Natural Product Sciences
26(4) : 311-316 (2020)
https://doi.org/10.20307/nps.2020.26.4.311

Chemicals Constituents from Leaves of *Diospyros iturensis* (Gürke) Letouzey & F. White and their Biological Activities

Hermann Marius Feumo Feusso[1,5,*], Jean de dieu Dongmo[1], Hermine Laure Maza Djomkam[1], Carine Mvot Akak[2], Mehreen Lateef[3], Ayaz Ahmed[4], Anatole Guy Blaise Azebaze[1], Alain François Kamdem Waffo[1], Muhammad Shaiq Ali[5], and Juliette Catherine Vardamides[1,*]

[1]Department of Chemistry, Faculty of Science, University of Douala P.O. Box 24157 Douala-Cameroon
[2]Department of Organic Chemistry, Faculty of Science, University of Yaounde I P.O. Box 812 Yaounde-Cameroon
[3]Multi-Disciplinary Research Laboratory Bahria University Medical and Dental College, Karachi, Pakistan
[4]Dr. Panjwani Center for Molecular Medicine and Drug Research International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan
[5]H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Karachi-75270, Pakistan

Abstract – The chemical investigation of the methanolic crude extract of leaves of *Diospyros iturensis* gave us 15 known secondary metabolites identified as mixture of α-amyrenone (**1**) and β-amyrenone (**2**), β-amyrin (**3**), mixture of β-sitosterol (**4**) and stigmasterol (**5**), betulin (**6**), uvaol (**7**), betulinic acid (**8**), ursolic acid (**9**), corosolic acid (**10**), actinidic acid (**11**),11-*O*-*p*-hydroxybenzoylbergenin (**12**), bergenin (**13**) and mixture of stigmasterol glucoside (**14**) and β-sitosterol glucoside (**15**) respectively. The structures of secondary metabolites were elucidated with the help of NMR and mass spectral data and by comparison of their spectral data with literature. Among the fifteen isolated compounds, four compounds were identified for the first time in *Diospyros* genus. These included uvaol (**7**), corosolic acid (**10**), actinidic acid (**11**) and 11-*O*-*p*-hydroxybenzoylbergenin (**12**). Crude methanolic extract of leaves and four isolated compounds including betulin (**6**), betulinic acid (**8**), 11-*O*-*p*-hydroxybenzoylbergenin (**12**) and bergenin (**13**) were evaluated for their antiproliferative activity against two cancer cell lines CAL-27 and NCI-H460 by the MTT assay, antioxidant potential and inhibitory activity against the lipoxygenase and urease enzymes, respectively. The results indicated that the methanolic crude extract of leaves exhibited moderate antioxidant activity and was inactive against the two cancer cell lines. Betulin (**6**), 11-*O*-*p*-hydroxybenzoylbergenin (**12**) and bergenin (**13**) exhibited moderate antioxidant and lipoxygenase inhibition with IC_{50} = 65.8, 85.6, 82.5 μM and IC_{50} = 58.5, 95.2, 76.2 μM, respectively. Furthermore, 11-*O*-*p*-hydroxybenzoylbergenin (**12**) and bergenin (**13**) exhibited moderate urease inhibition activity with IC_{50} values of 45.6 μM and 49.8 μM, respectively.

Keywords – *Diospyros iturensis*, secondary metabolites, leaves, biological activities

Introduction

Diospyros is a genus of about 450-500 species of deciduous and evergreen trees. Most are native to the tropics with only a few species extending into temperate regions.[1] They are commonly known as ebony or persimmon trees. It is the most important genus of the Ebenaceae family due to its high distribution and study for biological and phytochemical properties. In addition to *Diospyros*, the three other genera include *Euclea*, *Lassiocarpa* and *Royena*.[1] *Diospyros* species have turned out to be rich at pentacyclic triterpenes (ursane, lupane, oleanane), coumarins, steroids, diterpenes (ent-kaurane), naphtoquinones etc which possess various biological activities such as antiprotozoal, cytotoxicity, antimolluscocidal, antioxidant, anti-inflammatory, antibacterial activities.[2,3] *Diospyros iturensis* is a tree for a few meters high or small tree not exceeding 20 meters in height but up to 30-50 centimeters of diameter.[4] It is native to tropical central Africa and is mainly found in equatorial forest regions from Cameroon, Gabon, Congo Brazzaville and Kinshasa,

*Author for correspondence
Juliette Catherine Vardamides, Department of Chemistry, Faculty of Science, University of Douala P.O. Box 24157 Douala-Cameroon
Tel: +237 677 91 96 03; E-mail: jucathmas@yahoo.fr

Hermann Marius Feumo Feusso, Department of Chemistry, Faculty of Science, University of Douala P.O. Box 24157 Douala-Cameroon
Tel: +237 67707308; E-mail: feumo87@gmail.com

Angola and Equatorial Guinea and is used in traditional medicine for the treatment of bronchitis.[5] In Cameroon, *D. iturensis* is present in the Littoral region (Edea), South region (Akonetye, Ambam, kribi), Centre region (Eseka) South–West region (Mamfe) and East region (Lomie).[4] The purification of the leaves of *D. iturensis* furnished mixture of α-amyrenone (**1**) and β-amyrenone (**2**), β-amyrin (**3**), mixture of β-sitosterol (**4**) and stigmasterol (**5**), betulin (**6**), uvaol (**7**), betulinic acid (**8**), ursolic acid (**9**), corosolic acid (**10**), actinidic acid (**11**), 11-*O*-*p*-hydroxybenzoylbergenin (**12**), bergenin (**13**) and mixture of stigmasterol glucoside (**14**) and β-sitosterol glucoside (**15**). We report herein the structure elucidation of the above compounds as well as their biological activities.

Experimental section

General experimental procedures – Melting points were determined on a Büchi M-560 melting point apparatus, UV spectra were recorded on a Hitachi UV 3200 spectrophotometer in MeOH. The IR spectra were done with a Shimatzu FT/IR-8900 spectrophotometer, the NMR spectra (^{1}H, ^{13}C and 2D) were measured on a Bruker DRX NMR spectrometer operating at 400 MHz. The chemical shifts are given in δ values in ppm with TMS as internal standard and coupling constants (*J*) in Hz. The EIMS and HREIMS were recorded using a JOEL MS 600-I mass spectrometer. Silica gel (70 - 230 mesh, Merck) was used for column chromatography and thin layer chromatography was carried out on Merck pre-coated silica gel 60 F_{254} aluminum foils. Spots were visualized under UV light (254 and 365 nm) or by spraying with ceric sulfate followed by heating.

Plant material – The leaves of *D. iturensis* were collected at Lolabe 1 situated at 2°40'00" of latitude North and 9°51'00" longitude East at proximity of Kribi in the Southern region of Cameroon, in November 2015. The plant material was identified by M. Nana Victor at the National Herbarium of Yaounde where a plant specimen has been deposited under the voucher number (20226 SRF/CAM).

Extraction and isolation – The air-dried and powered leaves of *D. iturensis* were extracted at room temperature with methanol for 72 h. The extract was concentrated under reduced pressure to afford 190.1 g crude extract. The methanolic extract of leaves was subjected to column chromatography, successively eluting with *n*-hexane, mixtures of *n*-hexane-EtOAc and EtOAc-MeOH in increasing order of polarity. Elution with *n*-hexane-EtOAc (39:1) provided mixture α-amyrenone and β-amyrenone[6] (**1** and **2**; 10.1 mg; white powder) and β-amyrin[7] (**3**; 9.0 mg; white powder). Elution with *n*-hexane-EtOAc (19:1) afforded mixture of stigmasterol and β-sitosterol[2,7] (**4** and **5**; 18.1 mg; white needle. However, betulin[8,10] (**6**; 14.4 mg; white powder) and uvaol[11,12] (**7**; 12.6 mg; white powder) were obtained with elution at *n*-hexane-EtOAc (37:3). Elution with *n*-hexane-EtOAc (9:1) and *n*-hexane-EtOAc (7:1) afforded betulinic acid[13] (**8**; 20.5 mg; white powder) and ursolic acid[14] (**9**; 12.1 mg; white powder) respectively. Elution with *n*-hexane-EtOAc (3:1) yielded corosolic acid[14] (**10**; 10.2 mg; white powder) and actinidic acid[15] (**11**; 13.4 mg; white powder). Elution with *n*-hexane-EtOAc (1:1) afforded 11-*O*-*p*-hydroxybenzoyl bergenin[16] (**12**; 9.2 mg; white powder), bergenin[17,18] (**13**; 17.5 mg; white powder) and mixture of stigmasterol and β-sitosterol glucoside[2] (**14** and **15**; 52.7 mg; white powder).

Uvaol (7): White powder; (+)-EIMS *m/z*: 442.4 $[M]^+$ ($C_{30}H_{50}O_2$); mp: 226 - 227 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.13 (1H, *t*, *J* = 3.8 Hz, H-12), 3.52 (1H, d, *J* = 11.1 Hz, H-28β), 3.22 (1H, dd, *J* = 11.2, 5.0 Hz, H-3), 3.19 (1H, d, *J* = 11.0 Hz, H-28α), 1.08 (3H, s, H-27), 0.98 (3H, s, H-23), 0.97 (3H, s, H-25), 0.93 (3H, d, *J* = 5.8 Hz, H-30), 0.91 (3H, d, *J* = 5.8 Hz, H-29), 0.79 (3H, d, *J* = 11.2 Hz, H-5), 0.77 (3H, s, H-24). ^{13}C NMR (125 MHz; $CDCl_3$): δ 38.8 (C-1), 27.3 (C-2), 79.0 (C-3), 38.0 (C-4), 55.2 (C-5), 18.3 (C-6), 32.8 (C-7), 40.0 (C-8), 47.7 (C-9), 36.9 (C-10), 23.4 (C-11), 125.0 (C-12), 138.7(C-13), 42.1 (C-14), 26.0 (C-15), 23.4 (C-16), 38.0 (C-17), 54.0 (C-18), 39.4 (C-19), 39.4 (C-20), 30.6 (C-21), 35.2 (C-22), 28.1 (C-23), 16.8 (C-24), 15.7 (C-25), 17.3 (C-26), 23.3 (C-27), 69.9 (C-28), 17.3 (C-29), 21.3 (C-30).

Corosolic acid (10): White powder; (+)-EIMS *m/z*: 495.4 $[M+Na]^+$ ($C_{30}H_{48}O_4$); mp: 213-215 °C. ^{1}H NMR (400 MHz; CD_3OD+$CDCl_3$): δ 5.23 (1H, *t*, *J* = 3.8 Hz, H-12), 3.77 (1H, d, *J* = 10.4, H-28), 3.30 (1H, d, *J* = 10.8, H-2), 3.19 (1H, dd, *J* = 11.2, 4.8, H-3), 2.62 (1H, ddd, 11.2, 11.2, 6.0, H-19), 2.12 (3H, s, H-29), 2.05 (1H, d, *J* = 8.5, H-21), 2.02 (1H, d, *J* = 11.2, H-18), 1.91 (1H, d, *J* = 1.6, H-22), 1.63 (1H, d, *J* = 11.2, H-1), 1.59 (1H, d, *J* = 5.0, H-2), 1.54 (1H, ddd, *J* = 12.4, H-13), 1.40 (1H, d, *J* = 9.8, H-7), 1.40 (1H, d, *J* = 12.2, H-11), 1.35 (1H, d, *J* = 9.8, H-6), 1.27 (1H, dd, *J* = 12.2, H-9), 1.26 (1H, d, *J* = 2.7, H-16), 1.15 (1H, d, *J* = 12.4, H-12), 1.04 (1H, d, *J* = 2.7, H-15), 0.98 (6H, s, H-26, H-27), 0.95 (3H, s, H-23), 0.80 (3H, s, H-25), 0.74 (3H, s, H-24), 0.68 (1H, d, *J* = 10.8, H-5). ^{13}C NMR (100 MHz; CD_3OD+$CDCl_3$): δ 48.0 (C-1), 69.4 (C-2), 84.3 (C-3), 40.6 (C-4), 56.5 (C-5), 19.4 (C-6), 34.0 (C-7), 40.3 (C-8), 48.0 (C-9), 37.9 (C-10), 24.3 (C-11), 126.5 (C-12), 139.5 (C-13), 43.2 (C-14), 29.0 (C-15), 25.2 (C-16), 48.4 (C-17), 54.1 (C-18), 40.2

(C-19), 40.2 (C-20), 27.1 (C-21), 38.0 (C-22), 29.1 (C-23), 21.6 (C-24), 17.7 (C-25), 19.4 (C-26), 24.1 (C-27), 181.5 (C-28), 24.3 (C-29), 17.6 (C-30).

Actinidic acid (11): White powder: (+)-EIMS *m/z*: 486.3 $[M]^+$ ($C_{30}H_{46}O_5$); mp: 213-215 ºC. ^{1}H NMR (500 MHz; CD_3OD+$CDCl_3$): δ 5.28 (1H, t, *J* = 3.5, H-12), 4.62 (1H, s, H-30), 4.67 (1H, s, H-30), 3.90 (1H, ddd, *J* = 11.3, 9.6, 4.5, H-2), 3.60 (1H, d, *J* = 9.6, H-3), 3.54 (1H, d, *J* = 11.1, H-23), 3.40 (1H, d, *J* = 11.1, H-23), 2.34 (1H, d, *J* = 11.4, H-18), 1.19 (3H, s, H-27), 1.03 (3H, s, H-25), 1.01 (3H, d, *J* = 6.4, H-29), 0.84 (3H, s, H-26), 0.77 (3H, s, H-24). ^{13}C NMR (125 MHz; CD_3OD+$CDCl_3$): δ 40.8 (C-1), 67.2 (C-2), 78.7 (C-3), 44.2 (C-4), 56.5 (C-5), 18.9 (C-6), 33.7 (C-7), 40.8 (C-8), 49.6 (C-9), 48.3 (C-10), 24.4 (C-11), 127.0 (C-12), 139.5 (C-13), 43.5 (C-14), 29.1 (C-15), 25.3 (C-16), 49.6 (C-17), 54.3 (C-18), 39.1 (C-19), 154.5 (C-20), 33.7(C-21), 40.4 (C-22), 71.3 (C-23), 16.7 (C-24), 17.8 (C-25), 19.4 (C-26), 24.1 (C-27), 180.9 (C-28), 24.1(C-29), 105.3 (C-30).

11-*O-p*-hydroxybenzoylbergenin (12): White powder; (+)-EIMS *m/z*: 448.2 $[M]^+$ ($C_{21}H_{20}O_{11}$); mp: 213 - 215 ºC. ^{1}H NMR (400 MHz, CD_3OD+$CDCl_3$): δ 3.55 (1H, t, *J* = 9.4 Hz, H-3), 3.89 (3H, s, H-OCH_3), 3.87 (1H, dd, *J* = 10.5; 9.3 Hz, H-4), 3.97 (1H, ddd, *J* = 9.3; 7.5; 1.9 Hz, H-2), 4.13 (1H, t, *J* = 10.5 Hz, H-4a), 4.39 (1H, dd, *J* = 12.0; 1.6 Hz, H-11), 4.92 (2H, dd, *J* = 12.0; 1.6 Hz, H-11), 5.03 (1H, d, *J* = 10.5 Hz, H-10b), 6.84 (2H, d, *J* = 8.8 Hz, H-3', H-5'), 7.09 (1H, s, H-7), 7.94 (2H, d, *J* = 8.8 Hz, H-2', H-6'); ^{13}C NMR (100 MHz, CD_3OD+ $CDCl_3$): δ : 80.6 (C-2), 71.8 (C-3), 75.3 (C-4), 81.1 (C-4a), 165.6 (C-6), 119.2 (C-6a), 111.2 (C-7), 152.2 (C-8), 142.2 (C-9), 149.2 (C-10), 116.2 (C-10a), 74.3 (C-10b), 64.7 (C-11), 60.9 (9-OCH_3), 121.7 (C-1'), 132.9 (C-2'), 116.1 (C-3'), 163.6 (C-4'), 116.1 (C-5'), 132.9 (C-6'), 167.8 (COO).

***In vitro* anti-proliferative activity** – The anti-proliferative activities of crude extracts and isolated compounds were evaluated against two cancer cell lines CAL-27 (Human oral squamous cell carcinoma) and NCI-H460 (Human lung cancer cell line) using MTT assay.[19,20] Both cell lines were seeded in 96 well plate with the density of 15.000 and 10.000 cells per well, in DMEM supplemented with 10% FBS and incubation for 24 h at 37 ºC in a humidified incubator with 5% CO_2. After incubation, the compounds were serially diluted to the plate in incomplete medium. After 48 h of treatment, media was aspirated. MTT dye with concentration of 0.5 mg/ml was added in the plate and further incubated for 4 h. Subsequently, formazine crystals were dissolved with 100 μl DMSO per well after aspiration of media, absorbance was measured at 570 nm. According to the FDA, IC_{50} represents the concentration of a drug that is required for 50% inhibition *in-vitro*. In our study, IC_{50} is a concentration of drug at which 50% of cell population die. For primary screening, a threshold of 50% cell growth inhibition as a cut off for compound toxicity against cell lines was used. IC_{50} values were determined from plot of dose response curve between log of compound concentration and percentage (%) of cell growth inhibition. Graph was plotted by keeping log concentration of drug on X axis and % cell growth inhibition or % cytotoxicity Y axis. IC_{50} values were estimated as a concentration of drug at 50% position on Y axis. The relationship should be sigmoidal, log concentration of the drug on the X axis and 'response / measurement' of the Y axis. The prism web site has some good guides for this. So, we have used this software. IC_{50} values were calculated using the nonlinear regression program origin. The average of two (duplicates manner) were taken in determination. IC_{50} value has been derived using curve fitting methods with graph pad prism statistical software

Determination of DPPH Radical Scavenging activity – The free radical scavenging activity was measured by 1,1-diphenyl-2-picryl hydrazil (DPPH) using the method described by Gulcin.[21] The solution of DPPH of 0.3 mM was prepared in ethanol. Five microliters of each sample of different concentration (62.5 μg - 500 μg) was mixed with 95 μL of DPPH solution in ethanol. The mixture was dispersed in 96 well plate and incubated at 37 ºC for 30 min. The absorbance at 515 nm was measured by microtiter plate reader (Spectramax Plus 384 Molecular Device, USA) and percent radical scavenging activity was determined in comparison with the methanol treated control.[21] BHA was used as standard.

DPPH scavenging effect (%) = (Ac – As)/Ac × 100

Where Ac = absorbance of control (DMSO treated); As = absorbance of sample

Lipoxygenase Inhibition Assay – Lipoxygenase inhibitory activity was measured by modifying the spectrophotometric method developed by Tappel.[22] Lipoxygenase enzyme solution was prepared so that the enzyme concentration in reaction mixture was adjusted to give rates of 0.05 absorbance/min. The reaction mixture contained 160 μL (100 mM) sodium phosphate buffer (pH 8), 10 μL of test solution and 20 μL of LOX solution, in buffer. The contents were mixed and incubated for 10 min at 25 ºC. The reaction was then initiated by the addition of 10 μL substrate solution (linoleic acid, 0.5 mM, 0.12%w/v tween 20 in ratio of 1:2) and the change in absorbance at 234 nm was followed for 6 min. The

concentration of the test compound that inhibited lipoxygenase activity by 50% (IC_{50}) was determined by monitoring the effect of increasing concentrations of these compounds in the assays on the degree of inhibition. The IC_{50} values were calculated by means of EZ-Fit, Enzyme kinetics Program (Perrella Scientific In., Amhherset, USA).

Urease Inhibition Assay – Reaction mixtures comprising 25 μL of enzyme (Jack bean urease) solution and 55 μL of buffers containing 100 mM urea were incubated with 5 μL of test compounds (1 mM concentration) at 30 °C for 15 min in 96-well plates.[23] Urease activity was determined by measuring ammonia production using the indophenol method as described by Weatherburn.[24] Briefly, 45 μL each of phenol reagent (1% w/v phenol and

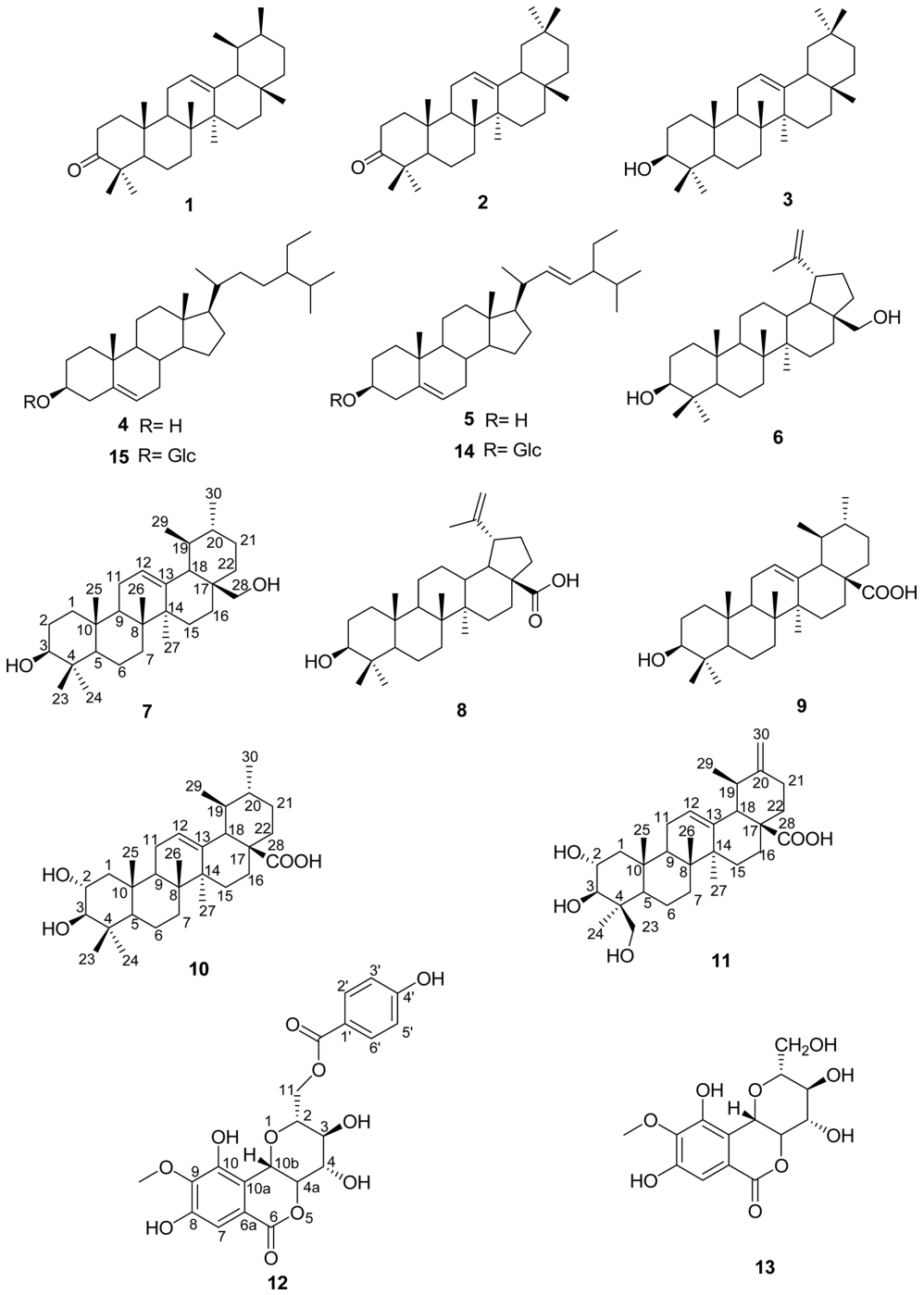

Fig. 1. Chemical structures of compounds isolated from leaves of *D. iturensis*.

0.005% w/v sodium nitroprusside) and 70 µL of alkali reagent (0.5% w/v NaOH and 0.1% active chloride NaOCl) were added to each well. The increasing absorbance at 630 nm was measured after 50 min, using a microplate reader (Molecular Device, USA). All reactions were performed in triplicate in a final volume of 200 µL. The results (change in absorbance per min) were processed by using Soft Max Pro software (Molecular Device, USA). All the assays were performed at pH 8.2 (0.01 M $K_2HPO_4.3H_2O$, 1 mM EDTA and 0.01 M $LiCl_2$). Percentage inhibitions were calculated from the formula $100 - (OD_{testwell} / OD_{control}) \times 100$. Thiourea was used as the standard inhibitor of urease.

Results and discussion

The methanolic crude extract of *D. iturensis* leaves was subjected to silica gel column chromatography to afford fifteen compounds. The structures of the isolated compounds **1 - 15** (Fig. 1) were unambiguously confirmed by spectroscopic methods, including ^{1}H NMR, ^{13}C NMR, 2D NMR, and MS analysis and by comparison of their spectroscopic data with those reported in the literature.

The methanolic crude extract and four isolated compounds including betulin (**6**), betulinic acid (**8**), 11-*O*-*p*-hydroxybenzoylbergenin (**12**) and bergenin (**13**) were evaluated for their antiproliferative activity against two cancer cell lines CAL-27 and NCI-H460 by the MTT assay, antioxidant potential and inhibitory activity against the enzymes lipoxygenase and urease, respectively. The results showed that betulin (**6**) presented moderate antioxidant and lipoxygenase inhibition with IC_{50} = 65.8 µM and IC_{50} = 58.5 µM, respectively (Table 1), confirming that the presence of the hydroxyl group at C-3 and C-28 on the lupane-type triterpenoids are necessary to exert the antioxidant and lipoxygenase inhibition activities.[8,9] Furthermore, 11-*O*-*p*-hydroxybenzoylbergenin (**12**) and bergenin (**13**) exhibited moderate urease inhibition activity with IC_{50} = 45.6 µM and IC_{50} = 49.8 µM, respectively (Table 1), suggesting that the presence of methoxyl group in C-9 and benzoyl function in C-11 in 11-*O*-*p*-hydroxybenzoylbergenin (**12**) as well as the methoxyl group in C-9 in bergenin (**13**) enhanced significantly the urease inhibition activity.[8] 11-*O*-*p*-hydroxybenzoylbergenin (**12**) and bergenin (**13**) presented moderate antioxidant activity with IC_{50} = 85.6 µM and IC_{50} = 82.5 µM, respectively; and moderate lipoxygenase inhibition activity with IC_{50} = 95.2 µM and 76.2 µM, showing that the presence of the methoxyl group in C-9 in the both compounds **12** and **13** reduced the antioxidant and lipoxygenase activities.[8] The crude methanolic extract is inactive against two cancer cell lines (Table 2).

In conclusion, fifteen compounds were isolated in the present study including nine triterpenoids, four steroids and two isocoumarins. Furthermore, four of these compounds namely uvaol (**7**), corosolic acid (**10**), actinidic acid (**11**) and 11-*O*-*p*-hydroxybenzoylbergenin (**12**) were obtained from *D. iturensis* for the first time. Moreover, we evaluated the anti-proliferative activity of the methanolic crude extract of leaves against two cancer cell lines CAL-27 and NCI-H460 by MTT assay, lipoxygenase inhibition and urease inhibition activities of some isolated compounds. The result indicated that the methanolic crude extract is inactive against two cancer cell lines, betulin (**6**) exhibited moderate antioxidant and lipoxygenase inhibition with IC_{50} = 65.8 µM and IC_{50} =

Table 2. IC_{50} values of crude extracts of *D. iturensis* leaves for NCI-H460 and CAL-27 cancer cell lines

Compounds	IC_{50} in µM	
	NCI-H460	CAL-27
DIL	>200	>200
5-Flurouracil	12.70	

DIL: *D. iturensis* leaves extract.

Table 1. IC_{50} values of pure compounds of leaves of *D. iturensis* for antioxydant and enzyme inhibition activities

Compounds	IC_{50} in µM		
	Antioxidant	Urease Inhibition	Lipoxygenase Inhibition
6	65.8 ± 0.54	Nil	58.5 ± 0.17
8	87.5 ± 0.69	Nil	>500
12	85.6 ± 0.18	45.6 ± 0.14	95.2 ± 0.18
13	82.5 ± 0.22	49.8 ± 0.26	76.2 ± 0.26
DIL	85.9 ± 0.46		
BHA	44.2 ± 0.06	-	
Baicalein		-	22.6 ± 0.08
Thiourea		21.6 ± 0.12	-

DIL: *D. iturensis* leaves extract.

58.5 μM, respectively. Furthermore, 11-*O*-*p*-hydroxybenzoylbergenin (**12**) and bergenin (**13**) inhibited moderate urease activity with IC_{50} = 45.6 μM and IC_{50} = 49.8 μM, respectively. This study revealed that *Diospyros* genus plants can be explored to discover new drugs for degenerative diseases.

Acknowledgments

The authors acknowledged TWAS and International Center for Chemical and Biological Sciences, Pakistan for the financial support, contribution in spectral registration and the biological screening. We also thank M. Nana Victor of the Cameroon National Herbarium for identification of plant material.

Conflicts of interest

The authors declare no conflict of interest.

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Received September 27, 2020
Revised November 10, 2020
Accepted November 17, 2020