

# Cytotoxicity of Lupeol from the Stem Bark of *Zanthoxylum gillettii* against Multi-factorial Drug Resistant Cancer Cell Lines

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## Abstract

**Background:** Drug resistance is one of the major hurdles in the treatment of cancer. Rather than single factors, drug resistance is caused by multiple mechanisms making it even more complex. In the present study, the cytotoxicity of six compounds isolated from *Zanthoxylum gillettii* were screened against drug-sensitive leukemia CCRF-CEM cells. The most cytotoxic compound, lupeol (1) was further tested in a panel of nine human cancer cells, including multi-drug resistant phenotypes with different mechanisms and one normal cell line.

**Methods:** The structures of isolated compounds were characterized using spectroscopic techniques and comparison of their thin layer chromatography (TLC) profiles with authentic samples. The cytotoxicity tests were carried out using the resazurin reduction assay.

**Results:** In preliminary tests, all compounds except lupeol (1) were inactive at a fixed concentration of 40 µg/mL against drug-sensitive leukemia CCRF-CEM cells, as they displayed inhibition of <70% of the cell population. The IC<sub>50</sub> values for lupeol (1) and doxorubicin were determined against a panel of cancer cell lines with drug-sensitive, multidrug-resistant (MDR) phenotypes and normal AML12 hepatocytes. Compound 1 displayed considerable cytotoxicity with IC<sub>50</sub> values ranging from 13.60 µM (towards glioblastoma U87MG.ΔEGFR cells) to 69.39 µM (towards multidrug-resistant CEM/ADR5000 leukemia cells) and from 0.02 µM (towards CCRF-CEM cells) to 66.83 µM (towards the multidrug-resistant CEM/ADR5000 leukemia cells) for doxorubicin. Compound 1 showed higher toxicity to HepG2 liver carcinoma cells than to normal AML12 hepatocytes, indicating some degree of selectivity to tumor cells relative to normal cells. It is important to note that 1 exhibited collateral sensitivity against 3/5 cases, thus displaying its capacity to inhibit drug resistance cells through different mechanisms.

**Conclusion:** Lupeol (1) displayed interesting cytotoxic potencies against a panel of drug-sensitive and MDR tumor cells *via* multiple mechanisms with marginal or no effect to normal cells at similar doses.

**Keywords:** Lupeol; cytotoxicity; collateral sensitivity; multidrug resistance.

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## Background

Multidrug resistance (MDR) is characterized with expulsion of chemically and functionally diverse drugs out of the tumor cells mainly by drug efflux pumps of the ATP-binding cassette [1]. Drug resistance is not caused by one or a few mechanisms but by many factors making it even more complex [2]. The multifactorial nature of drug resistance may require a multi-target approach, acting not only on one but several cellular targets [1, 3]. The compound investigated in the present study, lupeol (1) is a diet-based widespread natural pentacyclic triterpenoid of the lupane-type saponins present in fruits, vegetables and medicinal plants including plants from the genus *Zanthoxylum*. In our search for biologically active compounds from plants of this genus, including; *Z. chalybeum*, *Z. paracanthum* and *Z. gilletii*, lupeol (1), sesamin (5) and fagaramide (6) were isolated in substantial amounts in comparison with other compounds. As previously reported, this compound bears a wide range of pharmacological properties against human diseases, including; anticancer, antioxidant, anti-inflammatory, anti-arthritic, anti-mutagenic, and antimalarial activity in *in vitro* and *in vivo* systems among others [4-9]. Recently, several *in vivo* and *in vitro* studies have been carried out to evaluate the anticancer potential of lupeol (1) and to determine its mode of action in different cells. From these studies, lupeol (1) exhibited *in vitro* and *in vivo* anticancer potencies against various cancer cells including human breast cancer, prostate, colorectal, skin, liver and gastric cancer and leukemia, melanoma, and oral cavity cancer. Furthermore, the selectivity of lupeol (1) towards affected cells only was very high [10-15], making it a suitable candidate for further development into a potential anticancer prototype.

A number of current studies that are aimed at determining the modes of action of natural products usually target proteins that are involved in inhibiting apoptosis in humans. Studies on lupeol (1) have focused mainly on its safety profile in addition to evaluating its potential as an inhibitor of various resistance proteins especially the apoptosis regulators. It is evident from these studies that the Bcl-2 protein plays a major role in preventing apoptosis, and hence it has been linked to chemotherapy resistance observed in melanoma [7, 16]. However, lupeol (1) has been shown to initiate significant reduction in the expression level of this important protein in metastatic melanoma cells [17]. Furthermore, Saleem et al [7] established that lupeol (1) ameliorated the inefficiency of melanoma cells to undergo apoptosis and inhibit the human melanoma growth *in vitro* and *in vivo*. The compounds used for

the present studies were isolated from the stem bark of *Zanthoxylum gilletii* (De Wild) Waterman [18].

*Zanthoxylum gilletii* (De Wild) Waterman (syn. *Fagara macrophylla* (Oliv.) Eng. (Rutaceae), also known as the African satinwood is an indigenous deciduous tree that grows up to 35 m in height. The trunk and branches of this plant consists of characteristic aculeate bosses [19]. It belongs to the family Rutaceae and the genus *Zanthoxylum*. The genus *Zanthoxylum* is distributed worldwide from the tropics to the temperate zones and is represented by seven species in Kenya, including; *Z. holstianum* (Engl.) Waterman, *Z. usamarense* (Engl.) Kokwaro, *Z. chalybeum* (Engl.) var. *chalybeum*, *Z. gilletii* (De wild) P.G. Waterman, *Z. mildbraedii* (Engl.) P.G. Waterman, *Z. paracantum* (Mildbr) Kokwaro and *Z. rubescens* Planch. Ex Hook.f [19]. *Z. gilletii* is extensively used in Kenyan system of traditional medicine especially by habitants of the locations where it grows; that is, along the Coastal and Western parts of Kenya to manage coughs, stomachic, analgesic and antipyretic, antibiotic [20]. Previous phytochemical studies on a number of *Zanthoxylum* species revealed similar phytochemical profiles with chemotaxonomic importance to the genus. The diversity of alkaloids included; benzophenanthridine, protoberberine, bishordeninyl, aporphine [18], and also amides, coumarins, lignans, flavonoids, sterols and volatile oils have been characterized from these species as the common secondary metabolites [18]. These compounds are most probably responsible for the biological activities exhibited by plants in this genus, which include; larvicidal, analgesics, anthelmintic, anti-viral, antioxidant anti-fungal, anti-biotic, anti-inflammatory, anti-plasmodial and cytotoxicity [18, 21-24].

The assumption in the present investigation was that lupeol (1), which has been previously shown to exert promising anticancer potencies is capable of inhibiting drug-resistant cells with more than one different mechanisms.

Therefore, the present investigation was aimed at determining the anti-proliferative potencies of lupeol (1) isolated from *Z. gilletii* against a panel of ten cell lines. The cells used in the present study included different drug resistance phenotypes: cells specifically over-expressing the ABC transporter P-glycoprotein (ABCB1/MDR1) or BCRP (ABCG2) exhibiting multidrug resistance phenotypes, a p53 knockout cell line (HCT116 p53<sup>-/-</sup>) as a model for mutational loss-of-function of this tumor suppressor gene and U87MG cells stably transfected with a deletion-mutation-activated EGFR gene as model for drug resistance by oncogenes. These cell lines were compared to their corresponding wild-type counterparts. All these cell models are known to exert resistance to anti-cancer drugs [1, 25-28].

To the best of our knowledge there is scanty or no information on collateral sensitivity of lupeol (**1**) against resistant cancer cells. Previous studies have focused on evaluating the potential of lupeol (**1**) to inhibit expression of proteins responsible for apoptosis.

## Methods

### *Reagents and compounds*

The compounds used in the present investigation included; lupeol (**1**) [29]; dihydrochelerythrine (**2**), 8-acetyldihydrochelerythrine (**3**), norchelerythrine (**4**), sesamin (**5**) [30-32] and fagaramide (**6**) [18, 33]. The compounds were re-isolated from the stem bark of *Z. gillettii* (50% MeOH in CH<sub>2</sub>Cl<sub>2</sub>) and characterized using spectroscopic techniques (such as NMR and MS) coupled with comparison of their TLC profile with authentic samples from the Natural Products laboratory of the Chemistry Department, University of Nairobi, Kenya (Figure 1).

Doxorubicin 98.0% was provided by the University Pharmacy of the Johannes Gutenberg University (Mainz, Germany) and dissolved in Phosphate Buffer Saline (PBS; Invitrogen, Germany) at a concentration of 10 mM. Geneticin >98% was purchased from Sigma-Aldrich (Taufkirchen, Germany) and stored at a stock concentration of 72.18 mM.

### *Cell cultures*

The cell lines used in the current work, their origins and their treatments were previously reported. A panel of drug-sensitive and multidrug-resistant cell lines was used in the current experiments. These included drug-sensitive CCRF-CEM and multidrug-resistant P-glycoprotein-over-expressing CEM/ADR5000 leukemia cells [34-36], MDA-MB-231-pcDNA3 breast cancer cells and its resistant and transfectant subline MDA-MB-231-BCRP clone 23 [25], HCT116 (p53+/+) colon cancer cells and its knockout clone HCT116 (p53-/-), U87MG glioblastoma cells and its resistant and transfected subline U87MG.ΔEGFR [37-39].

### *Resazurin reduction assay*

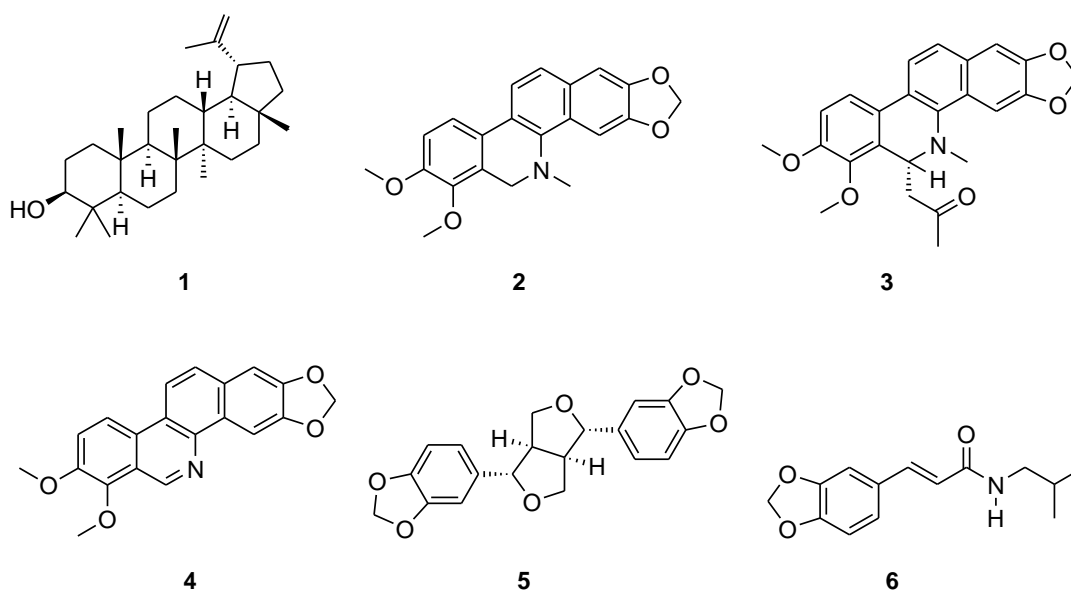
The cytotoxicity testing was performed by using the resazurin reduction assay as previously described [37-42]. Resazurin reduction assay [40] was

performed to assess the cytotoxicity of the test samples toward various sensitive and drug-resistant cancer cell lines. The assay is based on reduction of the indicator dye, resazurin, to the highly fluorescent resorufin by viable cells. Non-viable cells rapidly lose the metabolic capacity to reduce resazurin and thus produce no fluorescent signal. Briefly, adherent cells were detached by treatment with 0.25% trypsin/EDTA (Invitrogen, Darmstadt, Germany). An aliquot of  $1 \times 10^4$  cells was placed in each well of a 96-well cell culture plate (Thermo Scientific, Langensfeld, Germany) in a total volume of 200  $\mu$ L. Cells were allowed to attach overnight and then were treated with different concentrations of the studied sample. For suspension cells, aliquots of  $2 \times 10^4$  cells per well were seeded in 96-well-plates in a total volume of 100  $\mu$ L. The studied sample was immediately added in varying concentrations in an additional 100  $\mu$ L of culture medium to obtain a total volume of 200  $\mu$ L/well. After 24 h or 48 h, 20  $\mu$ L resazurin (Sigma-Aldrich) 0.01% w/v in double-distilled water (ddH<sub>2</sub>O) was added to each well and the plates were incubated at 37 °C for 4 h. Fluorescence was measured on an Infinite M2000 Pro™ plate reader (Tecan, Crailsheim, Germany) using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Each assay was done at least two times, with six replicate each. The viability was evaluated based on a comparison with untreated cells. IC<sub>50</sub> values represent the sample's concentrations required to inhibit 50% of cell proliferation and were calculated from a calibration curve by linear regression using Microsoft Excel.

## Results and discussion

*Isolated compounds:* The stem bark of *Z. gillettii* (50% MeOH in CH<sub>2</sub>Cl<sub>2</sub>) yielded six compounds which were characterized as: one triterpenoid, lupeol (**1**) [29]; three alkaloids with benzophenanthridine skeleton, dihydrochelerythrine (**2**), 8-acetyldihydrochelerythrine (**3**), norchelerythrine (**4**), one lignan, sesamin (**5**) [30-32] and one amide, fagaramide (**6**) [18, 33].

*Cytotoxicity of the studied samples:* In the current study, the cytotoxicity of six compounds characterized from *Zanthoxylum gillettii* was determined initially at 40  $\mu$ g/mL (Table 1). According to the established criteria, an IC<sub>50</sub> value threshold of 4  $\mu$ g/mL or 10  $\mu$ M [43-44] after 48 and 72 h incubations has been set to identify good cytotoxic compounds.



**Figure 1.** Compounds isolated from the stem bark of *Zanthoxylum gillettii*

The activity is moderate, if  $10 < IC_{50} < 50 \mu M$  [45]. All compounds except lupeol (**1**) were inactive at the tested concentration as they inhibition  $< 70\%$  of the cell population. These results concur with previous published studies, which provide evidence of the anticancer potency of **1**. At the initial concentration, lupeol (**1**) was more active than the standard drug, doxorubicin with cell inhibition rates of up to 94.16% relative to doxorubicin, which inhibited the growth of drug sensitive CCRF-CEM leukemia cells by 85.5%. The  $IC_{50}$  values of lupeol (**1**) was further determined on a panel of cancer cell lines with drug-sensitive and -resistant phenotypes and compared with doxorubicin as control drug (Table 1). In addition to the normal drug sensitive CCRF-CEM and multidrug-resistant P-glycoprotein (ABCB1/MDR1)-over-expressing CEM/ADR5000 cells, compound **1** was tested against MDA-MB-231 cells over-expressing breast cancer resistance protein (ABCG2/BCRP), HCT116 p53 knockout and wild type cells, and transfectant U87MG cells harboring a mutation-activated EGFR gene ( $\Delta EGFR$ ) as examples for resistance inducing ABC-transporters, tumor suppressors and oncogenes. To compare carcinoma cells with normal cells, **1**, was tested against HepG2 liver cancer cells and AML12 normal hepatocytes [1].

Compound **1** displayed considerable cytotoxicity as expected with  $IC_{50}$  values ranging from  $13.60 \pm 0.87 \mu M$  (towards glioblastoma U87MG. $\Delta EGFR$  cells) to  $69.39 \pm 7.43 \mu M$  (towards drug-sensitive CCRF-CEM leukemia cells) and from  $0.02 \mu M$  (towards CCRF-CEM cells) to  $66.83 \pm 2.29 \mu M$  (towards the drug-resistant CEM/ADR5000 leukemia) for doxorubicin as reference drug. It is important to note that collateral sensitivity (or

hypersensitivity: higher toxicity to drug resistant than sensitive cells with a degree of resistance of below 1) was observed for lupeol (**1**) in 3/5 cases, including against drug-resistant epidermal growth factor receptor-transfected US7MG $\Delta EGFR$  with a degree of resistance being only 0.66-fold; against drug-resistant human breast adenocarcinoma MDAMB-232-BCRP with a degree of resistance of 0.8-fold and finally against drug-resistant colon carcinoma HCT116 (p53<sup>+/+</sup>) with a degree of resistance of 0.88-fold compared to the drug-sensitive counterparts, thus displaying its capacity to inhibit drug resistance cells through different mechanisms. However, cross resistance (higher toxicity to drug sensitive than resistance cells with a degree of resistance of above 1) was observed with lupeol (**1**) in 2/5 cases, including against multidrug-resistant P-glycoprotein (ABCB1/MDR1)-over-expressing CEM/ADR5000 cells with a degree of resistant of 4.39-fold and against the AML12 normal hepatocytes with a degree of resistance of 1.45-fold with reference to the drug-sensitive counterparts. Interestingly, lupeol (**1**) showed higher toxicity to HepG2 liver carcinoma cells than to normal AML12 hepatocytes, indicating some degree of selectivity of lupeol (**1**) to tumor cells relative to the normal cells. This fact supports earlier studies on lupeol (**1**), which showed high selectivity on numerous cancer cells in comparison to normal cells [15]. This investigation is substantial as the current research efforts for novel anti-cancer agents focused on natural diet-based agents with preferential elimination of drug-resistant malignant cancer cells with marginal or no effect to normal cells at similar doses [7].

**Table 1:** Cytotoxicity of lupeol (**1**) from *Zanthoxylum gillettii* and doxorubicin towards sensitive and multi-drug resistant cancer cells as well as normal cells as determined by the resazurin assay

Cell lines	<b>1</b>		Doxorubicin	
	IC <sub>50</sub> (μM)	Degrees of resistance	IC <sub>50</sub> (μM)	Degrees of resistance
CCRF-CEM	15.82 ± 1.27		0.02 ± 0.00	
CEM/ADR5000	69.39 ± 7.43	4.39	66.83 ± 2.2	3341
MDA-MB-231-pcDNA	27.96 ± 4.71		0.07 ± 0.00	
MDA-MB-231-BCRP	22.36 ± 1.80	0.80	0.43 ± 0.10	6.14
HCT116 (p53+/+)	22.31 ± 1.50		0.26 ± 0.01	
HCT116 (p53-/-)	19.64 ± 2.09	0.88	0.97 ± 0.02	3.73
U87MG	20.58 ± 1.59		0.14 ± 0.01	
U87MG.ΔEGFR	13.60 ± 0.87	0.66	0.53 ± 0.08	3.79
HepG2	33.53 ± 2.30		2.15 ± 0.03	
AML12	48.58 ± 4.15	1.45	0.48 ± 0.01	0.22

The degree of resistance was determined as the ratio of IC<sub>50</sub> value in the resistant divided by the IC<sub>50</sub> in the sensitive cell line; CEM/ADR5000, MDA-MB-231-BCRP, HCT116 (p53-/-) U87MG.ΔEGFR and ML12 were used as the corresponding resistant counterpart for CCRFCEM, MDA-MB-231-pcDNA, HCT116 (p53+/+), U87MG and HepG2 respectively; **1**: lupeol

Furthermore, all drug-resistant cell lines exhibited much lower degrees of resistance to lupeol (**1**) as compared to doxorubicin suggesting the suitability of this compound as drug candidate for the management of drug-resistant cancer cells.

## Conclusions

Lupeol (**1**) displayed interesting anticancer potencies against a panel of drug-sensitive and MDR cells via multiple mechanisms with marginal or no effect to normal cells at similar doses. It is important to extend these studies to structure modification of lupeol (**1**) towards improving its cytotoxic activities and also determination of the mode of action of lupeol (**1**) in different drug-resistant cancer cell lines.

## Authors' Contribution

HK and MM carried out the isolation experiment; HON, MM V-AN-N, VM, EO, MH contributed to identification of the isolated compounds. LKO and VK wrote and edited the manuscript. LKO, TE and VK designed the experiments; ATM carried out the bioassay experiment. All authors read the manuscript and approved the final version.

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## Conflict of interest

The authors have no competing interest.

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