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Research Paper

Two steroidal saponins from *Agave franzosinii* and *Agave angustifolia* leaves and Biological activities of *Agave franzosinii.*

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ABSTRACT

Two steroidal saponins having the structures of hecogenin oligosaccharides were isolated and identified for the first time from the saponin fraction of both *Agave franzosini* (SFAF) and *Agave angustifolia* (SFAA). Cytotoxic, molluscicidal, schistosomicidal, miracidicidal and cercaricidal activities of the methanolic extract of *Agave franzosini* leaves, family *Agavaceae* (MEAF) and its saponin fraction (SFAF) were evaluated. MEAF and SFAF showed marked cytotoxic effect on EAC, HepG2 and Hella cells. SFAF showed Molluscicidal and Schistosomicdal activities, higher than that of MEAF.

Keywords: *Agavaceae*; Analgesic; Anti-inflammatory; Anti-cancer; Molluscicidal.

Family *Agavaceae* is native to southern and western United States, central and tropical South America (Baiely, et al., 1960). Studies on genus Agave , had been known for several years as a good source of steroidal saponins (Blunden, et al., 1986; Ding, et al., 1993) and that they have diverse biological activities like hemolytic , anti-inflammatory activities (Peana, et al., 1997), antimicrobial, anti-mitotic and molluscicidal activities (El-Sayed Mortada, 1997; Ferrer Lopez, et al., 1993; Kishor, 1990; Rana, 1993) anticancer activity (Yokosuka, et al., 2000) and anti-allergic activity (Kawai, et al., 2000). These activities are mainly attributed to the presence of saponins in Agave species. The most important economic use of genus Agave is the production of alcoholic drink mescal and tequila. These products are said to add what worth millions of dollars to Mexican economy (Bahre, et al., 1980). Also Agave species are good source of food, drink, soap, clothing, fibers and paper as well as military instruments, medicine and ornamental plants (Shahina, 1994). In continuation of our studies on bioactive saponins from *Agave* species as *A. lophantha* sheide (Abdel-Khalik, et al., 2002) and *Agave macroacantha* (Eskander, et al., 2010), we were encouraged to screen the anti-cancer, molluscicidal, schistosomicidal, cercaricidal and miracidicidal activities of the methanolic extract (MEAF) as well as the saponin fraction (SFAF) of *Agave franzosinii* leaves. Moreover this study includes the identification of two steroidal saponins **1** and **2** having a hecogenin nucleus

isolated for the first time from the saponin fraction (SFAF and SFAA) of the two species of Agave (*A. franzosinii* and *A. angustifolia*).

MATERIALS AND METHODS

Instruments and material: NMR spectra were recorded on a JEOL α 400, 399.65 MHz for PMR and 100.40MHz for ^{13}C NMR. The spectra were run in pyridine-d_5 and chemical shifts were given in ppm with TMS as internal standard. Diaion HP-20, Sephadex LH-20 and silica gel G_{60} were used for column chromatography. Preparative TLC was carried out on silica gel plates. The plates were visualized by spraying with 20% H_2SO_4 in MeOH. HPLC was carried out on a JASCO system and column of interstil ODS – 3 using solvent mixture MeOH-H_2O (60% - 80%). PC was carried out using Whatman paper No1 and spots were visualized by spraying with aniline phthalate reagent.

Plant material*: Agave franzosini* and *Agave angustifolia* leaves were obtained from Orman Public Garden, Giza, Egypt in February 2008. Two Voucher Specimens (no. NG-2- *Agave franzosinii* & NG-3 *Agave angustifolia*) have been deposited in the Herbarium of Pharmacognosy department, Faculty of Pharmacy, Helwan University.

Extraction, preparation of MEAF, SFAF and Isolation of Saponins from A. franzosinii : The air dried powdered leaves of *A. franzosinii* (4 kg) were defatted with n-hexane then extracted twice with MeOH. The solvent was evaporated from the combined extract under vacuum and a part (15g) of the residue obtained was freeze-dried (MEAF) and kept until use for pharmacological work. The remaining part of the residue (70g) was dissolved in distilled H_2O and the aqueous solution was passed through a column packed with porous polymer gel Diaion HP-20. Elution was carried out with distilled H_2O followed by 25%, 50%, 75% and finally 100% MeOH. The 75% and 100% eluates were combined after TLC and the combined fraction was evaporated to give SFAF (9.5 g). A part of this fraction (4.5g) was freeze-dried and kept until use for pharmacological work. The remaining part of the residues (5.0g) was chromatographed on silica gel column eluted with CH_2CL_2-MeOH (9:1), yielded compound **1** (18mg). Group of eluted fractions (0.5g) were combined and re-chromatographed on another silica gel column eluted with CH_2CL_2- ACOEt -MEOH mixture. Fractions eluted with CH_2CL_2- ACOEt- MeOH (9:10:0.9) were combined (0.295g) and subjected to repeated PTLC ($CHCL_3$-MEOH-H_2O, 6:3:0.5) to give compound **2** (0.21g).

Extraction, preparation of SFAA and Isolation of Saponins from A.angustifolia: The air dried powdered leaves of *A.angustifolia* (2kg) were defatted with n-hexane then extracted twice with MeOH. The solvent was evaporated from the combined extract under vacuum and the residue obtained (80g) was subjected to porous polymer gel Diaion HP-20 column chromatography. Elution was carried out with distilled H_2O followed by 25%, 50%, 75% and finally 100% MeOH. The methanol eluate was evaporated to obtain SFAA (10g) which was subjected to column chromatography packed with silica gel and eluted with CH_2CL_2-MeOH (9:1). Collected fractions were subjected to HPLC to give the same compounds **1** (0.20g) amorphous solid and **2** (0.29g) amorphous solid. ^1H NMR & ^{13}C NMR see Table 1.

General method of acid hydrolysis: Each saponin (2mg) dissolved in dioxan (50µl) and 2N HCL (1:1) was heated at 95°C for 30 minutes. Dioxan was evaporated and the residue was diluted with water and extracted with ethyl acetate. The remaining aqueous layer was concentrated and the monosaccharide content was detected by PC

using n-BuOH-AcOH-H_2O system (4: 1: 5 v/v, upper layer) .Sugar components were identified by comparison with standard samples after spraying with aniline phthalate.

Biological materials: Ehrlich ascites carcinoma cell line, Hepatocellular carcinoma cell line (HepG2), Hella cell line, adult male and female *Schistosoma mansoni* worms, *Schistosoma mansoni* cercariae, miracdiae, and *Biomphlaria alexandrina* snails, were used. Drugs were used praziquantel (Epico, Egypt) and niclosamide (Misr, Egypt).

Cytotoxic activity of MEAF and SFAF:(Trypan blue dye exclusion test on Ehrlich ascites carcinoma cells (EAC)) The cytotoxic activity was evaluated using EAC cells cultured in RPMI 1640 media at (37°C) in humidified CO_2 atmosphere using trypan blue dye exclusion test (Vijayan, et al., 2002).The effect of different concentrations of the test drugs (12.5, 25.0, 50.0, 100.0, 200.0 and 400.0µg/ml) on the survival of the EAC cells were evaluated. EAC cells were incubated with RPMI medium for 24 hours in tissue culture tubes, each tube contained 0.1ml cells + 0.9ml medium such that the final concentration of the cells was 2×10^n (n=5) cells/ml. After 24 hours incubation, the tubes were centrifuged and the cells were separated by aspiration of the supernatant. EAC cells were again re-incubated with the RPMI medium then the test drug (MEAF, SFAF) was added so that the content of each tube was (0.8ml) medium + 0.1ml cells + 0.1ml drug. After 24 hours incubation of the cells with the test drug, the tubes were centrifuged and the cells were separated by aspiration of the supernatant. The cells were stained with trypan blue dye and the percentage of survival cells was determined by counting the dead and the viable cells using haemocytometer. For each test drug regimen to be evaluated for its cytotoxic effect, three in vitro experiments were done. In each experiment, the drug was evaluated in a triple system (three culture tubes were used). Control experiment in which EAC cells were cultured without test drug was conducted.

Percent survival of cells = T/C × 100

- T = the number of viable cells in a unit volume of the test drug tube.
- C= the number of viable cells in a unit volume of the control tube.

Cytotoxic activity by MTT assay on Hepg2 and Hella cell lines: The cytotoxicity was carried out using MTT [3-(4,5-methylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay on HepG2 and Hella cell lines (Mossman, 1983). Different concentrations of the test drug (6.25, 12.5, 25.0, 50.0, 100.0, 200.0 and 400.0µg/ml) were used. Cells were plated at 5×10^n (n=3)cells/well in 96 well micro-titer plate in aliquot of (190µl) and incubated for 24 hours at 37°C and 5% CO_2 in a humidified atmosphere. The incubated cells were then allowed to become attached to the plate surface by growing in a drug free medium for 18 hours. The test drug was then dissolved in dimethyl sulfoxide (DMSO). Water solution in a ratio of 1:9 and added to aliquot of (10µl) then incubated for 48 hours. After 48 hours of exposure of the cells to the drug, (10µl) of MTT solution (5mg of MTT dissolved in 1 ml of sterile phosphate buffer saline) were added to each micro-titer well and incubated for 4 hours at 37°C. (100µL) Sodium dodecyl sulphate in hydrochloric acid (SDS-HCl) solution was added to each micro-titer well and mix thoroughly using a pipette then incubation of the micro-plate was allowed for 4 hours at 37°C.The optical densities were read on an automated SpectraMax micro-plate reader at λ=570nm and the data analysis were then generated automatically to give the survival fraction of the cells. For each drug regimen to be evaluated for its cytotoxic effect, two in vitro experiments were done. In each experiment, the drug was evaluated in a triple system (three culture tubes were used).

Molluscicidal activity of MEAF and SFAF: The molluscicidal activity was performed according to the standard method recorded by WHO (1965). In this

method, different concentrations measured in ppm were used. For each concentration, ten snails were kept for 24 hours at normal laboratory conditions (25°C). Three replicates were run and in each case and two control groups were used. The positive control was Niclosamide and the negative one was de-chlorinated water. After exposure, the snails were washed with de-chlorinated water and kept for 24 hours for recovery. Dead and living snails were then distinguished from one another by physical and chemical means. Further, the dead and the living snails were distinguished by immersing in 5% aqueous alkaline solution. Snails that were killed were then counted. The LC_{50}, LC_{90} and the slope function were determined according to the method of Lichfield and Wilcoxon's (1949). The materials under test were considered potent molluscicides when their LC_{50} is not more than (20ppm) after exposure for 24 hours.

Schistosomicidal activity of MEAF and SFAF*:* The method described by Yousif, et al. (2007) was used. A stock solution was prepared by dissolving the test drug in 100% DMSO and sterile de-mineralized water to get the required concentration. In each well of a tissue culture plate, (0.1ml) of this solution is placed and completed to (2.0ml) of RPMI 1640 medium containing antibiotics and 20% fetal calf serum inside a sterilization laminar flow. Two wells are used for each concentration and three pairs of *S. mansoni* worms, males and females equally represented are placed in each well. Praziquantel was used as a positive control and a clean media as a negative one. The test and control wells were incubated at 37°C±0.5°C and worms were examined daily for 5 days, using stereomicroscope for viability. The rate of worm mortality was then calculated for all wells. In each case, decreasing concentrations of each test solution were used to determine the LC_{50} and LC_{90} using the statistical program SPSS.

Cercaricidal activity of MEAF and SFAF*:* The method reported by Pellerongo and De Marie (1967) was used. A series of (2ml) de-chlorinated water containing 100 freshly shed cercariae in small Petri dishes (5cm in diameter) were mixed with (2ml) of fresh dilution of the test solution. The test concentrations used were equivalent to LC_{50}, 0.5 LC_{50}, 0.25 LC_{50} and 0.125 LC_{50}. Four ml of de-chlorinated water containing 100 freshly shed cercariae were used as control. Time in minutes was then measured after which 100% mortality of the cercariae occurred.

Miracidicidal activity of MEAF and SFAF: The miracidcidal activity was evaluated using the method described by Tchounwou et al. (1991). Laboratory tissue culture plates were used as test chambers to observe the viability and death of miracidiae under the dissecting microscope. One hundred schistosome miracidiae were picked up in 1 ml de-chlorinated water and placed in the test chamber well. A double strength concentration was then added, giving a total of (2ml) in each experimental well. A total of six replicates were made for each of test solution. The test concentrations were equivalent to LC_{50}, 0.5 LC_{50}, 0.25 LC_{50} and 0.125 LC_{50}. Two ml of de-chlorinated water containing one hundred miracidiae were used as control. Time in minutes was then measured after which 100% mortality of the miracidiae occurred.

RESULTS

Two saponins of spirostanol type were separated and identified from the saponin fractions of *A. fronzosinii* and *A. angustifolia*. Compound **1** was identified as hecogenin 3 -*O* – β- D - glucopyranosyl - (1→2) - [β - D - xylopyranosyl- (1→3)] -β- D- glucopyranosyl- (1→4) - β - D – galactopyranoside and the structure of compound **2** was concluded to be identical to hecogenin – 3 -*O* - β - D - xylopyranosyl (1→3) -

β -D -glucopyranosyl- (1→2) -[β -D - xylopyranosyl -(1→3)] -β-D - glucopyranosyl (1→4) - β - D – galactopyranoside.

The assessment of cytotoxic activity using the trypan blue dye exclusion test on EAC cells (Figure 2), showed that both of MEAF and SFAF had marked cytotoxic activity against EAC cells till a concentration of (200µg/ml). While its assessment against Hep-G2 (Figure 3) showed higher cytotoxic effect for MEAF than SFAF. Also, (Figure 4) showed that both test drugs possessed marked and nearly equal cytotoxic activity against Hella human tumor cell lines using MTT assay at concentrations of (50, 100 and 400µg/ml) as well as higher activity of SFAF at concentrations up to (12.5µg/ml).

The results of molluscicidal and schistosomicidal activities indicated the absence of significant effects of both tested drugs compared to niclosamide (LC_{50} 0.19ppm) and praziquantel (LC_{50} 0.27ppm). The cercaricidal and miracidal activities results were negligible even after an exposure period exceeding 3 hrs.

DISCUSION

Chemistry: The methanolic residues of both of *A. franzosinii* and *A. angustifolia* leaves were separately dissolved in water and passed through column chromatography packed with porous polymer gel Diaion HP-20 to remove sugars, phenolics and water soluble non-steroidal constituents. The saponin fractions obtained from both species were repeatedly separated and purified using CC, preparative TLC and HPLC to yield two saponins **1** and **2** .Their structures (Figure 1) were established on the basis of chemical hydrolysis as well as 1D and 2D NMR analysis (Table 1).

*Saponin **1*** was obtained as amorphous solid and its ^{1}H-NMR spectrum exhibited two-proton singlet signals at δ 0.68 and 1.08 indicating the presence of two angular methyl groups (Me-18 &19) as well as two-proton doublet signals at δ 0.73 (*J* =5.7 Hz) and 1.34 (*J* = 6.5 Hz) assignable to two secondary methyl groups (Me-21 & 27). The structure of **1**, was suggested to be a spirostanol derivative, based upon the above ^{1}H-NMR data and a quaternary carbon signal due to C-22 at δ 109.3 and a downfield carbonyl signal of C-12 at δ 212.6 in the ^{3}C-NMR spectrum. The observation of four anomeric proton signals at δ 4.82 (1H, *d*, *J* = 7.7Hz), 5.15 (1H, *d*, *J* = 7.6Hz), 5.19(1H, *d*, *J* = 7.6Hz) and 5.54 (1H, *d*, *J* = 7.3Hz) in the ^{1}H-NMR spectrum(Table1), suggested that saponin **1** posseses four sugar moieties which were identified from the acid hydrolysis as glucose, galactose and xylose. The^{13}C-NMR assignments of the aglycone of **1** were based on the combined use of 1D an 2D (H^{1}-H^{1} COSY, HMQC, HMBC) NMR and were in complete agreement with those of hecogenin substituted at C-3 position by a sugar chain (Agrawal, et al., 1985). The β configuration of all anomeric centers was deduced from the coupling constant values (*J*= 3.0-7.7 Hz). The^{13}C-NMR assignments of the sugar units using 1D and 2D NMR revealed the presence of 4- substituted β - galactopyranose, (Gal) 2,3-disubstituted β-glucopyranose (Glc I), as well as two terminal β – glucopyranose (Glc II) and β-xylopyranose (Xyl) units. The exact positions of the sugar units were determined by considering the long- range HMBC correlations. Thus, compound **1** was previously isolated from *Agave macroacantha* (Eskander, et al., 2010) and its structure was elucidated as hecogenin 3 -*O* – β- D - glucopyranosyl - (1→2) - [β - D - xylopyranosyl- (1→3)] -β- D- glucopyranosyl- (1→4) - β - D - galactopyranoside.

*Saponin **2*** was also obtained as amorphous solid. 1D and 2D NMR analysis of **2** in comparison with that of **1** indicated that **2** was also a 3-*O*-glycoside of hecogenin. Acid hydrolysis of **2** afforded the same sugar components obtained after hydrolysis of

1. The ^{1}H NMR spectrum of **2** displayed five sugar anomeric proton doublets at δ 4.81 (1H,*d*, *J* = 7.5Hz), δ 5.04 (1H,*d*, *J* = 7.3Hz), δ 5.11(1H,*d*, *J* = 7.5Hz), δ 5.13 (1H,*d*. *J* = 7.6Hz) and δ 5.53 (1H, *d*,*J* = 7.0Hz). The^{13}C-NMR assignments of the sugar units using 1D and 2D NMR, revealed the presence of the identical units in **2** as those of **1** with further β – xylopyranose (Xyl II) unit linked to C-3 position of the terminal β - glucopyranose moiety (Glc II). This conclusion was evident from the glycosylation shift effect and the HMBC corelation between the anomeric proton signal of Xyl II at δ 5.11and Glc II C-3 at δ 87.0. The structure of **2** was concluded identical to hecogenin – 3 -*O* - β - D - xylopyranosyl (1→3) - β -D -glucopyranosyl- (1→2) - [β -D - xylopyranosyl - (1→3)] -β-D - glucopyranosyl (1→4) - β - D – galactopyranoside. Saponin **2** was previously identified in *Agave macroacantha* (Eskander, et al., 2010)**.**

Pharmacology: The cytotoxic activity of MEAF and SFAF was evaluated using the trypan blue dye exclusion test on EAC cells. The concept of this test is based on the ability of the trypan blue dye to stain only dead cells which are then counted when examined microscopically by the haemocytometer. The cytotoxicities of MEAF and SFAF against Hep-G2 and Hella human tumor cell lines using MTT assay were also investigated and found that the activity of MEAF is higher than SFAF at large doses which may be accounted for that a synergistic activity of all the content of the extract rather than the action of the saponin fraction alone.

CONCLUSION

In conclusion, the present study revealed marked cytotoxic activity of the two tested fractions against EAC cells, HepG2 cells and Hella cells, although higher cytotoxicity was seen for MEAF. The structures of the two separated steroidal saponins are saponin 1 identified as hecogenin 3 -*O* – β- D - glucopyranosyl - (1→2) - [β - D - xylopyranosyl- (1→3)] -β- D- glucopyranosyl- (1→4) - β - D – galactopyranoside and saponin 2 identified as hecogenin – 3 -*O* - β - D - xylopyranosyl (1→3) - β -D - glucopyranosyl- (1→2) - [β -D - xylopyranosyl - (1→3)] -β-D - glucopyranosyl (1→4) - β - D – galactopyranoside. They are both separated for the first time from the two species *A. franzosinii* and *A. angustifolia*.

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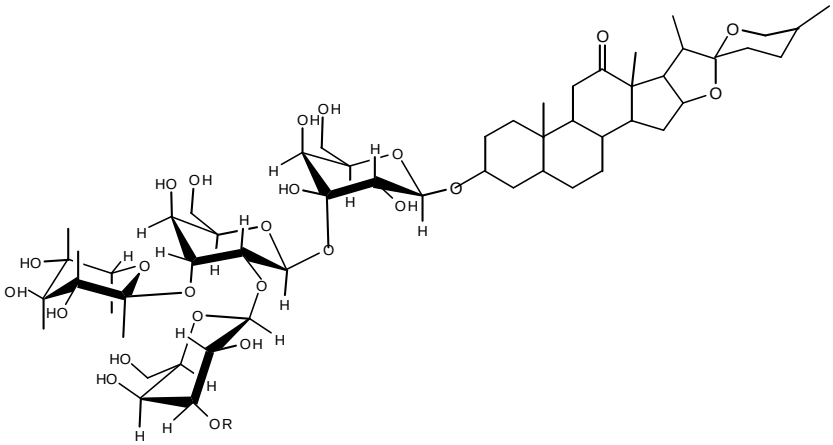
Table-1: ^{1}H NMR & ^{13}C NMR chemical shifts (ppm) for compounds 1 and 2 in pyridine d_5.

No.	H_1	C_1	H_2	C_2
Aglycone				
1	0.72,1.33	37.60	0.72, 1.33	36.70
2	1.5,1.99	29.70	1.55, 0.20	29.70
3	3.86	77.30	3.84	77.40
4	1.35, 1.77	34.80	1.35, 1.78	34.80
5	0.90	44.60	0.90	44.60
6	1.15	28.70	1.15	28.70
7	1.58, 0.80	31.80	1.58, 0.80	31.80
8	1.77	34.50	1.78	34.50
9	0.92	55.70	0.92	55.70
10	-	36.30	-	36.30
11	2.22(dd,14.0,4.5), 2.37 (t,13.5)	38.00	2.22(dd,14.0,4.5), 2.37 (t,13.5)	38.00
12	-	212.60	-	212.60
13	-	55.40	-	55.40
14	1.38	56.00	1.39	56.00
15	1.59, 2.09	31.50	1.59, 2.09	31.50
16	4.48	79.70	4.46	79.70
17	2.74 (dd,8.0,7.5)	54.40	2.70(dd,8.0,7.5)	54.40
18	1.08(s)	16.10	1.08 (s)	16.10
19	0.68(s)	11.80	0.69 (s)	11.80
20	1.92 (dq,7.5,6.7)	42.70	1.92 (dq,7.5,6.7)	42.70
21	1.34(d,6.5)	13.80	1.34 (d,6.7)	13.80
22	-	109.30	-	109.30
23	1.69	31.90	1.69	31.90
24	1.56	29.30	1.56	29.30
25	1.57	30.60	1.58	30.60
26	3.48(t,10.0), 3.57	67.00	3.48(t,11.0), 3.57	67.00
27	0.73(d,5.7)	17.30	0.73 (d,5.7)	17.30
3-O D- Gal				
1	4.82 (d,7.7)	102.6	4.81 (d,7.5)	102.60
2	4.35 (t,8.2)	73.10	4.34 (t,8.2)	73.10
3	4.05 (t,8.2)	75.70	4.06 (t,8.2)	75.60
4	4.56 (d,3.0)	79.80	4.55 (d,3.0)	79.60
5	3.96 (m)	75.40	3.95 (m)	75.30
6	4.18(dd,12.0,4.8)	60.70	4.18(dd,12.0,4.8)	60.70
6’	4.62 (t,9.5)		4.61 (t,9.5)	
D- Glc I				
1	5.15 (d,7.6)	105.10	5.13 (d,7.6)	104.80
2	4.38 (t,8.7)	81.30	4.33 (t,8.7)	80.70
3	4.12 (t,8.7)	87.10	4.07 (t,8.7)	86.90
4	3.78 (t,9.0)	70.50	3.78 (t,9.0)	70.50
5	3.86 (m)	77.70	3.83 (m)	77.60
6	4.33 (dd,12.4, 5.1)	62.60	4.24 (dd,12.4,5.1)	62.30
6’	4.50 (dd,12.1,5.0)		4.46 (dd,12.1,5.0)	

Table-1: Continue..

No	H_1	C_1	H_2	C_2
D- Glc II				
1	5.54 (d,7.3)	104.80	5.53 (d,7.0)	104.00
2	4.03 (t,8.1)	80.70	4.03 (t,8.1)	75.70
3	3.85 (t,8.1)	86.90	4.03 (t,8.1)	87.00
4	3.81 (t,8.1)	70.50	4.03 (t,8.1)	69.40
5	3.85 (m)	77.60	3.81 (m)	77.70
6	4.03 (dd,12.3,4.8)	62.30	4.04 (dd,12.3,4.8)	63.00
6’	4.51 (dd,12.2,4.9)		4.47 (dd,12.2,4.9)	
D-Xyl I				
1	5.19 (d,7.6)	105.00	5.04 (d,7.3)	106.10
2	3.92 (t,7.9)	75.10	3.91 (t,8.3)	75.10
3	4.03 (t,7.9)	78.70	4.00 (t,8.3)	78.40
4	4.08 (t,7.9)	70.80	4.06 (t,8.3)	70.80
5	3.64 (t,11.0)	67.40	3.62 (t,11.2)	67.30
5’	4.20 (dd,11.0,5.0)		4.19 (dd,11.2,5.2)	
D- Xyl II				
1	-		5.11 (d,7.5)	104.90
2	-		3.89 (t,8.0)	75.10
3	-		4.00 (t,8.0)	78.20
4	-		4.06 (t,8.0)	70.70
5	-		3.52 (t,10.8)	67.10
5’	-		4.19 (dd,10.8,4.9)	



Compound No.	**R1**
1	**H**
2	**D- Xylose**

Figure- 1: compound 1 and 2.

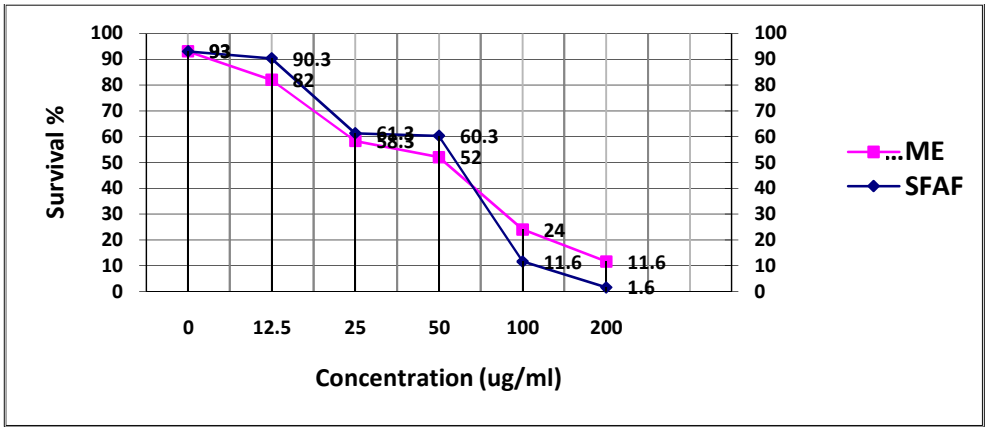


Figure -2: Viability percent of EAC cells of MEAF and SFAF.

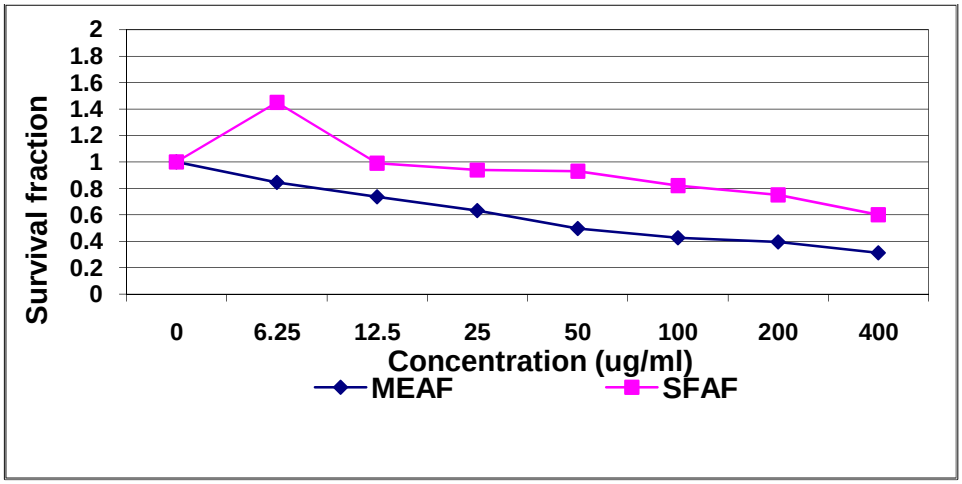


Figure- 3: Effect of MEAF and SFAF on proliferation of Hepg2 cells.

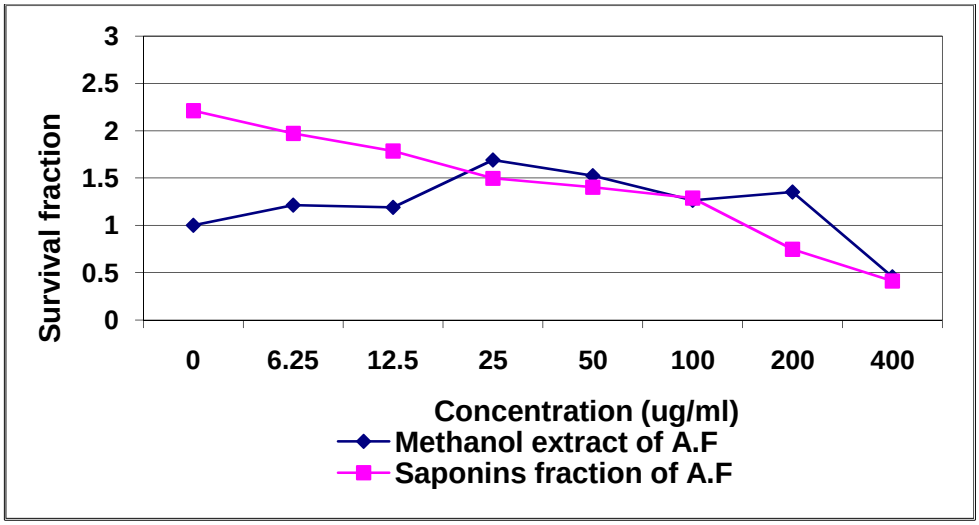


Figure- 4: Effect of MEAF and SFAF on proliferation of Hella cells.

