

# Antiproliferative Activity of Secondary Metabolites from *Zanthoxylum zanthoxyloides* Lam: *In vitro* and *in silico* Studies

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## ABSTRACT

**Background:** Plant derived compounds have provided promising leads in search for safer anticancer chemotherapies. *Zanthoxylum zanthoxyloides* is a common medicinal plant in Uganda whose bioactive composition has not been fully elucidated. The aim of this study was to evaluate the *in vitro* antiproliferative potential of compounds isolated from *Zanthoxylum zanthoxyloides* and their probable *in silico* anticancer mechanisms of action.

**Methods:** Column chromatography was used to isolate compounds from MeOH:CH<sub>2</sub>Cl<sub>2</sub> (1:1) extract of the stem bark extract of *Zanthoxylum zanthoxyloides*. The structures of the isolated compounds were elucidated by NMR and MS analyses. MTT assay was used to measure cell viability. Using *in silico* docking, the interaction of the compounds with key target proteins in the p53 pathway was determined. **Results:** From the root bark of this plant five compounds were isolated, namely; dihydrochelerythrine (1), skimmianine (2), tridecan-2-one (3), sesamin (4) and hesperidin (5). Dihydrochelerythrine (1) inhibited proliferation of liver cancer (HCC) cells (IC<sub>50</sub> 21.2), breast cancer (BT549) cells, (IC<sub>50</sub> 21.2 μM). Similarly, sesamin (4) exhibited moderate inhibitory activity against BT549 cancer cells (IC<sub>50</sub> 47.6 μM). Hesperidin (5) showed low inhibitory activity against A549 and HEp<sub>2</sub> (Larynx) cells but was significantly toxic to normal liver and lung cells.

*In silico* docking studies showed that all the compounds strongly bind to cyclin-dependent kinases (CDK2 and CDK6) and weakly bind to caspases 3 and 8 suggesting that they inhibit cancer cells by inducing cell cycle arrest and apoptosis. **Conclusion:** This study indicates that the compounds isolated from *Z. zanthoxyloides* hold promise as potential leads against cancer. Due to high toxicity of compound 5 against normal lung and liver cells, it deserves further toxicity investigations to assess its safety before *in vivo* trials.

**Key words:** Anticancer, *in vitro*, *in silico*, p53 Pathway, Rutaceae, *Zanthoxylum zanthoxyloides*.

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## INTRODUCTION

Cancer is a deadly disease, characterized by the uncontrolled multiplication of body cells. International Agency for Research on Cancer (IARC) statistics approximated 18.1 million new cases of cancer and 9.6 million deaths in 2018. Cancer cases are projected to rise from 18.1 million in 2018 to 29.5 million in 2040. The most common cancers diagnosed among men and women are lung, prostate, breast, colorectal, liver and cervical cancer.<sup>1</sup> Chemotherapy, surgery and radiotherapy are the standard techniques in cancer treatment and management.<sup>2</sup> All these conventional therapies are expensive to the common man and often laden with major side effects. In many cases, recrudescence occurs due to aggressive metastasis leading to resistance to chemo- and radiotherapies.<sup>2</sup> In cases where surgery has been used to remove cancerous tumors, only cancer follicles are removed leaving behind free cancer cells which are often the origin of relapse.<sup>3</sup>

Medicinal plants have shown to be potential sources of alternative drugs for cancer treatment and management. The early discovery of alkaloids such as vinblastine and vincristine from the Madagascan plant *Catharanthus roseus* captivated the search for plant-derived compounds for cancer treatment and management. To date, several natural products and their derivatives have successfully been used in cancer chemotherapy.<sup>4</sup> Thus, this study sought to determine the anticancer potential of secondary metabolites from *Zanthoxylum zanthoxyloides* Lam (Family Rutaceae).

The genus *Zanthoxylum* consists of over 200 species widely distributed along the tropics with 35 species found in tropical Africa.<sup>5,6</sup> Among the

different *Zanthoxylum* species, *Z. zanthoxyloides* is the most common species in Uganda, where a decoction from the root-bark is used traditionally to treat elephantiasis, toothache, sexual impotence, gonorrhoea, malaria, dysmenorrhoea and abdominal pain.<sup>7</sup> Elsewhere in Cameroon, extracts from different parts of *Z. zanthoxyloides* are indicated in the treatment of urinary tract infections, dysentery and sickle cell anemia.<sup>8</sup> Previous reports indicate the isolation of divanylloylquinic acids, amides, acridone, fluoroquinolone and benzophenanthridine alkaloids from the roots and fruits of *Z. zanthoxyloides*.<sup>8-10</sup> Despite the presence of phytochemical reports on the genus *Zanthoxylum*, investigation of the anticancer effects of compounds from *Z. zanthoxyloides* is limited. Where biological activities have been reported, they relate to the activity of crude extracts and, in most cases the activities have been tested on either a fewer number of cancer cell lines or other diseases.<sup>9-12</sup> In this study, therefore, we report the secondary metabolites isolated from the root bark of *Z. zanthoxyloides*, some of which show good antiproliferative activity against a panel of cancer cell lines. In a bid to understand the anticancer molecular mechanism, the isolated compounds were evaluated against target proteins/genes in the p53 pathway through *in silico* docking studies.

Protein 53 (p53) is a tumor suppressor that functions to eliminate and inhibit the multiplication of abnormal cells through induction of apoptosis, cell cycle arrest and senescence.<sup>13,14</sup> Normally, p53 is inactivated in cancerous cells as a means of evading apoptosis.<sup>15,16</sup> Studies have shown

that the p53 pathway is activated in the presence of stress factors in cells such as UV radiation, free radicals or expression of activated oncogenes.<sup>17</sup> Accumulation of p53 has been shown to occur upon DNA damage, which in turn causes cell cycle arrest or apoptosis of the affected cells.<sup>15</sup> MDM2 (murine double minutes-2) protein which is an E3 ligase binds p53 and regulates the functions of p53 through a negative feedback loop.<sup>16</sup> The negative feedback mechanism operates in such a way that an increase in nuclear p53 concentration activates MDM2 which in turn blocks the p53 transactivation domain targeting p53 for degradation.<sup>13,16,18</sup> Inhibiting p53-MDM2 interaction would thus activate p53 and thus induce cell-cycle arrest and apoptosis. The various functions of p53 are also mediated by a number of downstream targets some of which include the p21<sup>WAF1/CIP1</sup> and CDKs (CDK2, CDK6) genes involved in cell cycle control and Bcl-2 family proteins involved in programmed cell death. Secondary metabolites from different plants have been shown to have a modulatory effect on the expression and function of the p53 gene as well as some of the downstream targets in the p53 pathway.<sup>19,20</sup> Referring to the *in vitro* antiproliferative activities of the isolated compounds from *Z. zanthoxyloides*, we reasoned that these compounds may inhibit cancer cell proliferation by blocking some of the molecular targets in the p53 pathway. Therefore, *in silico* docking studies were carried out to understand the probable anticancer mechanism of action of the secondary metabolites isolated from *Z. zanthoxyloides*. The proteins in the p53 pathway evaluated herein included MDM2, cyclin-dependent kinases (CDK2 and CDK6), p21<sup>WAF1/CIP1</sup>, anti-apoptotic protein Bcl-2 and caspases (caspase 3 and caspase 8).

## MATERIALS AND METHODS

### Plant Collection and Processing

The root bark of *Z. zanthoxyloides* was collected from the hills of Ngora in Ngora district, Eastern Uganda in the month of January 2017. The plant was authenticated by Mr. Patrick Mutiso Chalo, a botanist at the Herbarium, School of Biological Sciences, the University of Nairobi, where a voucher specimen (Voucher number: AM/2016/02) was deposited. The samples were air-dried in the shade for 2 weeks and ground into powder.

### Phytochemicals

The phytochemicals used in this study were isolated from MeOH/CH<sub>2</sub>Cl<sub>2</sub> (1:1 vol/vol) extracts of dried, pulverized root bark of *Z. zanthoxyloides*. The isolated compounds include dihydrocherythrine C<sub>21</sub>H<sub>19</sub>NO<sub>4</sub> (1; white crystals, m.p.: 113-115°C, *m/z* 348.1229, [M-H]<sup>+</sup>),<sup>21</sup> skimmianine C<sub>14</sub>H<sub>13</sub>NO<sub>4</sub> (2, yellow crystals, m.p: 177 – 179°C, UV (λ<sub>max</sub>: MeOH: 248 nm)),<sup>22,23</sup> tridecan-2-one C<sub>13</sub>H<sub>26</sub>O (3, white amorphous solid, m.p: 24-27°C, *m/z*: 198.34, UV (λ<sub>max</sub>: 280 nm)),<sup>22</sup> Sesamin C<sub>20</sub>H<sub>18</sub>O<sub>6</sub> (4, white crystals, m.p: 121-122°C, *m/z*: 354.11),<sup>9,24</sup> and hesperidin C<sub>28</sub>H<sub>34</sub>O<sub>13</sub> (5, pale yellow solid, m.p: 258-262°C, *m/z*: 578.56, UV (λ<sub>max</sub>: MeOH: 258 and 321 nm)).<sup>10</sup> Detailed isolation procedure and spectroscopic data for these compounds are provided in the supporting information.

### General procedures

Column chromatography was carried out on silica gel (0.06-0.2 mm, Merck) to isolate compounds from the plant extracts. Pre-coated silica gel plates (60 F254, Merck) were used to perform analytical TLC. The spots on TLC plates were visualized using a UV lamp (254 and 360 nm). Gel filtration was performed on Sephadex LH-20. Mass measurements were done using LC-HRMS-ESI on an LTQ Orbitrap spectrometer (Thermo Scientific, USA). 1D and 2D NMR spectra were recorded on Bruker Avance 500 and Avance III 600 Spectrometers using standard pulse sequences and referenced with residual solvent peaks (<sup>1</sup>H: δ 5.32 for CD<sub>2</sub>Cl<sub>2</sub> and 2.50 for DMSO-d<sub>6</sub>); <sup>13</sup>C NMR 53.8 for CD<sub>2</sub>Cl<sub>2</sub> and 39.5

for DMSO-d<sub>6</sub>). Cells were cultured in standard media; Dulbecco's modified eagle medium (DMEM) or minimum essential medium (MEM) and supplemented with phosphate-buffered saline (PBS), Fetal bovine serum (FBS), penicillin and streptomycin in an atmosphere of 5% CO<sub>2</sub> at 37°C.

### *In vitro* antiproliferative activity

All test compounds were dissolved in DMSO at a final concentration of 50 mM and stored at -20°C before use. Cytotoxicity was assessed by using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml) assay as previously described.<sup>25</sup> Briefly, 4x10<sup>3</sup> cells per well were seeded in 96-well plates before drug treatments. After overnight incubation, the cells were exposed to different concentrations of selected compounds (0.19–100 μM) for 72 hr. Cells without drug treatment were used as control. Subsequently, 10 μl of 5 mg/mL MTT solution was added to each well and incubated at 37°C for 4 hr followed by addition of 100 μl solubilization buffer (10 mM HCl in a solution of 10% of SDS) and overnight incubation. Absorbance at 570 nm was then determined in each well on the next day. The percentage of cell viability was calculated using the following formula: Cell viability (%) = A treated / A control × 100. A representative graph of at least three independent experiments is shown in the supporting information.

### *In silico* molecular docking studies

Molecular operating environment (MOE) software, version 2008.10 (Chemical Computing Group ULC) was used for all *in silico* operations; including structure optimization, target preparation and molecular docking simulations.<sup>26</sup> Default settings in the MOE docking simulation tool were used for docking using the London dG scoring function. Before docking simulations were performed, ligands and proteins were prepared and the docking method was validated as described herein.

### Ligand preparation

2D structures of the molecules were drawn using Chembiodraw ultra software, version 12.0 (PerkinElmer) and imported into the MOE structure builder tool as SMILES string to generate 3D structures. The energy of the 3D molecules was minimized by applying the Hamiltonian MMFF94X force field. The minimized molecules were used to develop a local compound library for docking simulations.

### Protein preparation

The crystal structures of the following protein molecular targets involved in the p53 pathway namely; MDM2 (1RV1), P21<sup>WAF1/CIP1</sup> (2X4Z), CDK2 (1DI8, 1PYE), CDK6 (1XO2), Bcl-2 (2O2F), Caspase 3 (3DEI) and Caspase 8 (3KJQ) were retrieved from the protein data bank (PDB).<sup>27</sup> The proteins were protonated using the 3D protonate tool in MOE, partial charges were added, unbound water molecules were removed and the energy of the protein was minimized by applying the empirical Hamiltonian MMFF94X force field.

### Docking method validation

The docking method was first validated by self-docking the co-crystallized ligand into the active site of the native protein. Here an induced-fit docking mode was adopted (where both the ligand and the amino acid side chains were left flexible in order to achieve an optimal fit).<sup>26</sup> The reproducibility of the docking method was based on the RMSD between the re-docked conformation to the experimentally determined conformation of the native ligand. RMSD value < 2 Å was considered acceptable. The binding affinities measured in terms of S-score in Kcal/mol were used for comparison with the binding affinities of the test ligands.

## In silico Docking of Compound Library

The compound library of the secondary metabolites was docked into the inhibitor binding site of the target proteins. The resultant docking poses of the ligands were ranked based on the S-score, in this case, the more negative the S-score, the stronger the binding affinity to the target protein.

### Pharmacological and Drug Likeness Properties

Drug likeness and pharmacological properties such as absorption, distribution, metabolism, excretion and toxicity (ADMET) profiles of the compounds were evaluated using the online SWISS ADME program.<sup>28</sup>

### Statistical Analysis

All the experiments for antiproliferative activity were carried out in triplicate and the results represent an average of replicate measurements. The IC<sub>50</sub> values were processed using Graphpad Prism software Version 5.0.

## RESULTS

### Compounds Isolated from *Z. zanthoxyloides*

The MeOH:CH<sub>2</sub>Cl<sub>2</sub> extract of the root bark of *Z. zanthoxyloides* upon chromatographic separation yielded five compounds (Figure 1) and identified as dihydrochelerythrine (1),<sup>21</sup> skimmianine (2),<sup>22</sup> tridecan-2-one (3),<sup>29,30</sup> sesamin (4),<sup>9,24,30</sup> and hesperidin (5).<sup>10</sup> NMR spectral data for all the compounds are available as supporting information.

### In vitro cytotoxicity evaluation

Cytotoxicity of the isolated secondary metabolites was evaluated against a panel of 7 cancer cell lines including; renal cancer (786-0), breast cancer cells (MCF-7, BT549), liver cancer cells (HCC and HepG<sub>2</sub>), lung cancer cells (HEP<sub>2</sub>), adenocarcinoma human alveolar basal epithelial cells (A549) and three normal human cells namely; immortalized human bronchial epithelial cells (BEAS), immortalized human liver cells (LO<sub>2</sub>), immortalized breast cells (MCF-10a). The results are presented in Table 1.

Based on the proposed limits of cytotoxicity of secondary metabolites in the literature,<sup>31</sup> most of the compounds showed selective inhibitory activity against the tested cell lines. Dihydrochelerythrine (1) inhibited proliferation of breast cancer cells BT549 (IC<sub>50</sub> 21.2 μM), hepatocellular carcinoma (HCC) (IC<sub>50</sub> 8.9 μM) and showed low cytotoxicity against Hep2 cells (IC<sub>50</sub> 64.0 μM). Meanwhile, sesamin (4) showed moderate cytotoxicity against breast cancer cells BT549 (IC<sub>50</sub> 47.6 μM). On the other hand, hesperidin (5) showed low inhibitory activity against the proliferation of A549 and Hep2 cancer cells (IC<sub>50</sub> 64.7 and 67.5 μM respectively). However, 5 exhibited significant toxicity against immortalized lung cells (BEAS, IC<sub>50</sub> 7.1 μM) and normal liver cell LO<sub>2</sub> (IC<sub>50</sub> 30.6 μM). High toxicity against the normal cells (BEAS and LO<sub>2</sub>) was also observed for the reference drug, paclitaxel. Skimmianine (2) and tridecan-2-one (3) did not show any inhibitory activity against the tested cells. All the tested compounds did not show any significant cytotoxicity against the normal breast cell MCF-10a (IC<sub>50</sub> >100).

### In silico Docking Evaluation of Anticancer Mechanism of Action

#### Validation of the Docking Method

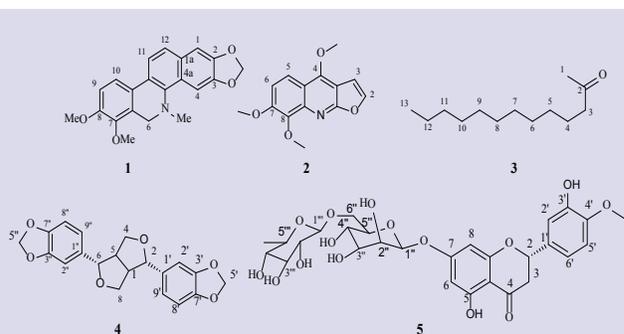
The co-crystallized ligands in the crystal structure of the target proteins were used as control ligands for docking reproducibility. The validity of the docking method was evaluated based on the Root Mean Square Deviation (RMSD) upon superposition of the best-docked pose to the conformation of the experimental native ligand. Table 2 shows RMSD values and binding affinities of the re-docked ligands. The low RMSD

values (<2 Å) of the docked and experimental conformations of the co-crystallized ligand indicates similarity in binding orientations of the co-crystallized ligand.<sup>32</sup> Figure 2 shows the best-docked conformations of the re-docked native ligands (red) superposed on their native conformations (grey) in the binding sites of the respective proteins.

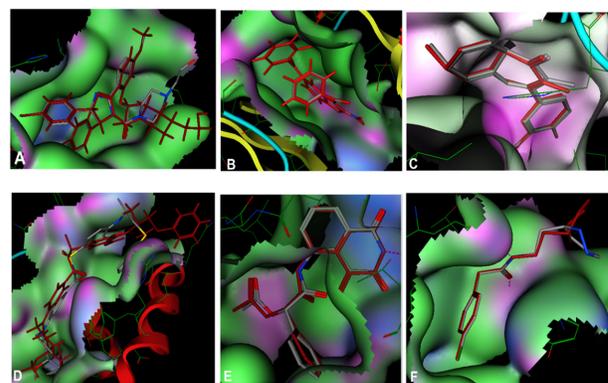
### In silico Docking Studies

Previous *in vitro* studies indicate that some of the compounds isolated from the genus *Zanthoxylum* exert their anticancer activity through induction of apoptosis and cell cycle arrest.<sup>33,34</sup> Based on the results of our *in vitro* antiproliferative activities, it was inferred that some of the isolated compounds reported herein inhibit cancer cell proliferation by causing cell cycle arrest and apoptosis. In that regard, we attempted to elucidate the probable anticancer mechanism of action of the isolated compounds against protein targets in the p53 pathway. Binding affinities and hydrogen bond interactions were used to measure the extent of protein-ligand interactions and compared with those of the co-crystallized ligands.

The docking results here show that all the docked compounds weakly bind to MDM2 with the best binding energy of -20.12 kcal/mol for hesperidin (5) as compared to the known inhibitor imidazoline (binding energy -26.74 kcal/mol) (Supplementary Information). Hesperidin (5) forms one hydrogen bond interaction with amino acid Lys94 in the binding site of MDM2 while similar amino acid residues are involved in hydrophobic interaction of MDM2 with the docked compounds and



**Figure 1:** Structures of compounds isolated from the root bark of *Z. zanthoxyloides*: dihydrochelerythrine (1), skimmianine (2), tridecan-2-one (3), sesamin (4), hesperidin (5).



**Figure 2:** Validation of docking method by superposition of co-crystallized ligand (grey/blue) with the pose of the re-docked native ligand (red) in the active site of the molecular targets. A: MDM2, B: CDK2, C: CDK6, D: Bcl-2, E: Caspase 3, F: Caspase 8.

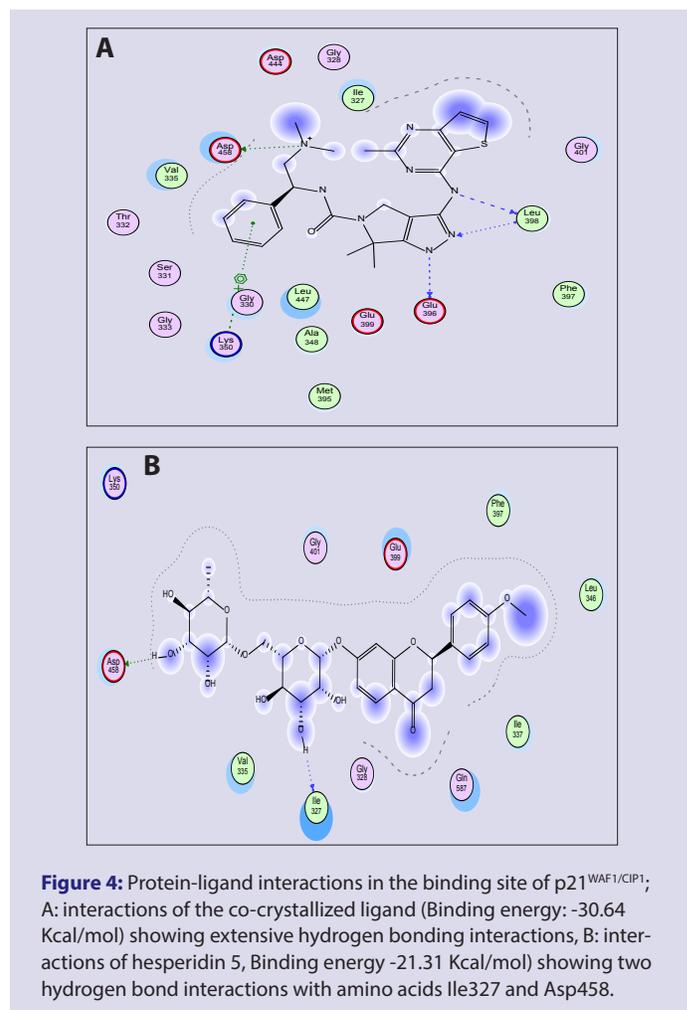
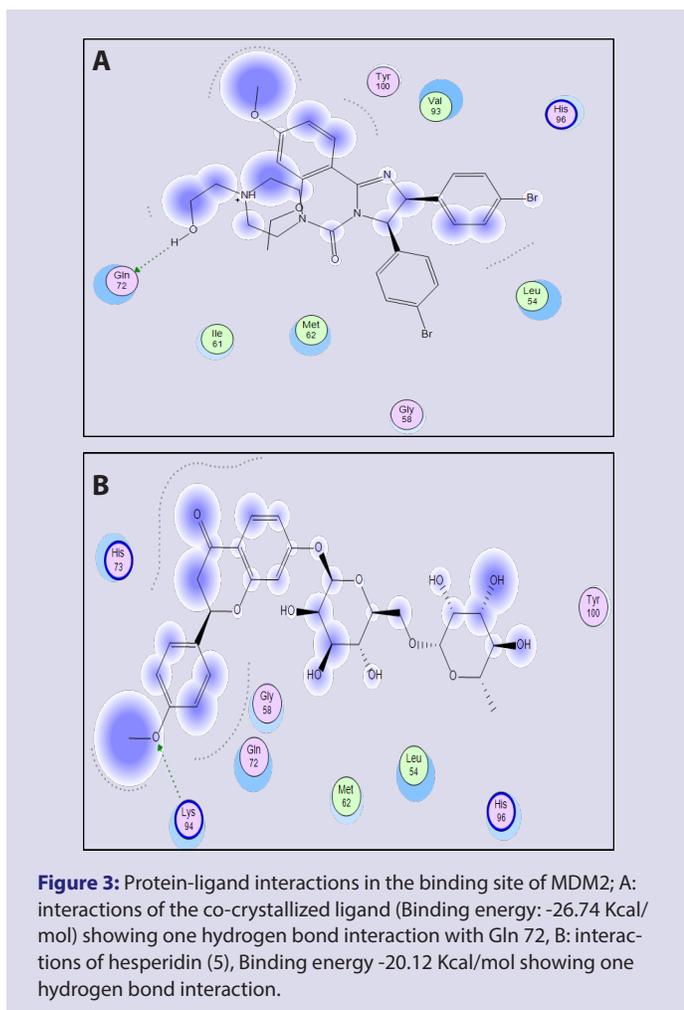
imidazoline (Figure 3). These results suggest that hesperidin (5) could inhibit p53-MDM2 interaction thereby activating the p53 pathway consequently inducing apoptosis.

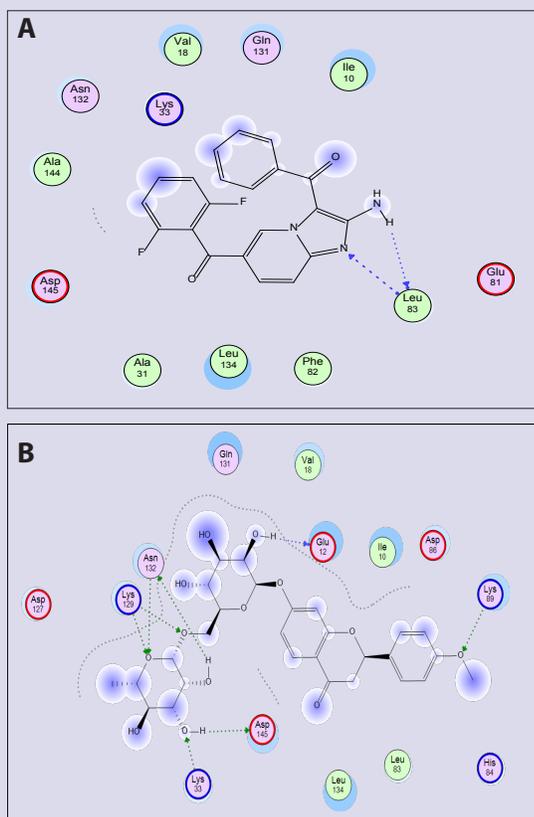
P21<sup>WAF1/CIP1</sup> is one of the downstream targets of p53 and an inhibitor of CDK which is activated upon DNA damage thus, inducing cell cycle arrest.<sup>35</sup> Previous studies indicate that p21<sup>WAF1/CIP1</sup> deficient thymocytes underwent rapid apoptosis upon damage.<sup>32</sup> Therefore p21<sup>WAF1/CIP1</sup> is a suitable target for anticancer drug development since, inhibiting p21<sup>WAF1/CIP1</sup> induces cell death by arresting the cell cycle. Docking simulations show that all the docked compounds had feeble interaction with the active site of p21<sup>WAF1/CIP1</sup> with the best binding affinity of -22.17 Kcal/mol for hesperidin (5) compared to the co-crystallized ligand (binding affinity -30.64 Kcal/mol) (Supporting Information). Similar amino acid residues are involved in hydrogen bonding and hydrophobic interactions with the native ligand and the best docked ligand (5) (Figure 4) suggesting that hesperidin (5) can be a potential inhibitor of p21<sup>WAF1/CIP1</sup> thus, inhibiting cell proliferation by causing cell cycle arrest.

Cyclin-dependent kinases (CDK2 and CDK6) are two cyclin-dependent Ser/Thr kinases which play important roles in cell cycle control, apoptosis, transcription and neuronal functions.<sup>36</sup> They are activated only when bound to cognate regulatory cyclins.<sup>36,37</sup> Thus, Inhibiting Cyclins (CDK2 and CDK6) would prevent binding to cognate regulatory cyclins consequently causing cell cycle arrest. The docking results indicate that compounds 1, 2, 4 and 5 strongly bind to the active site of CDK2 (binding energies -22.64, -18.54, -20.01 and -29.06, respectively as compared

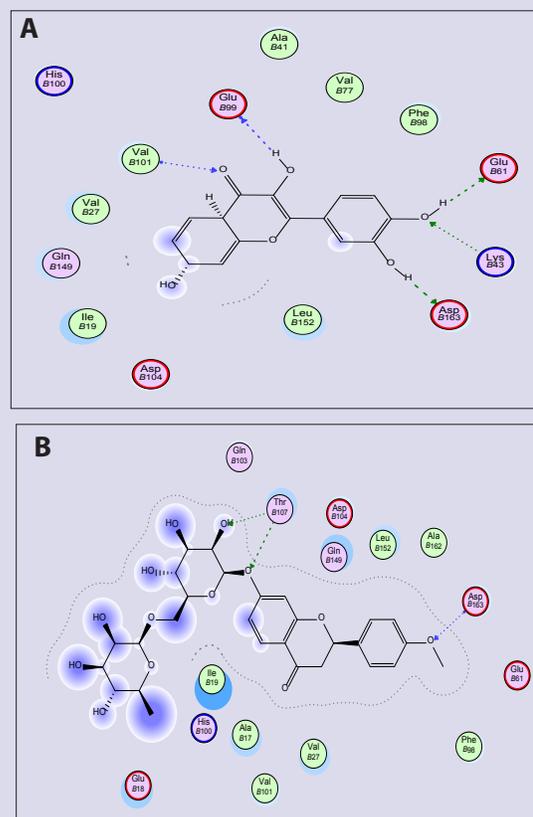
to the native ligand (binding energy -16.76 kcal/mol) (Binding affinities of the other compounds are available as supplementary information). It can be seen in Figure 5, that the best-docked ligand (hesperidin, 5) showed similar binding mode to the native ligand with extensive hydrogen bonding to similar amino acid residues as the native ligand. This suggests that these compounds can inactivate CDK2 preventing it from binding to its cognate regulatory cyclin consequently leading to cell cycle arrest. Similarly, all the compounds strongly bind to the active site of CDK6 (binding energies > -12.34 kcal/mol) compared to the co-crystallized inhibitor (binding energy -12.34 kcal/mol) (Figure 6, Supplementary Information). These results, therefore, suggest that these compounds could induce cell death by causing cell cycle arrest.

The BCL-2 family of proteins are key regulators of apoptosis process. However, most cancer cells maintain their immortality through over-expression of anti-apoptotic Bcl-2 proteins which oppose the action of pro-apoptotic proteins BAX and BAK.<sup>38</sup> Thus the Bcl-2 family is interesting targets for most anticancer drugs. Results of the docking simulations indicate that hesperidin (5) strongly binds to the active site of Bcl-2 (binding energy -25.88 kcal/mol) compared to the co-crystallized ligand (binding energy -25.15 kcal/mol) (Binding energies for other ligands are presented in the Supporting Information). This suggests that hesperidin (5) can inhibit Bcl-2 allowing the pro-apoptotic proteins such as BAX and BAK to oligomerize in the mitochondrial membrane consequently facilitating release of caspases thus inducing apoptosis. Similar amino acid residues are involved in the protein-ligand interactions between hesperidin (5) and the native ligand L10 (Figure 7). All the compounds





**Figure 5:** Protein-ligand interactions in the binding site of CDK2; A: interactions of the co-crystallized ligand (Binding energy: -19.82 Kcal/mol), B: interactions of the best docked ligand (hesperidin (5), Binding energy -27.56 Kcal/mol) showing extensive hydrogen bonding network with several amino acid residues in the binding site of CDK2.



**Figure 6:** Protein-ligand interactions in the binding site of CDK6; A: interactions of the co-crystallized ligand (binding energy: -12.34 Kcal/mol), B: interactions of the best docked ligand (Hesperidin, binding energy: -32.08 Kcal/mol) showing three hydrogen bonding networks with amino acid residues in the binding site.

**Table 1:** *In vitro* antiproliferative activity of the compounds isolated from the root bark of *Z. zanthoxyloides* (n=3).

Compound	Cell line and IC <sub>50</sub> (μM)									
	RCC-896-0	MCF10a	MCF <sub>-7</sub>	BT <sub>-549</sub>	HCC	HeP <sub>2</sub>	HepG <sub>2</sub>	LO <sub>2</sub>	BEAS	A549
1	> 100	> 100	> 100	21.2	8.9	64.0	ND	ND	ND	ND
2	> 100	>100	>100	>100	ND	ND	ND	ND	ND	ND
3	>100	>100	>100	>100	>100	ND	ND	ND	ND	ND
4	>100	>100	>100	47.6	>100	>100	ND	ND	ND	ND
5	ND	ND	ND	ND	>100	67.6	>100	30.6	7.1	64.7
Paclitaxel							0.19	<0.1	<0.1	0.003

ND: Not determined

had low binding affinity to the effector caspases 3 and 8 (Table 3) as compared to their known inhibitors thereby allowing caspases 3 and 8 to function as normal executioners of apoptosis.

### Pharmacological and Drug Likeness Properties

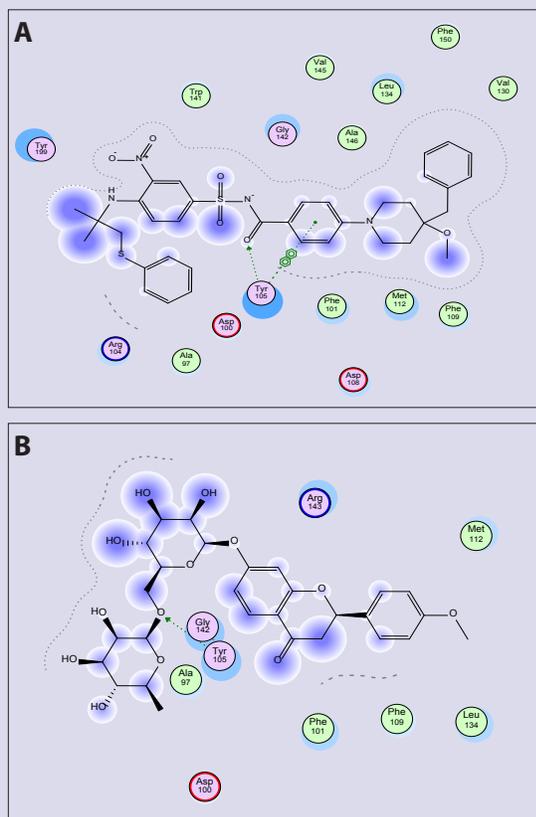
To support further evaluation and development of the tested compounds, their pharmacological properties (ADME) were analyzed using the on-line SWISSADME program.<sup>28</sup> Results of the key ADME predictions are presented in Table 4. Generally, most of the compounds show acceptable pharmacological properties except hesperidin (5) which violates Lipinski rule of 5 with 3 violations. Complete pharmacological properties are

shown in the supporting information.

## DISCUSSION

The role of natural products in the treatment of different diseases cannot be over emphasized. It is evident that over 60% of drugs used in cancer treatment are natural products or derivatives thereof.<sup>39</sup> Plants are still a focal point of investigation as sources of new drugs for the treatment of cancer and other diseases.

*Z. zanthoxyloides* is a medicinal plant which has been shown to exhibit several biological activities and a host of compounds have already been



**Figure 7:** Protein-ligand interactions in the binding site of the anti-apoptotic protein Bcl-2. A: interactions of the co-crystallized ligand showing one hydrogen bond and  $\pi$ - $\pi$  interaction with Tyr 105 (binding energy -25.15 Kcal/mol), B: interactions of the best docked ligand (hesperidin (5), binding -25.88 Kcal/mol) showing one hydrogen bond interaction with Tyr 105.

reported from this species.<sup>9,10</sup> Some previous studies have reported anticancer activity of extracts and pure compounds isolated from plants in the genus *Zanthoxylum*. For example, sesamin (4) exhibits inhibitory activity against lung carcinoma (A549) and pancreatic carcinoma (MIA-PaCa) cells with  $IC_{50}$  values of 37.46 and 34.04  $\mu$ g/mL.<sup>40</sup> Skimmianine (2) inhibited proliferation of acute lymphoblastic leukemia cancer cell lines (Jurkat), HEP-2, KG-1a and RAJI. In the present study, the antiproliferative activity of compounds from the root bark of *Z. zanthoxyloides* against other cancer cells is reported. The results demonstrate that dihydrochelerythrine (1) showed significant to moderate inhibitory activity against the proliferation of HCC and breast cancer cell lines (BT549) and no toxicity against normal mammary cells (MCF-10a). Activity of (1) against liver (HCC) and breast (BT549) is reported here for the first time and thus providing additional information on its potential as a lead compound against different cancer cell lines. Whereas hesperidin (5) exhibited low inhibitory activity against the proliferation of HeP<sub>2</sub> and A549 cells, it exhibits high toxicity against normal lung (BEAS) and liver (LO<sub>2</sub>) cells. Previous studies described in the review by Garg *et al.*<sup>41</sup> suggest that hesperidin is considered generally safe. There are no reports in the literature about the toxicity of hesperidin (5) against normal lung and liver cells. Only slight side effects were reported in placebo experiments cited in the same review.<sup>41</sup> It has been shown to exert cytotoxic activity against human melanoma (HT-144) and breast cancer (MCF-7) cells with  $IC_{50}$  of 11  $\mu$ M. The high toxicity of hesperidin (5) against normal

**Table 2:** Docking method validation showing RMSD and binding affinities of the re-docked ligands.

Target	PDB ID	Ligand	RMSD	S-Score Kcal/mol
MDM2	1RV1	IMZ	0.60	-27.25
CDK2	1DI8	DTQ	0.50	-19.80
CDK2	1PYE	PM1	0.04	-16.76
CDK6	1XO2	FSE	0.48	-12.34
P21	2X4Z	7KC	0.78	-30.64
Bcl2	2O2F	L1O	2.88	-25.15
Caspase 3	3DEI	RXB	0.75	-19.34
Caspase 8	3KJQ	B94	1.89	-17.60

**Table 3:** Binding affinities of docked ligands as compared to the native ligand when docked in the active site of caspase 3 and caspase 8.

Caspase 3		Caspase 8	
Compound	S-score; Kcal/mol	Compound	S-score. Kcal/mol
Hesperidin (5)	-18.12	Hesperidin (5)	-16.3
Skimmianine (2)	-13.85	Sesamin (4)	-15.36
Sesamin (4)	-13.71	Skimmianine(2)	-14.29
Dihydrochelerythrine (1)	-12.21	Dihydrochelerythrine (1)	-11.85
Tridecan-2-one (3)	-11.40	Tridecan-2-one (3)	-10.38
RXB	-19.34	B94	-17.60

lung (BEAS) and liver (LO<sub>2</sub>) cells observed in the present study indicates that hesperidin could be harmful to some tissues. We did not observe any significant inhibitory activity of skimmianine (2) and tridecan-2-one (3) against the tested cells. The results of the current study provide more information and highlight the potential of these compounds to be further evaluated as anticancer agents. In studies reported thus far, some of the compounds exert their anticancer effect by causing cell cycle arrest and apoptosis.<sup>42-44</sup> The results of *in-silico* investigations demonstrate that most of the compounds can potentially inhibit cancer cell proliferation by causing cell cycle arrest as evidenced by their high binding affinities to CDK2 and CDK6 compared to the native ligands. Feeble binding interactions of all the test compounds with MDM2 and p21<sup>WAF1/CIP1</sup> compared to the known inhibitors further demonstrates that some of the compounds probably inhibit cancer cell proliferation by inhibiting cyclin-dependent kinases (CDK2 and CDK6) or a totally different pathway. Thus, high binding affinity of hesperidin (5) to the anti-apoptotic protein Bcl-2 and low binding affinity to caspases 8 and 3 illustrates its ability to induce apoptosis by possibly overexpressing caspases 8 and 3. Moreover, all the compounds show low binding affinities to the effector caspases (caspase 8 and caspase 3) suggesting their possible involvement in inducing apoptotic cell death. Although the *in silico* predictions are not cancer cell-specific, they complement the *in vitro* antiproliferative activities reported herein. These results, therefore, indicate that some of the compounds from *Z. zanthoxyloides* are potential lead compounds for further development as anticancer agents. Predictions of the pharmacological properties (ADME) indicate that most of the compounds exhibit acceptable pharmacological properties making them potential lead molecules for further development and evaluation.

**Table 4: Predicted ADME properties of the isolated compounds.**

Compound	Molecular weight	Log <sub>P<sub>o/w</sub></sub>	Gastro-intestinal absorption	Blood brain barrier	CYP2D6 inhibition	Plasma protein substrate (P-gp)	Lipinski rule of 5 violations
1	349.38	3.66	high	Yes	Yes	Yes	0 violations
2	259.26	2.58	High	Yes	Yes	No	0 violations
3	184.32	3.85	High	Yes	No	No	0 violations
4	354.35	2.79	High	Yes	Yes	No	0 violations
5	578.56	-0.36	Low	No	No	Yes	3 violations

Log<sub>P<sub>o/w</sub></sub> octanol/water partition coefficient

## CONCLUSION

This study demonstrates that compounds 1, 4 and 5 hold promise as potential anticancer agents. *In silico* docking studies suggest that some of the compounds inhibit cancer cell proliferation by inducing cell cycle arrest and apoptosis. The results provide additional information on the anticancer activity of compounds from *Z. zanthoxyloides* against breast, renal, liver and lung cancer cell lines. High *in vitro* toxicity of hesperidin (5) to normal cells (LO<sub>2</sub> and BEAS) compared to the cancer cells warrant further investigations before any *in vivo* trials can be conducted. *In vitro* and *in vivo* evaluation of the anticancer mechanism of action is warranted to further understand the anticancer mode of action of these compounds.

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## Authors' Contribution

AM, PC, LJY, VW, CMN, MH carried out experiments, PC, AJN, AY, SD conceptualized the research, reviewed methods, supervised the study and corrected the manuscript. AM, PC, CMN AJN and SD wrote the manuscript. All authors read and approved the manuscript.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ABBREVIATIONS

**B94:** (3S)-3-(((5S,8R)-2-(3-carboxypropyl)-8-(2-(((4-chlorophenyl)acetyl)amino)ethyl)-1,3-dioxo-2,3,5,8-tetrahydro-1H-[1,2,4]triazolo[1,2-a]pyridazin-5-yl)carbonyl)amino)-4-oxopentanoic acid; **CDK:** Cyclin-dependent kinase; **DMSO:** Dimethylsulfoxide; **DTQ:** 4-[3-hydroxyanilino]-6,7-dimethoxyquinazoline; **FSE:** 3,7,3',4'-Tetrahydroxyflavone; **IMZ:** Imidazoline; **LC-HRMS-ESI:** Liquid chromatography coupled high resolution mass spectroscopy with electro spray ionization; **L1O:** 4-[4-methoxy-4-(phenylmethyl)piperidin-1-yl]-N-[4-[(2-methyl-1-phenylsulfonyl-propan-2-yl)amino]-3-nitro-phenyl]sulfonyl-benzamide; **MeOH:** Methanol; **MMFF94X:** Merck molecular force field 94X; **PMI:** [2-amino-6-(2,6-difluoro-benzoyl)-imidazo[1,2-A]pyridine-3-yl]-phenyl-methanone; **p21<sup>WAF1/CIP1</sup>:** Cyclin-dependent kinase inhibitor protein 21; **RXB:** (1S)-2-oxo-1-phenyl-2-[(1,3,4-trioxo-1,2,3,4-tetrahydroisoquinolin-5-yl)amino]ethylacetate; **SDS:** Sodium dodecyl sulfate; **SMILES:** Simplified molecular-input line-entry system;

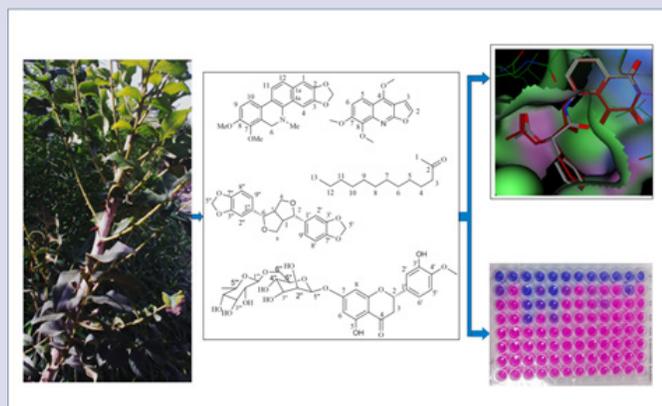
**7KC:** {N}-[([1~{S}]-2-(dimethylamino)-1-phenyl-ethyl)-6,6-dimethyl-3-[(2-methylthieno[3,2-d]pyrimidin-4yl)amino]-1,4-dihydropyrrolo[3,4-c]pyrazole-5-carboxamide.

## REFERENCES

- Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018;68(6):394-424.
- Chen Y, Chen H, Shi J. Inorganic nanoparticle-based drug codelivery nanosystems to overcome the multidrug resistance of cancer cells. *Mol Pharm.* 2013;11(8):2495-510.
- He Y, Du Z, Lv H, Jia Q, Tang Z, Zheng X, et al. Green synthesis of silver nanoparticles by *Chrysanthemum morifolium* Ramat. extract and their application in clinical ultrasound gel. *Int J Nanomedicine.* 2013;8:1809.
- Cragg GM, Newman DJ. Natural products: A continuing source of novel drug leads. *Biochimica et Biophysica Acta (BBA)-General Subjects.* 2013;1830(6):3670-95.
- Chase MW, Morton CM, Kallunki JA. Phylogenetic relationships of Rutaceae: A cladistic analysis of the subfamilies using evidence from RBC and ATP sequence variation. *Am J Bot.* 1999;86(8):1191-9.
- Zhang M, Wang J, Zhu L, Li T, Jiang W, Zhou J, et al. *Zanthoxylum bungeanum* Maxim. (Rutaceae): A Systematic Review of Its Traditional Uses, Botany, Phytochemistry, Pharmacology, Pharmacokinetics and Toxicology. *Int J Mol Sci.* 2017;18(10):2172.
- Ogwal-Okeng JW, Obua C, Anokbonggo WW. Acute toxicity effects of the methanolic extract of *Fagara zanthoxyloides* (Lam.) root-bark. *Afr Health Sci.* 2003;3(3):124-6.
- Wouatsa VN, Misra L, Kumar S, Prakash O, Khan F, Tchoumboungang F, et al. Aromatase and glycosyl transferase inhibiting acridone alkaloids from fruits of Cameroonian *Zanthoxylum* species. *Chem Cent J.* 2013;7(1):125.
- Chaaib F, Queiroz EF, Njoko K, Diallo D, Hostettmann K. Antifungal and antioxidant compounds from the root bark of *Fagara zanthoxyloides*. *Planta Med.* 2003;69(4):316-20.
- Queiroz EF, Hay AE, Chaaib F, Diemen DV, Diallo D, Hostettmann K. New and bioactive aromatic compounds from *Zanthoxylum zanthoxyloides*. *Planta Med.* 2006;72(08):746-50.
- Itemire OA, Ogbimi OA, Idu M. Phytochemistry and antimicrobial activity of *Zanthoxylum zanthoxyloides* root used as chewing stick in Nigeria. *J Phyto-pharmacol.* 2013;2(6):1-7.
- Kassim OO, Loyevsky M, Elliott B, Geall A, Amonoo H, Gordeuk VR. Effects of root extracts of *Fagara zanthoxyloides* on the *in vitro* growth and stage distribution of *Plasmodium falciparum*. *Antimicrob Agents Chemother.* 2005;49(1):264-8.
- Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature.* 1997;387(6630):296.
- Sun Y. p53 and its downstream proteins as molecular targets of cancer. *Mol Carcinog.* 2006;45(6):409-15.
- Hainaut P, Hollstein M. p53 and human cancer: The first ten thousand mutations. *Adv Cancer Res.* 1999;77:81-137.
- Harris SL, Levine AJ. The p53 pathway: Positive and negative feedback loops. *Oncogene.* 2005;24(17):2899.
- Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. *Nature.* 2000;408(6810):307-10.
- Ray-Coquard I, Blay JY, Italiano A, Le CA, Penel N, Zhi J, et al. Effect of the MDM2 antagonist RG7112 on the P53 pathway in patients with MDM2-amplified, well-differentiated or dedifferentiated liposarcoma: An exploratory proof-of-mechanism study. *Lancet Oncol.* 2012;13(11):1133-40.
- Aderonke ST, Babatunde JA, Adesola OT, Okereke OU, Innocent C, Elisha MO, et al. Evaluation of retinoblastoma (Rb) and protein-53 (p53) gene expression

- levels in breast cancer cell lines (MCF-7) induced with some selected cytotoxic plants. *J Pharmacognosy Phytother.* 2013;5(7):120-6.
20. Yazdanpanahi N, Behbahani M, Yektaeian A. Effect of boswellia thurifera gum methanol extract on cytotoxicity and p53 gene expression in human breast cancer cell line. *Iran J Pharm Res.* 2014;13(2):719-24.
  21. Joshi BS, Moore KM, Pelletier SW, Puar MS. Alkaloids of *Zanthoxylum burdunga* wall.: NMR assignments of dihydrochelyerythrine,(±)-evodiamine and zanthobungeanine. *Phytochem Anal.* 1991;2(1):20-5.
  22. Adesina SK. Further Novel Constituents of *Zanthoxylum zanthoxyloides* Root and Pericarp. *J Nat Prod.* 1986;49(4):715-6.
  23. Graham EJ, William EC, Bette D. Two Dimensional NMR Study of 8-Methoxyflindersine, Skimmianine and Monocrotaline. *Spectrosc Lett.* 1990;23(8):971-82.
  24. Mukhija M, Dhar KL, Kalia AN. Bioactive Lignans from *Zanthoxylum alatum* Roxb. stem bark with cytotoxic potential. *J Ethnopharmacol.* 2014;152(1):106-12.
  25. Coghi P, Yaremenko IA, Prommana P, Radulov PS, Syroeshkin MA, Wu Y J, et al. Novel Per oxides as Promising Anticancer Agents with Unexpected Depressed Antimalarial Activity. *Chem Med Chem.* 2018;13(9):902-8.
  26. Herrmann F, Sivakumar N, Jose J, Costi M, Pozzi C, Schmidt T. *In silico* identification and *in vitro* evaluation of natural inhibitors of *Leishmania* major pteridine reductase I. *Molecules.* 2017;22(12):2166.
  27. RCSB Protein Data Bank. Available online. Retrieved 10/6/2019 <http://www.rcsb.org/pdb/home/home.do>
  28. Daina A, Michielin O, Zoete V. SwissADME: A free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Sci Rep.* 2017;7:42717.
  29. Bisht D, Chanotiya CS. 2-undecanone rich leaf essential oil from *Zanthoxylum armatum*. *Nat Prod Commun.* 2011;6(1):111-4.
  30. Luong NX, Hac LV, Dung NX. Chemical composition of the leaf oil of *Zanthoxylum alatum* Roxb. from Vietnam. *J Essent Oil-Bear Plants.* 2003;6(3):179-84.
  31. Kuete V, Efferth T. African flora has the potential to fight multidrug resistance of cancer. *Bio Med Research International.* 2015;2015:914813.
  32. Al-Khodairy FM, Khan MKA, Kunhi M, Pulicat MS, Akhtar S, Arif JM. *In silico* prediction of mechanism of Erysolin-induced apoptosis in human breast cancer cell lines. *Am J Bioinform Res.* 2013;3:62-71.
  33. Li K, Zhou R, Jia WW, Li Z, Li J, Zhang P, et al. *Zanthoxylum bungeanum* essential oil induces apoptosis of HaCaT human keratinocytes. *J Ethnopharmacol.* 2016;186:351-61.
  34. Satria D, Silalahi J, Haro G, Ilyas S, Hasibuan PAZ. Cell Cycle Inhibition of Ethyl-acetate Fraction of *Zanthoxylum acanthopodium* DC. Fruit against T47D Cells. *Open Access Maced J Med Sci.* 2019;7(5):726-9.
  35. El-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, et al. WAF1/CIP1 Is Induced in p53-mediated G1 Arrest and Apoptosis. *Cancer Res.* 1994;54(5):1169-74.
  36. Shapiro GI. Cyclin-dependent kinase pathways as targets for cancer treatment. *J Clin Oncol.* 2006;24(11):1770-83.
  37. Graña X, Reddy EP. Cell cycle control in mammalian cells: Role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene.* 1995;11(2):211-20.
  38. Frenzel A, Grespi F, Chmelewskij W, Villunger A. Bcl<sub>2</sub> family proteins in carcinogenesis and the treatment of cancer. *Apoptosis.* 2009;14(4):584-96.
  39. Gordaliza M. Natural products as leads to anticancer drugs. *Clin Transl Oncol.* 2007;9(12):767-76.
  40. Deng P, Wang C, Chen L, Wang C, Du Y, Yan X, et al. Sesamin induces cell cycle arrest and apoptosis through the inhibition of signal transducer and activator of transcription 3 signalling in human hepatocellular carcinoma cell line HepG<sub>2</sub>. *Biol Pharm Bull.* 2013;36(10):1540-8.
  41. Garg A, Garg S, Zaneveld L, Singla A. Chemistry and pharmacology of the citrus bioflavonoid hesperidin. *Phytother Res.* 2001;15(8):655-69.
  42. Harikumar KB, Sung B, Tharakan ST, Pandey MK, Joy B, Guha S, et al. Sesamin Manifests Chemopreventive Effects through the Suppression of NF-κB-Regulated Cell Survival, Proliferation, Invasion and Angiogenic Gene Products. *Mol. Cancer Res.* 2010;8(5):751-61.
  43. Vrba J, Doležel P, Vičar J, Modrianský M, Ulrichová J. Celerythrine and dihydrochelyerythrine induce G1 phase arrest and bimodal cell death in human leukemia HL-60 cells. *Toxicology in vitro.* 2008;22(4):1008-17.
  44. Yokota T, Matsuzaki Y, Koyama M, Hitomi T, Kawanaka M, Enoki-Konishi M, et al. Sesamin, a lignan of sesame, down-regulates cyclin D1 protein expression in human tumor cells. *Cancer Sci.* 2007;98(9):1447-53.

### PICTORIAL ABSTRACT



### SUMMARY

- Secondary metabolites were isolated from the root bark of *Z. zanthoxyloides*.
- 1D, 2D NMR and Mass spectral analysis used to determine structure.
- The compounds were tested for cytotoxicity against seven cancer cell lines .
- Anticancer mechanism of action was evaluated using *in silico* docking studies against molecular targets in the p53 pathway.

### ABOUT AUTHOR

**Moses Andima**, is a Lecturer at the Department of Chemistry in Busitema University. He is at the final stages of his Ph.D studies. His research interest are in Natural products chemistry, Drug discovery and design and Nanomedicine.