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LEAVE EXTRACT OF Syzygium malaccenseAS GREEN INHIBITOR OF MILD STEEL IN ACIDIC MEDIUM

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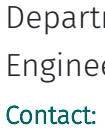
-  Bassim H. Hammadi

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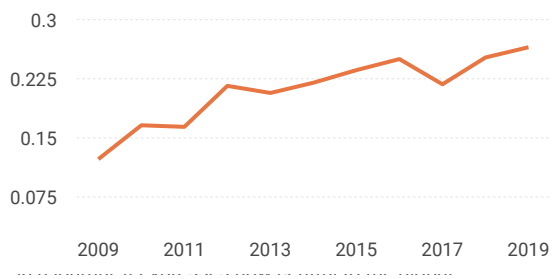


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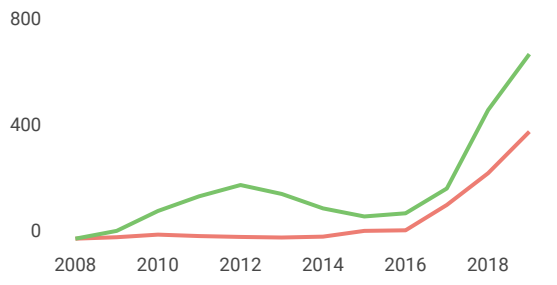


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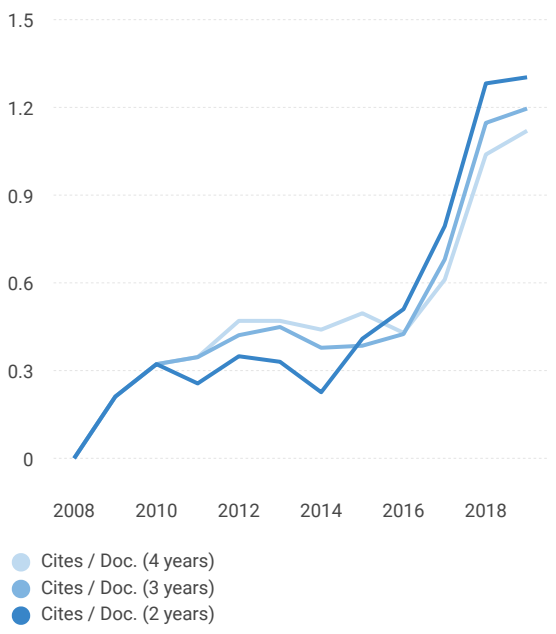

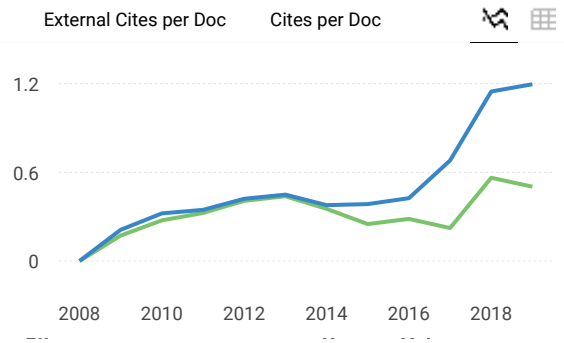



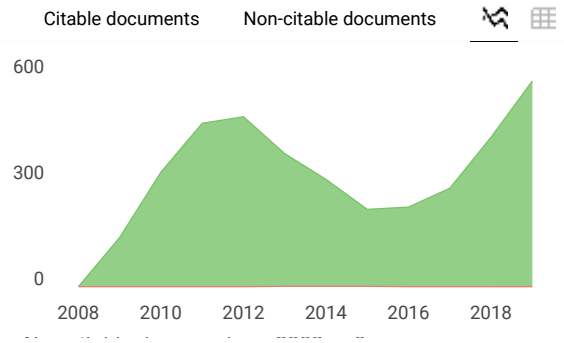


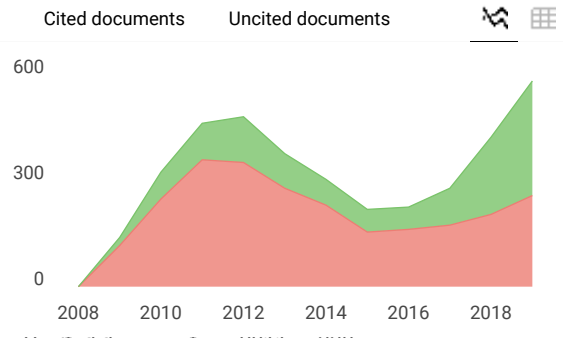



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# ANTICANCER ACTIVITY OF ISOLATED COMPOUNDS FROM *Syzygium aqueum* STEM BARK

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**ABSTRACT**

From stem bark of *Syzygium aqueum*, oleanolic acid, and β-sitosterol were isolated successfully. Their structure was identified using spectroscopic methods such as UV-Visible, Fourier Transform Infrared and Nuclear Magnetic Resonance. The isolated compounds were then tested for their anticancer activity against cervical, and breast cancer cell lines using the MTT method. The presence of these two compounds isolated from the stem bark of *S. aqueum* is the first to be reported.

**Keywords:** *Syzygium aqueum*, Stem Bark, Oleanolic Acid, β-sitosterol, T47D and HeLa Cancer Cell

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## INTRODUCTION

In the world, the second biggest killer disease for humans is cancer. According to the World Health Organization, fourteen million people have cancer, and eight million die worldwide.[1] Some cancer treatments are surgery, radiation therapy, chemotherapy, and radiotherapy, which depend on the specific type, location, and stage.[2] Chemotherapy and radiation therapy are primary clinical treatments at an early stage. Various anticancer medicines are also used in the present. However, these treatment methods and anticancer drugs have severe side effects such as destroying cancer cells and healthy cells.[1,3]

In therapeutic, phytochemical constituents derived from nature or living organisms play a significant role. Numerous substances have converted into drug candidates as paclitaxel, theophylline, doxorubicin, morphine, penicillin G, digoxin and vitamin A, etc.[4] Moreover, herbal medicines have been reported as an essential cancer treatment because this treatment is found to have high-quality effects and common side effects. Medicinal plants are recognized as one of the common alternatives for cancer treatment in many countries worldwide.[5] Medicinal plants contain various kinds of critical phytochemical constituents such as alkaloids, terpenoids, flavonoids, pigments, tannins, etc., which have antioxidant, anti-inflammatory, anticancer, and other bioactivity properties.[6,7]

The genus *Syzygium* has been reported to be a rich source of phytochemical constituents. This genus belongs to the Myrtaceae family, a woody flowering family consisting of 144 genera and 5500 species. Approximately 1,200 species of this family are species in the genus *Syzygium*.[8,9] They are widely found in South Asia, Southeast Asia, Australia, New Caledonia, tropical Africa, sub-tropical, and tropical Asia.[10] *S. samarangense*, *S. malaccensis*, *S. aqueum*, *S. aromaticum*, *S. jambos* and *S. polyanthum* are reported as public large. *Syzygium* species are well-known species in this genus, and they are cultivated because of the edible fruit or used as traditional medicine.[10,11]


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*S. aqueum*, which is native to Indonesia and Malaysia, is better known as watery rose apple or water apple.[12-14] This species is also used as traditional medicine, especially bark, leaves, and roots.[13,15] Most researchers have already presented the phenolic content, antioxidant, and antidiabetic activities of leaves and fruit extract of *S. aqueum* using various methods.[16-18] Tannin and flavonoids from the leaves have already been reported in some international research papers.[19,20] The bioactive components of the stem bark have never been noted. From this study, one terpenoid and one steroid were isolated and anticancer activity was evaluated using an MTT assay.

## EXPERIMENTAL

### Material and Methods

### Plant Materials

The stem bark of *S. aqueum* was obtained from Wage, Taman, Sidoarjo, East Java, Indonesia. The plant material was determined by the Department of Biology, Faculty of Science and Technology, Universitas Airlangga. It was crushed into small pieces like a powder. The powdered sample was then extracted and separated.

### Chemicals and Instrumentation

Silica gel 60 (700-200 mesh ASTM) was applied for column chromatography. Analytical TLC was conducted on a pre-coated silica gel 60 F254 (25 Aluminium sheets 20×20 cm, Merck). The U.V. spectrum was recorded on a UV-visible spectrometer (Shimadzu). FT-IR (Fourier Transform Infrared spectroscopy) spectra were measured on Tracer-100 spectrophotometer (Shimadzu). I.R. spectrum (KBr) was showed in cm-1. Nuclear Magnetic Resonance ($^{1}$H-NMR and $^{13}$C -NMR) were recorded on BRUKER 600 Hz in $CDCl_3$. δ (ppm) was used to measure the chemical shift, and Hertz (Hz) was used to measure the coupling constant (*J*). Anticancer activity assay was measured on ELISA (Enzyme-Linked Immunosorbent Assay) reader

### General Procedure

### Extraction and Separation

The powdered stem bark of *S. aqueum* (1kg) was extracted with methanol (40L) at room temperature for 3×24 hr. The methanol extract was evaporated with Buchi rotary vacuum evaporator. The extract (450g) was partitioned with n-hexane (6L) three times. N-hexane extract (50g) was put into silica gel column chromatography and then eluted with n-hexane-ethyl acetate mixture, in which polarity was increased gradient. The similar fractions were combined to obtain 19 main fractions (SA-1 to SA-19). Fraction (SA-11) was re-chromatographed with eluent n-hexane: ethyl acetate (7:3), gave 13 fractions (SA-11-1 to SA-11-13). Fraction (SA-11-3) yielded a pure compound, namely compound 1 (22mg). Fraction SA-9 was separated more using column chromatography with a similar eluent (8:2), yielding 6 fractions (SA-9-1 to SA-9-6). Fraction SA-9-5 gave compound 2 (25mg).

### Bioassay

### Cell Culture

Cervical (HeLa) and breast (T47D) cell lines were collected from ATCC (American Type Culture Collection). Cells were cultured at 37 ºC and 5% $CO_2$ for 24 hours and 100% humidified in medium supplemented with 1% L-glutamine, 10% FBS, and 1% penicillin/streptomycin. The process of cell culture (HeLa and T47D) for the anticancer test was carried out in Cancer Chemoprevention Research Center, Faculty of Pharmacy, Gajah Mada University, Indonesia.

### MTT Assay

Anticancer activity test of isolated pure compounds was executed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay method. Cancer cells were cultured in a 96well plate at 213 x $10^4$ cell/well density. The compound was prepared as serial dilutions (1.5625-100 μg/mL) dissolving in 0.1 M of DMSO, and it's 100 μL were treated with cells at 37 ºC in a $CO_2$ incubator for 24 hours. A 100 μL of MTT reagent (50 mg in 10 mL phosphate buffered saline) was filled in each well, followed by incubation in $CO_2$ incubator for 2-4 h until the forming of the purple formazan crystals. The formed formazan was proportional to the total number of viable cells that read its absorbance value at the wavelength 560 nm using ELISA (Enzyme-Linked Immunosorbent Assay) reader. The following formula calculated the % cell viability and IC50 value were calculated using SPSS 17, IBM Analytics.[21,22.] Doxorubicin is used as a standard drug.

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treatment} - \text{Absorbance of media control}}{\text{Absorbance of negative control} - \text{Absorbance of media control}} \times 100\%$$

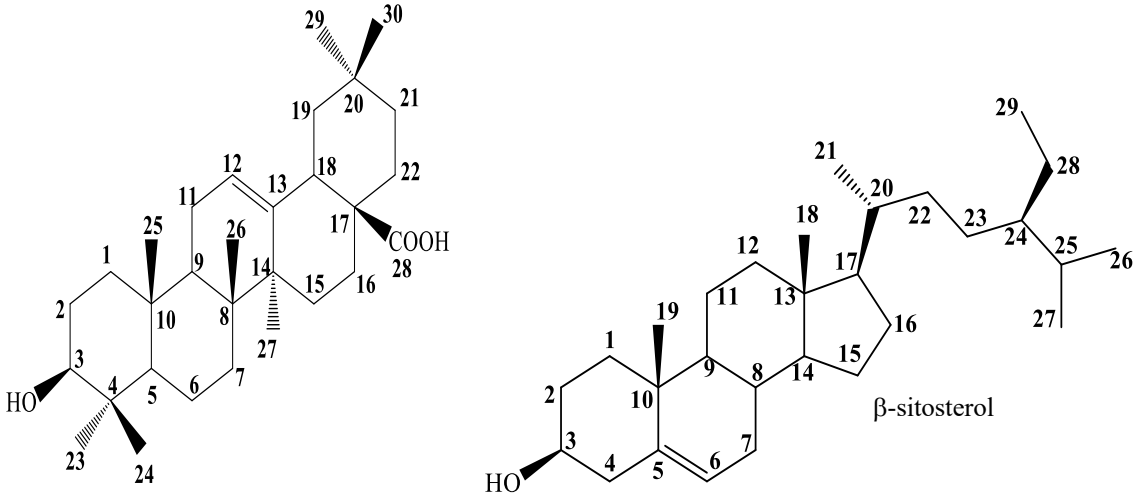
## RESULTS AND DISCUSSION

**Compound 1**

White powder. The structure was determined according to FTIR spectrum, $^{1}$H-NMR and $^{13}$C-NMR (including 2D: HMBC, HSQC and COSY). FTIR spectra showed some bands at 3421 cm$^{-1}$ (-OH group), 2931-2870 cm$^{-1}$ (sp$^3$ C-H stretching), 1689 cm$^{-1}$ (C=O). The $^{1}$H-NMR spectra indicated some signals at δH (ppm): 0.73 (m, 1H), 0.76 (s, 3H), 0.78 (s, 3H), 0.91 (s, 3H), 0.93 (s, 6H), 0.99 (s, 3H), 1.09 (m, 2H), 1.14 (s, 3H), 1.17 (m, 1H), 1.22 (m, 1H), 1.30 (m, 1H), 1.35 (m, 2H), 1.43 (m, 1H), 1.56 (m, 2H), 1.59 (m, 1H), 1.61 (m, 2H), 1.63 (m, 4H), 1.74 (m, 1H), 1.79 (m, 1H), 1.91 (m, 1H),1.99 (m, 1H), 2.83 (dd, *J*= 4.2 and 13.8 Hz, 1H), 3.22 (dd, *J*= 4.2 and 11.4 Hz, 1H), 5.29 (t, *J*= 3.5 Hz, 1H). $^{13}$C NMR spectra revealed some signals at δC (ppm): 15.5, 15.3, 17.1, 18.3, 22.9, 23.4, 23.6, 25.9, 27.2, 27.7, 28.1, 30.6, 32.4, 32.6, 33.0, 33.8, 37.0, 38.4, 38.7, 39.2, 41.0, 42.6, 45.8, 46.5, 55.2, 79.0, 122.6, 143.5, 182.8. Moreover, $^{1}$H-$^{1}$H COSY correlations and $^{1}$H-$^{13}$C HMBC correlations were used for confirmation of compound 1 structure (Fig-2). Compound 1 was recognized as oleanolic acid ($C_{30}H_{48}O_3$) according to the above data and comparison with literature data.[23] All NMR spectra data (600 MHz, $CDCl_3$) were presented in Table-1.

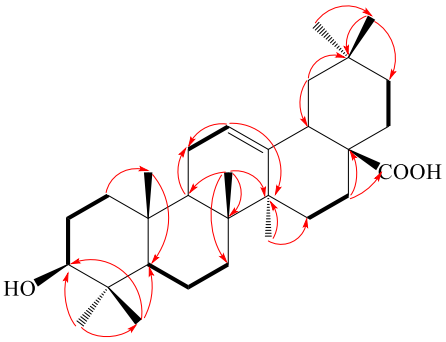
**Compound 2**

White powder. UV spectrum, FTIR spectrum, $^{1}$H-NMR and $^{13}$C-NMR spectrum (including 2D: HMBC, HSQC and COSY) were used for identification of its structure. UV spectrum (in MeOH) showed the $\lambda_{max}$ 260 nm. In FTIR spectrum, some bands were appeared at 3421 cm$^{-1}$ (-OH), 2958-2868 cm$^{-1}$ (sp$^3$ C-H stretching). The signals on $^{1}$H-NMR spectrum were appeared at chemical shift δH (ppm): 0.69 (s, 3H), 0.82 (d, *J*= 6.8 Hz, 3H), 0.83 (d, *J*= 6.8 Hz, 3H), 0.85 (m, 3H), 0.93 (m, 5H), 0.97 (m, 1H), 1.01 (s, 3H),1.03 (m, 1H), 1.09 (m, 3H), 1.17 (m, 3H), 1.26 (m, 2H), 1.28 (m, 1H), 1.33 (m, 1H), 1.36 (m, 1H), 1.50 (m, 3H), 1.58 (m, 1H), 1.67 (m, 1H), 1.85 (m, 3H), 1.98 (m, 2H), 2.01 (m, 2H), 2.24 (m, 1H), 2.30 (m, 1H), 3.53 (m, 1H), 5.36 (m, 1H). The $^{13}$C-NMR spectrum suggested as twenty-nine carbon at δC (ppm): 11.8, 11.9, 18.8, 19.0, 19.4, 19.8, 21.1, 23.1, 24.3, 26.1, 28.3, 29.2, 31.6, 31.9, 33.9, 36.2, 36.5, 37.3, 39.8, 42.3, 45.8, 50.2, 56.1, 56.8, 71.8, 121.7, 140.8. Furthermore, the structure of compound 2 was proved with the correlations of $^{1}$H-$^{1}$H COSY and $^{1}$H-$^{13}$C HMBC correlations (Fig-2). Compound 2 was recognized as β-sitosterol ($C_{29}H_{50}O$) according to the above data which were compared with literature.[24] All NMR spectra data (600 MHz, $CDCl_3$) were showed in Table-2.




Oleanolic Acid

Fig.-1: Molecular Structure of Compound 1 and Compound 2

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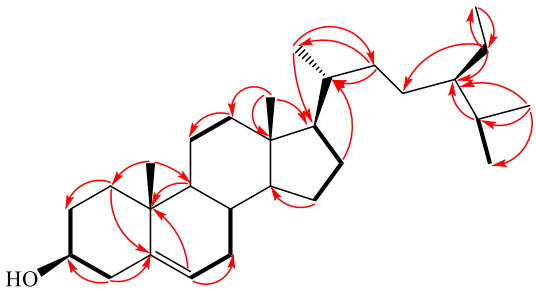


β-sitosterol

Fig.-2: HMBC ( ) and COSY ( ) Correlations of Compound 1 and Compound 2

Table-1: NMR Spectral Data of Compound-1 (Oleanolic Acid)

| Position | $\delta_H$ (ppm) {m, *J* (Hz)} | $\delta_C$ (ppm) | HMBC |
|---|---|---|---|
| 1 | 1.63 2H (*m*) | 38.4 | C-25 |
| 2 | 1.61 2H (*m*) | 27.2 | - |
| 3 | 3.22 1H (*dd*, *J*= 11.4 & 4.2 Hz) | 79.0 | - |
| 4 | - | 38.7 | - |
| 5 | 0.73 1H (*m*) | 55.2 | C-4 |
| 6 | 1.56 1H (*m*) | 18.3 | - |
| | 1.35 1H (*m*) | | |
| 7 | 1.79 1H (*m*) | 32.4 | - |
| | 1.59 1H (*m*) | | |
| 8 | - | 39.2 | - |
| 9 | 1.56 1H (*m*) | 46.5 | C-11 |
| 10 | - | 37.0 | - |
| 11 | 1.91 1H (*m*) | 23.4 | - |
| | 1.09 1H (*m*) | | |
| 12 | 5.29 1H (*t*, *J*= 3.5 Hz) | 122.6 | C-11; C-14 |
| 13 | - | 143.5 | - |
| 14 | - | 42.6 | - |
| 15 | 1.09 1H (*m*) | 27.7 | - |
| | 1.74 1H (*m*) | | |
| 16 | 1.99 1H (*m*) | 22.9 | C-17; C-28 |
| | 1.63 1H (*m*) | | |
| 17 | - | 46.5 | - |
| 18 | 2.83 1H (*dd*, *J*= 4.2 &13.8 Hz) | 41.0 | - |
| 19 | 1.63 1H (*m*) | 45.8 | C-20; C-18 |
| | 1.17 1H (*m*) | | |
| 20 | - | 30.6 | - |
| 21 | 1.35 1H (*m*) | 33.8 | - |
| | 1.22 1H (*m*) | | |
| 22 | 1.43 1H (*m*) | 32.6 | - |
| | 1.30 1H (*m*) | | |
| 23 | 0.99 3H (*s*) | 28.1 | C-3; C-4; C-5; C-24 |
| 24 | 0.78 3H (*s*) | 15.5 | C-3; C-4; C-5; C-23 |
| 25 | 0.93 3H (*s*) | 15.3 | C-5; C-9 |
| 26 | 0.76 3H (*s*) | 17.1 | C-7; C-8; C-9; C-14 |

| Position | $\delta_H$ (ppm) {m, *J* (Hz)} | $\delta_C$ (ppm) | HMBC |
|---|---|---|---|
| 27 | 1.14 3H (*s*) | 25.9 | C-8; C-13; C-14; C-15 |
| 28 | - | 182.8 | - |
| 29 | 0.91 3H (*s*) | 33.0 | C-21; C-30 |
| 30 | 0.93 3H (*s*) | 23.6 | C-20; C-21 |

Table-2: NMR Spectral Data of Compound-2 (β-sitosterol)

| Position | $\delta_H$ (ppm) {m, *J* (Hz)} | $\delta_C$ (ppm) | HMBC |
|---|---|---|---|
| 1 | 1.85 1H, (*m*) | 37.3 | C-2; C-5 |
| | 1.09 1H (*m*) | | |
| 2 | 1.98 1H (*m*) | 31.9 | - |
| | 1.50 1H (*m*) | | |
| 3 | 3.53 1H (*m*) | 71.8 | - |
| 4 | 2.24 1H (*m*) | 42.3 | C-3; C-5 |
| | 2.30 1H (*m*) | | |
| 5 | - | 140.8 | - |
| 6 | 5.36 1H (*m*) | 121.7 | C-4; C-8; C-10 |
| 7 | 1.85 1H (*m*) | 31.6 | - |
| | 2.01 1H (*m*) | | |
| 8 | 1.98 1H (*m*) | 31.9 | - |
| 9 | 0.93 1H (*m*) | 50.2 | C-10 |
| 10 | - | 36.5 | - |
| 11 | 1.50 2H (*m*) | 21.1 | - |
| 12 | 2.01 1H (*m*) | 39.8 | C-11 |
| | 1.17 1H (*m*) | | |
| 13 | - | 42.3 | - |
| 14 | 0.97 1H (*m*) | 56.8 | - |
| 15 | 1.09 1H (*m*) | 24.3 | C-14 |
| | 1.58 1H (*m*) | | |
| 16 | 1.85 1H (*m*) | 28.3 | C-17; C-20 |
| | 1.26 1H (*m*) | | |
| 17 | 1.09 1H (*m*) | 56.1 | - |
| 18 | 0.69 3H (*s*) | 11.9 | C-12; C-13; C-17 |
| 19 | 1.01 3H (*s*) | 19.4 | C-1; C-9 |
| 20 | 1.36 1H (*m*) | 36.2 | - |
| 21 | 0.93 3H *(m)* | 18.8 | C-17; C-22 |
| 22 | 1.33 1H (*m*) | 33.9 | C-20; C-21 |
| | 1.03 1H (*m*) | | |
| 23 | 1.17 2H (*m*) | 26.1 | - |
| 24 | 0.93 1H (*m*) | 45.8 | - |
| 25 | 1.67 1H (*m*) | 29.2 | C-24 |
| 26 | 0.82 3H (*d*, *J*= 6.8 Hz) | 19.8 | C-24; C-25; C-27 |
| 27 | 0.83 3H (*d*, *J*= 6.8 Hz) | 19.0 | C-26 |
| 28 | 1.28 1H (*m*) | 23.1 | C-23; C-24; C-29 |
| | 1.26 1H (*m*) | | |
| 29 | 0.85 3H *(m)* | 11.8 | C-28 |

In an anticancer assay, compound 1 was considered moderate active for both cancer cell lines. For compound 2, it was significantly toxic on the HeLa cell line. Its cytotoxicity was almost as toxic as the standard drug (doxorubicin), although it has moderate cytotoxicity against the T47D cell line. Anticancer activity of compound 1 and compound 2 was reported in Table-3, and the percentage of cell viability of these two compounds against HeLa cell and T47D were presented in Fig-3. The pure

compound is deemed as highly toxic when $IC_{50} \leq 4$ µg/mL and toxic when $IC_{50} \leq 20$ µg/mL. Moderately toxic were considered when $IC_{50}$ 20-100 µg/mL and $IC_{50}$ above 100 µg/mL were suggested as non-toxic in literature.[25-26.]

Table- 3: Anticancer Activity of Compound 1 and Compound 2 on Hela and T47D Cell Lines

| | $IC_{50} \pm SD$ (µg/mL) | | |
|---|---|---|---|
| | Doxorubicin | Compound 1 (Oleanolic acid) | Compound 2 (β-sitosterol) |
| HeLa cell line | 2.67 ± 0.25 | 64.38 ± 0.952 | 3.43 ± 0.002 |
| T47D cell line | 0.035 ± 0.012 | 20.15 ± 0.011 | 90.80 ± 5.710 |

*SD= Standard Deviation

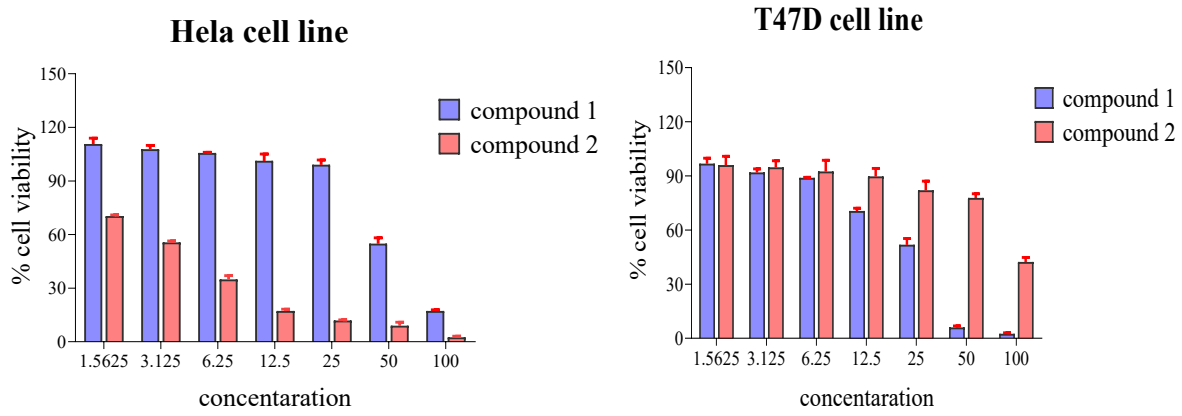


Fig.-3: Percentage of Cell Viability of Compound 1 acid and Compound 2 on HeLa Cell and T47D

## CONCLUSION

In this study, oleanolic acid and β-sitosterol were isolated from the stem bark of *S. aqueum*. The two compounds showed anticancer activity against both HeLa and T47D cell lines with MTT assay. Oleanolic acid was more active on the T47D cell line, but β-sitosterol was more active on the HeLa cell line.

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