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Research Article

Isolation and Biological Testing of Constituents from *Ilex kaushue* S.Y.Hu (Aquifoliaceae) Vietnam

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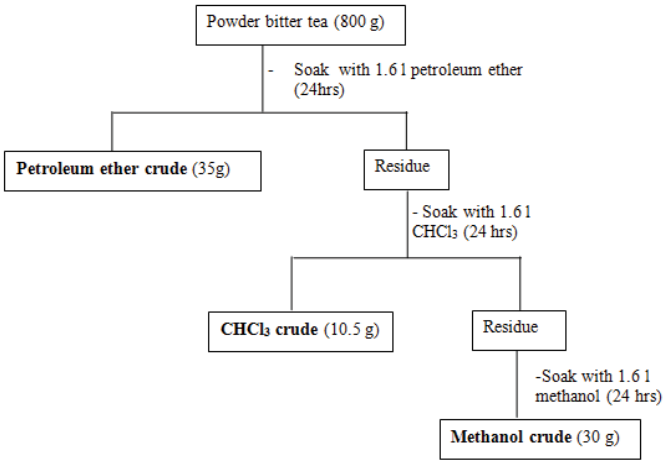
ABSTRACT

Compounds from the leaves of *Ilex kaushue* have been isolated: uvaol, 3*β*, stigmast-5-en-yl-*β*-D-glucopyranoside and three kudinosides C, D and E. Biological testing both for antimicrobial and cytotoxic activity of crude fractions showed activity possibly supporting the common belief that tea of these leaves is potentially healthy.

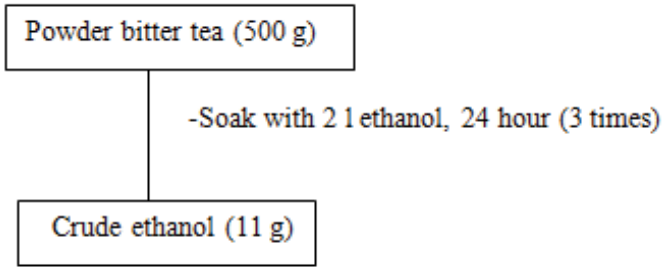
Keywords: *Ilex kaushue* S.H.Hu; Triterpenoids; Biological testing

INTRODUCTION

Leaves from *Ilex kaushue S. Y. Hu*, collected in the Cao Bang province, North Vietnam are used as an alternative to common tea (*Cammellia sinensis*). Though much more bitter than common tea, the beverage, named Cao Bang bitter tea, has an underlying sweet aftertaste, which makes it attractive. A similar feature is found in the China based one *Ilex kudingcha* (Schemes 1 and 2). This tea was found to have good antioxidant properties [1,2]. Other healthy extracts are based on *Ilex paraguariensis* [3] or *Ilex latifolia* [4]. *Ilex kaushue* presumably gives considerable health benefit and is used in folk medicine as a diuretic agent, and a remedy for common cold and sore throat. In previous papers six triterpenoids have been reported as well as 3,5-dicaffeonylquinic acid [7]. The present paper reports on an extraction of these leaves both at room temperature and with hot solvents (Soxhlet) leading to structural identification of five triterpenoids (Schemes 3-5). The crude mixtures were tested for biological activity both with respect to antimicrobial activity, cell toxicity and antioxidant activity.



Scheme 1: Procedure for creation of crude fractions for biological testing. Exemplified by the soak process.



Scheme 2: Extraction with ethanol.

MATERIALS AND METHODS

Melting points were determined using a Maquenne block and were uncorrected. IR spectra (KBr discs) were measured on a Bruker Quinox 55 spectrometer. NMR spectra (1D, extensive 2D-COSY, HMQC, HMBC) were recorded (TMS is used as reference) with a Bruker 500 MHz

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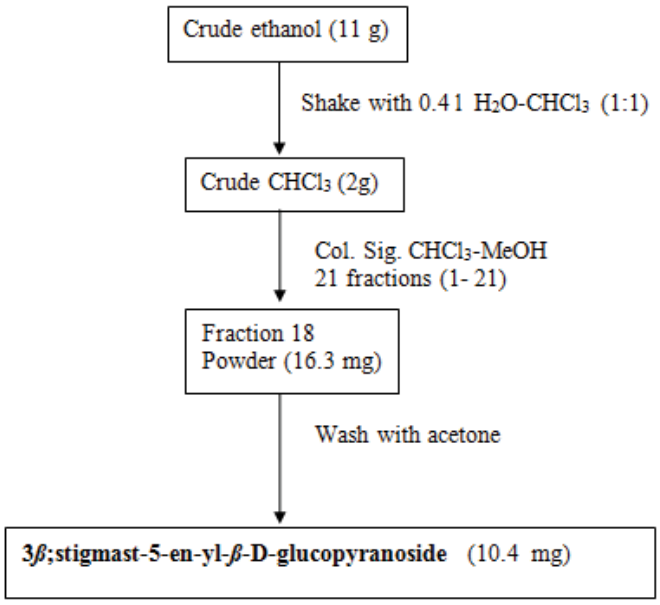
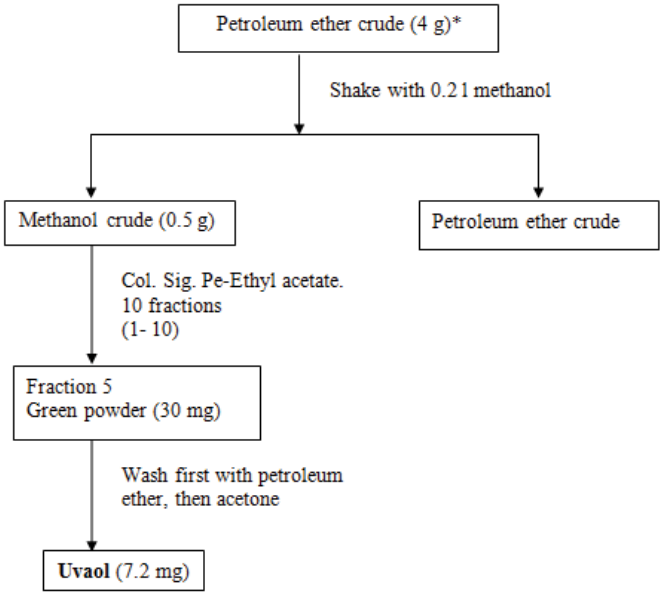


Scheme 3: Isolation of 3*β*; stigmast-5-en-yl-*β*-D-glucopyranoside. Col. Sig means column silica gel.



Scheme 4: Isolation of uvaol. *4 g out of 35 g petrol ether crude from Scheme 1.

spectrometer. Isolation procedures were monitored by thin layer chromatography on silica gel 60 (70-230 mesh, Merck).

Solvents

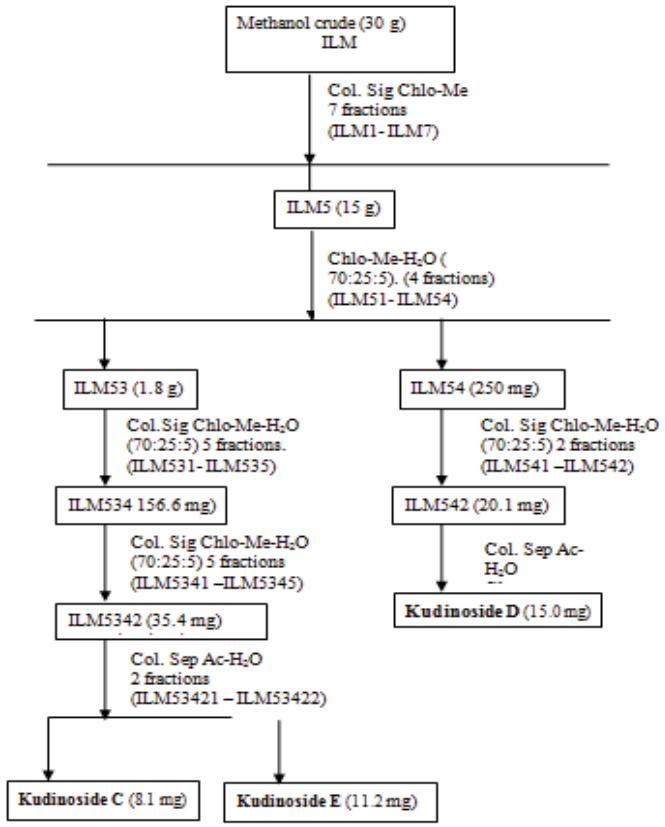
Absolute ethanol (Chemsol). Petroleum ether boiling point interval 60-90°C. Methanol b.p 66°C and chloroform, from Labscan (analytical grade).

Chromatography

Silica gel 40-60 m (Merck and Scharlau). Sephadex (25-100 m) LH20100 (Sigma-Aldrich).

Materials

Leaves of *Ilex kaushue* S. H. Hu were collected from Vuon Cam in the Cao Bang province, North Vietnam. The specie is identified by Prof. Nguyen Tien Ban and a specimen is kept at the Natural



Chlo: Chloroform; Me: Methanol; Ac: Acetone; Sig: Silica gel; Sep: Sephadex

Scheme 5: Isolation of kudinosides.

Science University of Ho Chi Minh city. Leaves were dried at room temperature and then grinded to a powder.

Extracts

Biological testing

Antibacterial activity: The antibacterial activity of the seven crude extracts was examined by use of the dilution method [8]. A stock solution of each extract was prepared in DMSO and the solvent concentration never exceeded 0.5 Mc. Fland. The dilutions were made and plated in standard 96-well microtiter plate (1-200 µg/ml) including wells with solvent only as controls. The Minimum Inhibitory Concentration (MIC) was estimated as the lowest concentration of the extracts that do not show any visible growth compared with control. The bacteria were incubated in the Eugon broth medium for 24 hours at 37°C and fungi in Mycophyl broth medium, for 48 hours at 30°C. The following microorganisms were included in the test: *E. coli* and and *P. aeruginosa* for Gr(-); *B. subtilis* and *S. aureus* for Gr(+); *A. niger* and *F. oxysporum* for molds and *S. cerevisiae* and *C. albicans* for yeasts. After that, they were made again with agar medium and had CFU (Colony Forming Unit) value<5.

- Ampicilline 50 mM for Gr(+)
- Tetracyline 10 mM for Gr(-)
- Nystatine 0.04 mM for molds and yeasts
- The samples 4 mg/mL.

- The concentration of DMSO in the samples<0.5%.

Antioxidant activity: The method used was that of Gorinstein et al. [9]. The antioxidant activity is determined by the DPPH (1,1 diphenyl-2-picrylhydrazyl) method with ascorbic acid as positive control (5 mM in DMSO). Negative control is DMSO (Dimethyl sulfoxide) solvent. Seven concentrations of the extract are mixed with the DPPH ethanol stock solution on a 96 well microtiter plate and the change in color (purple to yellow) after 30 minute of incubation is measured at 515 nm on an ELISA microtiter plate reader. From the absorbance value (Abs), the Scavenging Capacity (SC) in % is calculated as:

$$SC\% = [100 - \frac{Abs_{sample} - Abs_{blank}}{negative\ control} \times 100]$$

Cytotoxic activity:

- The cytotoxic activity was determined using the dye binding assay, the Sulforhodamine B (SRB) method [10,11] with ellipticine as a positive control to quantify the 50% growth inhibitory concentration (IC_{50}) against two human cancer cell lines Hep-2 and RD.
- Cells were obtained from the National Institute of Hygienic and Epidemiology of Vietnam. Cells were grown in Dolbeco Modified Eagle's medium, from Gibco and subcultivated twice a week. For the cytotoxicity assay, cells were seeded in 96 well-microtiter plate with a density 3-4 × 10^4 ml, after 24 hours, cells were exposed to the test extracts in various concentrations from 4-200 µg/ml (solvent concentration is below 0.5%), the plate was incubated in the 5% CO_2 incubator for 72 hours. After that, the old medium was removed; the cells were fixed with 30% trichloroacetic acid, washed, incubated with Sulforhodamine dye, then diluted in trisbase and read in an ELISA reader at 515-540 nm. The number of cell survival is expressed as means of 6 wells ± SD standard deviation.

RESULTS

Crude extracts from the leaves of *Ilex kaushue* (Aquifoliaceae) are prepared both by extraction at room temperature, called soaking, using petroleum ether, chloroform and methanol and ethanol [5] and by consecutive extraction with a Soxhlet apparatus starting with the non-polar solvent petroleum ether followed by chloroform and finally methanol (Figures 1 and 2). The soak with ethanol and the Soxhlet extraction with petroleum ether, chloroform and methanol have previously been described [5,6]. Soak with ethanol resulted in 3 β; stigmast-5-en-yl-β-D-glucopyranoside [12].

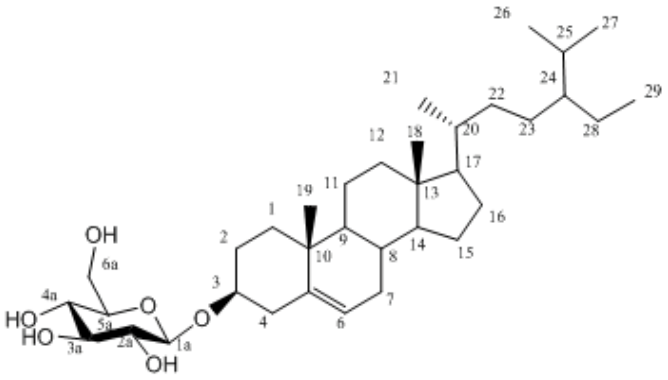


Figure 1: 3β;stigmast-5-en-yl-β-D-glucopyranoside.

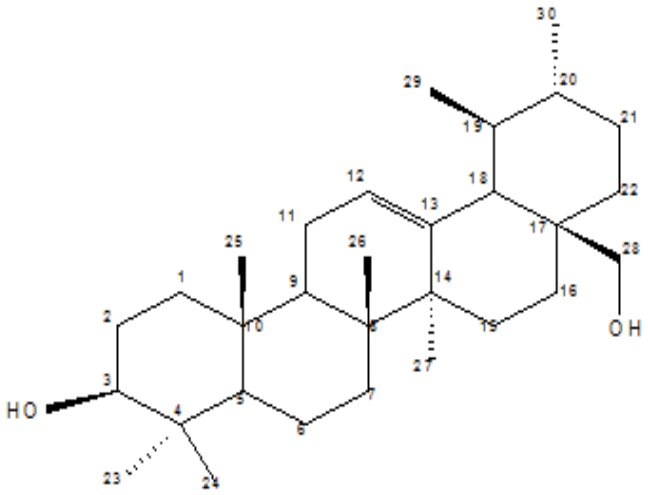


Figure 2: Uvaol.

White powder. 10.4 mg. m.p. 276-278°C.

IR υ_{max} (cm^{-1}) 3423 (OH), 1638(C=C).

LC/MS (+) m/z=577 $[M+H]^+$.

^1H NMR data of 3*β*; stigmast-5-en-yl-*β*-D-glucopyranoside are given in Table 1 together with ^{13}C data. DEPT data show:

-6 methyl carbons, 12 methylene carbons, 13 methine carbons and 3 quaternary carbons are identified.

Uvaol [14] was isolated as shown in Figures 3 and 4.

White powder. 7.2 mg. m.p. 190-192°C.

IR υ_{max} (cm^{-1})**:** 3442 (OH), 1640 (C=C), 1040 (C-O).

LC/MS (+) m/z=443.1 $[M+H]^+$.

^1H NMR data are given in Table 2 together with ^{13}C data. DEPT data shows:

-7 methyl carbons, 10 methylene carbons, 7 methine carbons and 6 quaternary carbons are identified.

-Soxhlet extraction with petro ether, chloroform and finally methanol resulted in three kudinosides (Figure 5).

Kudinoside D [1]

White powder. 15.0 mg. m.p. 336-338°C.

IR υ_{max} (cm^{-1}) 3429 (OH), 1727 (C=O), 1633 (C=C).

LC/MS-(-) m/z=907 [M-H].

^1H NMR data are given in Table 3 together with ^{13}C data. DEPT data gave:

8 methyl carbons, 10 methylene carbons, 19 methine carbons and 10 quaternary carbons are identified.

Kudinoside C [2]

White powder. 8.1 mg. M.p. 264-266°C.

IR υ_{max} (cm^{-1}) 3418 (OH), 1726 (C=O), 1635 (C=C), 1075 (C-O).

LC/MS–(-)m/z=1087 $[M-H]^-$.

^1H NMR data are given in Table 4 together with ^{13}C data. DEPT data gave:

Table 1: ^{1}H, ^{13}C and HMBC NMR spectral of 3*β*; stigmast-5-en-yl-*β*-D-glucopyranoside.

Number of C	3*β*;stigmast-5-en-yl-*β*-D-glucopyranoside[j] (C_5D_5N)[12,13]	3*β*;stigmast-5-en-yl-*β*-D-glucopyranoside ($CDCl_3$ and MeOD)		
		^{13}C (δppm)	^{1}H (δppm, *J* Hz)	HMBC (C)
1	38.8	37.6	1.08 m; 1.89 m	2, 3, 10, 19
2	33.5	32.2	1.97 m	
3	79.8	79.4	3.59 m	
4	41.3	39.0	2.27 m; 2.40 ddd (2.0; 4.5)	2, 3, 5, 6, 10
5	142.3	140.2		
6	123.2	122.4	5.37 m	4, 8, 10
7	31.6	29.9	1.27 m; 1.88 m	
8	33.4	32.2	1.50 m	
9	51.7	50.9	0.95 m	8, 10
10	38.2	37.1		
11	22.7	21.4	1.45 m; 1.53 m	8
12	40.7	40.1	1.19 m; 2.01 m	11, 13, 17
13	43.8	42.7		
14	58.1	57.1	1.02 m	
15	25.8	24.6	1.05 m	
16	29.9	28.5	1.26 m	
17	57.7	56.4	1.13 m	
18	13.5	12.0	0.69 s	12, 13, 14, 17
19	20.7	19.5	1.02 s	1, 5, 9, 10
20	37.7	36.5	1.35 m	23
21	20.3	19.0	0.93 d (6.5)	17, 20, 22
22	35.5	34.3	1.18 m	
23	27.7	26.4	1.07 m; 1.15 m	20
24	47.4	46.2	0.95 m	22, 27
25	30.7	29.5	1.66 m	24, 26, 27
26	21.3	19.9	0.84 d (6.5)	24, 25, 27
27	20.5	19.2	0.82 d (6.5)	24, 25, 26
28	24.7	23.4	0.85 m; 1.34 m	
29	13.3	12.1	0.85 d (7.5)	24, 28
1a	103.9	101.0	4.41 d (7.5)	3
2a	76.3	73.9	3.23 m	1a, 3a
3a	79.9	76.9	3.41 m	2a,4a
4a	73.0	70.6	3.41 m	3a, 5a, 6a
5a	79.4	76.0	3.30 m	4a
6a	64.1	62.1	3.74 dd (5.0; 12.0) 3.84 dd (2.0; 12.0)	4a, 5a

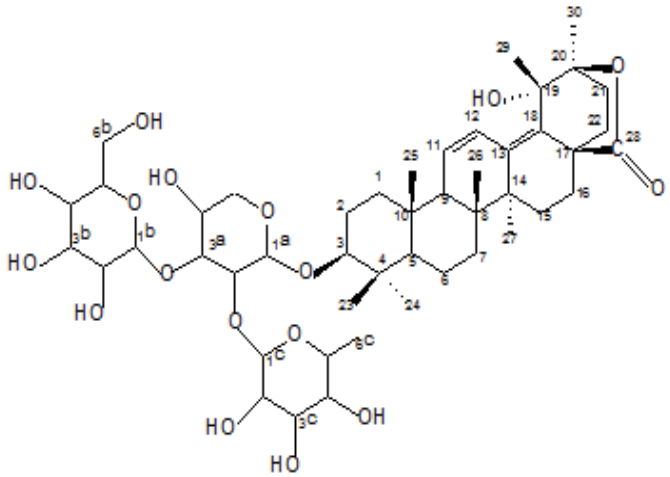


Figure 3: Kudinoside D.

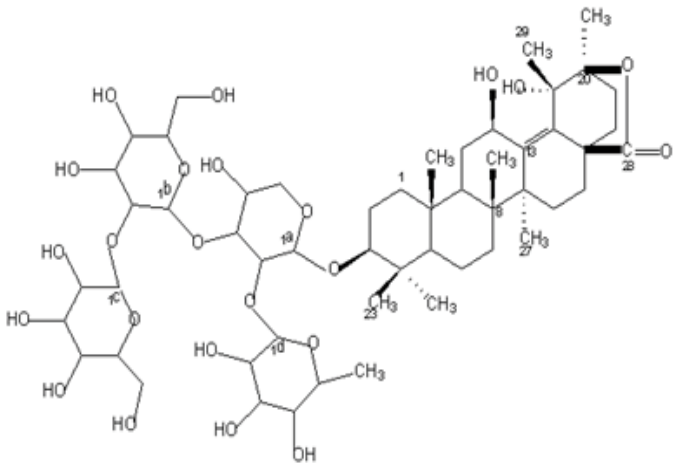


Figure 4: Kudinoside C.

Table 2: ^{1}H, ^{13}C NMR and HMBC Uvaol.

Number of C	Uvaol ($CDCl_3$) [15]	Uvaol (Acetone-d_6)		
		^{13}C (δ ppm)	^{1}H (δppm, *J* Hz)	HMBC (C)
1	38.7	39.6	1.02 m; 1.69 m	9
2	27.3	28.1	1.56 m; 1.61 m	3
3	79.0	78.6	3.16 ddt (11; 6; 5)	
4	38.7	39.5		
5	55.1	56.1	0.82 m	
6	18.3	19.1	1.43 m; 1.57 m	8
7	32.8	33.7	1.35 m; 1.59 m	
8	39.9	40.8		
9	47.7	48.6	1.62 m	
10	36.8	38.8		
11	23.2	23.9	1.38 m; 1.94 m	12; 13
12	125.2	125.6	5.14 t (7.5; 4)	
13	138.7	140.0		
14	42.0	42.8		
15	25.9	26.8	0.98 m; 1.89 m	13; 16
16	23.2	24.0	0.88 m; 1.89 m	22
17	37.9	37.6		
18	54.0	55.1	1.39 m	12; 13
19	39.4	40.2	1.44 m	
20	39.3	40.4	0.93 m	
21	30.6	36.3	1.70 m	22
22	35.1	31.5	1.28 m	
23	28.1	28.6	0.99 s	3; 24
24	15.6	16.1	0.79 s	3; 4; 5; 23
25	15.5	16.2	0.98 s	1; 5; 9; 10
26	16.7	17.3	0.84 s	
27	23.3	23.7	1.14 s	8; 13; 14; 15
28	69.9	69.6	3.53 d (10); 3.56 d (10)	
29	17.3	17.2	1.02 s	
30	21.3	21.6	0.94 s	
	OH_3		3.31 d (5)	
	OH_{28}		3.28 brd	

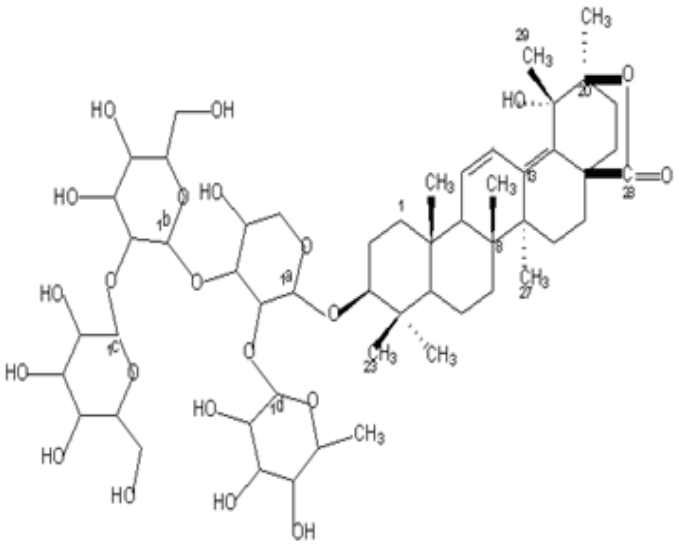


Figure 5: Kudinoside E.

8 methyl carbons, 12 methylene carbons, 23 methine carbons and 10 quaternary carbons are identified.

Kudinoside E [1]

White powder. 11.2 mg.

IR υ_{max} (cm^{-1}) 3443 (OH), 1736 (C=O), 1643 (C=C), 1076 (C-O).

LC/MS-(-) m/z=1069 $[M-H]^-$.

^{1}H NMR data are given in Table 5 together with ^{13}C data. DEPT data reveal:

8 methyl carbons, 11 methylene carbons, 24 methine carbons and 10 quaternary carbons are identified.

Antimicrobial activity

Seven crude extracts of *Ilex kaushue* (Aquifoliaceae) were tested for antimicrobial activity using *Escheriche coli*, *Pseudomonas aeruginosa*, *Bascillus subtilis*, *Saccharomyces cerevisiae*, *Aspergillus niger*, *Fusarium*

Table 3: ^{1}H,^{13}C NMR and HMBC of Kudinoside D.

Number of C	Kudinoside D [2] (C_5D_5N)	Kudinoside D (MeOD-d_4)		
		^{13}C (δppm)	^{1}H (δppm; J Hz)	HMBC (C)
1	38.4	39.5	1.95 d (*J* = 3.5 Hz); 1.09 d (3.0)	3; 10
2	28.3	27.1	1.93 m; 1.81 s	
3	88.5	89.5	3.21 dd (4.5, 12.0)	1a
4	39.6	40.5		
5	55.2	56.5	0.90 s	
6	18.7	19.2	1.67 m; 1.49 m	
7	33.0	33.4	1.79 m; 1.82 m	
8	42.2	43.1		
9	54.6	55.6	2.06 s	8; 10; 11; 12; 25
10	37.1	37.6		
11	127.3	129.6	5.84 dd (10.5; 1.5)	8; 9; 10; 13
12	128.5	127.7	7.02 dd (10.5; 3.0)	9; 14
13	140.8	142.7		
14	42.2	43.2		
15	25.9	26.4	1.60 m; 1.18 m	27
16	26.3	26.8	1.42 m; 2.28 m	
17	43.8	44.6		
18	135.2	134.2		
19	74.2	75.0		
20	85.9	87.2		
21	28.5	29.0	1.71 m; 2.33 m	20
22	32.9	33.8	1.82 m; 1.41 m	17; 18; 28
23	16.2	16.7	0.88 s	3; 4; 5; 24
24	28.0	28.3	1.05 s	3; 23
25	18.4	18.9	0.97 s	1; 9; 10
26	16.6	16.9	0.76 s	7; 8; 9
27	18.7	18.8	1.04 s	13; 14; 15
28	175.4	177.8		
29	23.7	23.4	1.41 s	18; 20
30	19.4	19.4	1.37 s	20; 21
Sugar				
1a	105.3	105.1	4.53 d (5.0)	3; 3a
2a	74.4	75.0	3.30 m	
3a	82.3	82.1	3.90 m	
4a	68.4	68.5	4.05 dt	
5a	65.0	64.6	3.53 dd (12.0; 2.5); 3.93 m	
1b	104.9	104.3	4.52 d (7.5)	
2b	75.0	75.2	3.93 m	
3b	78.1	78.0	3.41 m	
4b	71.7	71.2	3.36 m	
5b	78.6	78.0	3.33 m	
6b	62.5	62.4	3.87 m; 3.71 m	
1c	102.0	101.9	5.23 d (1.0)	2a; 2c; 3c; 5c
2c	72.4	72.1	3.72 m	
3c	72.5	72.1	3.93 m	
4c	74.0	73.8	3.42 m	
5c	70.2	70.2	3.88 m	
6c	18.4	18.0	1.24 m	

Table 4: ^{1}H,^{13}C NMR and HMBC data of Kudinoside C.

Number of C	Kudinoside C (C_5D_5N)	Kudinoside C (MeOD)		
		^{13}C (δ ppm)	^{1}H (δ ppm, J Hz)	HMBC (C)
1	39.3	40.1	1.77 m; 1.11 m	
2	28.2	27.3	1.92 m; 1.73 m	
3	88.5	89.8	3.20 dd (12.0; 4.0)	
4	39.6	40.4		
5	56.3	57.5	0.85 s	
6	18.6	19.2	1.62 m; 1.41 m	
7	35.5	36.3	1.56 m; 1.51 m	
8	41.7	42.8		
9	44.9	45.7	1.89 m	
10	37.1	37.9		
11	28.9	28.5	1.68 m	
12	66.1	67.6	5.54 d (2.5)	8
13	146.4	147.1		
14	43.9	44.7		
15	28.9	29.5	1.62 m; 1.21 m	
16	26.8	26.6	2.27 m; 1.36 m	14; 15; 17; 18
17	41.7	42.7		
18	137.8	138.5		
19	74.4	75.0		
20	85.7	86.9		
21	28.3	28.7	1.68 m	19; 20
22	32.9	33.2	1.83 m	18; 28
23	17.2	17.2	0.88 s	3; 4; 5; 24
24	28.2	28.2	1.06 s	1; 3; 5; 23
25	16.8	17.0	0.96 s	
26	18.2	17.8	1.25 s	
27	23.5	23.5	1.37 s	
28	175.4	177.6		
29	25.2	25.1	1.36 s	19; 20
30	19.5	19.3	1.38 s	
Sugar				
Ara				
1a	105.1	106.0	4.41 d (6.5)	3
2a	74.4	75.3	3.91 m	
3a	83.0	83.0	3.89 m	1a; 2b; 1d
4a	69.4	70.6	4.07 m	
5a	65.8	66.5	3.61 m; 3.86 m	
Glc				
1b	103.1	103.3	4.67 d (7.0)	
2b	84.9	83.7	3.62 m	1b; 3b; 1c
3b	78.4	78.2	3.32 m	
4b	70.9	71.1	3.39 m	
5b	78.4	78.0	3.59 m	
6b	62.5	62.5	3.89 m; 3.69 m	
Glc				
1c	106.4	105.7	4.77 brd	2b
2c	76.2	75.8	3.39 m	
3c	78.2	77.9	3.37 m	
4c	70.5	70.8	3.39 m	
5c	76.9	78.5	3.32 m	
6c	62.0	62.2	3.73 m; 3.91 m	

Rha				
1d	101.0	101.2	5.60 d (1.5)	2a; 2d; 4d; 5d
2d	72.4	72.2	3.71 m	
3d	72.6	72.2	3.94 m	
4d	74.0	74.0	3.43 m	
5d	69.8	69.9	4.07 m	
6d	18.3	18.4	0.85 s	

Table 5: ^{1}H, ^{13}C NMR and HMBC of Kudinoside E.

Number of C	Kudinoside E [1] (C_5D_5N)	Kudinoside E (MeOD)		
		^{13}C (δ ppm)	^{1}H (δ ppm, J Hz)	HMBC (C)
1	38.5	39.5	1.91 m; 1.10 m	
2	28.6	27.1	1.06 m	
3	88.4	89.7	3.20 dd (11; 4)	
4	39.7	40.4		
5	55.6	56.7	0.80 s	
6	18.6	19.2	1.66 m	
7	33.0	33.4	1.86 m; 1.82 m	
8	42.3	43.1		
9	54.6	55.7	2.10 s	8, 10, 12, 26
10	37.2	37.6		
11	127.3	127.7	7.02 d (11; 3)	8, 9, 10, 13
12	128.6	129.6	5.82 d (11)	9, 14
13	140.9	142.7		
14	42.3	43.2		
15	26.0	26.4	2.30 m	
16	26.4	26.8	1.80 m; 1.96 m	
17	43.9	44.6		
18	135.0	134.2		
19	74.2	75.1		
20	86.1	87.2		
21	28.7	29.0	1.68 m; 2.32 m	30
22	33.0	33.8	1.40 m	
23	16.6	16.8	0.88 s	3, 4, 5, 24
24	27.9	28.2	1.06 s	3, 4, 5, 23
25	18.4	18.9	0.95 s	1, 9, 10
26	16.7	17.0	0.75 s	7, 8,9
27	18.8	18.8	1.03 s	13, 15
28	175.5	177.9		
29	23.8	23.4	1.40 s	18, 20
30	19.6	19.4	1.36 s	19, 20, 21
Sugar				
Ara				
1a	105.2	106.0	4.41 d (6.5)	3
2a	74.5	75.4	3.92 m	
3a	82.8	82.9	3.90 m	
4a	69.5	70.6	3.39 m	
5a	65.8	66.5	3.58 d (11); 3.88 m	
Glc				
1b	103.1	103.3	4.67 d (7)	
2b	84.5	83.7	3.61 m	
3b	78.4	78.2	3.62 m	
4b	71.0	71.2	3.37 m	
5b	78.5	78.6	3.33 m	

6b	62.5	62.5	3.69 m; 3.86 m	
Glc				
1c	106.3	105.8	4.77 m	2b
2c	76.2	75.8	3.39 m	
3c	78.3	78.0	3.41 m	
4c	70.7	70.9	3.37 m	
5c	78.9	78.1	3.63 m	
6c	62.1	62.2	3.75 d (5); 3.90 d (2.5)	
Rha				
1d	101.2	101.2	5.60 s	2a, 2d, 4d, 5d
2d	72.5	72.2	3.95 m	
3d	72.6	72.3	3.72 m	
4d	74.0	73.9	3.44 s	6d
5d	69.9	70.0	4.07 m	
6d	18.4	17.8	1.24 d (6)	4d, 5d

Table 6: Minimum inhibitory concentrations.

Samples	Minimum Inhibitory Concentration: MIC (µg/ml)							
	Gr (-)		Gr (+)		Molds		Yeasts	
	E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*	*A. niger*	*F. oxysporum*	*S. cerevisiae*	*C. albicans*
Soxhlet								
A	(-)	200	200	(-)	(-)	200	200	(-)
B	(-)	200	200	(-)	(-)	(-)	200	200
C	(-)	(-)	200	(-)	100	(-)	200	(-)
Soak								
D	(-)	200	200	(-)	200	(-)	200	(-)
E	(-)	200	200	(-)	200	(-)	200	200
F	(-)	(-)	200	(-)	(-)	(-)	(-)	(-)
H	(-)	(-)	200	(-)	(-)	(-)	(-)	(-)

A, D: Crude petroleum ether
B, E: Crude chloroform
C, F: Crude methanol
H: Crude ethanol

oxysporum and *Candida albicans*. The results in Table 6 showed that seven among them exhibited activity against bacteria, molds and yeast.

By Soxhlet (Figure 1).

- The petroleum ether extract exhibited an inhibitory effect against *P. aeruginosa* against *B. subtilis S. cerevisiae* and against *F.oxysporum, all* showed MIC (minimum Inhibitory Concentration) of 200 µg/ml.
- The chloroform extract showed MIC values of 200 µg/ml against *P. aeruginosa*, against *B. subtilis*, against *S. cerevisiae* and against C. *albicans*.
- The methanol fraction had MIC value of 200 µg/ml against *B. subtilis*, against *S. cerevisiae*, whereas *A. niger* showed the lowest inhibition concentration MIC: 100 µg/ml.
- By soak (Figure 1).
- The petroleum ether extract exhibited an inhibitory effect against *P. aeruginosa*, *B. subtilis*, *A. niger* and *S. cerevisiae*. All showed a MIC: 200 µg/ml MIC (Table 6).
- The chloroform extract exhibited an inhibitory effect against *P. aeruginosa*, against *B. subtilis*, against *A. niger*, against *S. cerevisiae* MIC: 200 µg/ml and against C. *albicans*, all MIC: 200 µg/ml.
- The methanol and ethanol fractions exhibited an inhibitory effect against *B. subtilis* MIC: 200 µg/ml (Table 6).

Antioxidant activity

The antioxidative activity of the petroleum ether (soak) extract from the leaves of *Ilex kaushue* was determined at 167.79 µg/ml and compared with the antioxidative activity of 23.28 µg/ml ascorbic acid. None of the extracts showed an antioxidant activity except those from petroleum ether and methanol (soak) [13,14].

Cytotoxic activity

Tables 7 and 8 show the cytotoxicity of the seven crude extracts from *Ilex kaushue*. The chloroform extracts (Soxhlet and soak) and petroleum ether (soak) are cytotoxic against both human cancer cell lines used here: Hep-2 and RD. The crude petroleum ether (Soxhlet) is only cytotoxic against the human cancer cell line Hep-2.

Table 7: Cell survival.

Samples	Cell survival%		
	Hep-2 (*)	RD (**)	LU(***)
DMSO	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
(+) control	2.05 ± 0.0	1.5 ± 0.02	2.35 ± 0.07
Soxhlet			
A	44.3 ± 0.4	62.5 ± 0.74	84.3 ± 1.6
B	24.5 ± 1.1	31.7 ± 0.01	77.04 ± 1.4
C	72.7 ± 0.1	82.5 ± 0.65	95.9 ± 1.01
Soak			
D	28.8 ± 0.25	18.5 ± 0.84	78.9 ± 0.24
E	34.2 ± 0.49	8.4 ± 0.09	78.6 ± 0.01
F	58.9 ± 1.01	86.7 ± 0.89	83.2 ± 2.6
H	85.5 ± 0.2	100.1 ± 0.01	98.7 ± 0.5

A, D: Petroleum ether
B, E: Chloroform
C, F: Methanol
H: Ethanol

Table 8: $IC_{50.}$

Samples	IC_{50}^{*} (µg/ml)		
	Hep-2	RD	LU
(+) control	0.32	0.25	0.35
Soxhlet			
A	13.38	>20	>20
B	6.1	14.8	>20
Soak			
D	7.3	9.96	>20
E	9.67	11.1	>20

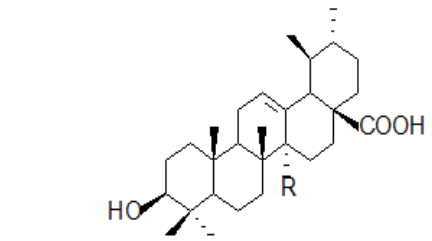
A, D: Petroleum ether; B, E: Chloroform

DISCUSSION

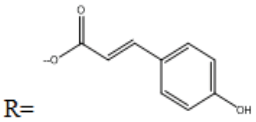
All extracts did not exhibit antioxidant activity except the ones from petroleum ether and methanol (soak) (weak effects). However, all seven crude extracts exhibited antibacterial activity (Table 6).

From Tables 7 and 8 it is seen that the crude chloroform and petroleum ether (by soak) showed cytotoxic activity against the tested cell-lines: Hep-2 (human Hepatocellular carcinoma) and RD (Rhabdosarcoma human heart). The crude petroleum ether extract (by Soxhlet) showed cytotoxicity activity against the cell-line Hep-2 and not cytotoxicity against cell-line RD. None of the extracts showed cytotoxicity activity against cell-line LU (Lung cancer).

A comparison of the isolated compounds and the cytotoxicity of the crude fractions revealed that the crude petrol ether fraction contained uvaol (Figure 4). However, the most active fractions are those extracted with chloroform. These compounds were isolated earlier as ursolic acid and 27-*p*-(E)-cumaryoloxyursolic acid [6]. Ursolic acid is known to have many useful biological effects (Figure 6) [16]. In contrast to the chloroform extract, neither the ethanol extract containing lupanes [5] such as lupeol (Figure 7) nor the methanol extract containing kudinosides showed any activity.

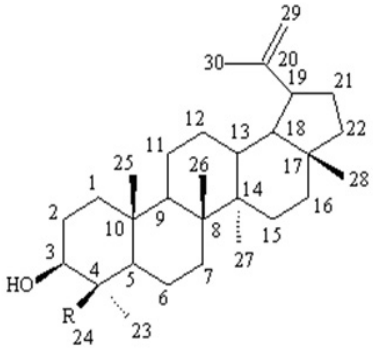


R= H : Ursolic acid



R= 27-*p*-(*E*)-Coumaroyloxyursolic acid

Figure 6: Ursolic acid and 27-*p*-(E)-Coumaroyloxyursolic acid.



R= CH_3 : Lupeol
R= $COOCH_3$: Lup-20(29)-en-3β-hydroxy-24-methyl carboxylate
R= CH_2OH : Lup-20(29)-en-3β, 24-diol

Figure 7: Lupanes isolated by ethanol extraction [5].

CONCLUSION

The seven crude extracts of *Ilex kaushue* S. Y. Hu all exhibited antibacterial activity. The crude chloroform and petroleum ether (by soak) showed a cytotoxicity activity against the tested cell-lines: Hep-2 and RD. The crude petroleum ether (by Soxhlet) showed cytotoxicity activity against tested cell-line Hep-2. Some of the active compounds isolated are uvaol (Figure 2) and ursolic acid (Figure 6). In addition, 27-*p*-(E) cumaroyloxyursolic acid (Figure 7) shows potent inhibitory activity in the acyl CoA cholesteryl acyl transferase (ACAT) assay [17].

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