Antibacterial and cytotoxic activities of undescribed cassiaric acid and other constituents from *Cassia arereh* stem barks

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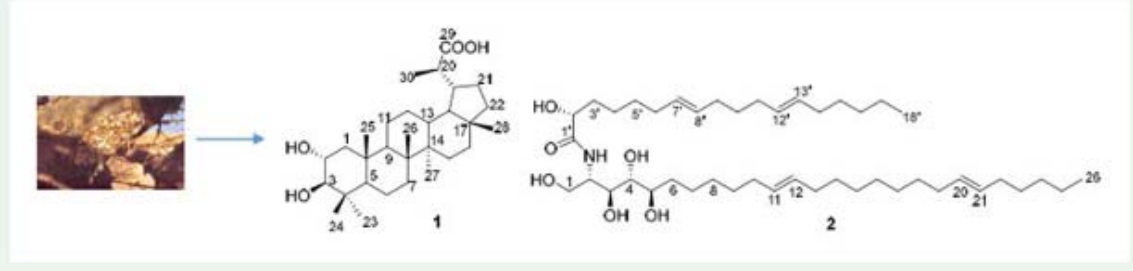
Abstract

A new lupane-type triterpene, 2*α*,3*β*-dihydroxylupan-29-oic acid (**1**), and one new ceramide derivative: (2*S**,2'*R**,3*S**,4*R**,5*R**,7'*E*,11*E*,12'*E*,20*E*)-*N*-[2'-hydroxyoctadeca-6,11-dienoyl]-2-aminohexacosa-11,20-diene-1,3,4,5-tetrol (**2**) were isolated from the ethyl acetate fraction obtained from the methanol extract of the stem barks of *Cassia arereh* together with seven known compounds. Their structures were characterized using two-dimensional NMR, mass spectrometry, and compared with reported data. To date, this is the first report of the isolation of a multiple double bonds sphingolipid type **(2)** from this genus. The ethyl acetate extract and selected isolates were examined for antimicrobial and cytotoxic activities *in vitro*. Betulinaldehyde (**5**) has shown to be active against all bacterial strains whereas, cassiaric acid (**1)** and betulinic acid (**6)** have demonstrated to be moderately active. In addition, cassiaric acid (**1**) showed the best cytotoxic result against HeLa and MCF-7 cell lines tested with IC_{50} 75.00 µM, while lupeol (**3)** and betulinic acid (**6)** displayed weak cytotoxicity at 100.00 µM.



Keywords: *Cassia arereh*; Fabaceae; cassiaric acid; lupane triterpenoid derivative; cassiaramide; sphingolipid; antimicrobial and cytotoxicity

1. Introduction

Cancer is a life-threatening disease; more than 100 different types of cancers have resulted due to underlying molecular changes within cells (Shahat et al. 2019). It is the second leading cause of death worldwide and accounted for an estimated 9.6 million (or one in six) death in 2018. The World Health Organization (WHO) predicts the number of cancer related deaths to double by 2040 in both developed and developing countries (WHO 2020). Parkin et al. (2014) estimated that 650,000 of 965 million indigenous Africans are diagnosed with cancer annually. In Cameroon, approximately 3273 new cancer cases were detected and in 2018 an estimate of 10,533 mortalities from 15,769 total cases of cancer were reported. Among the different cancer types, breast and cervical cancers remain the most common (WHO 2020). Cervical cancer cells may be vulnerable to grow under the influence of infectious agents such as bacteria, fungi, viruses and parasites. According to a recent study, infection with some particular strains of Human papillomavirus (HPV) is a well-known risk for cervical cancer (Moradi et al. 2017). Breast cancer is an invasive tumor that develops in mammary glands, it is the leading cause of death among women in European countries and low-and middle-income countries (GLOBOCAN 2012). Moreover, the spread of antibiotic-resistant bacteria represents a substantial threat to morbidity and mortality worldwide. Multidrug-resistant infections are being considered by WHO as a global priority to develop new drugs (Tacconelli et al. 2018). Historically, plants have provided a good source of anti-infectious and anti-cancer agents. For this reason, ethnomedical reports are considered of great value in drug discovery (Gezici and Şekeroğlu 2019). The genus *Cassia* (Fabaceae), comprises approximately 600 species, widely used in different communities across the globe to treat various ailments. Its ethnopharmacological values are globally acknowledged as antioxidants which have shown experimentally to neutralize or trap reactive oxygen species. A direct effect of this finding prevents cellular damage caused by the reaction of these species with proteins and nucleic acids (Jacob et al. 2002). *Cassia arereh* is an aesthetics medicinal tree widely distributed in India and Africa (Arbonnier 2004; Olusola et al. 2011). This tree is used in folk medicine and other practices to treat cancer, bacterial infections, diarrhea, dysentery, cough, dermatitis, pneumonia, yellow fever, malaria, rheumatism, and liver diseases. It is also a diuretic, an antipyretic and an analgesic agent, and sought after for its use in religious practices (Arbonnier 2004; Musa et al. 2011; Ngulde et al. 2015). *C. arereh* has shown to be a good source of alkaloids, anthraquinones, carbohydrates, bufadienolides, flavonoids, phenols, saponins, steroids, tannins and terpenoids (De et al. 2009; Ngulde et al. 2010, 2013; Olusola et al. 2011; Akanbi and Nnakaogu 2012; Imam et al. 2013; Ado et al. 2014). Furthermore, prior findings have shown-cased the excellent anticancer and antibacterial properties displayed by *C. arereh* (Ngulde et al. 2015). This finding has prompted our research team to further investigate and extend the anticancer and antibacterial potential of a wider selection of C. *arereh* related plant species.

In this present study, we intend to investigate the antibacterial and cytotoxic properties of the phytoconstituents isolated and purified from the EtOAc fraction. This paper describes the isolation and elucidation of various secondary metabolites, which include a highly unsaturated sphingolipid, from the *Cassia* genus, and bio-evaluation of the antibacterial and cytotoxic potentials of the isolates and extracts.

2. Results and discussion

A proportion of MeOH/H_2O (80:20, *v/v*) extract, prepared from the dried stem barks of *C. arereh* was partitioned with EtOAc and the resulting portion was repeatedly subjected to

column chromatography, to afford two new compounds, 2*α*,3*β*-dihydroxylupan-29-oic acid (**1**) and a ceramide derivative, (2*S**,2′*R**,3*S**,4*R**,4*R**,7′*E*,11*E*,12′*E*,20*E*)-*N*-[2′-hydroxyoctadeca-6,11-dienoyl]-2-aminohexacosa-11,20-diene-1,3,4,5-tetraol (**2**). Based on spectroscopic analysis and comparison to literature values, the structures of the known compounds were elucidated as lupeol (**3**) (Prakash and Prakash 2012), betulin (**4**) (Chaniad et al. 2019), betulinaldehyde (**5**) (Zhong et al. 1984), betulinic acid (**6**) (Bisoli et al. 2008), ceanothic acid (**7**) (Rambabu et al. 2011), *β*-sitosterol-3-*O*-*β*-D-glucoside (**8**) (Kojima et al. 1990) and epicatechin (**9**) (Spek et al. 1984) (Figure 1).

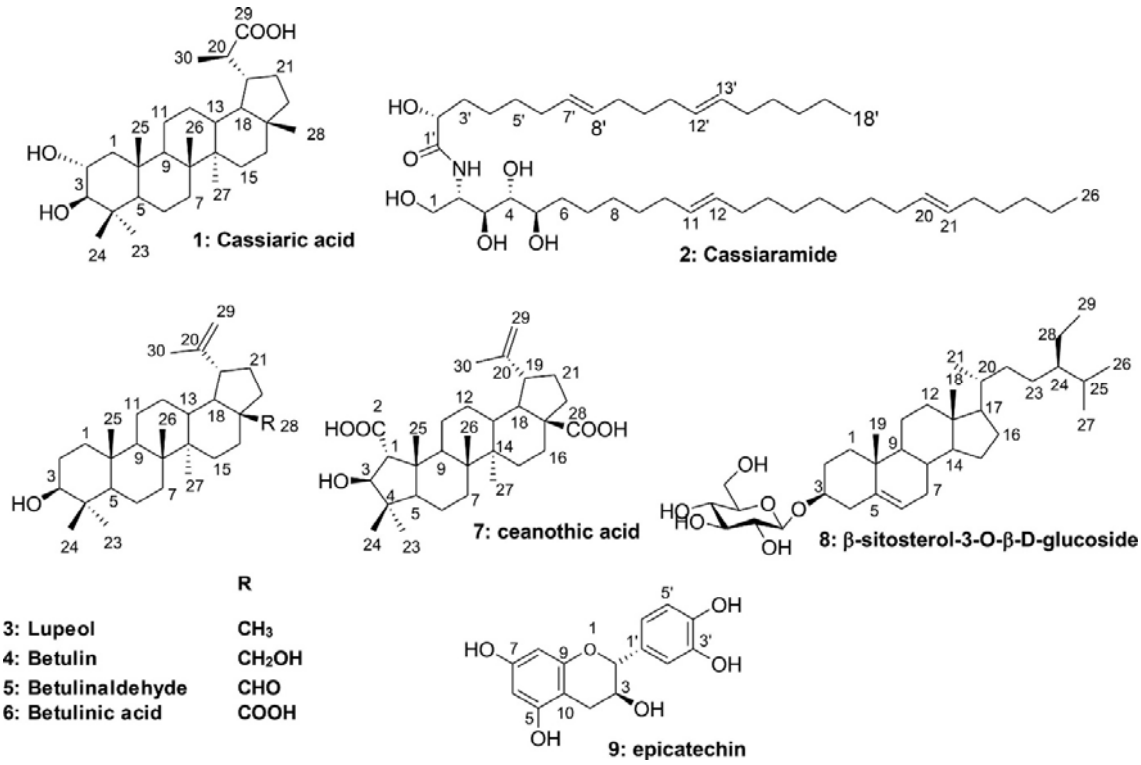


Figure 1. Structures (**1–9**) of the isolated compounds from Cassia arereh.

Compound **1**, obtained as a white solid, gave a positive result in the Liebermann–Burchard test for triterpenes. It was assigned the molecular formula $C_{30}H_{50}O_4$ based on HR-ESI-MS at *m/z* 473.3649 $[M–H]^-$ (calcd. for $C_{30}H_{49}O_4$: 473.3650), indicating six degrees of unsaturation. The ^{1}H-NMR spectrum of **1** confirmed the characteristic features for a triterpene lupane base skeleton: six tertiary methyl groups at δ_H 0.70, 0.75, 0.81, 0.85, 0.86 and 0.97 (each 3H, s, Me-28, Me-24, Me-26, Me-23, Me-27 and Me-25), one secondary methyl at δ_H 1.00 (3H, *d*, *J* = 6.9 Hz, Me-30), and two carbinolic protons at δ_H 3.14 and 3.75 (1H each, bd, H-3 and m, H-2). The methine broad doublet at δ_H 3.14 (H-3) coupled to the one at δ_H 3.75 (H-2), indicates that the extra hydroxyl group is at position 2 in ^{1}H-^{1}H COSY experiment. In addition, we have noticed the absence of the characteristic lupenyl type proton derivative H$_\beta$-19 at δ_H 2.38 described for the lupeol derivative compounds (Pereira et al. 1996). For compound **1**, of the isoprenyl moiety has been replaced by an isopropyloic group, characterized by the downfield shift of methine H-20 at δ_H 2.57 (1H, m) and the upfield shift of methyl at position 28. The ^{13}C-NMR and DEPT 135 spectra revealed 30 carbon signals. In addition, we unambiguously confirmed the skeleton of **1** to correspond to a lupane triterpenoid class of substructure, using ^{1}H- and ^{13}C-NMR analysis (see Supplementary material, Table S1). Thus, instead of observing

the characteristic isoprenyl carbons at δ_C 151.9 (C-20) and 110.3 (C-29) respectively, (Pereira et al. 1996; Prakash and Prakash 2012) for the isoprenyl moiety; six quaternary methyl carbons, two oxymethine carbons at δ_C 65.2 and 78.2, and one carboxyl at δ_C 177.1 signals were assigned to the isopropyloic group. The signals that correspond to the α and β-hydroxyl groups at C-2 and C-3 were distinctly assigned by comparing their spectral data with literature values (Schmidt et al. 1995; Rungsimakan and Rowan 2014). Firstly, the configurations of these hydroxyl groups were assigned 2α and 3β, based on the absence of interactions between H-2 and H-3 (NOESY), and secondly, by the absence of diaxial spin-spin coupling (small J value of H-3, $J = 2.5$ Hz) which indicates an equatorial position of both protons H-2 and H-3 (Culioli et al. 2003) (Supplementary material, Figure S15). In the HMBC spectrum, the H-1 proton signal at δ_H 1.46 correlated with carbons C-2 (δ_C 65.2), C-3 (δ_C 78.2), C-5 (δ_C 47.8), and C-10 (δ_C 38.2); proton H-2 at δ_H 3.75 (1H, m) correlated with carbon C-1 (δ_C 42.0) confirming the correct position of these alcoholic methine protons. The position of the carboxylic acid functional group at C-29 was confirmed by the observation on the HMBC spectrum of $^{2,3}J_{C\text{-}H}$ interactions between H-19 at δ_H 1.70 and H-20 at δ_H 2.57 to carbons C-29 (δ_C 177.1) and C-30 (δ_C 17.5); proton H-30 at δ_H 1.00 correlated to carbon C-29 (δ_C 177.1) (Supplementary material, Figure S14 and Table S1). Moreover, the ^{1}H–^{1}H COSY spectrum of **1** revealed the presence of structural characteristics of C(18)–C(19)–C(20)–C(30) as shown in Figure S1 (Supplementary material). The presence of the carboxyl group attached to C-20 was also supported by the downfield shift observed for H-20 and C-20; thus, the isopropyloic moiety in **1** was fully confirmed. The relative configuration of **1** was assigned from the NOESY spectrum (Supplementary material, Figure S15), in which the correlations from H-3 to H-23/H-27, H-23 to H-27, and H-3 to H-5/H-18 showed that the H-3, H-5, H-18 and two methyl groups (C-27 and C-26) were α-oriented, while the NOESY correlations from H-2 to H-13/H-24/H-25/H-26, H-25 to H-28/30 indicated that the H-2, H-13 and C-24, C-25, C-26, C-28 and C-30 methyl groups were β-oriented (Peng et al. 2019). Subsequently, the structure of **1** was established as shown and proposed the name 2α, 3β-dihydroxylupan-29-oic acid, for which the trivial name cassiaric acid was suggested.

Compound **2** was isolated as a white amorphous powder. The negative HR-ESI-MS analysis showed a *quasi*-molecular ion at *m/z* 718.6014 $[M–H]^–$, consistent with the formula $C_{44}H_{80}NO_6$ (calcd. for $C_{44}H_{80}NO_6$: 718.6017), which accounted for five degrees of unsaturation. The IR spectrum showed the presence of hydroxyl (3331 cm^{-1}), amide (1622 and 1544 cm^{-1}) and methylene (722 cm^{-1}) groups in the molecule (Figure 1). A broad shoulder-like band, which appeared at the higher frequency (1067 and 1022 cm^{-1}), as opposed to carbonyl absorption which appeared at 964 cm^{-1}, corresponded to a *trans* carbon-carbon double bond (Svatos and Attygalle 1997). Subsequently, the structure was fully elucidated by ^{1}H and ^{13}C NMR spectroscopy. The ^{13}C NMR spectrum revealed a signal at δ_C 51.4 (C–N) and a carbonyl signal at δ_C 175.6, suggesting the presence of an amide group. The ^{1}H NMR spectrum (Supplementary material, Table S2) of **2** indicated a broad signal in the range of δ_H 1.28–2.02 (m, CH_2 group) and a triplet at 0.89 (6H; $J = 6.7$ Hz, two-terminal Me groups, Me-26 and Me-18′) assigned to two long aliphatic chains. A signal at δ_H 5.40 (8H, m) suggested a double bond of olefinic protons. Hence, amongst the five unsaturations of **2**, four of them could unambiguously be assigned to the olefinic double bonds and the last one to the carbonyl group. Finally, the presence of four oxygenated methines (δ_H 3.52, 3.54, 3.56 and 4.04; δ_C 72.0, 72.1, 74.9 and 71.6 respectively) in compound **2** was consistent with the structure of a ceramide (Sharma et al. 2014; Khedr et al. 2018). Also, in agreement with the above-mentioned structure, we observed the characteristic peak of an oxymethylene at δ_H 3.77 (H-1) correlating in the HSQC spectrum with a carbon at δ_C 60.8 and a signal at δ_H 7.38 (d, $J = 8.0$ Hz), identified as an amide hydrogen due to the absence of correlation in the HSQC spectrum. Moreover, the assignments

of various protons and carbons in the NMR spectra were based on $^{1}H–^{1}H$ COSY and HMBC (Supplementary material, Figures S21 and S24) experiments. The COSY spectrum revealed coupling for the methine attached to the nitrogen at δ_H 4.12 (H-2) with the oxymethylene at δ_H 3.77 (H-1), the oxymethine at δ_H 3.56 (H-3) and nitrogen proton at δ_H 7.38 (N-H) respectively. The positions of the hydroxyl groups were confirmed based on the HMBC spectrum in which the proton of oxymethine signal at δ_H 3.56 (H-3) showed correlations with the carbon signals at δ_C 72.0 (C-4) and 72.1 (C-5). Methine signal at δ_H 4.12 (H-2) exhibited correlations with the carbonyl at δ_C 175.6 (C-1′); moreover, the HMBC correlation between the proton signal at δ_H 4.04 (H-2′) and the carbonyl at δ_C 175.6 (C-1′) confirmed the presence of a α-hydroxylated long-chain fatty acid. The position of the double bond in the alkenyl side chain was deduced from mass spectral analysis of the corresponding α,β-bis-methyl thiolated derivatives, which showed distinct characteristic fragments on electron bombardment (Supplementary material, Figures S2 and S27).In addition a, the presence of double bonds was supported by two ethylenic carbon values at δ_C 129.9 and 130.4. In this case, the derivatization reaction led to methyl thiolated derivatives, which mass spectrum showed two fragments at *m/z* 131 (cleavage between C(12′) and C(13′), as well as between C(20) and C(21), respectively) and *m/z* 199 (C(7′) and C(8′)), indicative of the presence of double bonds separated by three or several methylene groups. The above evidence was further supported by HMBC (Figure 2): cross-peaks of the olefinic protons at δ_H 5.40 with a carbon atom at δ_C 32.3 and 32.4 (C-10, C-13, C-19 and C-22) on the one hand, and between unsaturated adjacent methylene protons at δ_H 1.97 and 2.02 with olefinic carbons at δ_C 129.9 and 130.4 on the other. The double bonds were determined to be *trans*, according to the chemical shifts of allyl carbons at δ_C 32.3 and 32.4 (Ngono et al. 2011; dos Santos et al. 2012). Despite the double bond at position 11 of sphingosine moiety not demonstrating through dimethyl disulfide (DMDS) derivative reaction, the occurrence of *trans* olefinic double bond predominates at this position for long alkyl chain (Hay and Morrison 1973). This was confirmed by HMBC spectrum in which H-8 (δ_H 1.31) correlated with C-6 (δ_C 31.9) and C-10 (δ_C 32.4), thus, verifying the aforementioned assertion. While the stereochemistry of chiral centers has already been established in molecules (Honda et al. 1991), the comparison was made with analogous compounds (dos Santos et al. 2012). The NOESY correlation (Supplementary material, Figure S26) observed between H-2′ (δ_H 4.04) and H-2 (δ_H 4.12), and H-2 and H-4 (δ_H 3.52) indicated the common orientation on the same side of the molecule for these protons. Thus, the relative stereochemistry for the stereocenters C-2, C-2′, C-3, C-4 and C-5 was presumed to be 2*S**, 2′*R**, 3*S**, 4 *R** and 5 *R**, respectively and also confirming the presence of a long-chain base and a α-hydroxylated long-chain fatty acid in **2**. Additionally, a comparison with literature data of natural sphingamines (Nana et al. 2012; Tian et al. 2014) led to the conclusion that the optical rotation, $[\alpha]_D = +16.9°$ supported the (2*S*,2'*R*,3*S*,4*R*) configuration. The 1,3,4,5 *tetra*hydroxylated long-chain base was confirmed by the ion at *m/z* 440 ($C_{26}H_{50}NO_4$). Methanolysis of **2** produced only one fatty acid methyl ester, which was identified by LC-MS (APCI and UPLC). The MS showed one *pseudo* molecular ion peak $[M+H]^+$ at *m/z* 311.1715 (calcd. 311.1884) confirmed as methyl 2-hydroxy-octadeca-7,12-dienoate. Furthermore, the molecular formula was confirmed by the fragment ions peaks at *m/z* = 166, 252, 279, 382, 440 and 649 in its UPLC (Supplementary material, Figure S2). In conclusion, compound **2** was established to be (2*S**,2'*R**,3*S**,4*R**,5*R**,7'*E*,11*E*,12'*E*,20*E*)-*N*-[2′-hydroxyoctadeca-7,12-dienoyl]-2-aminohexacosa-11,20-diene-1,3,4,5-tetrol (**2**), for which the trivial name cassiaramide was proposed. To the best of our knowledge, there is no evidence in the literature concerning research done on this type of ceramide, making this study the first report to conduct a detailed investigation of the isolation and characterization of this compound.

The antibacterial activities of three isolated compounds and the crude MeOH extract against Gram-negative bacteria are summarized in Table S3 (Supplementary material). All three triterpenes tested displayed variable antibacterial activity. The presence of polar substituents in positions 2, 17 and 20 led to the loss of inhibitory potency as exemplified in compounds **1** (which is the most polar triterpene) and **6** (which comes second in terms of polarity) with MICs values ranging between 31.25 and 62.50 µg/mL. Both exhibited moderate activity against all microorganisms and these results are in accordance with the published data, reported by Vàzquez et al. (2012). In this research he highlighted the antimicrobial activity of pentacyclic triterpenes with MIC values ranging from 64 − 1088 µg/mL against similar microorganisms. The presence of aldehyde substituent at the C-17 position (less polar than acidic substituent) in **5** may lead to an increased activity (Supplementary material, Table S3), verifying the findings in previous studies which demonstrated that the good inhibitory effects observed may be attributed to the presence of less polar substituents on triterpenes of this class, evaluated against the similar microorganisms (Selm and Litinas 2015; Gossan et al. 2016). Evident from the MBC/MIC ratio values and according to the findings proposed by Fauchère (2002), we concluded that compounds **1** and **6** showed bactericidal effect ($1 \leq$ MBC/MIC ≤ 2) against all the strains assessed, while compound **4** and the crude extract displayed either bactericidal or bacteriostatic effects ($4 \leq$ MBC/MIC ≤ 16). However, we cannot fully explain the mode of action of antimicrobial activity of these triterpenes. For triterpenoids containing an aldehyde function, only the pharmacologic activity has been established, demonstrating their high toxicity effect (O'Brien et al. 2005), however concrete reasoning for their molecular mechanism of action could not be proposed. The cytotoxic activities of **1**, **3**, **6**, **7** and **9** were evaluated against the two human cancer cell lines (HeLa and MCF-7) at different concentrations by means of MTT method (Mosmann 1983); compounds **2**, **4**, **5** and **8** were excluded from the evaluation in this study due to their low amounts. Compound **1** exhibited the best cytotoxic activity against Hela and MCF-7 cell lines compared to other tested compounds. As shown in Figure S4 (Supplementary material,), compound **1** displayed moderate cytotoxicity against all cell lines tested, with IC_{50} value 75.00 µM. Compounds **3** and **6** showed weak or were inactive ($IC_{50} \geq 100$ µM) against the same cancer cell lines. Although previous studies of lupane-type triterpenes such as betulin, betulinic acid and lupeol showed a range of moderate to good bioactivities against various cancer cell lines (lung, liver, stomach, colorectal and breast cancers) (Cháirez-Ramírez et al. 2016), our findings reveals a more superior cytotoxic inhibitory activity of isolated triterpenes (**1**, **3** and **6**) than the those reported. Possible reasoning for this observation, could be due to these inhibitors perturbing the cell cycle and inducing apoptotic cell death in cancer cells (Rajavel et al. 2017). A lack of cytotoxic activity against MCF-7 cell lines at concentrations below 100 µM and Hela cell lines at 75 µM was noted for compounds **7** and **9**. Percentage of living cells (cell viability) present after treatment with the tested compounds at different concentrations are given in Figures S4 and S5 (Supplementary material) and show an example of three replicate of MCF-7 cell viability data for compound **6** at different concentrations. Cell viabilities were converted from absorbances of formazan formed after MTT treatment. It is clear that cell viability decreased as the concentration of test compounds increased.

3. Experimental (in supplementary data)

4. Conclusion

Two new compounds cassiaric acid (**1**) and cassiaramide (**2**) were isolated from *Cassia arereh*. Our findings suggest that most of the isolated compounds may have originated from lupane triterpene derivatives. Even though, triterpenes of this type have been isolated from several

Cassia species as mentioned before, except for ceanothic acid, the isolation of C-29 acid triterpenes and ceramides from *Cassia* genus have never been reported. Thus, our study revealed unambiguously the isolation of triterpenes as major components from *Cassia* genus.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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