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Anticancer, Anti HIV-1 and Antimicrobial Potentials of Methanol Extract and Non Polar Fractions of *Citrus volkameriana* Leaves and Phytochemical Composition

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ABSTRACT

This study investigated anticancer, anti HIV-1, antimicrobial potentials and the phytochemical composition of the total methanol extract, petroleum ether (60-80°C) and dichloromethane fractions of *Citrus volkameriana* leaves. Anticancer activity was measured by a sulforhodamine B (SRB) assay. Syncytia formation assay was used to evaluate anti-HIV-1 potential and antimicrobial assay was performed according to Buwa and Van staden with some modifications. The results showed that petroleum ether fraction exerted growth inhibitory effect on melanoma cancer cell line (UACC62), it inhibited the syncytia formation of HIV-1 with EC_{50} of 17.4 μg mL^{-1} and also it inhibited the growth of *Micrococcus luteus* B-287 and *Bacillus subtilis* with Minimum Inhibitory Concentrations (MIC) of 31.25 and 62.5 μg mL^{-1}, respectively. While dichloromethane fraction inhibited the growth of *Micrococcus luteus* with MIC of 62.5 μg mL^{-1}. Phytochemical investigation of petroleum ether fraction yielded six compounds, (1) α-amyrin, (2) β-sitosterol, (3) 5-O-demethylnobiletin, (4) 4', 5-dihydroxy-6, 7, 8, 3'-tetramethoxy flavone, (5) Tangeretin and (6) Nobiletin while five compounds were isolated from dichloromethane fraction including, (7) 4', 5-dihydroxy-3', 6, 7-trimethoxyflavone (cirsilineol), (8) luteolin 7-O-methyl ether, (9) Hesperitin, (10) 4'-hydroxy -5, 6, 7-trimethoxy flavone and (11) β-sitosterol 3-O-β-D-glucoside. The compounds 7, 8 and 10 were isolated for the first time from Rutacea family. This study provides promising results for *C. volkameriana* leaves in treatment of tumors, microbes and HIV/AIDS-related opportunistic infections.

Key words: *Citrus volkameriana*, bioactivities, sterols, methylated flavones

INTRODUCTION

Emerge of multi drug resistant diseases has made the search for new strategies and novel drug entities necessary and on the top of the list come tumors and microbial infections. Natural products and medicinal plants tend to provide an open source for drug discovery due to the large biodiversity

of their components (Dias *et al*., 2012). These secondary metabolites were found to play an important role in protection of plant against diseases besides having a positive influence on human health. Among these metabolites are flavonoids which are small molecular weight polyphenols exists in all plants to provide protection against pathogens and UV radiation (Ferreyra *et al*., 2012). Experimental studies have reported the effectiveness of flavonoids as antiviral, antibacterial and anticancer however, *in vivo* studies did not match with these results (Galvez *et al*., 2001; Walle, 2007b). The difference in results was attributed to the poor absorption and extensive metabolism of the flavonoid nucleus (Kumar and Pandey, 2013). Recent studies have been focusing on the effect of methylation of flavonoids on pharmacokinetics and bioactivities of flavonoids. It was found that methylation of flavonoids has positive effects on oral bioavailability and metabolic resistance of flavonoids besides enhancing their cytotoxic activities (Walle, 2007a, b; Walle *et al*., 2007; Wen and Walle, 2006b). One of the rich sources of methylated flavonoids is citrus genus. Recently, a lot of researches have been done concerning the bioactivities citrus byproducts. Results have shown that citrus extract exerted many bioactivities including anticancer (Visalli *et al*., 2014), antioxidant, analgesic, anti-inflammatory (Sood *et al*., 2009), antiulcer (Bhavitavya *et al*., 2012), hepatoprotective (Abirami *et al*., 2015), cardioprotective (Campelo *et al*., 2011), anxiolytic (Carvalho-Freitas and Costa, 2002), antiviral (Balestrieri *et al*., 2011), anti-microbial (Shende *et al*., 2015) antiobesity (Cardile *et al*., 2015) and antidiabetic activity (Parmar and Kar, 2007). These actions were correlated to the wide variety of secondary metabolites produced by citrus including polyphenolic compounds, alkaloids, limonoids, pectins and dietary vitamin C (Benavente-Garcia and Castillo, 2008; Marti *et al*., 2009; Murakami *et al*., 1997; Silalahi, 2002; Tundis *et al*., 2014; Visalli *et al*., 2014). However, some citrus trees have been not investigated for these activities as Volkamer lemon. *Citrus volkameriana* was reported for being used as rootstock due to resistance to many plant infections (Chapot, 1965). Very few reports about the presence of flavonoids, nitrogenous bases, volatile terpenes in *Citrus volkameriana* (Avula *et al*., 2005; Tirado *et al*., 1995).

The aim of this present research is to investigate cytotoxic, anti HIV-1, antimicrobial potentials and phytochemical composition of the total methanol extract, petroleum ether and dichloromethane fractions of *C. volkameriana* leaves.

MATERIALS AND METHODS

Experimental: Apparatus for spectral analysis: UV-Visible Spectrophotometer: UV-Visible Jasco V-670 spectrophotometer, EI-MS were measured using Jeol JMS-Ax 500 spectrometer. NMR spectra were measured using JOEL-FT NMR-AL 300 MHz spectrophotometer, JOEL ECX-400 NMR spectrometer for ^{1}H-NMR and 100, 75 MHz for ^{13}C-NMR. Melting points were determined in open capillary tubes on Electrothermal 9100 digital melting point apparatus (Electrothermal Engineering Ltd, Serial No. 8694, Rochford, UK). Silica gel G (E. Merck, Darmstadt, Germany) 60 mesh for column chromatography, Sephadex LH-20 for CC (Pharmacia, Uppsala, Sweden), Silica gel aluminum sheets G 60 (F254-Merck, Darmstadt, Germany) for TLC. Solvents: Petroleum ether (60-80°C), n-hexane, acetone, ethyl acetate, dichloromethane, 2-propanol, methanol (absolute and 95%), ethanol (absolute and 95%), n-butanol, acetic acid, distilled water and DMSO.

Plant identification and collection: Leaves of *Citrus volkameriana* were collected from the Horticulture Research Institute, Giza, Egypt in June 2011. The plant was kindly authenticated by senior botanist Dr. Mohammed El-Gibali. A dried specimen was placed at the museum of the pharmacognosy department, faculty of pharmacy, Cairo University with voucher number 130501.

Plant extraction: Air dried leaves (1.9 kg) of *Citrus volkameriana* were exhaustively extracted using methanol 70%. The total methanol extract was then filtered and concentrated under vacuum to yield 142 g of a viscous residue. The residue was suspended in water and partitioned with petroleum ether (60-80°C), dichloromethane, ethyl acetate and butanol, respectively. The total extract and fractions were phytochemically screened (Qadir *et al*., 2015).

***In-vitro* anticancer screening:** The human cell lines, renal (TK10), melanoma (UACC62) and breast (MCF7) were previously obtained from National Cancer Institute (NCI) in the framework of a collaborative research program between Council for Scientific and Industrial Research (CSIR) in South Africa (CSIR) and NCI. Cell lines were routinely maintained as monolayer cell cultures in RPMI containing 5% foetal bovine serum, 2 mM L-glutamine and 50 μg mL^{-1} gentamicin. The cells were inoculated in 96-well microtiter plates at plating densities of 7-10000 cells/well and were incubated for 24 h. After 24 h the plate was fixed with trichloroacetic acid to represent a measurement of the cell population for each cell line at the time of drug addition (T_0). The other plates with cells were treated with the total extract and fractions which were previously dissolved in DMSO as 10000 μg mL^{-1} stocks and diluted in medium to a final concentration 100 μg mL^{-1}. Cells without the total extract or fractions served as controls. Blank wells contained complete medium without cells. Emetine was used as a reference standard. The plates were incubated for 48 h after addition of the total extract and fractions. At the end of the incubation period, the cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried and dyed with SRB. Unbound dye was removed and protein-bound dye was extracted with 10 mM Tris base for optical density determination at a wavelength 540 nm using a multi well spectrophotometer. Optical density measurements were used to calculate the net percentage cell growth. The optical density of the wells after 48 h period of exposure to tested material is Ti, the optical density at time zero is T_0 and the control (untreated cells) optical density is C (Monks *et al*., 1991). Percentage cell growth is calculated as:

$$[(T_i-T_0)/(C-T_0)]\times100 \text{ for concentrations at which } T_i \geq T_0$$

$$[(T_i-T_0/T_0]\times100 \text{ for concentrations at which } T_i<T_0$$

Anti HIV-1 assay

Cells and virus: C8166 cells and HIV-1IIIB were kindly donated by Medical Research Council, AIDS Regent Project. The cells were maintained at 37°C in 5% CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivating FBS (Gibco). HIV-1IIIB was prepared from the supernatants of H9/HIV-1IIIB cells. The 50% HIV-1 tissue culture infectious dose (TCID50) in C8166 cells was determined and calculated (Reed and Muench, 1938). Virus stocks were stored in small aliquots at -70°C. The titer of virus stock was 1.0×108 TCID50 per mL.

Cytotoxicity assay: The cellular toxicity of the total extract and fractions on C8166 cells was assessed by MTT colorimetric assay. Briefly, 100 μL of 4×10^5 cells were plated into 96-well plates, 100 μL of various concentrations of the extract and fractions was added and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 72 h. Discard 100 μL supernatant, MTT reagent was added and incubated for 4 h, 100 μL 50% DMF-15% SDS was added. After the formazan was dissolved completely, the plates were read on a Bio-Tek ELx 800 ELISA reader at 570/630 nm 50% Cytotoxicity Concentration (CC_{50}) was calculated.

Inhibition of syncytia formation: The effect of the extract and fractions on acute HIV-1 infectivity was measured by the syncytia formation assay. In the presence or absence of various concentrations of the total extract and fractions, 4×10^4 C8166 cells were infected with HIV-1 at a Multiplicity Of Infection (MOI) of 0.04 and cultured in 96-well plates at 37°C in 5% CO_2 for 3 days. AZT was used as a positive control. At 3 days post-infection, the cytopathic effect (CPE) was measured by counting the number of syncytia (multinucleated giant cell) in each well of 96-well plates under an inverted microscope (100×). The inhibitory percentage of syncytia formation was calculated by the percentage of syncytia number in treated culture compared to that in infected control culture. 50% Effective Concentration (EC_{50}) was calculated (Reed and Muench, 1938), 50 Cytotoxic Concentration (CC_{50}) and 50 Effective Concentration (EC_{50}) was determined from dose-response curve.

Therapeutic index (TI) of anti-HIV active is CC_{50}/EC_{50}

$$\text{Cell viability (\% control)} = \frac{\text{ODtest-ODblk}}{\text{ODctrl-ODblk}}\times100$$

$$\text{CPE inhibition (\%)} = \frac{\text{1-CPEtest}}{\text{CPEctrl}}\times100$$

***In vitro* antimicrobial screening:** Antimicrobial activity was carried out against four gram positive bacteria, Bacillus subtilis NRRL-B-4219, Micrococcus luteus B-287 (clinical isolate; identified and obtained from Chemistry of Natural and Microbial Product Department, National Research Centre, Egypt), *Staphylococcus aureus* ATCC 29213, *Streptococcus faecalis* ATCC 19433, four gram negative bacteria, *Alcaligenes faecalis* B-170 (clinical isolate; identified and obtained from Chemistry of Natural and Microbial Product Department, National Research Centre, Egypt), *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 10131, *Pseudomonas aeruginosa* ATCC 27953, gram stain resistant microbe *Mycobacterium tuberculosis* (clinical isolate; identified and obtained from Chemistry of Natural and Microbial Product Department, National Research Centre, Egypt) and against four fungal yeasts, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019, *Candida tropicalis* ATCC 750, *Saccharomyces cerevisiae* ATCC2180-1A. The assay was performed according to Buwa and van Staden (2006) with some modifications. The bacterial cultures were refreshed on Nutrient Agar (NA) media and the fungal yeasts were refreshed on Potato Dextrose Agar (PDA) media for 24 h at 37°C. After incubation period, the microbial colonies were scrapped off the agar and transferred to nutrient and potato dextrose broth solutions for bacteria and yeast respectively, to prepare 0.5 McFarlane of microbial cultures with turbidity standard 107 Colony-Forming Units (CFU mL^{-1}). The total extract and fractions were prepared by dissolving 1 mg of extract or fraction in 1 mL of DMSO and then diluted with deionised water to prepare stock solutions of 10% DMSO. The total extract and fractions were initially tested at 250 μg mL^{-1} by addition of 100 μL of the total extract and fractions to the first well of 96-well microplates and were serially diluted down (50%) with sterile water to 1.95 μg mL^{-1}. After that, 100 μL of inoculated broths were added to each well. The inoculated 96-well plates were incubated at 37°C for 24 h. The currently used antibiotic drug dipenacid (CID pharmaceutical company),

composed of two bactericidal agents ampicillin and dicloxacillin, was included as positive control reference in each assay. Extract-free solution was used as a negative control and 10% DMSO solution was used as blank control. As an indicator of microbial growth, 40 μL of p-iodonitrotetrazolium violet salt (INT, Sigma) (0.2 mg mL^{-1}) dissolved in water were added to the wells. The plates were incubated again at 37°C for 1 h until the development of purple color of formazan. The MIC values were recorded as the lowest concentration of the extract or fraction that completely inhibited bacterial growth, i.e., a clear well.

Isolation of the compounds from petroleum ether fraction: Petroleum ether fraction (7 g) was subjected to silica gel column chromatography using hexane: Acetone as mobile phase to yield four fractions (P-I, P-II, P-III, P-IV). Fraction P-I was eluted with 5% acetone and further purified over silica gel using petroleum ether: dichloromethane to yield two compounds 1 and 2.

Fraction P-II eluted with 10% acetone and then purified with hexane: Ethyl acetate to afford two subfractions from which compounds 3 and 4 were recrystalized by acetone.

Fraction P-III eluted with 15% acetone was evaporated and washed with methanol several times to afford compound 5.

Fraction P-IV eluted with 25% acetone was subjected to normal phase preparative thin layer chromatography using hexane: Ethyl acetate (1:1) to obtain compound 6.

Isolation of the compounds from dichloromethane fraction: Dichloromethane fraction (14 g) was subjected to flash column chromatography over silica gel using hexane: acetone as solvent system to yield 3 main fractions (C-I, C-II, C-III).

Fraction C-II was further subjected to sephadex LH_{20} column using methanol as eluent to give compound (7) and a subfraction A. Subfraction A was then chromatographed over normal phase silica gel using n-hexane-EtOAc to give two compounds (8) and (9). Fraction C-III was separated over silica gel column using chloroform: acetone to yield compounds (10) and (11).

RESULTS AND DISCUSSION

***In vitro* anticancer screening:** Petroleum ether fraction exerted the highest growth inhibition on melanoma cancer cell line (UACC62) with the value of -50.41%. Anticancer results of the total methanol extract and the fractions of *C. volkameriana* leaves are in Table 1 and represented in Fig. 1.

Anti HIV-1 assay: Plant extract and fractions of *C. volkameriana* leaves showed anti-HIV activity, especially petroleum ether fraction. It showed EC_{50} of 17.40 μg mL^{-1} on inhibiting syncytium formation. However, CC_{50} of petroleum ether fraction on C8166 cell line was 37.72 μg mL^{-1}. The Therapeutic Index (TI) of petroleum ether fraction was about 2.17. The results of anti-HIV activity of the total extract and fractions of *C. volkameriana* leaves are shown in Table 2.

Table 1: Cytotoxic results of *Citrus volkameriana* leaves total methanol extract, petroleum ether and dichloromethane fractions

Plant extracts	Con. (μg mL^{-1})	Growth TK10 (%)	SD	Growth UACC62 (%)	SD	Growth MCF7 (%)	SD
70% methanol extract	100	78.98	0.118	44.59	0.028	51.11	0.038
Petroleum ether fraction	100	0.05	0.061	-50.41	0.030	-29.55	0.054
Dichloromethane fraction	100	17.98	0.037	-39.73	0.026	16.53	0.003
Emetine	10 μM	-61.35	0.007	-86.66	0.006	-46.41	-61.035

Con: Concentration, SD: Standard deviation

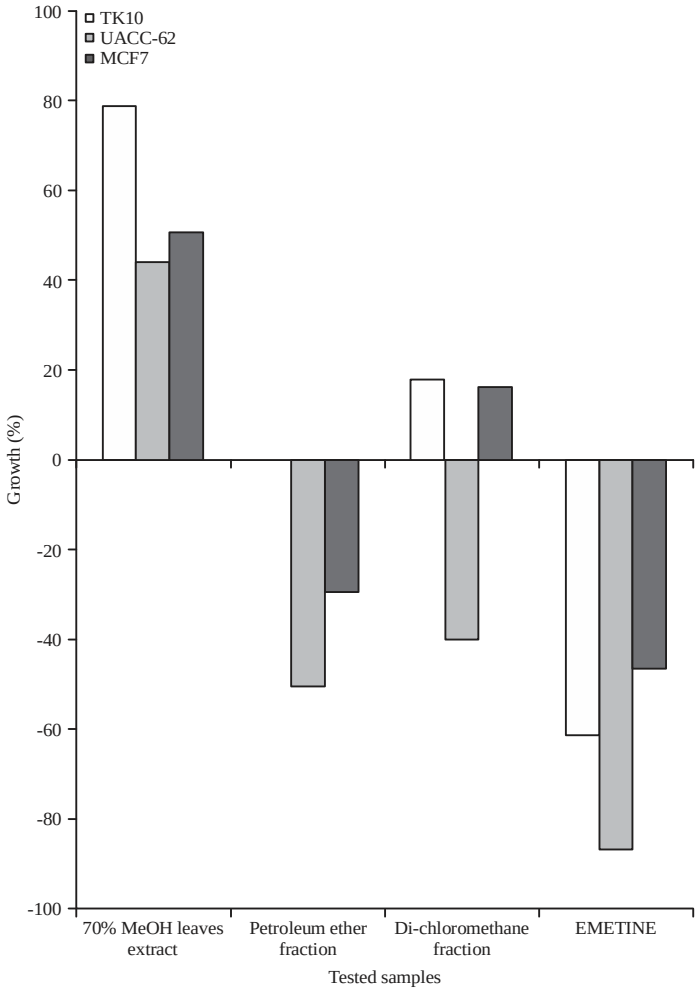

Fig. 1: Cytotoxic results of *Citrus volkameriana* leaves total 70% methanol extract, petroleum ether and dichloromethane fractions

Table 2: Cytotoxicity and anti-HIV-1 results of *Citrus volkameriana* leaves total extract, petroleum ether and dichloromethane fractions

Plant extracts	Experiments	Methods	CC_{50} (μg mL^{-1})	EC_{50} (μg mL^{-1})	Therapeutic index
70% methanol extract	Cytotoxicity assay	MTT	200<	-	2.65<
	Inhibition of synchytrium formation	CPE	-	75.56	
Petroleum ether fraction	Cytotoxicity assay	MTT	37.27	-	2.17
	Inhibition of synchytrium formation	CPE	-	17.40	
Dichloromethane fraction	Cytotoxicity assay	MTT	105.32	-	1.68
	Inhibition of synchytrium formation	CPE	-	62.87	
AZT	Cytotoxicity assay	MTT	1043	-	459471
	Inhibition of synchytrium formation	CPE	-	2.27	

AZT: Azido-thymidine, MTT: Microcuhure terazolium, CPE: Chemical penetration enhancer

***In vitro* antimicrobial screening:** Plant extract and fractions showed a broad antimicrobial spectrum and exerted a much stronger antimicrobial effect against gram-positive bacteria than gram-negative bacteria. The most susceptible bacteria was *Micrococcus luteus* B-287 whereas, the highest MIC value was 31.25 μg mL^{-1} for petroleum ether fraction and 62.5 μg mL^{-1} for dichloromethane fraction. Petroleum ether fraction was also found to inhibit the growth of *Bacillus subtilis* NRRL-B421 with MIC of 62.5 μg mL^{-1}. The results of antimicrobial activity are in Table 3.

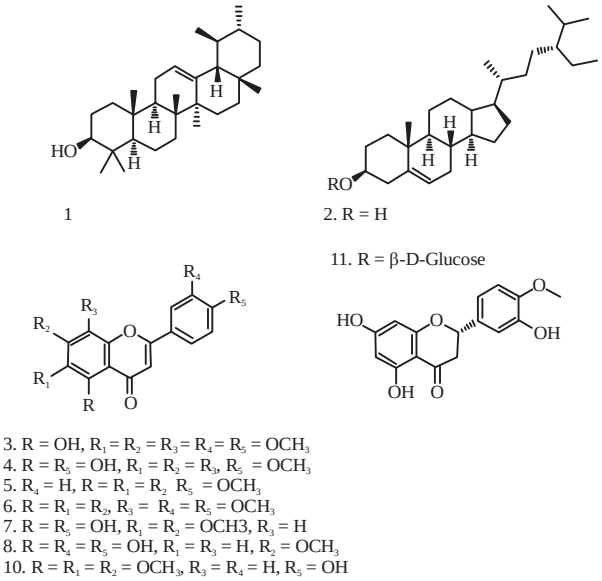

Fig. 2: Chemical structures of the compounds isolated from *Citrus volkameriana* leaves

Table 3: Antimicrobial assay results of *Citrus volkameriana* leaves total 70% methanol extract, petroleum ether and dichloromethane fractions

Samples μg mL^{-1}	70% methanol extract	Petroleum ether fraction	Dichloromethane fraction	Positive control (Dipenacid)
Gram positive				
Bacillus subtilis NRRL-B4219	250	62.5	125	<3.9
Micrococcus luteus B-287	-	31.25	62.5	<3.9
Staphylococcus aureus	-	250	250	<3.9
ATCC 29213	-	250	250	<3.9
Streptococcus faecalis	-		250	<3.9
ATCC 19433	-	-	250	<3.9
Gram negative				
Alcaligenes faecalis B-170	-	250	-	<3.9
Escherichia coli ATCC25922	-	250	250	<3.9
Klebsiella pneumoniae	-	-	-	<3.9
ATCC 10131	-	250	250	<3.9
Pseudomonas aeruginosa	125	250	250	-
ATCC 27953	-	-	-	-
Gram resistant				
Mycobacterium tuberculosis	-	-	250	-
Yeast				
Candida albicans ATCC10231	125	-	250	-
Candida parapsilosis ATCC 22019	-	-	250	-
Candida tropicalis ATCC 750	-	-	250	-
Saccharomyces cerevisiae ATCC2180-1A	-	-	-	-

Phytochemical investigation of the total 70% methanol extract and fractions of *C. volkameriana* leaves: Phytochemical analysis of the total methanol extract of *C. volkameriana* leaves revealed the presence of carbohydrates, triterpenes and/or sterols, tannins, coumarins and flavonoids while phytochemical investigation of petroleum ether and dichloromethane fractions proved the presence of flavonoids and triterpenes and/or sterols as shown in Table 4.

Identification of the isolated compounds: The structures of the isolated compounds were established by means of UV, NMR, MS spectral analyses, melting point and co-chromatography and the chemical structure of the isolated compounds were shown in Fig. 2.

Table 4: Phytochemical investigation of the total 70% methanol extract and fractions of *Citrus volkameriana* leaves

Constituents	70% methanol (%)	Petroleum ether	Dichloromethane
Triterpenes and/or sterols	+	+	+
Carbohydrates and/or glycosides	+	-	-
Flavonoids	+	+	+
Coumarins	+	-	-
Alkaloids and/or nitrogenous compounds	-	-	-
Tannins	+	-	-
Saponins	-	-	-

+: Presence of constituents, -: Absence of constituents

Compound 1: The 5.6 mg. White amorphous solid, m.p. 193°C. ^{1}H-NMR ($CDCl_3$, 400 MHz): 5.07 (t, J = 3.2, H-5), 3.13 (dd, J = 5.1,11.2 Hz, H-3), 1.01 (s, CH_3-27), 0.95 (s, CH_3-28), 0.94 (s, CH_3-23), 0.91 (s, CH_3-26), 0.86 (d, J = 6.0, CH_3-29), 0.74 (s, CH_3-24), 0.73 (s, CH_3-25), 0.73 (d, J = 7.0, CH_3-30). EI/MS (rel. int.): m/z 426.39 which in accordance with Molecular formula $C_{30}H_{50}O$. It appeared as brown spot under UV light and changed to purple upon spraying with anisaldehyde-sulfuric acid and heating in an oven at 110°C for 5 min. NMR spectral data showed signals very close to α-amyrin (Toriumi *et al*., 2003).

Compound 2: The 15.7 mg. White crystals, m.p. 135°C, ^{1}H-NMR ($CDCl_3$, 300 MHz): δ ppm 5.33 (t, 1H, J = 6.4 Hz, H-5), 3.53 (m, 1H, H-3), 0.98 (s, 3H, CH_3-29), 0.89 (d, 3H, J = 6.5 Hz, CH_3-19), 0.81 (t, 3H, J = 7.2 Hz, CH_3-24), 0.79 (d, 3H, J = 6.4 Hz, CH_3-26), 0.78 (d, 3H, J = 6.4 Hz, CH_3-27), 0.65 (s, 3H, CH_3-28); EI/MS (rel. int.): m/z 414 which in accordance with Molecular formula $C_{29}H_{50}O$. This compound gave a dark spot under UV light changed to violet on spraying with anisaldehyde/sulfuric acid reagent and heating in an oven at 110°C for 5 min and comparison with published data allowed us to identify that compound 2 is β-sitosterol (El-Alfy *et al*., 2012).

Compound 3: The 32.1 mg. Yellow granules. UV λmax (MeOH): 283, 339; (NaOMe): 291, 322; ($AlCl_3$): 290, 366; ($AlCl_3$/HCl): 290, 361; (NaOAc): 283, 340; (NaOAc/ H_3BO_3): 283, 340. ^{1}H NMR ($CDCl_3$, 300 MHz): δ ppm 12.52 (OH, s, H-5), 7.56 (1H, dd, J = 2.2, 8.7Hz, H-6), 7.39 (1H, d, J = 2.2 Hz, H-2), 6.98 (1H, d, J = 8.7Hz, H-5), 6.68 (1H, s, H-3), 4.1, 3.95, 3.94, 3.9, 3.8 (5 s, five methoxy groups at C-6, 7, 8, 3 , 4). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ ppm 182.95 (C-4), 163.88 (C-2), 152.40 (C-7), 152.36 (C-4'), 149.50 (C-3'), 149.27 (C-9), 145.75 (C-5), 136.50 (C-6), 132.90 (C-8), 123.60 (C-1'), 120.11 (C-6'), 111.16 (C-5'), 108.58 (C-2'), 106.90 (C-10), 103.94 (C-3), 62.01, 61.78, 61.19, 56.19, 55.91 (5 s, five methoxy groups at C-6, 7, 8, 3', 4'). EI/MS (rel. int.): m/z 388, which in accordance with molecular formula $C_{20}H_{20}O_8$. It appeared as a dark purple spot under UV light and with on spraying with anisaldehyde/sulfuric acid reagent and heating in an oven at 110°C for 5 min, it yield yellow spot and its spectral analyses were in accordance with previously published data of 5-O-demethylnobiletin (Hamdan *et al*., 2011).

Compound 4: The 25.7 mg. Yellow granules. UV λmax (MeOH): 276,342; (NaOMe): 268,413; ($AlCl_3$): 284, 368; ($AlCl_3$/HCl): 284,368; (NaOAc): 272,411; (NaOAc/H_3BO_3): 276, 342. $_{1}$H-NMR ($CDCl_3$, 300 MHz): δ ppm: 12.60 (OH, s, H-5), 7.51 (1H, dd, J = 8.5, 2.2 Hz, H-6'), 7.38 (1H , d, J = 2.2 Hz, H-2'), 7.02 (1H, d, J = 8.5 Hz, H-5), 6.57 (1H, s, H-3), 4.09, 3.97, 3.95, 3.91 (4 s, four methoxy groups at C-5, 7, 8, 3'); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ ppm 182.97 (C-4), 163.99 (C-2), 153.15 (C-7), 149.48 (C-5), 149.40 (C-3'), 146.86 (C-4'), 145.73 (C-9), 136.47 (C-6), 132.86 (C-8), 123.19 (C-1'), 120.74 (C-6'), 115.08 (C-5'), 108.20 (C-2'), 106.91 (C-10), 103.77 (C-3), 62.16, 61.80, 61.24, 56.00 (4 s, four methoxy groups at C-6, 7, 8, 3'). It appeared as a dark purple spot under UV

light and with anisaldehyde-sulfuric acid, it yield yellow spot. Spectral data are very close to previously published data of 4', 5- dihydroxy-6, 7, 8, 3'-tetramethoxy flavone (Hamdan *et al*., 2010).

Compound 5: The 4.3 mg. Yellow amorphous powder. UV λmax (MeOH): 271, 321 (NaOMe): 271, 321; ($AlCl_3$): 271, 321; ($AlCl_3$/HCl): 271, 321; (NaOAc): 271, 321; (NaOAc/H_3BO_3): 271, 321. $_1$H-NMR ($CDCl_3$, 300 MHz): δ ppm 7.85 (2H, d, *J* = 8.9Hz, H-2', 6), 7.0 (2H, d, *J* = 8.9Hz, H-3 ,5'), 4.07, 4.01, 3.92×2, 3.86 (5 s, five methoxy groups at C-5, 6, 7, 8, 4'); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ ppm 177.35 (C-4), 162.22 (C-2), 161.13 (C-4'), 151.26 (C-7), 148.25 (C-9), 147.57(C-8), 143.79 (C-5), 138.18 (C-6), 127.66 (C-2',C-6'), 123.71 (C-1'), 114.46 (C-10), 114.45 (C-3', C-5'), 106.62 (C-3), 62.28, 61.98, 61.70, 61.62, 55.51 (5 s, five methoxy groups at C-5, 6, 7, 8, 4'). It appeared as a pale yellow spot under UV light and with anisaldehyde-sulfuric acid, it yield yellow spot. Its spectral signals are similar with previously published data of 5, 6, 7, 8, 4 -pentamethoxyflavone (Tangeretin) (Hamdan *et al*., 2011).

Compound 6: The 15.2 mg. Yellowish-white needle-shaped crystals. UV λmax (MeOH): 271, 333; (NaOMe): 271,333; ($AlCl_3$): 271,333; ($AlCl_3$/HCl): 271, 333; (NaOAc): 271,333; (NaOAc/H_3BO_3): 271, 333. $_1$H-NMR ($CDCl_3$, 300 MHz): δ ppm 7.54 (1H, dd, *J* = 2.1, 8.5 Hz, H-6), 7.38 (1H, d, *J* = 2.1 Hz, H-2), 6.96 (1H, d, *J* = 8.5Hz, H-5), 6.59 (1H, s, H-3), 4.07, 3.99, 3.95, 3.94, 3.92×2 (6s, six methoxy groups at C-5, -6, 7, 8, 3 , 4); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ ppm 177.31 (C-4), 161.00 (s, C-2), 151.90 (C-4'), 151.39 (C-7), 149.27 (C-3'), 148.39 (C-9), 147.69 (C-8), 144.07 (C-5), 137.98 (C-6), 123.99 (C-1'), 119.58 (C-6'), 114.96 (C-10), 111.21 (C-5'), 108.54 (C-2'), 106.86 (C-3), 62.22, 61.93, 61.80, 61.63, 56.05, 55.94 (6 s, six methoxy groups at C-5, 6, 7, 8, 3', 4'). EI/MS (rel. int.): m/z 402 which in accordance with molecular formula $C_{21}H_{22}O_8$. It appeared as a yellowish green spot under UV light and with anisaldehyde-sulfuric acid, it yielded yellow spot. Its spectral signals were in accordance with previously published data of 5, 6, 7, 8, 3, 4-hexamethoxy flavone (Nobiletin) (Hamdan *et al*., 2011).

Compound 7: The 3.2 mg. Yellow powder; UV λmax (MeOH): 274, 346; (NaOMe): 266, 412; ($AlCl_3$): 283, 306sh, 361, 402sh; ($AlCl_3$/HCl): 283, 306sh, 361, 402sh; (NaOAc): 274, 354; (NaOAc/H_3BO_3): 274, 346. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ ppm 12.60 (OH, s, H-5), 7.53 (1H, dd, *J* = 8.5, 2.2 Hz, H-6'), 7.39 (1H , d, *J* = 2.2 Hz, H-2'), 7.04 (1H,d, *J* = 8.5 Hz, H-5'), 6.55 (1H, s, H-3), 6.40 (1H, s, H-8), 3.98, 3.93, 3.92 (3 s, three methoxy groups at 6, 7, 3'). EI/MS: (rel.int.): m/z 344 which in accordance with molecular formula of $C_{18}H_{16}O7$. It appeared as dark purple spot under UV light and with anisaldehyde-sulfuric acid, it yielded yellow spot. Its spectral data are very close to that previously published data of 4', 5-dihydroxy-3', 6, 7-trimethoxyflavone (Cirsilineol) (Kikhanova *et al*., 2013).

Compound 8: The 64.2 mg. Yellow granules. UV λmax (MeOH): 269, 344; (NaOMe): 269,405; ($AlCl_3$): 277, 297sh, 385; ($AlCl_3$/HCl): 261, 385; (NaOAc): 273, 362; (NaOAc/H_3BO_3): 271, 344. $_1$H-NMR (DMSO-d_6, 300 MHz): δ ppm: 12.96 (1H, s, 5-OH), 7.54 (1H, d, *J* = 2.1 Hz, H-2), 7.54 (1H, dd, *J* = 8.4, 2.1 Hz, H-6), 6.92 (1H, d, *J* = 8.4 Hz, H-5), 6.92 (1H, s, H-3), 6.49 (1H, d, *J* = 2.1 Hz, H-8), 6.17 (1H, d, *J* = 2.1 Hz, H-6), 3.87 (3H, s, methyl group at C-7). ^{13}C-NMR (DMSO-d_6, 75 MHz): δ ppm 181.81 (C-4), 164.16 (C-7), 163.66 (C-2), 161.44 (C-9), 157.33 (C-5), 150.71 (C-4), 148.02 (C-3), 121.49 (C-1), 120.36 (C-6), 115.75 (C-5), 110.14 (C-2), 103.70 (C-10), 103.19 (C-3), 98.83 (C-6), 94.06 (C-8), 55.94 (7-OCH3). EI/MS (rel.int.): m/z 300 which in accordance with molecular formula of $C_{16}H_{12}O6$. It appeared as a dark purple spot under UV light and with anisaldehyde-sulfuric acid, it yield yellow spot. Its spectral signals are similar with previously published data of luteolin 7-O-methylether (Saewan *et al*., 2011).

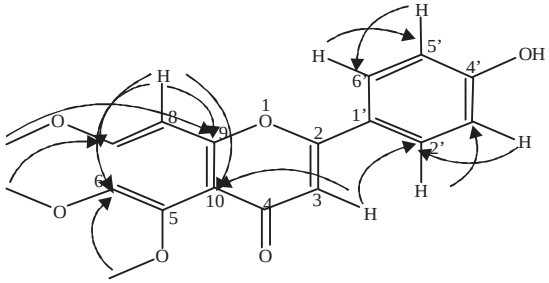

Fig. 3: Selected multiple bond interactions in compound 10 as observed in the HMBC experiment

Compound 9: The 5.6 mg. White powder. UV λmax (MeOH): 287, 328; (NaOMe): 324; ($AlCl_3$): 310, 380; ($AlCl_3$/HCl): 310, 380; (NaOAc): 287sh, 324; (NaOAc/H_3BO_3): 287, 328. ^{1}H-NMR ($CDCl_3$, 300 MHz,): δ ppm 12.03 (1H, s, 5-OH), 6.93 (1H, d, *J* = 8.2 Hz, H-5'), 6.91 (1H,d, *J* = 1.6Hz, H-2'), 6.89 (1H, dd, *J* = 8.2, 1.6 Hz, H-6'), 5.88 (1H, d, *J* = 1.9 Hz, H-8), 5.87 (1H, d, *J* = 1.9 Hz, H-6), 5.27 (1H, dd, *J* = 12.3, 3.0 Hz, H-2), 3.84 (3H, s, methoxy group at C-4'), 3.04 (1H, dd, *J* = 17.2, 12.3 MHz, equatorial H-3), 2.70 (1H, dd, *J* = 17.2, 3 Hz, axial H-3); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ ppm:197.58 (C-4),168.33(C-7), 165.43 (C-9), 164.72 (C-5), 149.32 (C-4'),147.74 (C-3'), 133.10 (C-1'), 119.00 (C-6'), 114.52(C-2'), 112.55 (C-5'), 103.36 (C-10), 97.08 (C-6), 96.19 (C-8), 80.52 (C-2), 56.42 (methoxy group at C-4'), 44.03 (C-3). It appeared as a dark purple spot under UV light and with spraying with anisaldehyde-sulfuric acid, it yield purple colour and its spectral data was in accordance with previously published data of Hesperetin (Erenler *et al*., 2015).

Compound 10: The 18.1 mg. White needles crystals, It appeared as yellow spot under UV light. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ ppm 6.93 (2H, d, *J* = 8.9 Hz, H-3', 5'), 6.86 (2H, d, *J* = 8.9 Hz, H-2', 6'), 6.67 (1H, s, H-8), 6.59 (1H, s, H-3), 3.97, 3.86, 3.83 (3s, three methoxy groups at C-5, 6, 7). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ ppm 176.42 (C-4), 161.11 (C-4'), 160.49 (C-2), 156.70 (C-7), 156.11 (C-9),151.5 (C-5), 130.4 (C-6), 128.27 (C-2', C-6'), 122.02 (C-1'), 116.51 (C-3', C-5'), 108.30 (C-10), 106.16 (C-3), 94.07 (C-8), 61.58, 56.87, 56.76 (3 s, three methoxy groups at C-5, C-6, C-7). Positions of H-3, H-8 and methoxy substitutions have been determined from ^{1}H-NMR, ^{13}C-NMR shifts, HMQC and HMBC as shown in Fig. 3. This compound was identified as 4'-hydroxy-5, 6, 7-trimethoxyflavone. It was previously isolated from *Zeyheria tuberculosa* (Bastos *et al*., 2009).

Compound 11: The 6.3 mg. Yellowish amorphous powder, m.p. 279-280ºC. ^{1}H-NMR (Pyridine, 400 MHz): δ ppm 5.33 (1H, br. s, H-6), 5.01 (1H, d, anomeric proton), 4.56 (1H, m, H-3), 4.53-4.03 (6H, m, glucosidal protons), 2.70 (1H, br. dd, *J* = 4.5, 2.7 Hz, H-4) , 1.01 (3H, s, H-19), 0.92 (3H, d, *J* = 6.4 Hz, H-21), 0.88 (3H, s, H-29), 0.86 (3H, s, H-26), 0.84 (3H, s, H-27), 0.64 (3H, s, H- 18). ^{13}C-NMR (Pyridine, 100 MHz): δc ppm 140.76 (C-5), 121.78 (C-6), 102.44 (C-1'), 78.48 (C-5'), 78.36 (C-3), 77.94 (C-3'), 75.20 (C-4'), 71.54 (C-2'), 62.69 (C-6'), 56.68 (C-14), 56.01 (C-17), 50.19 (C-9), 45.89 (C-24), 42.34 (C-13), 39.80 (C-12), 37.33 (C-1), 36.78 (C-10), 36.25 (C-20), 34.06 (C-22), 32.03 (C-7), 31.91 (C-8), 30.11 (C-2), 29.48 (C-4), 29.31 (C-25), 28.40 (C-16), 26.23 (C-23), 24.36 (C-15), 23.24 (C-28), 21.14 (C-11), 19.84 (C-26), 19.27 (C-18), 19.06 (C-27), 18.86 (C-21), 12.02 (C-29), 11.83 (C-19). It appeared as violet spot upon spraying with anisaldehyde/sulfuric acid reagent and its spectral analyses was in accordance with previously published data of β-sitosterol 3-O-β-D-glucoside (El-Alfy *et al*., 2012).

The content of polymethoxylated flavonoids isolated from petroleum ether and dichloromethane fractions may be the reason for anticancer, antimicrobial and anti-HIV-1 activities observed in these extracts. Methylation of flavonoids was reported to enhance absorption, retard metabolic conjugation, decrease side effects and enhance anticancer activities (Walle, 2007b; Wen and Walle, 2006a). It was stated before that they can inhibit cancer initiation and promotion phases and exhibit anti proliferative activities (Walle, 2007a). Regarding antimicrobial activities several reports have mentioned the antimicrobial activities of flavonoids. Recent studies reported that hydroxylation at position 5 in flavones and flavanones are important for activity against methicillin-resistant *Staphylo-coccus aureus*. Some methoxylated flavonoids, 5, 6, 7, 8-tetramethoxyflavone and 4'-hydroxy-5, 6, 7, 8-tetramethoxyflavone, which have four methoxyl groups in the ring A, were actives for S. aureus and C. albicans while 5, 6, 7-trimethoxyflavone, which lacks a methoxyl group at C-8 of this ring, was active only for C. albicans. On the other hand, 4'-hydroxy-5,6,7-trimethoxyflavone, which lacks a methoxyl group at C-8 and has a hydroxyl group at C-4', in comparison with the similar 4'-hydroxy-5, 6, 7, 8-tetramethoxyflavone, did not affect the growth of the tested microorganisms. These results suggested that the presence of methoxyl group at C-8 can be responsible for the antibacterial activity (Bastos *et al*., 2009). Several mechanisms have been studied for antimicrobial activities of flavonoids including inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function and inhibition of energy metabolism (Cushnie and Lamb, 2005). Polymethoxylated flavones, nobiletin and tangeretin are among the most effective compounds at inhibiting cancer cell growth of melanoma and lung and it is also the reason why these dietary polyphenols have emerged as potential drug candidates for treatment of these malignancies. Nobiletin and tangeretin were reported to destroy the permeability of the cell membrane and inhibit the protein synthesis, leading to metabolic dysfunction and eventually to cell death (Yao *et al*., 2012). There is a structure–activity relationship between the number of the methoxy groups in polymethoylated flavonoids and anticancer activity. Increase of the number of methoxy groups on the B-ring moiety lowers the anticancer activity with the same A-ring methoxylation pattern (Kawaii *et al*., 2012). In the contrary, an increase in the number of A-ring methoxyl groups tended to enhance the activity. Typically, nobiletin which had the tetramethoxylated A-ring structure, demonstrated significant anticancer activity (Kawaii *et al*., 2012). The β-sitosterol potently induces apoptosis in U937 cells (leukemia cells) and that β-sitosterol induced apoptosis is related to the selective activation of caspase-3 and induction of Bax (an apoptosis promoter)/Bcl-2 (an apoptosis inhibitor) ratio and it induced apoptosis in the leukemia cells in a dose-dependent manner (Park *et al*., 2007).

In vitro studies of anti-HIV-1 activity of flavonoids proved that some flavonoids are active against HIV-1 through inhibition of virus protease and transcriptase enzymes (Cushnie and Lamb, 2005).

CONCLUSION

These results revealed that petroleum ether and dichloromethane fractions of *Citrus volkameriana* leaves exerted significant antimicrobial activity. Also, petroleum ether fraction exhibited anti-melanoma and anti HIV-1 potentials. However, further studies for the observed bioactivities of the extracts should be done for the isolated compounds to determine the bioactive compounds in these bioactive extracts.

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