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Antibacterial and cytotoxic triterpenoids from *Lantana viburnoides* ssp. *viburnoides* var. *kisi*

Lantana viburnoides ssp. *viburnoides* var. *kisi* kaynaklı antibakteriyel ve sitotoksik triterpenoidler

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SUMMARY

BACKGROUND: *Lantana viburnoides* ssp. *viburnoides* var. *kisi* is used in traditional medicine as a mosquito repellent and sometimes chewed for treatment of gastrointestinal problems. Gastrointestinal problems are varied and may include microbial infections, parasitic infestation; inflammatory bowel disease, malignancies, peptic ulcers, or simply colic pains. This work seeks to establish proof of the concept on safety and efficacy for treatment of bacterial infections.

METHODS: Root and stem bark ethanol extracts, extract fractions and two isolated compounds were tested for antibacterial activity against five standard Gram positive and two Gram negative bacteria using the broth micro-dilution method. The extracts and isolated compounds were also tested for cytotoxic activity against brine shrimp larvae (*Artemia salina*).

RESULTS: The stem and root bark ethanol extracts exhibited strong antibacterial activity against *Pseudomonas aeruginosa* (195.3 µg/ml) and *Staphylococcus aureus* (390.6 µg/ml). Camaric acid (**1**), isolated from the dichloromethane fraction of root bark extract, exhibited antimicrobial activity against *Salmonella typhi* (MIC = 19.5 µg/ml), *Streptococcus feacalis* (MIC = 19.5 µg/ml), *Pseudomonas aeruginosa* (MIC = 9.76 µg/ml), *Staphylococcus aureus* (MIC = 4.88 µg/ml), and *Bacillus subtilis* (MIC = 19.5 µg/ml), and was toxic to the shrimps (LC_{50} = 4.1 µg/ml). Betulinic acid (**2**) also from the same fraction exhibited poor anti-bacterial activity against all bacteria tested but showed high cytotoxic activity against brine shrimp larvae (LC_{50} = 2.4 µg/ml).

CONCLUSION: Detection of antibacterial activity and isolation of an antibacterial compound, camaric acid, from extracts of *L. viburnoides* ssp. *viburnoides* var. *kisi* supports the traditional use of extracts of the plant for treatment of gastrointestinal problems. Betulinic acid and camaric acid are already established to have anticancer activity.

Key words: *L. viburnoides* ssp. *viburnoides* var. *kisi*; Verbenaceae; triterpenoids; camaric acid; betulinic acid; antibacterial; cytotoxic

ÖZET

GİRİŞ: *Lantana viburnoides* ssp. *viburnoides* var. *kisi,* bir sivrisinek kovucu olarak geleneksel tıpta kullanılmakta ve bazen gastrointestinal sorunların tedavisi için çiğnenmektedir. Gastrointestinal sorunlar, mikrobiyal enfeksiyonlar, parazitik enfestasyonlar, enflematuar bağırsak hastalıkları, kanserler, peptic ülser veya basit kolik ağrılar olmak üzere değişkendirler. Bu çalışma, bakteriyel enfeksiyonların tedavisinde güvenli ve etkin bir kavramın kanıtını ortaya koymak istemektedir.

YÖNTEM: Gövde ve kök yaprak etanol ekstreleri, ektre fraksiyonları ve izole edilen iki bileşik, broth mikrodilüsyon yöntemi kullanılarak, beş standart gram pozitif ve iki gram negative bakteriye karşı antibakteriyel etki bakımından test edildi. Ekstreler ve izole edilen bileşikler, tatlı su karidesi larvasına (*Artemia salina)* sitotoksik etkisi açısından da test edildi.

BULGULAR: Kök ve gövde yaprak etanol ekstreleri, *Pseudomonas aeruginosa* (195,3 µg/ml) ve *Staphylococcus aureus* (390,6 µg/ml)'a karşı güçlü bir antibakteriyel aktivite sergiledi. Gövde yaprak ekstrelerinin diklormetan fraksiyonlarından izole edilen Kamarik asit, *Salmonella typhi* (MIC = 19,5 µg/ml), *Streptococcus feacalis* (MIC = 19,5 µg/ml), *Pseudomonas aeruginosa* (MIC = 9,76 µg/ml), *Staphylococcus aureus* (MIC = 4,88 µg/ml), ve *Bacillus subtilis*'e (MIC = 19,5 µg/ml) karşı antimikrobiyal aktivite sergiledi ve karideslere toksik etkiliydi (LC_{50} = 4,1 µg/ml) . Aynı fraksiyondan elde edilen diğer bileşik olan Betulinik asit de test edilen tüm bakterilere karşı kötü antibakteriyel aktivite sergilerken, tuzlu su karidesi larvasına karşı yüksek sitotoksik aktivite gösterdi (LC_{50} = 2,4 µg/ml).

SONUÇ: *L. viburnoides* ssp. *viburnoides* var. *kisi*'nin ekstrelerinden antibakteriyel bir bileşik olan kamarik asitin izolasyonu ve antibakteriyel aktivitenin saptanması, gastrointestinal sorunların tedavisi için bitki ektrelerinin geleneksel kullanımını desteklemektedir. Betulinik asit ve kamarik asit zaten antikanser aktiviteye sahip olarak oturmuş bileşiklerdir.

Anahtar kelimler: *L. viburnoides* ssp. *viburnoides* var. *kisi*, Verbenaceae, triterpenoidler, kamarik asit, betulinik asit, antibakteriyel, sitotoksik

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INTRODUCTION

Lantana viburnoides ssp. *viburnoides* var. *kisi* (A. Rich) Verdc. (Verbenaceae) is a 0.3-2.5 m tall shrub, with discolourous leaves that are densely thick and very rugose above [1-2]. The plant is indigenous to Tanzania, where the leaves are used as mosquito repellent in Iringa region, and sometimes chewed to relieve gastrointestinal problems [3-5]. The Jaluo of Northern Tanzania report that the plant is poisonous if eaten in large amounts, but it is non-poisonous to sheep and goats, being eaten mostly by these domestic animals [5]. The plant has larvicidal activity against *Anopheles gambiae s.s* that is reported to be due to lantadene triterpenoids and furanonaphthaquinones that were recently isolated from the plant [3]. Literature search indicates that, several lantadene triterpenoids from *Lantana* species exhibit strong activity against both gram positive and gram negative bacteria [6-8].

Gastrointestinal problems are varied and may include microbial infections, parasitic infestation; inflammatory bowel disease, malignancies, peptic ulcers, or simply colic pains. In the present study we investigated the toxicity and antibacterial activity of the extracts and compounds isolated from *L. viburnoides* sp. *viburnoides* var. kisi as a means to establish proof of the concept for treatment of gastrointestinal conditions associated with bacterial infections. The brine shrimp lethality test was also used as a preliminary tool to determine safety.

MATERIAL AND METHODS

General instruments and materials used

CC: silica gel (Merck, 230-400 Mesh, pet ether/ethyl acetate) or Sephadex® LH-20 (Pharmacia), 1:1 v/v MeOH/ $CHCl_3$); VLC: silica gel (Merck, 230-400 Mesh), CH_2Cl_2 then EtOH; TLC: silica gel (60 F_{254}, Merck) precoated on plastic or aluminium plates; visualization: UV/VIS or anisaldehyde spray; FT-IR: Shimadzu 8400; UV-VIS: 168 diode array detector; 1D and 2D NMR: Bruker Avance DPX 300 NMR spectrometers, operating at 300 for ^{1}H NMR, and 75 MHz for ^{13}C NMR (δ= 0; TMS internal standard); MS: high resolution TOF MS EI mass spectrometer operating at 70 eV. Soyabean Casein Digest Agar (Oxoid Ltd., Hampshire, England); Tryptone Soya Broth (Oxoid Ltd., Hampshire, England); Iodonitrotetrazolium chloride (0.2%, Sigma Aldrich, United States); Dimethylsulfoxide (Lab Pak Ltd., Mill Lane, Fillongley); Gentamicin (Vital Healthcare, Canada).

Collection of plant materials and extraction

Root and stem barks were collected in October, 2008 from Lugaga village in Mufindi district, Tanzania, and voucher specimen identified by Mr. F.M. Mbago a botanist in the Department of Botany of the University of Dar es Salaam. Voucher specimen no. FMM 3290 is deposited at the Herbarium of the Department of Botany, University of Dar es Salaam. The plant materials were air-dried, pulverized and powder soaked in ethanol twice each for 72 h. The crude extracts were then fractionated on silica gel by flashing with dichloromethane twice followed by ethanol.

Antimicrobial studies

Extracts were tested against representative Gram-positive and Gram-negative bacteria. The Gram-negative bacteria included *Pseudomonas aeruginosa* (NCTC 10662) and *Salmonella typhi* (NCTC 8385), while Gram-positive bacteria included *Streptococcus faecalis* (Clinical isolate), *Bacillus anthraci*s (NCTC 10073), *Bacillus subtilis* (Clinical isolate), *Staphylococcus aureus* (NCTC 25923) and *Streptococcus agalactiae* (NCTC 8181). Selection of these bacteria was guided by their availability at the time of the study.

Using a 96 well microtitre plate, the bacteria were tested in duplicate against each of the extracts, fractions and compounds. Two rows were designated for a negative control (DMSO), two for the positive control (gentamicin), two rows for only microbes and broth, and the remaining rows were reserved for placement of samples. Broth (50 µl) was added to each well of the first rows of each category, followed by 50 µl of 100 µg/ml gentamicin (positive control rows), and DMSO in the solvent control rows. The other first rows received 50µl of extracts, extract fractions (50 mg/ml), or pure compounds (2.5 mg/ml). Subsequently 50 µl were transfered from the first rows of each category to the second rows, from second rows to the third rows, and the same repeated down to the end of each column. The 50 µl from the last rows were discarded. After this double dilution 50 µl of over night broth cultures (0.5 MacFarland standard turbidity) of test bacteria were added to each well and then incubated at 37°C for 24 h. The presence of bacterial growth was determined visually using *p*-iodonitrotetrazolium (INT) chloride dye. After 24 h of incubation at 37°C, 40µl of 0.02% INT solution was added to each well followed by incubation for 1h at 37°C. Bacterial growth was indicated by a change in colour of INT (emergence of pink colour) in the wells. Absence of bacterial growth was indicated by no colour change of the

dye. The lowest concentration which showed no bacterial growth was taken as MIC. Grading of the activity of crude extracts was done according to the criteria proposed by Aligiannis et al., 2001 [9]. According to this arbitrary criterion crude extracts with MICs up to 500 µg/ml are considered to have strong inhibitory activity; MICs between 600 µg/ml and 1.5 mg/ml as having moderate inhibitory activity and MICs above 1.6 mg/ml as having weak inhibitory activity.

Brine shrimp test

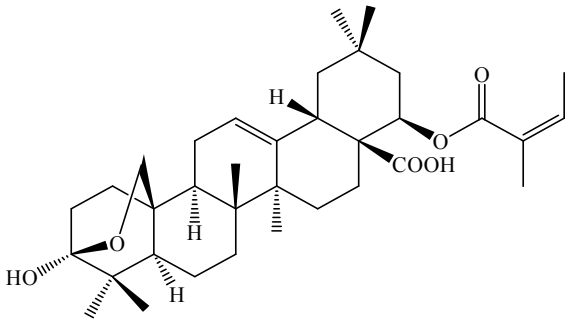
The brine shrimp lethality test (BST) was set as described by Meyer et al., 1982 and Moshi et al., 2009 [10,11]. Briefly, solutions of the extracts were made in DMSO, and then in artificial sea water at concentrations of 8, 24, 40, 80 and 240 µg/ml for the extracts and 2.5, 5, 10 and 20 µg/ml for pure compounds and then incubated for 24 h in triplicate vials. Ten brine shrimp larvae were put in 5 ml of a mixture containing the sample, seawater and DMSO, for treatment experiment and only seawater and DMSO for control experiment. The average number of survived larvae in each triplicate was recorded after 24 h and the mean percentage mortality obtained was used in calculation of LC_{50} using SAS program [12].

Fractionation of compounds

Repeated fractionation on silica gel column of the dichloromethane root extract yielded camaric acid (**1**) as a white amorphous compound. The compound was obtained by eluting with a mixture of ethyl acetate and n-hexane (2:3 v/v) followed by column chromatography over Sephadex® LH-20 eluting with a mixture of methanol and chloroform (1:1 v/v) and then purified by column chromatography on silica gel eluting with a mixture of ethyl acetate and n-hexane (1:1 v/v gradient). Betulinic acid (**2**) was obtained as white amorphous after column chromatography eluting with a mixture of ethyl acetate and petroleum ether (1:9-3:7 v/v gradient) followed by washing with cold hexane.

***Camaric acid* (1)**. White amorphous (MeOH), 188-189 ^{o}C [14]; Yield, 325 mg; anisaldehyde - greenish-yellow; IR (KBr) ν_{max} cm-1, 3430 br, 2951, 2877, 1716, 1649, 1556, 1513, 1462, 1376, 1235, 1152, 1042, 938 and 845; MS, *m/z* (% rel. int.) 568 ([M]+, 0.1), 469 (30), 468 (100), 450 (20), 424 (18), 407 (10), 285 (34), 239 (28), 201 (27), 189 (40), 119 (42), 83 (100), 69 (42) and 55 (94); ^{1}H and ^{13}C NMR [13-14]

***Betulinic acid* (2)**. White amorphous (MeOH), Yield, 308 mg; anisaldehyde - black; IR (KBr) ν_{max} cm-1, 3438 br, 3072, 2945, 2872, 1696, 1646, 1558, 1377, 1232, 1187, 1031 and 887; MS, *m/z* (% rel. int.) 456 ([M]+, 12), 438 (7), 410 (5), 248 (45), 219 (20), 220 (20), 207 (50), 189 (100), 175 (32), 161 (20), 147 (25), 135 (45), 119 (47), 107 (48), 95 (57), 69 (55) and 55 (65); ^{1}H and ^{13}C NMR [15-16].



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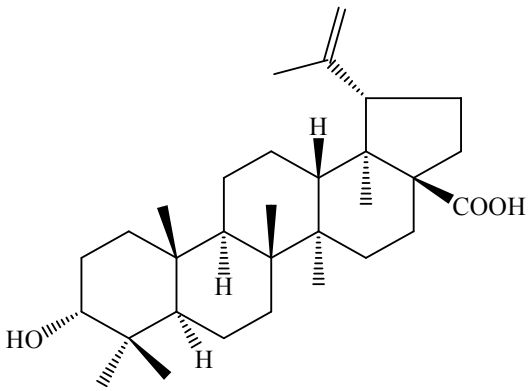

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Figure 1: Chemical structure of camaric acid (1) and betulinic acid (2)

RESULTS

Antibacterial activity

The ethanol extract of the stem barks exhibited strong antibacterial activity against *Pseudomonas aeruginosa* (195.3 µg/ml) and *Staphylococcus aureus* (390.6 µg/ml), while the root bark ethanol extract exhibited strong antibacterial activity against *Pseudomonas aeruginosa* (195.3 µg/ml) and *Staphylococcus aureus* (390.6 µg/ml), and weak activity against *Salmonella typhi* (MIC 2500 µg/ml). After fractionation with dichloromethane and

Table 1: Antibacterial activity expressed as Minimum inhibitory concentrations (MICs)

Sample name	MIC* (µg/mL)						
	S. typhi	***P. aeruginosa***	***S. aureus***	***S. agalactiae***	***B. anthracis***	***B. subtilis***	***S. faecalis***
L.virbunoides stem bark EtOH extract	NA	195.3	390.6	NA	NA	NA	NA
L.virbunioides root bark EtOH extract	2500	195.3	390.6	NA	NA	NA	NA
L.virbunoides root bark EtOH fraction	NA	195.3	390.6	NA	NA	NA	NA
L.virbunoides root bark DCM fraction	NA	1562.5	390.6	NA	NA	1562.5	NA
L.virbunoides stem bark EtOH fraction	NA	NA	NA	NA	NA	NA	NA
L.virbunoides stem bark DCM fraction	NA	1562.5	390.6	NA	NA	NA	NA
Camaric acid	19.5	9.76	4.88	2500	625	9.76	19.5
Betulinic acid	NA	NA	NA	NA	NA	NA	NA
Gentamicin	≤ 1.0	≤ 1.0	≤ 1.0	50	≤ 1.0	3.12	1.56

*MIC value less than 500 µg/ml shows strong inhibition; Between 500-1600 µg/ml- moderate inhibition; Greater than 1600 µg/ml- weak inhibition or not active (NA) [19]

Table 2: Yields (%) and toxicity activity of extracts and pure compounds

Sample name	Yield	Toxicity (µg/ml)	
	g (%)	LC_{50}	95% CI
1. *L.virbunoides* stem bark EtOH extract	54.7 (2.2)	10.6	7.1 - 15.8
2. *L.virbunioides* root bark EtOH extract	60.3(2.4)	10.6	7.5 - 15.5
3. Betulinic acid (**2**)	0.31 (0.12)	2.4	1.4 - 4.0
4. Camaric acid (**1**)	0.33 (0.13)	4.1	2.9 - 5.8
5. *Cyclophosphamide [16]		16.30	10.6 - 25.15

Positive cytotoxic standard; Amount of plant materials used was 250 g

ethanol the total extract resulted in an inactive stem bark ethanol fraction, while the dichloromethane fraction retained activity against *Pseudomonas aeruginosa* (MIC 2500 µg/ml) and *Staphylococcus aureus* (MIC 390.6 µg/ml) and remained inactive against the other bacteria tested. The ethanol fraction of the root ethanol extract retained activity against *Pseudomonas aeruginosa* (195.3 µg/ml) and *Staphylococcus aureus* (390.6 µg/ml). The dichloromethane fraction showed lower activity against *Pseudomonas aeruginosa* (1562.5 µg/ml), and retained the same level of activity for *Staphylococcus aureus* (390.6 µg/ml). The root dichloromethane extract was also minimally active against *Bacillus subtilis* (MIC 1562 µg/ml).

Column chromatography (TLC) of the dichloromethane root bark fraction yielded camaric acid (**1**) and betulinic acid (**2**) as major compounds together with a series of furanonaphthaquinones as minor constituents. The chemical structures of compound 1 and 2 (***Figure 1***) were established on the basis of interpretation of their ^{1}H and ^{13}C NMR, and mass spectral data in comparison with those reported in the literature for camaric acid (**1**) [13-14]and betulinic acid (**2**) [15-16].

Camaric acid (**1**) exhibited broad and strong antibacterial activity against both Gram positive and Gram negative bacteria with MICs ranging from 4.88-19.5 µg/ml, except for *Bacillus agalactiae* and *Bacillus anthracis* which had MIC of 2500 µg/ml and 625 µg/ml, respectively (***Table 1***). Betulinic acid did not exhibit significant antibacterial activity against any of the tested bacteria.

Brine shrimp toxicity

The brine shrimp results show that the stem and root bark ethanol extracts were equally toxic to the shrimps with LC_{50} value of 10.6 µg/ml (Table 2). The two isolated compounds, camaric acid (LC_{50}=2.4 µg/ml) and betulinic acid (LC_{50}=4.1 µg/ml) were more toxic than the crude extracts. The pure compounds were more toxic to the *Artemia salina* larvae than the cytotoxic drug cyclophosphamide (LC_{50}=16.3 µg/ml) which is used in our laboratory as a standard positive control.

DISCUSSION

The results suggest that ethanolic extracts of *Lantana viburnoides* root and stem barks have both antibacterial and cytotoxic activity, and that of the two isolated triterpenoids camaric acid (**1**) significantly contributes to antibacterial activity. These results are in agreement with a previous report that camaric acid (**1**) exhibited antibacterial activity against *Bacillus subtilis, Escherichia coli* and *Micrococcus luteus* [6]. Camaric acid (**1**) has been reported to possess nematicidal activity against *Meloidogyne incognita,* antimycobacterial activity against *Mycobacterium tuberculosis,* [17], larvicidal activity against *Anopheles gambiae s.s* [3] and antitumor activity [18], but showed no significant *in vitro* antiplasmodial against chloroquine resistant (K1) and sensitive (T9-96) *Plasmodium falciparum* [7]. Camaric acid (**1**) was also highly cytotoxic (LC_{50} LC_{50}=4.1 µg/ml) to brine shrimps.

Betulinic acid (**2**) did not show anti-bacterial activity against any of the tested bacteria (**Table 1**) but had high cytotoxic activity (LC_{50}=2.4 µg/ml) (**Table 2**). These results are consistent with an earlier report that betulinic acid exhibited weak antimycobacterial activity [19]. The literature also report that betulinic acid exhibit a variety of other biological activities, including inhibition of human immunodeficiency virus (HIV) [20-21], anti-inflammatory [22] and antioxidant [23] properties.

The cytotoxic properties of betulinic acid shown in these results support literature reports for the anticancer activity of this compound [20]. Because betulinic acid (**2**) has strong anti-HIV activity, selective cytotoxicity against tumor cells and favorable therapeutic index, it is a very promising new chemotherapeutic agent for the treatment of HIV infection and cancer [20-22].

The results also support the use of *L. virbunoides* extracts for the treatment of gastrointestinal problems related to bacterial infections [3, 4], and given the known biological activities of betulinic acid [20-22], and camaric acid the extracts are indeed indicated to be a valuable traditional remedy for gastrointestinal ailments and other conditions. However, potentially this plant is likely to contain a lot of other tritepenoids [23], probably in smaller amounts, and therefore the full potential of this plant remains to be further explored. The fact that the cytotoxic activity is selective against cancer cells [20, 22] suggests that the extracts may be safe for use by human beings.

CONCLUSION

Detection of antibacterial activity and isolation of an antibacterial compound, camaric acid, from extracts of *L. viburnoides* ssp. viburnoides var. *kisi* supports the traditional use of extracts of the plant for treatment of gastrointestinal problems. Betulinic acid and camaric acid are already established to have anticancer activity.

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