of the 70% ethanol extract of *Bligha sapida* seeds led to the isolation and characterization of 3,4-dihydro-6,7-dimethoxy-2*H*-isoquinolin-1-one (an isoquinoline/isoquinolone derivative). The alkaloidal portion (500 μg/cm^3) and isolated quinolone derivative (100 μg/cm^3) in comparison with Ciprofloxacin (10 μg/cm^3) exhibited no activity against *Salmonella typhi* and *Salmonella paratyphi* A, but was active against *S. paratyphi* B and C. This indicates that they both might be useful in the treatment of paratyphoid fevers. Also, the alkaloidal portion and isolated alkaloid along with maybe other phytoconstituents present in the seeds of *Bligha sapida* could probably be acting synergistically to account for the ethnomedicinal use of the plant in the treatment of fevers and as an antibacterial agent.

Declaration of Interest

The authors declare no competing interest anywhere.

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