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# The Effects of Phytosterols Isolated from *Omphalocarpum elatum* (Sapotaceae) on Mouse Peritoneal Cells

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**Abstract**

Although some herbal medicines have promising potential and are widely used, many of them remain untested and their use is not monitored. *Omphalocarpum elatum* is a plant that is native to Cameroon in West Africa and ranges from Sierra Leone to Zaire. It has been used traditionally for the treatment of sterility of males and it increases lactation in women. The main objective of this study was to determine the toxicity profile of phytosterols isolated from *O. elatum*. Phytosterols were isolated by column chromatography on silica gel and their structure were deduced from NMR and MS data. Isolated phytosterols were exposed to mouse peritoneal cells and their effects were determined by assessing for cell viability after 72hours. Cells were exposed to four different phytosterols namely OEW34, OEWCP34, OESH146, and OESE55 at concentrations of 0, 6.3, 12.5, 25 and 50µg/ml in their respective equimolar equivalence (µM). OEWCP34 was combined with reduced Glutathione (GSH) at 81.35µM and with the anticancer drug, daunorubicin at 0.47, 0.95, 1.90, 3.79 and 9.48µM. GSH at 81.35µM was also combined with daunorubicin at 0.47, 0.95, 1.90, 3.79 and 9.48µM. OEWCP34 and OESH146 decreased cell viability by 25 % and 12.6 % respectively. OEWCP34 antagonised the efects of daunorubicin and increased cell viability when there were combined. Incubation of GSH with OEWCP34 provided an additive effect, and thus, increased cell proliferation. Phytosterols isolated from *O. elatum* were not toxic and were able to induce cell proliferation and this may provide a basis for the ethnomedicinal of the plant.

**Keywords**: *Omphalocarpum elatum*; Mouse peritoneal cells; Phytosterols; Toxicity; Reduced glutathione

## Background

*Omphalocarpum elatum* is a tall, tropical African tree, native to Cameroon in West Africa and ranges from Sierra Leone to Zaire, belonging to the family Sapotaceae, notable for the large fruits growing directly from the trunk [1]. In Africa, plants of the genus *Omphalocarpum* are prepared for various purposes such as decoctions, powders, macerations, and are used for years in traditional medicine to treat headaches, wounds, skin diseases, constipation, elephantiasis, fever, cough, and rheumatism [2]. *O. elatum* has been used traditionally for the treatment of sterility of males and increases lactation in women. The wood is used for planks, implements such as mortars and bowls, handles, seats and drums as well as dugout canoes. The latex has been used as an adulterant of rubber [3]. Phytosterols, which encompass plant sterols and stanols, are phytosteroids similar to cholesterol which occur in plants and vary only in carbon side chains and or presence or absence of a double bond [4]. They are more likely precursors to steroids which act as hormones and sterols that might be involved in the structural arrangement of membrane [5].

With nearly 80% of people living in developing countries still depend on plant-based traditional medicine for their primary health care [6]. To this effect, research has become focused on the identification and isolation of compounds from natural products. Toxicological research and testing of natural compounds helps us to live safely and to derive benefit from natural and synthetic substances while avoiding harm [7]. Toxicologists use *in vitro* methods and animal models that have been approved by the scientific community and acknowledged by regulatory bodies so that the population that are exposed to the natural medicines that have been tested can enjoy the benefits with a minimum of risk [8]. The primary aim of toxicological assessment of any herbal medicine is to identify adverse effects and to determine limits of exposure level at which such effects occur [9]. Therefore, cell viability and cytotoxicity assays are essential for drug screening and *in vitro* safety evaluation of drug molecules and herbal products. For majority of these products in use, very little is known about their active and or toxic constituents. In many countries including the U.S, herbal medicines are not subjected to the same regulatory standards as orthodox drugs in terms of efficacy and safety [10]. This raises concern on their safety and implications for their use as medicines.

Toxicity testing can reveal some of the risks that may be associated with use of herbs, therefore, avoiding potential harmful effects when used as medicine. In Africa, knowledge of traditional




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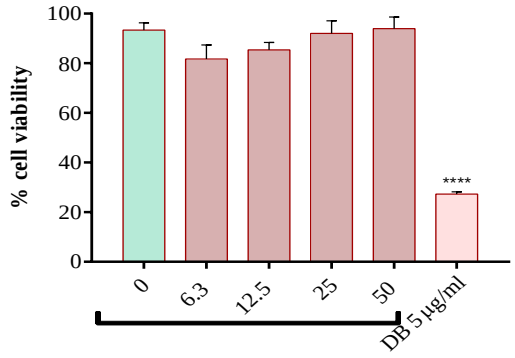



**Figure 1:** The effect of OEW34 on cell proliferation. OEW 34 generally had no effect on the cell viability. Error bars represent standard deviation from the mean. *p<0.05, **p<0.01, ***p<0.001.

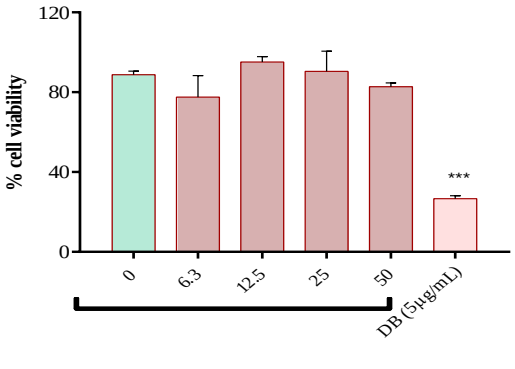



**Figure 2:** The effect of OESH 146 on cell proliferation. OESH did not have an effect on the cell viability. Error bars represent standard deviation from the mean. *p<0.05, **p<0.01, ***p<0.001.

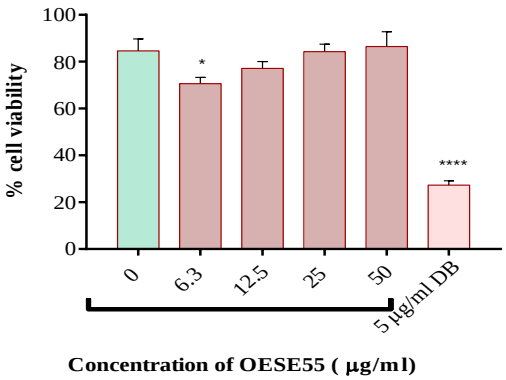



**Figure 3:** The effect of OESE 55 on cell proliferation. A lower concentration of OESE caused a decrease in cell viability. Error bars represent standard deviation from the mean *p<0.05, **p<0.01, ***p<0.001.

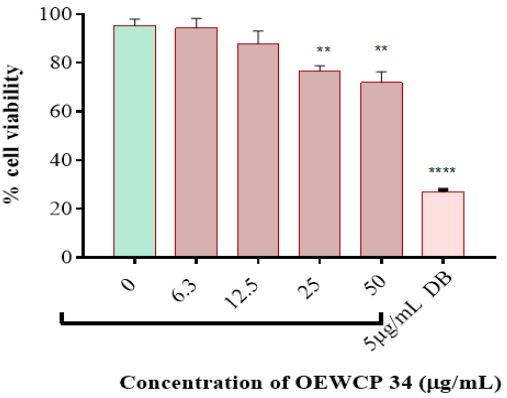



**Figure 4:** The effect of OEWCP 34 on cell proliferation. Error bars represent standard deviation from the mean. *p<0.05, **p<0.01, ***p<0.001.

medicine as part of holistic system was passed through generations by oral communication and indigenous practices [11]. The preclinical toxicity testing on various biological systems reveals the species-, organ- and dose- specific toxic effects of an investigational product. The toxicity of substances can be observed by studying the accidental exposures to a substance, *in vitro* studies using cells or cell lines, *in vivo* exposure on experimental animals [12]. The assessment parameters for cytotoxic effects include inhibition of cell proliferation, cell viability markers (metabolic and membrane), morphologic and intracellular differentiation markers [13]. This study was carried out to determine the toxicity profile of phytosterols that were isolated from *Omphalocarpum elatum.*

## Materials and Methods

### Reagents

All chemicals used in this study were acquired from Sigma-Aldrich Chemicals Company in Munich, Germany. Roswell Park Memorial Institute (RPMI), Daunorubicin, Ethanol, Foetal Bovine Serum (FBS), Phosphate Buffer Saline (PBS) and antibiotics were used when carrying out the assays. Water used for all experiments was distilled locally in the laboratory. The phytosterols (Table 1) were isolated in Cameroon and sent to University of Zimbabwe, Mt Pleasant Biochemistry Department. The use of animal cell lines in this study was approved by the Joint Parirenyatwa Group of Hospitals and College of Health Sciences Research Ethics Committee (JREC/327/14, Harare, Zimbabwe). For the toxicity studies, mouse peritoneal cells were used. Mouse peritoneal cells were extracted from 5 male Balb/c from the Animal House, Department of Anatomy, (University of Zimbabwe Mt. Pleasant, and Harare, Zimbabwe).

### Isolation, purification and elucidation of the chemical structures of phytochemicals from the *Omphalocarpum elatum* bark and heart wood

The air-dried and powdered stem bark (2.3kg) and heart wood (2.4kg) were separately macerated twice in a mixture of $CH_2Cl_2$/MeOH (1:1, 10L) for 48h. The solvent was then evaporated 40˚C under vacuum to afford 90g and 52g of dark red residue respectively. Solid- liquid extraction of the above crudes with chloroform yielded a chloroform fraction, OEW (23g) for the heart wood, OES (42g) for the stem bark and a dark red residue from each crude extract. OEW34 and OEWCP34 were isolated from the heart wood extract of *O. elatum* while OESE55 and OESH146 were got from the stem bark extracts. The compounds were isolated from their extracts by liquid column chromatography using silica gel as stationary phase and the mobile phase composed of a gradient of ethyl acetate in hexane. OEW34 was eluted from Hex-EA [9-1] and re-crystallised

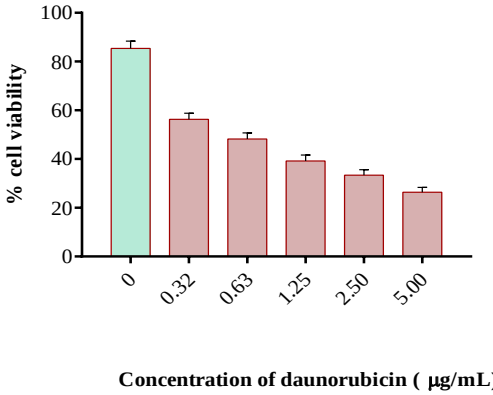



**Figure 5:** Effect of daunorubicin on cell proliferation. There was a dose-dependent effect of daunorubicin on the cells. Error bars represent standard deviation from the mean. *p<0.05, **p<0.01, ***p<0.001.

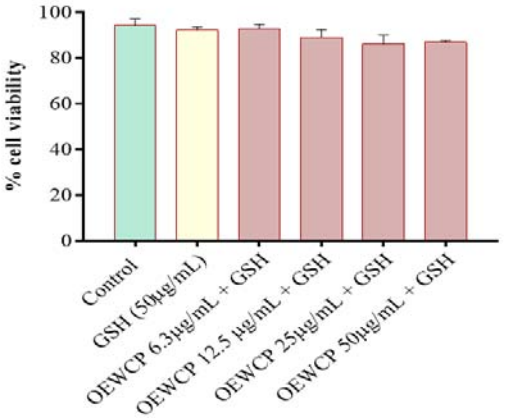



**Figure 6:** The effect of combining OEWCP 34 and reduced glutathione on cell proliferation. Error bars represent standard deviation from the mean. *p<0.05, **p<0.01, ***p<0.001.

in a mixture of chloroform-methanol as white crystals. OEWCP34 was got from Hex-EA [25-75] as a white amorphous powder and further purified by silica gel column chromatography on a narrow column using chloroform-methanol [95-5]. OESE55 was isolated as white amorphous powder from directly from Hex-EA [25-75] and OESH146 as colourless powder from Hex-EA [6-4]. The structures of these compounds were determined by analysis of their physical and spectroscopic data (MS and NMR data) from a Bruker Advance 400 spectrometer (IET, Illinois, USA) in conjunction with data published in literature. UV spectra were recorded on a Carry 300 spectrophotometer (Santa Clara, CA, United States). IR spectra were recorded on a JASCO Fourier Transform IR spectrometer (Easton, MD, USA). Compound OEW34 was found to be spinasterol [14] OEWCP34 as clethric acid [15]; OESE55 rotundic acid [16] and OESH146 elatumic acid [17].

**OEW34:** (24S)-stigmasta-7, 22(E)-dien-3a-ol or spinasterol MF: $C_{29}H_{48}O$, m.p.:168-169˚C, IR$\nu_{max}$ ATR ($cm^{-1}$): 3425, 2964, 2895; HREIMS: m/z 412.3733 (calculated for $[M+Na]^+$, 435.3603)

$^1$H NMR ($CDCl_3$, 300MHz) δ (ppm): 5.12 (1H, dd, J= 8.7Hz, H-22), 5.07 (1H, m, H-7), 4.98 (1H, dd, J = 8.4Hz, H-23), 3.52 (1H, m, J = 7.2, 4.5, 4.2, H-3), 0.96 (3H, d, J = 6.6Hz, H-21), 0.79 (3H, d, J = 6.3Hz, H-27), 0.73 (3H, t, J = 7.5Hz, H-29), 0.73 (3H, s, H-26), 0.73 (3H, s, H-19), 0.48 (3H, s, H-18)

$^{13}$C NMR ($CDCl_3$, 75MHz) δ (ppm): 139.58 (C-8), 138.20 (C-22), 129.47 (C-23), 117.49 (C-7), 71.08 (C-3), 55.93 (C-14), 55.15 (C-17), 51.28 (C-24), 49.43 (C-9), 43.31 (C-13), 40.86 (C-20), 40.29 (C-5), 39.49 (C-12), 37.17 (C-1), 34.24 (C-16), 31.90 (C-25), 31.49 (C-2), 29.67 (C-6), 28.54 (C-16), 25.43 (C-28), 23.05 (C-11), 21.57 (C-15), 21.41 (C-21), 21.12 (C-26), 19.02 (C-27), 13.07 (C-19), 12.23 (C-29), 12.08 (C-18).

**OEWCP34:** 3α, 19α, 23, 24-tetrahydroxyurs-12-en-28-oic acid or Clethric acid, white amorphous powder, MF: $C_{30}H_{48}O_6$, m.p.: 283-285˚C, IR$\nu_{max}$ ATR ($cm^{-1}$): 3374, 2965, 2894, 1685, 1250; EIMS: m/z 504.60 (calculated for $[M+Na]^+$, 527.35);

$^1$H NMR ($CDCl_3$, 300MHz) δ (ppm): 5.32 (1H, brs, H-12), 4.10 (1H, s, H-3), 3.95 (1H, d, J = 11.1Hz, H-23), 3.77 (1H, d, J = 11.1Hz, H-23), 3.73 (1H, d, J = 11.1Hz, H-24), 3.62 (1H, d, J = 11.1Hz, H-24), 2.60 (1H, dt, J = Hz, H-16), 2.52 (1H, s,H-18), 1.38 (3H, s, H-27), 1.23 (3H, s, H-29), 0.97 (3H, s, H-26), 0.96 (3H, d, J = 8.1 Hz, H-30), 0.80 (3H, s, H-25), 0.73 (3H, s, H-).

$^{13}$C NMR ($CD_2Cl_2$, 75MHz) δ (ppm): 180.8 (C-28), 139.9(C-13), 127.9(C-12), 69.8 (C-3), 72.6 (C-19), 69.1 (C-23), 54.5 (C-18), 47.8 (C-9), 45.0 (C-5), 48.2 (C-17), 42.3 (C-20), 33.7 (C-1), 46.0 (C-4), 40.4 (C-8), 38.4 (C-22), 37.2 (C-10), 33.7 (C-7), 29.2 (C-15), 26.3 (C-21), 27.0 (C-29),26.3 (C-2), 26.9 (C-16), 24.5 (C-27), 24.2 (C-11), 19.1 (C-6), 17.1 (C-26), 15.6 (C-25), 64.5 (C-24).

**OESE55:** 3β, 19α, 23-trihydroxyurs-12-en-28-oic acid or Rotundic acid, white amorphous powder, MF: $C_{30}H_{48}O_5$, m.p.: 272-273°C, IR$\nu_{max}$ ATR ($cm^{-1}$): 3375, 2964, 2895, 1686, 1249; EIMS: m/z 488.37 (calculated for $[M+Na]^+$, 511.37);

$^1$H NMR (MeOD, 300MHz) δ (ppm): 5.30 (1H, brs, H-12), 3.64 (1H, dd, J = 12.0, 4.2Hz, H-3), 3.35 (1H, d, J = 10.8 Hz, H-23), 3.31 (1H, d, J = 10.8Hz, H-23),2.59 (1H, dt, J = 4.2, 9.0Hz, H-16), 2.51 (1H, s, H-18), 1.36 (3H, s, H-27), 1.21 (3H, s, H-29), 1.00 (3H, s, H-26), 0.95 (3H, d, J = 7.2Hz, H-30), 0.82 (3H, s, H-25), 0.73 (3H, s, H-24).

$^{13}$C NMR (MeOD, 75 MHz) δ (ppm): 180.9 (C-28), 138.6 (C-13), 128.0 (C-12), 72.6 (C-3), 72.2 (C-19), 66.1 (C-23), 53.8 (C-18), 48.7 (C-9), 48.2 (C-5), 47.1 (C-17), 41.7 (C-20), 41.2 (C-1), 39.6 (C-4), 38.1 (C-8), 37.6 (C-22), 36.5 (C-10), 32.3 (C-7), 29.3 (C-15), 28.2 (C-21), 25.9 (C-29), 25.7 (C-2), 25.2 (C-16), 23.5 (C-27), 23.3 (C-11), 17.9 (C-6), 16.1 (C-26), 14.9 (C-25), 11.3 (C-24).

**OESH146:** 3α, 6α, 19α-trihydroxyurs-12-en-28-oic acid-23-carboxylic acid methyl ester or elatumic acid, colourless powder, MF: $C_{31}H_{48}O_7$, m.p: 229.3 – 230.2˚C $[\alpha]^{20D}$: -5.6 (c 0.12, MeOH), UV ($CH_2CH_2$) ($\lambda_{max}$, log ε): 230 (3.27), 261 (3.28), 287 (2.91) nm. IR$\nu_{max}$ ATR ($cm^{-1}$): 2964, 1778, 1734, 1698, 1375, 1249; HRESIMS: m/z 555.329 (calculated. For $[M+Na]^+$, 555.3292);

$^1$H (MeOD, 300MHz) δ (ppm): 5.35 (1H, t, J = 3.7 Hz, H-12), 3.70 (1H, pseudo-t, J = 2.9 Hz), 3.68 (3H, s, H-OCH3), 2.59 (1H, dt,J = 4.2, 9.0Hz, H-16a), 2.54 (1H, s, H-18), 1.41 (3H, s, H-27), 1.22 (3H, s, H-29), 1.13 (3H, s, H-26), 0.96 (3H, d, J = 6.7 Hz, H-30), 1.40 (3H, s, H-25), 1.55 (3H, s, H-24).

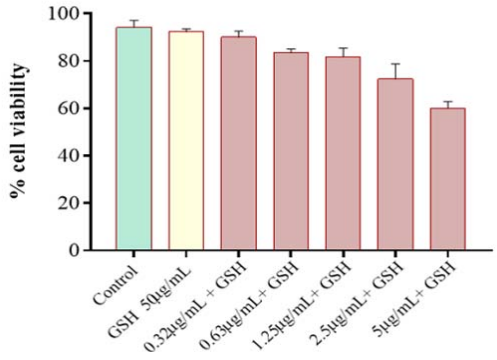



**Figure 7:** Effect of combining daunorubicin with reduced glutathione. Glutathione reduced the antiproliferative effects of daunorubicin. Error bars represent standard deviation from the mean. *p<0.05, **p<0.01, ***p<0.001.

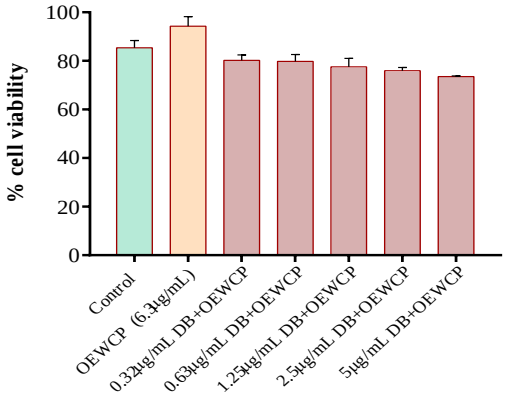



**Figure 8:** The effect of combining daunorubicin and OEWCP 34 on cell proliferation. Error bars represent standard deviation from the mean with n=2, *p<0.05, **p<0.01, ***p<0.001.

$^{13}$C NMR (MeOD, 75 MHz) δ (ppm): 182.5 (C-28), 139.5 (C-13), 129.7 (C-12), 75.6 (C-3), 73.6 (C-19), 178.9 (C-23), 53.3 (C-18), 48.8 (C-9), 45.9 (C-5), 48.8 (C-17), 43.1 (C-20), 35.4 (C-1), 53.3 (C-4), 40.8 (C-8), 39.1 (C-22), 37.5 (C-10), 41.4 (C-7), 29.6 (C-15), 27.3 (C-21), 27.0 (C-29), 26.0 (C-2), 26.7 (C-16), 25.1 (C-27), 24.5 (C-11), 71.4 (C-6), 18.8 (C-26), 17.3 (C-25), 20.0 (C-24).

## Cell viability assay

The Trypan blue dye exclusion assay was used in all the cell viability determinations carried out. Cells were incubated in replicates of 3 per treatment in 12-well plates. For cell counting, each sample from the 12-well plates was counted by taking 200µL of cells and adding 100µL Trypan blue 4% in a 1.5mL microtube. A cell count would then be conducted under a Celestron digital light microscope (Celestron, Los-Angeles, USA) using a haemocytometer whereby dead and live cell numbers were recorded. The percentage cell viability was calculated using the following formula:

% Cell viability = Number of live cells /Total number of cells × 100%. [18]

## Determination of the Effects of the pure compounds on mouse peritoneal cells

Cell count was performed using the Trypan blue dye exclusion assay to determine cell viability in a volume of cells that contained 1 × $10^5$cells/mL. Assays were done on 4 pure compounds namely, OEW 34, OEWCP 34, OESH 146, and OESE 55.

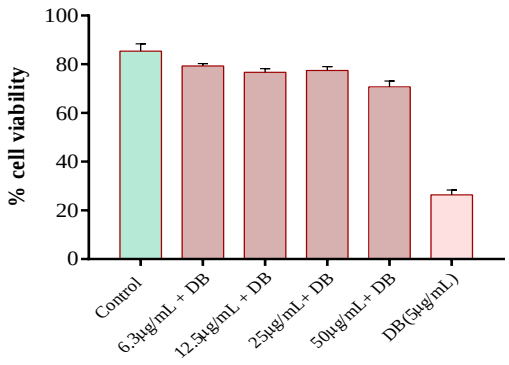



**Figure 9:** The effect of combining daunorubicin and OEWCP 34 on cell proliferation. Error bars represent standard deviation from the mean with n=2, *p<0.05, **p<0.01, ***p<0.001.

The effects of the pure compounds were determined in duplicate and triplicate in a 12-well plate. The total volume for each well was 3mL. Initially the cells were exposed to concentrations of 50, 25, 12.5, 6.3 and 0µg/mL of the pure compound. The US NCI compound screening program shows that for a compound, it is generally considered to have in vitro cytotoxic activity, if the IC50 value following incubation between 48 and 72h is less than 150$\mu$g/mL [19]. In this study, we reduced the cutoff point to 50µg/mL. This concentration of the pure compounds used is considered to be very low, according to the American National Cancer Institute, a value of 150µg/mL is considered promising when searching for activity in pure compounds [20]. The cells were incubated at 37°C in a humidified atmosphere with 5 % carbon dioxide. Cell counts were done every 72hours using the Trypan blue dye exclusion assay. Cell viability was determined as described before.

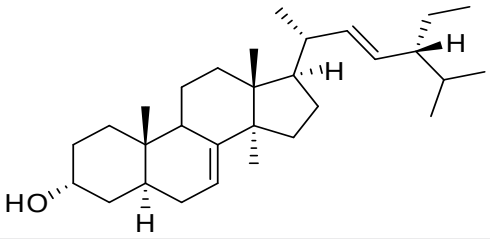
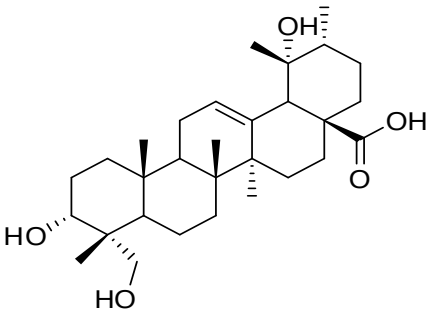
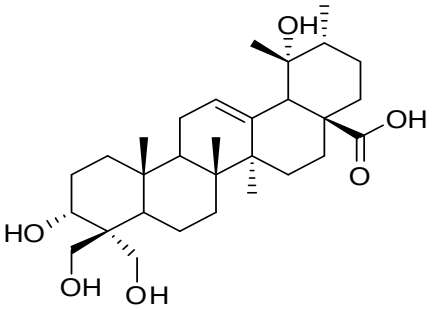
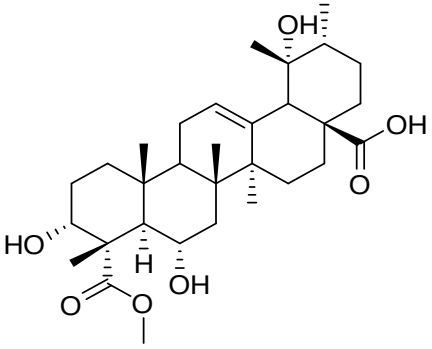
## Determination of the effects of combining OEWCP34 with reduced glutathione

Glutathione is an endogenous antioxidant and as such would possibly antagonise the pro-oxidant role of electrophilic compounds. In order to determine a possible mechanism of action of the compound, the effects exposing cells to the most potent compound and Reduced Glutathione (GSH) were determined. Cells were exposed to media, GSH (50 µg/mL), GSH (50µg/mL) + OEWCP 34 (50µg/mL ), GSH (50µg/mL) + OEWCP 34 (25µg/mL), GSH (50µg/mL) + OEWCP 34 (12.5µg/mL) and GSH (50µg/mL) + OEWCP 34 (6.3µg/mL). In addition, a positive control for GSH and daunorubicin was carried out, were a constant concentration of GSH (50µg/mL) was combined with daunorubicin concentrations of 5.0, 2.5, 1.25, 0.63 and 0.32µg/mL. The total volume for each well was 3mL. The cells were incubated at 37°C in a humidified atmosphere with 5% carbon dioxide. Cell counts were done every 12 hours using the Trypan blue dye exclusion assay. Cell viability was determined as described before.

## Determination of the effects of combining OEWCP 34 with an anticancer compound, daunorubicin

Cells were exposed to a constant concentration of anticancer compound, daunorubicin of 5µg/mL plus OEWCP 34 at varying

**Table 1:** Structure of some phytochemicals isolated from stem bark of *Omphalocarpum elatum.*

| Code | Structure | Name |
|---|---|---|
| OEW34 |  | spinasterol |
| OESE55 |  | 3*β*,19*α*,23-Tihydroxyurs-12-en-28-oic acid<br>or<br>Rotundic acid |
| OEWCP34 |  | 3*α*,19*α*,23,24-Tetrahydroxyurs-12-en-28-oic acid or<br>Clethric acid |
| OESH146 |  | 3α, 6α, 19α-trihydroxy-urs-12-en-28-oicacid-23-carboxylic acid methyl ester |

concentrations of 50, 25, 12.5 and 6.3µg/mL.

OEWCP 34 at 6.3µg/mL was found to have a high cell viability percentage alone, thus, was combined with varying concentrations of daunorubicin, 5.0, 2.5, 1.25, 0.63 and 0.32µg/mL. Cell counts were done every 72hours using the Trypan blue dye exclusion assay. Cell viability was determined as described before.

### Statistical analyses

One-Way Analysis Of Variance Test (ANOVA) with Dunnett's Multiple Comparison Post Test was used to analyse the results. All columns of treatments were compared to the control. The values with a p-value < 0.05 or less were considered statistically significant. Graphical and Statistical analyses were carried out using Graphpad Prism 5® Software (Version 5.0, Graph pad Software Inc, San Diego, USA).

## Results

Effects of the Phytosterols OEW34, OESH146, OESE55, OEWCP 34 and daunorubicin on mouse peritoneal cells.

The effects of OEW34 on mouse peritoneal cells were determined by assessing for cell viability. The percentage cell viability increased with an increase in concentration of OEW34. At the lowest concentration of 6.3µg/mL, the cell viability was 81.75% and increased to 93.93% at 50µg/mL which was the highest concentration. The positive control which had daunorubicin had 31.82% cell viability and the negative control which contained cells only had 93.38% as illustrated in (Figure 1). The effects of OESH146 on mouse peritoneal

cells were determined by assessing for cell viability. The cell viability increased from 77.60% at the lowest concentration of 6.3µg/mL to 95.20% at 12.5µg/mL and decreased slightly to 82.79 % cell viability at 50µg/mL. The decline in cell viability was 12.5 % comparing it with the negative control. The positive control which was daunorubicin had 18.12% cell viability and the negative control which contained unexposed cells had 88.75% as shown in (Figure 2).

The effects of OESE55 on mouse peritoneal cells were determined by assessing for cell viability. The percentage cell viability increased with an increase in concentration of compound. At the lowest concentration of 6.3µg/mL, the cell viability was 70.62% and increased to 86.46% at 50µg/mL which was the highest concentration. The positive control which had daunorubicin (10µg/mL) had 27.25% cell viability and the negative control which contained unexposed cells had 84.60% as illustrated in (Figure 3). The effects of OEWCP 34 on mouse peritoneal cells were determined by assessing for cell viability. There was no definite trend on the cell proliferation. The cell viability was 94.32% at 6.3µg/mL and decreased to 71.88% at 12.5µg/mL and increased again to 87.75% at 25µg/mL then decreased slightly to 76.57% at 50µg/mL. OEWCP 34 exhibited a decline in cell viability by 25% comparing it with the negative control. This was found to be the most potent compound in terms of decreasing the cell proliferation. The positive control which had daunorubicin (10µg/mL) had 10.56% cell viability and the negative control which contained cells that were in medium had 88.95% as illustrated in (Figure 4). As the concentration of the anticancer drug, daunorubicin increased, a decrease in cell proliferation was noted for instance from a concentration of 0.32µg/mL the cell viability decreased from 56.31 % to 26.39% at a concentration of 5µg/mL as illustrated in (Figure 5).

### Effect of combining OEWCP 34 with reduced glutathione on cell proliferation

OEWCP 34 was the most potent compound in reducing cell viability since it exhibited a decrease in cell viability of 25%. It was, therefore, combined with reduced GSH and results obtained showed that the percentage cell viability increased. After exposure to GSH alone, cell viability was high at 92.32%. As expected, cells exposed to GSH combined with OEWCP 34 increased in proliferation for example at 12.5µg/mL of OEWCP 34 (Figure 4), viability was 85.42% whilst at 12.5µg/mL + GSH 50µg/mL it was 89%. A combination of OEWCP, 6.3µg/mL + GSH 50 µg/mL produced the highest cell viability of 92.71 % and OEWCP 50µg/mL + GSH 50µg/mL the lowest cell viability of 86.95% (Figure 6).

### Effect of combining daunorubicin with reduced glutathione

Exposure of cells to a combination of GSH and daunorubicin resulted in an increase in percentage cell viabilities as compared to cells exposed to daunorubicin that had an average cell viability of 30 %. It was also found that the cell viability decreased with an increase in concentration of daunorubicin. Cells exposed to GSH had a cell viability of 92.32%. A concentration of daunorubicin at 0.32µg/mL when combined with GSH, produced a cell viability of 90.07 %. Exposure of cells to a combination of daunorubicin at 5µg/mL with glutathione resulted in a cell viability of 33% (Figure 7).

### Effect of combining daunorubicin and OEWCP 34 on cell proliferation

When cells were exposed to a concentration of 6.3µg/mL of OEWCP34 a percentage cell viability of 94.32% was noted. When OEWCP34 was combined with daunorubicin, the cell viability decreased slightly. At a concentration of daunorubicin of 5µg/mL plus OEWCP34 the viability produced was 73.51% showing a decline in cell count. At 0.32 µg/mL of daunorubicin, cell viability was 80.20% reflecting a decrease by 14.12% (Figure 8).

### Effect of combining daunorubicin and OEWCP 34 on cell proliferation

There was a decline in cell viability after combining anticancer drug at a high concentration of 5µg/mL and the test compound, OEWCP34. At 6.3µg/mL of OEWCP34 plus 5µg/mL of daunorubicin it cell viability was found to be 79.29% and at the highest concentration of 50µg/mL of OEWCP34 plus daunorubicin the viability was reduced to 70.69% (Figure 9).

## Discussion

As the global use of herbal medicinal products continues to grow and new products are introduced into the market, public health issues, and concerns surrounding their safety are increasingly recognized [17]. Although herbal medicines have promising potential and are widely used, many of them remain untested and their use is also unmonitored [18]. The scientific evaluation of safety and efficacy of herbal products and medicinal preparation is, thus, of vital importance from both medicinal and economic perspectives [21]. Raditional medicines are not necessarily safe simply because they are "natural" and have a long history of use [22]. As such, an evaluation of toxicity of plant-derived phytochemicals is of importance in the use of plants as medicine.

The toxicity profile of phytochemicals isolated from *O. elatum* was determined by assessing for cell viability using the trypan blue dye exclusion assay. Some of the phytosterols extracted from *O. elatum* namely, OEW34, OESE131, OESE55 and EC149 generally showed an increase in cell proliferation as the concentration of compound was increased. However, two compounds OEWCP34 and OESH146 deviated from the pattern of increasing cell growth as concentration increased. OEWCP34 was the most potent compound in reducing cell viability but was considered not to be toxic because it has been shown that, for a compound to be stated as toxic it should at least exhibit 30% decrease in cell viability [23]. There compounds OEWCP34 and OESH146 increased cell viabilities.

The increase in cell proliferation may be due to the phytosterols that induce or promote optimum conditions for cell growth, thus, high percentage cell viability [24]. The potency of increasing cell proliferation increased with increase in concentration of compounds. There is evidence in the literature that sterols might be one of the necessary factors for cell growth [25]. Bouic [26] discovered three kinds of sterols; campesterol, stigmasterol and β-sitosterol in Ginkgo cells which are partially similar to the phytosterols from *O. elatum* in terms of the chemical structure. Addition of β-sitosterol restored the incorporation of medium components and provided an optimum condition for cell growth hence phytosterols contributed to an increase in proliferation of cells. It has also been shown that sterols modulate the functions of the T cells both *in vitro* and *in vivo* by enhancing their cellular division and their secretion of these important regulatory soluble factors called Lymphokines (IL2 and IFN) [27]. The sterols isolated from *O. elatum* promoted cell division

of mouse peritoneal cells and thus, high percentage viabilities noted.

From previous studies, it was found that sterols with minor modifications of the side chain such as Campesterol, β-Sitosterol, and desmosterol supported long-term growth of mutant cells [28]. For instance, compound OEW34 (spinasterol) closely resembles the chemical structure of sterols mentioned above that were shown to support proliferation of cells. Isolated compounds from *O. elatum* may be working via the same mechanism of stimulating cell growth as reported about *Calophyllum Brasiliense* [29]. In the evaluation of the proliferative activity of extracts from six medicinal plants in murine spleen cells, *Calophyllum Brasiliense* stimulated T lymphocyte proliferation, thereby activating the cellular immune response [30].

OEWCP34 and OESH146 caused a decline in cell viability by 25% and 12.6% respectively. The difference of these two compounds from the rest of the other compounds maybe due to oxidation to Phytosterol oxides that probably took place *in vitro*. Phytosterol oxides have been documented to be slightly cytotoxic *in vitro* [30-31]. One of the reported cytotoxic effects included decreased cell viability [31] and this was also was noted in this project. Also, another study reported the same information pertaining phytosterol oxides exhibiting some toxicity, however the effects were less severe [32] hence, OEWCP34 and OESH146 oxidized forms caused that decrease in cell viability. The oxidation may be due to the hydroxyl groups attached to the rings in close proximity that pose a large electron withdrawing effect, thus, inducing a negative inductive effect. This effect results in a partially positive charged on the ring, therefore, making the compounds more susceptible to oxidation. The other compounds e.g. OEW34, may not undergo oxidation because of the less number of hydroxyl groups that are spatially arranged and, thus, leading them to less likely undergo oxidation [33].

A combination of OEWCP34 and reduced Glutathione (GSH) resulted in high percentage cell viability compared to OEWCP34 alone. GSH protects cells from oxidative stress and contribute a favorable redox environment both inside and outside the cell [34]. Glutathione has an effect on cell proliferation process because different studies have shown that glutathione is involved in cells proliferation of such cells as human fibroblast cells, lymphocyte, hepatocytes, mouse bone marrow cells and intestinal epithelial cells [35]. When GSH was combined with OEWCP34, there was an additive effect for cell proliferation. Since GSH is a powerful intracellular antioxidant, it was expected that if the OEWCP34 was combined with GSH cell proliferation would increase.

GSH is a scavenger of free radicals such as Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) and in its presence, there is no accumulation of the oxidants [36]. Therefore, it is possible that GSH quenched the oxidants that accumulate in the cell resulting in a high cell proliferation that was observed.

Daunorubicin is an Anthracycline antibiotic which damages DNA by intercalating between base pairs resulting in uncoiling of the helix, ultimately inhibiting DNA synthesis and DNA-dependent RNA synthesis [37]. It may also act by inhibiting polymerase activity, affecting regulation of gene expression and generating free radicals [34]. The combination of daunorubicin and GSH resulted in increased percentage cell viability and this was expected as previous studies have shown that cells become less sensitive to anticancer agents in the presence of glutathione [38]. Alternatively, the GSH could be reacting directly with the daunorubicin as this is a standard reaction of quinones and reduced glutathione [39], consequently increasing cell viability. Reactive oxygen species from daunorubicin could have been quenched by GSH, thus, producing high cell proliferation [34].

The antiproliferative effect of daunorubicin was dose-dependent. However, when OEWCP34 was combined with daunorubicin, the antiproliferative effect of the anticancer agent decreased. This may be due to the compound OEWCP34 blocking or antagonising the effects of daunorubicin because it has been shown that the preventive effects of natural products e.g. phytosterols, are primarily due to their antioxidant and free radical-scavenging activities [40].

## Conclusion

Phytosterols from *O. elatum* increased cell proliferation of mouse peritoneal. OEWCP34 was the most potent compound in reducing cell viability but was not cytotoxic. Glutathione provided an additive effect to OEWCP34 in terms of cell proliferation. OEWCP34 antagonized the effects of the anticancer drug, daunorubicin, thus, promoting cell viability. It can be concluded that phytosterols isolated from *O. elatum* are not toxic to mammalian cells and they can be enhance cell proliferation of mouse peritoneal cells in the presence of reduced glutathione. These results support the use of the plant in ethnomedicine.

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## Availability of Data and Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Authors' Contributions

TN, CDF and SS conducted all the experimental studies and data analysis. SM conceptualised, designed and directed the study. TN, CDF, SS and SM finalized the manuscript. All authors read and approved the final manuscript.

## Ethics Approval and Consent to Participate

The use of animal cell lines in this study was approved by the Joint Parirenyatwa Group of Hospitals and College of Health Sciences Research Ethics Committee (JREC/327/14, Harare, Zimbabwe).

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