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Iridoid glucosides from *Vitex grandifolia* displayed Anti-inflammatory and Antileishmania Effects and Structure Activity Relationship

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ABSTRACT: In this study, two iridoids i.e. agnuside (**1**) and bartioside (**2**) were isolated from *Vitex grandifolia* for the first time. These potent flavonoids were purified using fractionation and were separated by Sephadex LH 20, Silica gel 60, RP C-18 and Diaion HP-20 columns. Their structure was elucidated using FTIR, 1D and 2D NMR data, these were further evaluated for anti-inflammatory and antileishmania effects. Agnuside, isolated for the first time from *V. grandifolia* exhibited good activity against NF-kB assay with IC_{50} (ug/mL) of 8.9 while bartsioside showed an IC_{50} (ug/mL) of 12. Agnuside (1), an iridoid showed the highest IC_{50} (µg/mL) of 5.38 and corresponding IC_{90} (µg/mL) of 7.07 against *L. donovani* (intracellular amastigotes in THP1 cells). Positive standards were employed in these studies. The results allowed us to establish the relationship between the structure and the activities on the basis of the different patterns of substitution, particularly hydroxylation. This study gave credence to the view that there is a link between low incidence of some diseases and consumption of vegetables hence advantages of these vegetables are beyond nutritional gains.

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The family called Lamiaceae, this is mostly referred also to as mint family consist of mainly herbs, medicinal plants and shrubs which include about 230 genera and 7100 species mainly found in tropical and subtropical regions (Harley *et al*., 2004; Bello *et al.,* 2017). Members of Lamiaceae are believed to be important because their cosmetics, culinary advantages, ornamental, therapeutic uses and temperate regions of the world contains only a few species of this family (Bello *et al*., 2017). Amongst this family are; *Ajuga* (50 species), *Lantana* (150 species), *Marrubium* (40), *mentha* (25 species), *Tecurium* (100 species) and *Vitex* (750 species) (Akgül *et al.,* 2008). One of the well-known genus in the family of Lamiaceae is *Vitex*, this genus contains of mostly herbs, shrubs and small trees, and they are popular to tropical and subtropical region like Africa, Asia and Latin America belonging to this family. *Vitex* genus has been considered rich source of iridoids (Corrêa & Pena, 1984).

In present times, research on medicinal plants has globally increased tremendously, and volumes of reputable evidence have been gathered to portray the enormous prospects of medicinal plants used in traditional systems (Fiala *et al.,* 1985; Tapsell *et al.,* 2006). Many of these herbal plants have been identified and studied using current scientific methods and ways, the results revealed the immense promise of medicinal plants in the field of medical science (Triggiani *et al.,* 2006). *Vitex grandifolia* is a tagged as wild vegetable though it is popular for its ethnomedicinal uses. Its bark is used as a treatment against stomach ache and to treat diarrhea, bronchial complaints, rickets, sore, and fever, alcoholic drink is made from its fruits though they are edible too. The leaves are used in medication against colic, infections of the umbilical cord, toothache, rheumatism, and orchitis (Burkill, 1997). Unexpectedly, *V. grandifolia*'s phytochemistry have not being investigated at all nor its compounds biological activities investigated. So, the anti-inflammatory and antiprotozoal effects of the isolated constituents from this neglected vegetable will be considered worthwhile to investigate. Hence, the present study reports the isolation, characterization and the *in-vitro* anti-inflammatory and antiprotozoal of the constituents from the polar extract of the *Vitex grandifolia*.

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MATERIAL AND METHODS

*Collection of Plant Material***:** The leaves with the stem of *Vitex grandifolia* was collected in April-October 2015 from Ilorin metropolis, Kwara State, Nigeria. The collected plant was identified by a taxonomic botanist in the department of Plant Biology, University of Ilorin, Ilorin where voucher number was obtained after the deposit of the specimen. The leaves and stem were air dried, powdered and stored for further analysis.

Extraction of the Plant Material: The air-dried leaves of *Vitex grandifolia* (400 g) was successively extracted with methanol (3 × 1 litres for 72 h) by maceration at room temperature and evaporated under vacuum to dryness, the concentrated methanol extract weighs 45.6 g.

Isolation of Compounds: The methanol extract was chromatographed using vacuum layer chromatography (VLC) with reverse phase silica gel (RP C-18), using gradients of H_2O/MeOH [100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 80:20, 10:90, 0:100] to obtain eleven (11) fractions, the first fraction (H_2O only) was picked for further fractionation. The aqueous fraction was subjected to column chromatography (cc) over Diaion HP-20 (80 × 6 cm), using gradients of H_2O/MeOH with increasing methanol and the eluates were collected and concentrated, twelve (12) fractions were collected and monitored by TLC. The first fraction was subjected to a Sephadex column, with methanol (100 %) used as eluting solvent, six fractions were obtained. From these, the second and third fractions were combined, two compounds (**1** and **2**) were purified from the combined fractions using column chromatography with a solvent system of CH_3Cl: MeOH: H_2O (65:35:10) as eluting solvent. Compounds **1** and **2** were isolated.

FTIR and NMR analysis: Pre-coated TLC plates (AluO), Silica gel 60 F_{254}, layer thickness 0.25 mm (Merck), Pre-coated TLC plates (Glass), RP-18, F_{254} S, layer thickness 0.25 mm (Merck), Silica gel 60, 40-63 µm mesh size (Merck), RP-18, 40-63 µm mesh size, Sephadex LH 20, 25-100 µm mesh size (Merck) Silica gel 60 and RP C-18, Diaion HP-20 was used for column chromatography. ^{1}H NMR, ^{13}C NMR and 2D NMR were recorded on 400, 500 and 600 (MHz) instrument (Agilent and Bruker Inc., California). Chemical shifts were expressed in parts per million (δ) using TMS as internal standard. Values of coupling constant *J* are reported in Hz. Infra-Red spectroscopy was done using Perkin Elmer FT-IR Spectrum. Two spectrometer and the mass of the compounds were determined using Agilent 1260 liquid chromatography (Agilent, USA) equipped with a quaternary solvent delivery system, and Triple quad 6410 MS system and Agilent technologies 6540 UHD Accurate Mass Q-TOF Liquid chromatography-mass spectrometer (Agilent, USA). Chemical shifts were referenced to the residual solvent signal (DMSO: δ_H 2.49 ppm, δ_C 39.7 ppm and MeOD: δ_H 3.31 ppm, δ_C 49.3 ppm).

Anti-inflammatory Activity: Inhibition of iNOS activity: The assay was performed using mouse macrophages 915 (RAW 264.7, obtained from ATCC). Cells were cultured in phenol 916 red free RPMI medium supplemented with 10 % bovine calf serum and 100 U/mL penicillin G sodium, and 100 µg/mL streptomycin at 37 ^{o} C in an atmosphere of 5 % CO_2 and 95 % humidity. Cells were seeded in 96-well plates at 5×10^4 cells/well and incubated for 24 h. Test compounds diluted in serum free medium were added to the cells. After 30 minutes of incubation, LPS (5 µg/mL) was added and the cells were further incubated for 24 h. The concentration of nitric oxide (NO) was determined by measuring the level of nitrite released in the cell culture supernatant by using Griess reagent (Quang *et al.,* 2006). Percent inhibition of nitrite production by the test compound was calculated in comparison to the vehicle control. IC_{50} values were obtained from dose curves. Parthenolide was used as positive control (Zhao *et al.,* 2014; Al-Taweel *et al.,* 2015).

Inhibition of NF-kB activity: The assay was performed in human chondrosarcoma (SW1353, obtained from ATCC) cells as described earlier. Cells were cultured in 1:1 mixture of DMEM/F12 supplemented with 10 % FBS, 100 U/mL penicillin G sodium and 100 µg/mL streptomycin at 37 ° C in an atmosphere of 5 % CO_2 and 95 % humidity. Cells (1.2×10^7) were washed once in an antibiotic and FBS-free DMEM/F12, and then reintroduced in 500 µL of antibiotic-free DMEM/F12 containing 2.5 % FBS. NF-κB luciferase plasmid construct was added to the cell suspension at a concentration of 50 µg/mL and incubated for 5 min at room temperature. The cells were electroporated at 160 V and one 70-ms pulse using BTX disposable cuvettes model 640 (4-mm gap) in a BTX Electro Square Porator T 820 (BTX I, San Diego, CA). After electroporation, cells were plated to the wells of 96-well plates at a density of 1.25×10^5 cells per well. After 24 h, cells were treated with different concentrations of test compound for 30 min prior to the addition of PMA (70 ng/mL) and incubated for 8 h. Luciferase activity was measured using the Luciferase Assay kit (Promega). Light output was detected on a Spectra-Max plate reader. Percent inhibition of luciferase

activity was calculated as compared to vehicle control and IC_{50} values were obtained from dose curves. Sp-1 was used as a control transcription factor which is unresponsive to inflammatory mediators (such as PMA). This is useful in detecting agents that nonspecifically inhibit luciferase expression due to cytotoxicity or inhibition of luciferase enzyme activity (Al-Taweel *et al.,* 2015).

Antileishmania and Antitrypanosoma Assay: All fractions were tested for their antiprotozoal activities against *Leishmania donovani* Promastigote, *L. donovani* Amastigote, *L. donovani* Amastigote/THP1 cells and *T. b. brucei* using the method described by Manda *et al.*, (2014). The *in vitro* antileishmanial and antitrypanosomal assays were done on cell cultures of *L. donovani* promastigotes, axenic amastigotes, THP1-amastigotes, and *T. brucei* trypomastigotes by Alamar Blue assays as described earlier (Croft and Yardley, 2002). The assays have been adapted to 384 well micro-plate formats. In a 384 well micro-plate, the samples with appropriate dilution were added to the *L. donovani* promastigotes or *L. donovani* axenic amastigotes or *T. brucei* trypomastigotes cultures (2 × 106 cell/mL). The compounds were tested at three concentrations ranging from 40 to 1.6 µg/mL or 10–0.25 µg/mL. The plates were incubated at 26 °C for 72 h (37 °C for axenic amastigotes and *T. brucei* trypomastigotes) and growths of the parasites in cultures were determined by Alamar Blue assay (Croft and Yardley, 2002). The compounds were also tested against *L. donovani* intracellular amastigotes in THP1 cells employing a parasite-rescue and transformation assay. The compounds were simultaneously tested for cytotoxicity against THP1 cell cultures. The compounds were also tested against *L. donovani* intracellular amastigotes in THP1 cells employing a parasite-rescue and transformation assay. IC_{50} values were computed from the dose response curves using XLfit software. Pentamidine, amphotericin B and difluoromethylornithine (DFMO) were used as a control

RESULTS AND DISCUSSION

Elucidation of Compounds: Compound 1 (22 mg) was isolated as a yellow amorphous compound with UV (MeOH) 232 nm, and melting point range: 150 – 152 °C. IR (Nujol): 1708 (C-O), 1640 (C-C), 3400 (-OH), 1590, 1455 (C C) cm^{-1}, [α]D 33 −91.5°. ^{1}H NMR (400 MHz, DMSO): δ 6.28 {*dd, J* = 2,7, H-3), 5.10 (*dd, J* = 4,6, H-4), 2.90 (m, H-5), 4.38 (m, H-6) 5.80 (s, H-7), 2.90 (m, H-9), 5.76 (s, H-10), 4. 69 (*d, J* = 8, H-l'), 3.65 (m, H-2′), 7.86 (*dd, J* = 2,7, H-2 “, 6″) 6.86 (*dd, J* = 7, 2, 7, H-3”, 5″). ^{13}C NMR (DMSO): δ = 98.0 (C-1), 139.8 (C-3), 105.6 (C-4), 46.4 (C-5), 82.9 (C-6), 132.5 (C-7), 140.2 (C-8), 48.4 (C-9), 63.7 (C-10), 100.3 (C-1′), 74.9 (C-2′), 78.0 (C-3′), 71.5 (C-4′), 78.3 (C-5′), 62.8 (C-6′), 120.1 (C-1″), 131.4 (C-2″, C-6″), 115.3 (C-3″, C-5″), 162.1 (C-4″), 165.1 (COO) This compound was identified as agnuside (1) by comparison with spectroscopic data (^{1}H, ^{13}C, COSY, HMQC and HMBC NMR experiments) values in literature (Bello *et al.,* 2017; Li *et al.,* 1995 and Ersoz *et al.,* 2001).

Compound 2 (16 mg) was isolated as a white pure/amorphous powder, its UV ƛmax. (MeOH) 210 nm, IR (KBr) u max: 3369 (-OH), 2929 (C-H), 1653 (C-C), 1369, 1227 cm−1 (C-O-C), $[\alpha]^D$ 33 −90.0° (c = 0.3, MeOH). ^{1}H NMR (400 MHz, MeOD): δ = 5.28 (1H, *d, J* = 3.5, H-1), 6.06 (1H, *d, J* = 6, H-3), 4.65 (1H, dt, H-4), 2.95 (1H, m, H-5), 5.61 (1H, s, H-7), 4.33 (*d, J* = 14), 4.78 (1H, *d, J* = 8, H-1′ of glc). ^{13}C NMR (MeOD): δ = 93.8 (C-1), 139.62 (C-3), 101.7 (C-4), 38.4 (C-5), 47.6 (C-6), 125.3 (C-7), 144.22 (C-8), 48.7 (C-9), 61.9 (C-10), 99.2 (C-1′), 73.1 (C-2′), 77.1 (C-3′), 70.5 (C-4′), 76.7 (C-5′), 59.6 (C-6′). Therefore, by comparing with spectroscopic data (^{1}H, ^{13}C, COSY, HMQC and HMBC NMR experiments) values in published data literature, it was found to be bartioside (**2**) (Bello *et al.,* 2017; Bianco *et al.,* 1976; Venditti *et al.,* 2013). Agnuside (**1**) and bartsioside (**2**) are reportedly isolated from *Vitex grandifolia* for the first time to the best of our knowledge.

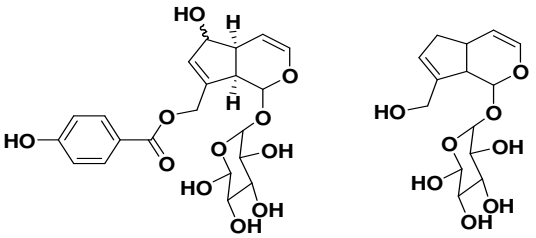

Fig 1: Iridoids isolated from *Vitex grandifolia*

Anti-inflammatory: Iridiods are renowned anti-inflammatory agents hence these iridoids in the showed significant result against different employed assays. Agnuside, isolated for the first time from *V. grandifolia* exhibited good activity against NF-kB assay with IC_{50} (ug/mL) of 8.9 while bartsioside showed an IC_{50} (ug/mL) of 12. Bartsioside showed a moderate activity against Sp-1 assay with IC_{50} (ug/mL) of 23 while agnuside showed a poor activity with an IC_{50} (ug/mL) of 63. The two iridoids displayed a moderate activity against iNOS assay with an IC_{50} (ug/mL) of 18 and 24 respectively. Parthenolide was employed as a positive standard (as Table 1 revealed).

Anti-leishmania and anti-trypanosomiasis Activity: These two compounds were active against *L. donovani* (promastigotes, axenic amastigotes and intracellular amastigotes in THP1 cells) as shown in the Table 2. Agnuside (1), an iridoid showed the highest IC_{50} (µg/mL) of 5.38 and corresponding IC_{90} (µg/mL) of 7.07 against *L. donovani* (intracellular amastigotes in THP1 cells) though it displayed a moderate activity against *L. donovani Amastigote* with IC_{50} (µg/mL) of 16.98 and corresponding IC_{90} (µg/mL) of >25. Bartsioside, one of the iridoids isolated from this wild vegetable exhibited good activity against *L. donovani Promastigote* with IC_{50} (µg/mL) of 9.09 and IC_{90} (µg/mL) of >25, agnuside (1) displayed a moderate activity against *T. brucei brucei* with IC_{50} (µg/mL) of 16.98. The positive standard employed were amphotericin B, pentamidine, α-difluoro methylornethine (see Table 2).

Table 1: IC_{50} Values of Isolated Compounds as Anti-inflammatory agents

	Test Compounds	NF-kB	Sp-1	iNOS	% cell death at the highest conc (100 µg/mL)
1	Agnuside (**1**)	8.9	63	18	63.89
2	Bartsioside (**2**)	12	23	24	41.2
3	Parthenolide	0.9	6.5	0.18	
4	Parthenolide	0.6	8	0.15	

Table 2: IC_{50} Values of Isolated Compounds against *Leishmania donovani* and *T. brucei brucei*

	Test Compounds	*L. donovani Promastigote (µg/mL)*		*L. donovani Amastigote (µg/mL)*		*L. donovani Amastigote + THP(µg/mL)*		*T. brucei brucei (µg/mL)*	
		IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
1	Agnuside (**1**)	>25	>25	16.98	>25	5.38	7.07	13.7	16.8
2	Bartsioside (**2**)	9.09	>25	16.90	>25	>25	>25	>25	>25
3	Amphotericin B	0.214	0.250	1.140	NA	0.18	0.31	NT	NT
4	Pentamidine	1.498	2.207	9.997	NA	3.17	5.20	.002	.003
5	α-difluoro methylornethine	NT	NT	NT	NT	NT	NT	2.9	7.4

NT = Not tested NA= Not active

Structure Activity Relationship (SAR) for Anti-inflammatory Activity: The following preliminary structure-activity relationship (SAR) profile is proposed based on the anti-inflammatory effects of the iridoids that were isolated from *V. grandifolia;* these are summarized as following: (a) the hydroxyl group on C-6 (-OH) might contribute to the activity of agnuside since the latter displayed more significant anti-inflammatory activity than bartsioside, Recio *et al*., 1994 reported some positive features that increases anti-inflammatory activity in iridoids are hydroxyl substitution at C-5, C-6, unsaturation at C7-C8 and methyl substitution of carboxyl C-11. The authors further reiterated that the most positive characteristic for anti-inflammatory activity is a double bond between C-7 and C-8 (Recio *et al*., 1994); (b) the bulk moiety at C-8 may also contribute to the positive activity seen in agnuside i.e. when C-8 is a tertiary carbon.

*Structure Activity Relationship (SAR) for Anti-leishmania and Anti-trypanosomiasis Activity***:** The following preliminary structure-activity relationship (SAR) profile is proposed based on the anti-leishmania and anti-trypanosomiasis activity of the iridoids that were isolated from *V. grandifolia;* it seem (a) the hydroxyl group on C-6 (-OH) might contribute to the more significant activity noticed in agnuside to bartsioside (b) the bulk moiety at C-8 may also contribute to the positive activity seen in agnuside i.e. when C-8 is a tertiary carbon.

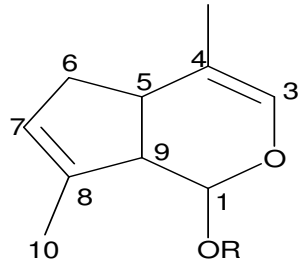

Fig 2: Basic Skeleton or Structure of Iridoids

The proton and carbon spectral of the compound 54 is similar to that of aucubin except for resonances ascribed to an additional p-hydroxybenzoyl group δ 6.84 and 7.92, J=8.6 Hz and the corresponding carbon signals i.e. δ_C 167.9, 163.7, 133 x 2, 122.2, 115 x 2 which is as same as the new compound, which is frequently encountered in *Vitex* species. This acyl group was attached to C-10 methylene protons resonances which is characteristically shifted downfield by 0.77 ppm. This was confirmed by the downfield with δ_H 2.2 ppm of C-10 signal in the ^{13}C NMR spectrum when compared to that of aucubin, hence this compound is agnuside (**1**). The ^{1}H NMR spectrum of the compound 2 showed a significant

relationship with that of aucubin except for the signals for H-6. The two multiplet signals appearing at 2.66 and 2.11 clearly indicated the absence of any substitution at C-6. Therefore, by comparing the ^{1}H NMR spectral data of compound **2** with published data, we confirm that this compound is bartsioside (**2**). Hence these constituents were identified. Beside that agnuside has been identified as a chemotaxonomy marker for Vitex family (Bello *et al.,* 2017a), agnuside belong to a group of compounds called iridoids. This class of compounds represent monoterpenoids which are cyclopenta[c]pyran in structure, they are mostly found in dicotyledonous plant families such as the apocynaceae, lamiaceae, loganiaceae, rubiaceae, scrophulariaceae, and verbenaceae etc (Villaseñor, 2007). Iridoids are renowned as anti-inflammatory agents, many authors have reported significant anti-inflammatory effects of iridoids isolated from natural sources (Awale *et al.,* 2005; Viljoen *et al.,* 2012). This study reaffirms the anti-inflammatory activity of iridoids, as agnuside and bartsioside displayed a significant effect through *in vitro* evaluation. Bello *et al.,* (2017b) carried out antiprotozoal activity of some selected medicinal plants employed traditional especially in West Africa against malaria, leishmania and trypanosomiasis. The authors further reported that methanol fraction of *Vitex grandifolia* exhibited activity against *T. brucei brucei* blood stage trypamastigotes with IC_{50} value of 8.73 (µg/mL). Agnuside (**1**) may be the compound responsible for this activity in this medicinal plant as revealed in this study.

Conclusion: Raising awareness on the so called neglected vegetables by popularizing its importance and usefulness is the thrust of this study. Two known iridoids were isolated from the polar fraction of this wild vegetable hence tagged neglected, their biologically activity were evaluated i.e. inflammation and antiprotozoal. These compounds displayed significant anti-inflammatory effect and only agnuside displayed a moderate antileishmania and antitrypanosomiasis activity. The structural activity relationship of these compounds vis-à-vis these activities were proposed. Further work on phytochemistry of the moderately polar and non-polar fraction of this plant will be commendable and more studies (*in vitro* and *in vivo*) is needed to ascertain the advantages this vegetable possesses beside nutritional gains. This study may give credence to the fact that these neglected vegetables should not be neglected after all.

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