



THE LATENT CYTOTOXICITY EFFICIENCY OF THE CHEMICAL CONSTITUENTS OF *Morus Rubra* LINN. BARK ON CANCER CELL LINES

(Keberkesanan Kesitotoksian Laten Bahan Kimia daripada Jujukan Kulit *Morus rubra* Linn. ke atas Titisan Sel Kanser)

Maria Carmen Tan^{1*}, Jasmine Ting¹, and Glenn Oyong²

¹Chemistry Department,

De La Salle University, 2401 Taft Avenue, Manila 1004, Philippines

²Molecular Science Unit Laboratory Center for Natural Sciences and Environmental Research,

De la Salle University, 2401 Taft Avenue, Manila 1004, Philippines

*Corresponding author: maria.carmen.tan@dlsu.edu.ph

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Abstract

Owing to the intricate nature of cancer treatment, traditional herbal medicines are being regarded as potential anticancer agents, primarily due to their reported low toxicity in normal cells. With this in mind, this study's primary objective was to identify the constituents present in the semi-purified 15% ethyl acetate fraction of *Morus rubra* bark (**M1**) and to investigate its antiproliferative property on immortalized cell lines (HT-29, HepG2, BxPC-3, MCF-7, HeLa, and THP-1) by an *in vitro* cell viability assay with Zeocin and dimethylsulfoxide (DMSO) as the positive and negative controls. The chemical constituents present in **M1** were identified using gas chromatography-mass spectrometry (GC-EI-MS). The NIST library v 2.0 was used to putatively identify the thirteen (13) compounds observed in the GC-EI-MS chromatogram which were primarily tocopherols (27.58%) and phytosterols (44.06%). **M1** exhibited high cytotoxicity in HepG2 cell lines ($IC_{50} = 7.09 \mu\text{g/mL}$) and was followed by MCF-7 ($IC_{50} = 7.12 \mu\text{g/mL}$), HT-29 ($IC_{50} = 7.98 \mu\text{g/mL}$), HeLa ($IC_{50} = 16.63 \mu\text{g/mL}$), BxPC-3 ($IC_{50} = 16.89 \mu\text{g/mL}$) and THP-1 ($IC_{50} = 17.43 \mu\text{g/mL}$). **M1** did not exhibit cytotoxicity on normal HDFn while Zeocin on normal cells were exceedingly disruptive. The data generated from this work suggests that **M1** could be an effective chemotherapeutic agent due to the presence of phytosterols and tocopherols.

Keywords: Moraceae, *Morus rubra*, gas chromatography-electron ionization-mass spectrometry, retention index, cell viability assays

Introduction

Mulberry, which is under the botanical order Rosales, family Moraceae, and genus *Morus*, is found in many areas of the world specifically in the sub-tropic regions of Asia such as Japan, India, China, and Korea as well as other regions, for instance North America and Africa

[1]. The practice of mulberry cultivation using diverse morphological cultivars is estimated to have started around 400-500 years ago [2]. Several studies have documented the aqueous, methanolic, and ethanolic extracts of *Morus* species. The phytochemicals obtained were characterized to demonstrate several bioactivities

such as anti-oxidative, anti-diabetic, anti-stress, nephroprotective, antimicrobial, anti-mutagenic, anticancer, anxiolytic, hepatoprotective, anthelmintic, antimicrobial, immune-modulatory and cholesterol lowering effects [1].

Organic acids, sugars, flavonoids, alkaloids, steroids, some vitamins, and minerals such as Na, K, Fe, P, Mg, S were found in the extract of the *Morus* genus [2, 3]. It was also indicated that genetic, cultural, and ecological factors can influence the percentage composition of the compounds isolated from extracts of the *Morus* genus. Among the species under the genus, *M. nigra* (black mulberry), *M. rubra* (red mulberry), and *M. alba* (white mulberry) are the most recognized cultivated species of mulberry due to its availability and use in traditional medicines for diseases such as rheumatoid arthritis and the common cold [1].

The fruit of the mulberry species specifically *M. rubra* (purple), *M. nigra* (black) and *M. alba* (white), were found to contain higher vitamin C content for lighter colored species compared to the darkly colored species. The darkly colored berries demonstrated a higher total antioxidant capacity (TEAC) compared to the lighter colored berries due to the presence of anthocyanins. Previous reports have shown that anthocyanin pigments are medically beneficial due to their antioxidant capacity which can prevent or reduce free radical induced damage and inflammation [4-7]. Flavonoids such as rubraflavones A, B, C, and D were likewise isolated from *M. rubra*. These compounds are known to be antihyperglycemic phytochemicals which induce insulinogenic action [8, 9]. Further, the presence of phenolic acids in the fruit, such as cinnamic acid, were found to contribute to the antioxidant property of the plant and to neutralize carcinogenic substances such as nitrosamine. Among the three species, *M. nigra* exhibited the highest phenolic acid content [5, 7].

Glucopyranosides isolated from an ethanolic extract from the leaves of *M. alba* inhibited HL60 cell line proliferation and initiated an immune response which caused the differentiation in the expression of the CD66b and CD 14 antigens [7]. The rich quercetin content in the leaf of *M. alba* prevented P-glycoprotein

(P-gp) production in the tumor's ATP-dependent pump which was construed to be responsible for exporting xenobiotic compounds out of the cell which may be the cause of multidrug resistance (MDR) of some aggressive malignant tumors [7].

M. rubra leaf extract's anticancer property was correlated with its ability to cause the accumulation of cells at the G1 phase via inhibitory action or through down-regulation of cyclins and kinases (CDKs) [10]. As a result, the said extract attenuated cell population at the S phase where DNA synthesis occurs. Additionally, *M. rubra* leaf extracts induced apoptosis by matrix metalloproteinase (MMP) depolarization in a dose-dependent manner as well as the inhibition of human telomerase reverse transcriptase (hTERT) expression, which caused the cessation of the proliferation of cancer cells [10]. *M. rubra* extract presented modest discriminating cytotoxicity on WiDr cells assessed along with foreskin fibroblast cells [11]. In fact, the aforementioned *M. rubra* extracts at 600 mg/mL dilution considerably amplified cell numbers at the G0/G1 phase [11].

A study by Abbas et al. [12] reported a new stilbene, 2',3',4',5',5'-pentahydroxy-cis-stilbene isolated from the ethanol fraction which was obtained from the methanol extract of *M. nigra* stem bark. The new stilbene exhibited an effective antioxidant activity with an IC₅₀ of 4.6 µM. The isolation of prenylated flavonoids from the stem bark of *M. nigra* extract from several distinct fractions (n-hexane, dichloromethane, and ethanol) was also reported. To the best of our knowledge, this is the first study aimed to provide the characterization and the investigation of the cytotoxicity on selected immortalized cell lines from a semi-purified extract prepared from the dried bark of the red mulberry species.

Materials and Methods

Plant material

M. rubra bark (Figure 1) was gathered from Abucay, Bataan, Philippines (14.7128° N, 120.4934° E) on well-cultivated arable land. The plant identity was established and validated by the Bureau of Plant Industry in Malate, Manila, Philippines.



Figure 1. Images of *Morus rubra* Linn

GC-EI-MS analysis

Dried *M. rubra* bark (3 g) was ground and extracted with methanol. The mixture was filtered using filter paper (Whatman 91) and then desolvated under nitrogen for one hour. The crude extract was reconstituted in 5% ethyl acetate in petroleum ether (PE). The resultant solution underwent separation using 5% increments of 5% ethyl acetate in PE as an eluent through column chromatography. The semi-purified fraction collected from 15% ethyl acetate in PE (2.9 mg) was then reconstituted and filtered through a syringe with a 0.45 μm nylon membrane and labeled as **M1**.

M1 was explored using an Agilent gas chromatograph-mass spectrometer (GC-MS) 7890B, by an HP-5ms (5% phenyl methyl siloxane) Ultra Inert column (30 m x 250 mm x 0.25 mm) and ultra-pure helium gas as the carrier gas to examine the vaporizable components. Helium gas constraints were stipulated using the following parameters: flow rate of helium gas was at 1 mL/min, the pressure kept at 8.2 psi, with an average velocity of 36.62 cm/sec and a holdup time of 1.37 min. The splitless inlet was conserved at 250 °C at 8.2 psi, with a total flow of 24 mL/min, and a septum purge flow speed of 3 mL/min. The injector temperature was maintained at 250 °C. The temperature gradient was initially at 70 °C with an automated linear ramp of 2 °C / min until 135 °C and was continued at this temperature for 10 minutes. Additional temperature changes were attained at 4 °C / min until 220 °C and were retained for 10 minutes. The

temperature was then augmented to 270 °C at a 3.5 °C / min rate and was continuous for 37 minutes [13].

NIST library, v. 2.0 was used to identify the compounds, and the average percentage of the peak area was managed from the subsequent total ion chromatograms. The consequent data was established by assessing the analytes according to their elution succession on a non-polar stationary phase. A homologous series of *n*-alkanes was used to calculate the retention indices for each compound's entirety observed in the spectrum. The experiments were undertaken in three replicates.

Cell viability assay

The **M1** fraction obtained through the extraction technique previously described in the GCMS experiments was used for the PrestoBlue-based cytotoxicity assays on the preceding human cancer cell lines (ATCC, Manassas, Virginia, U.S.A.): colon adenocarcinoma (HT-29), hepatocarcinoma (HepG2), pancreatic adenocarcinoma (BxPC-3), breast adenocarcinoma (MCF-7), cervical adenocarcinoma (HeLa), and acute monocytic leukemia (THP-1). The primary culture of normal human dermal fibroblast neonatal (HDFn) (ThermoFisher Scientific, Gibco®, USA) was utilized as normal cell control. The cells were maintained and provided by the Cell and Tissue Culture Laboratory, Molecular Science Unit, Center for Natural Sciences and Environmental Research, De La Salle University. Cells were cultured in complete DMEM

(Dulbecco's Modified Eagle Medium with 10% fetal bovine serum and 1X antibiotic-antimycotic) (Thermo Fisher, Invitrogen, USA) and were incubated at 37°C with 5% CO₂ in a humidified chamber. The *in vitro* assays' constraints were described in our formerly published work [12] and stringently followed the protocols described therein. Zeocin (Thermo-Fisher, Invitrogen, USA), a cytotoxic intercalating agent, was used as positive control while DMSO was applied as negative vehicle control. All tests were performed in three replicates. Optical density (O.D._{570 nm}) readings were utilized to determine cytotoxicity indexes, which were plotted against log concentrations from which the half-maximal inhibitory concentrations (IC₅₀) were derived. GraphPad Prism version 8.02 (GraphPad Software, Inc., USA) was applied to plot the non-linear regressions and was also employed for statistical analysis using ANOVA with Tukey's post-hoc assessment in an alpha of 0.05 level of significance.

Results and Discussion

GC-EI-MS characterization of M1 fraction

GC-EI-MS was used to identify the chemical constituents present in the 15% ethyl acetate in PE of **M1** fraction. As observed in the GC chromatogram,

thirteen volatile components were identified and verified through the NIST library for spectral library matching and comparison of retention indices (RI) [14] (Figure 2). Identified compounds (Table 1) were listed according to their elution succession through a HP-5ms column. The **M1** fraction comprised the following constituents: tocopherols: [2R-[2R*(4R*,8R*)]]-2H-1-benzopyran-6-ol,3,4-dihydro-2,8-dimethyl-2-(4,8,12 trimethyl tetradecyl) or δ -tocopherol (3.36%), γ -tocopherol (7.40%), vitamin E (20.18%); diterpene: phytol (16.42%); diterpene acetate: phytol acetate (2.04%); and triterpenes: squalene (3.87%), γ -sitosterol (11.61%), β -amyrin (1.36%), lanosterol (3.87%), Lupeol (6.14%) and 3 β -D:B-friedo-B':A'-neogammacer-5-en-3-ol (6.81%); triterpene acetate:3 β -lanosta- -8-24-dien- 3-ol acetate (9.50%) and 3 β -lup-20(29)-en-3-ol acetate (6.81%).

Major classes of compounds that were detected from the **M1** fraction included triterpenes and tocopherols, which are known to have several biochemical properties. The constituents identified from the extract are also known to exhibit cholesterol-lowering, anti-inflammatory, antibacterial, anti-ulcerative, and antiproliferative effects.

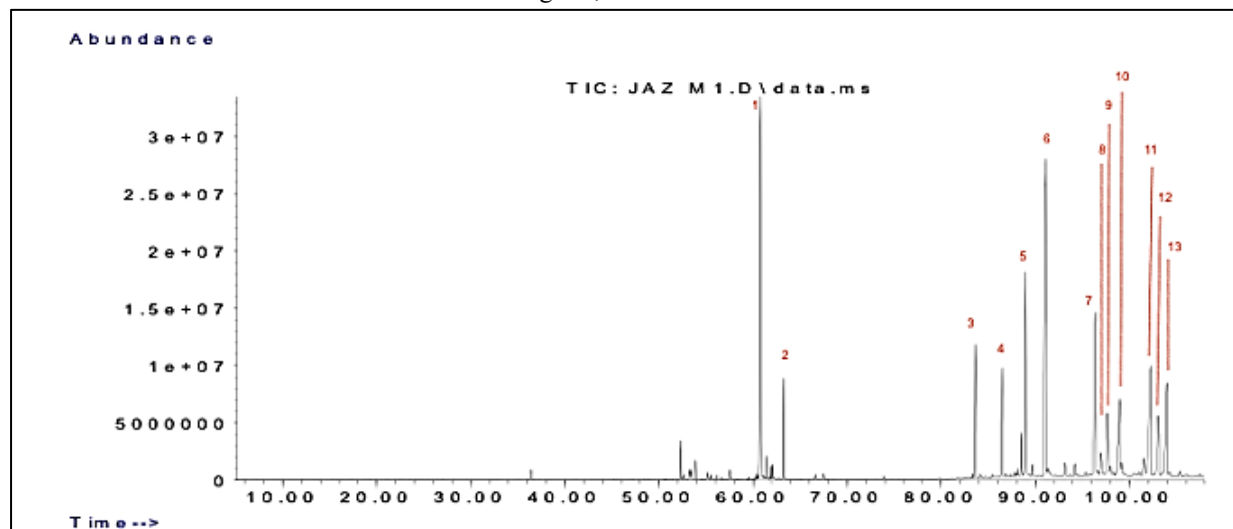


Figure 2. Total ion GCMS chromatogram of **M1**

Table 1. Constituents of **M1** fraction (15% ethyl acetate in PE)

	Compound	RT (min.)	RI ^a	% Peak Area	Functionality
1	Phytol	60.81	2117±0.25	16.42	Diterpene
2	Phytol Acetate	63.29	2119±0.00	2.04	Diterpene acetate
3	Squalene	83.62	2824±0.13	3.87	Triterpene
4	[2R-[2R*(4R*,8R*)]]-2H-1-Benzopyran-6-ol,3,4-dihydro-2,8-dimethyl- 2-(4,8,12-trimethyltridecyl)/ δ -tocopherol	86.46	2837±0.26	3.36	Tocopherol
5	γ -tocopherol	88.96	2843±0.23	7.40	Tocopherol
6	Vitamin E	91.15	3429±0.51	20.18	Tocopherol
7	γ -Sitosterol	96.40	3492±0.18	11.61	Triterpene
8	β -Amyrin	97.010	3408±0.07	1.36	Triterpene
9	Lanosterol	97.69	3423±0.15	3.87	Triterpene
10	Lupeol	98.98	3453±0.20	6.14	Triterpene
11	3 β -Lanosta-8-24-dien-3-ol acetate	102.30	3425±0.23	9.50	Triterpene acetate
12	3 β -D:B-Friedo-B':A'-neogammacer-5-en-3-ol	103.08	3439±0.11	4.77	Triterpene
13	3 β -Lup-20(29)-en-3-ol acetate	103.99	3456±0.16	6.81	Triterpene acetate

^aRetention Index (HP-5ms column)

Cytotoxicity assessment of **M1**:

The examination of the antiproliferative capacity of **M1** on normal HDFn cells ensued no observable cytotoxicity, which afforded an IC₅₀ value exceeding 100 μ g/mL. Figures 3 and 4 show graphical representations of the percent cell viability as a function of the logarithm (log₁₀) of the concentrations. The characteristic inhibitory dose-response sigmoidal curve or distribution was exhibited in the majority of the plots. Charts reflecting the inhibitors' cytotoxic action on the specific cell lines' viability are displayed in Figure 4. The half-maximal inhibitory concentrations (IC₅₀) of the agents used are presented in Table 3.

One-way ANOVA on HT-29 trials showed that the paired treatments of **M1** versus Zeocin were similar ($p > 0.05$) and **M1** versus DMSO were dissimilar ($p < 0.05$). Statistical evidence also pointed out that there was similarity in the mean of **M1** (M = 7.98, SE = 1.15) and the mean of Zeocin (M = 11.60, SE = 1.08) but was different for DMSO trials (M > 100, SE = 37.93). The outcome revealed that the cytotoxicity of the inhibitors **M1** and Zeocin was analogous.

HepG2 tests presented that **M1** cytotoxic action was akin to Zeocin activity and found no significant differences ($p > 0.05$) as seen in post hoc data. Conclusions for the nonlinear fitting of the log (inhibitor) vs. normalized response using variable slope analyses illustrated that **M1** (M = 7.09, SE = 1.13) and the mean of Zeocin (M = 1.05, SE = 1.05) were similar in action but was markedly unlike DMSO trials (M > 100, SE = 5.85).

The cytotoxic action of **M1** on BxPC-3 experiments was typical of Zeocin activity ($p > 0.05$) as established in multiple comparisons. The means and standard error of the **M1** trials in BxPC-3 cells using the negative control DMSO (M > 100, SE = 2.33) were seen to be non-cytotoxic to these cells, whereas **M1** (M = 16.89, SE = 1.07) and the mean of Zeocin (M = 11.83, SE = 1.05) were comparable and were significantly distinct to DMSO tests.

Data generated using the inhibitor **M1** on MCF-7 cell lines indicated a half-maximal inhibitory concentration

of 7.09 $\mu\text{g/mL}$ with a standard error of 1.07. Tukey's post hoc multiple comparison conveyed that there were considerable differences ($p < 0.05$) in the means of the inhibitors **M1** and Zeocin ($M = 8.85$, $SE = 1.06$) to the negative control DMSO ($M = >100$, $SE = 43.15$). MCF-7 treated with **M1** and Zeocin were construed to be statistically alike ($p > 0.05$).

One-way ANOVA analyses of the potential inhibitors on HeLa cells showed that the paired trials of **M1** vs. Zeocin were analogous ($p > .05$), and **M1** vs. DMSO were unrelated ($p < 0.05$). Similarity the means of **M1** ($M = 16.63$, $SE = 1.08$) and Zeocin ($M = 13.71$, $SE = 1.07$) were noticeably different for DMSO trials ($M > 100$, $SE = 1.96$). The cytotoxic capacity of the inhibitors **M1** and Zeocin corresponded to each other. THP-1 presented the following IC_{50} data on the inhibitors: **M1** ($M = 17.43$, $SE = 1.05$); Zeocin ($M = 8.15$, $SE = 1.05$); and DMSO ($M = >100$, $SE = 1.57$). Multiple comparisons or Tukey's post hoc analyses expressed considerable differences ($p < 0.05$) in **M1** vs. DMSO and Zeocin vs. DMSO trials. The antiproliferative activity of **M1** and Zeocin on acute monocytic leukemia cells were found to be similar.

M1 was not cytotoxic to normal HDFn, with an IC_{50} value greater than 100 $\mu\text{g/mL}$. Post hoc comparisons of **M1** and human cell lines trials established that there were substantial variances, and that the null hypothesis (one curve for all data sets) was excluded (log IC_{50} the equivalent for all data sets). This was confirmed by the extra sum-of-squares F-test of the best-fit-parameter (IC_{50} concentration) between individual cell line experiments, $F(6, 49) = 1.978$, $P = 0.0869$, and to the observations in the dose-response curve fits $F(12, 154) = 121.4$, $p < 0.0001$. Zeocin trials with each cancer cell line were observed to be similar ($p > 0.05$). Zeocin a recognized cytotoxic proponent furnished IC_{50} values of 4.40, 11.60, 6.10, 11.83, 8.85, 13.71 and 8.15 $\mu\text{g/mL}$ for HDFn, HT-29, HepG2, BxPC-3, MCF-7, HeLa, and THP-1, respectively and one-way ANOVA data disclosed similarity in the trials ($p > 0.05$). Post hoc comparison of the half-maximal inhibitory concentration for the cancer cell line treatments also verified the results' congruency.

M1 presented the highest effectiveness towards HepG2 with a half-maximal inhibitory concentration of 7.09 $\mu\text{g/mL}$ which was followed by MCF-7, HT-29, HeLa, and BxPC-3, which gave IC_{50} values of 7.12, 7.98, 16.63, 16.89 $\mu\text{g/mL}$, correspondingly. Substantial variances, as obtained in Tukey's post hoc multiple comparisons, were established in HT-29 vs. HDFn, HepG2 vs. HDFn, and MCF-7 vs. HDFn ($p < 0.05$). The other paired treatments were deduced to be statistically similar ($p > 0.05$). The figures of the subgroups of all the human cell lines and Zeocin demonstrated that the subcategories were comparable ($p > 0.05$). It was found that HepG2, MCF-7, and HT-29 cancer cell lines were positively affected by **M1** and exhibited minimal half-maximal inhibitory concentrations ($< 8 \mu\text{g/mL}$), which were acquired for all of the trials. HeLa, BxPC-3, and THP-1 were moderately responsive to **M1**. Normal HDFn trials treated with **M1** were substantiated to be non-cytotoxic and presented half-maximal inhibitory concentrations of more than 100 $\mu\text{g/mL}$; while Zeocin on normal cells were exceedingly damaging as manifested in the $IC_{50} = 4.40 \mu\text{g/mL}$.

Results obtained from the cell viability assay of **M1** can be explained by the bioactivities reported from previous studies suggesting that phytosterols and tocopherols present in the extract can affect the signal transduction pathway in cancer cells. Targeted analyses have shown that phytosterols and tocopherols can prevent angiogenesis and induce apoptosis in tumor cells by an up-/down-regulating expression such as NF- κ B [15] or by cell cycle arrest [16]. As observed in published studies, phytosterols are cytotoxic to common cancer cell lines such as breast (MCF-7) and colon (HT-29) which deemed phytosterols to be promising compounds against cancer. This is further confirmed by the ability of phytosterols to affect the following: inhibit angiogenesis; induce apoptosis (by controlling the cell proliferation process by cell cycle arrest); increase ceramide activities, protein phosphatase 2A (PP2A), and pro-apoptotic MAP kinase; and limiting the supply of nutrients to cancer cells [17, 18]. γ -Sitosterol was found to induce cell arrest in the G₂/M phase and cause apoptosis for MCF-7 and A549 cell lines by reducing the c-Myc expression [19]. The ability of phytosterols, such as those found in the isomers of sitosterol, to induce cell

arrest in the G₀/G₁ phase was believed to manifest the cytotoxic effect on the HT-29 cell line [20]. Another common phytosterol present in plants, lupeol, is known to have multiple-targeting capabilities that have the following properties: anti-inflammatory capability through different mechanisms; anti-diabetic effect by inhibiting the alpha-amylase; and anti-carcinogenic action as linked to the arrest of the cell cycle of cancer cells in G₂-M-phase. Lupeol was also established to induce apoptosis on different cancer cell lines through the following signal transduction pathways: Ras-induced protein kinase C-alpha/ornithine decarboxylase; p13K/Akt, MAPKs; and NF-κB signaling. Furthermore, lupeol activated an extrinsic apoptotic pathway by increasing the Fas receptor on prostate cancer. Lupeol has been heralded as a probable chemo preventive and anti-inflammatory agent by inhibiting hepatocellular carcinoma receptor 3 (DR 3) expression and reducing the production of IL-4 through T-helper type 2 cells, respectively [16].

The mechanisms linked to β-amyrin as an anti-inflammatory agent was found to be involved in the inhibition of NF-κB, activation of CREB, reduction of neutrophil filtration, curtailment of oxidative stress, and the depletion of the production of proinflammatory cytokine TNF-α for acute periodontal inflammation. Furthermore, β-amyrin induced a cytotoxic effect against human bladder cancer cells (NTUB1) when treated with cisplatin after 24 hrs. β-amyrin, which was isolated and purified from plants, hampered collagen-induced platelet aggregation with an IC₅₀ value of 4.5ug/mL, which was comparable to the effects of aspirin (11 μg/mL) [21]. A polyunsaturated hydrocarbon triterpene, squalene, found to be present in the bark extract has been known to be an essential precursor for synthesizing phytosterols in plants specifically growth hormones, terpenoids, sterol, steroids, pigments, essential oils, and other defense compounds in the body. While the acyclic structure of squalene is responsible for connecting the lipid layers to the biological membrane's intermembrane, the structure of the molecule can scavenge free radicals by either donating or receiving electrons thereby preventing bio membrane damage [22, 23].

In addition to triterpenes, diterpenes have also been reported to exhibit antitumor effects. Phytol and its derivatives have often been reported as notable constituents of essential oils present in plants. Aside from being one of the components used in the formulation of fragrances, phytol is known to have multiple therapeutic functions such as anti-inflammatory, immune-modulating, antimicrobial, and cytotoxic as well as apoptosis-inducing effects in cancer cells. Since PPARs and NF-κB are known to be important regulators of inflammatory, immune response, and antiproliferative agents of cancer, phytol is perceived to be a modulator in the treatment of degenerative diseases [15].

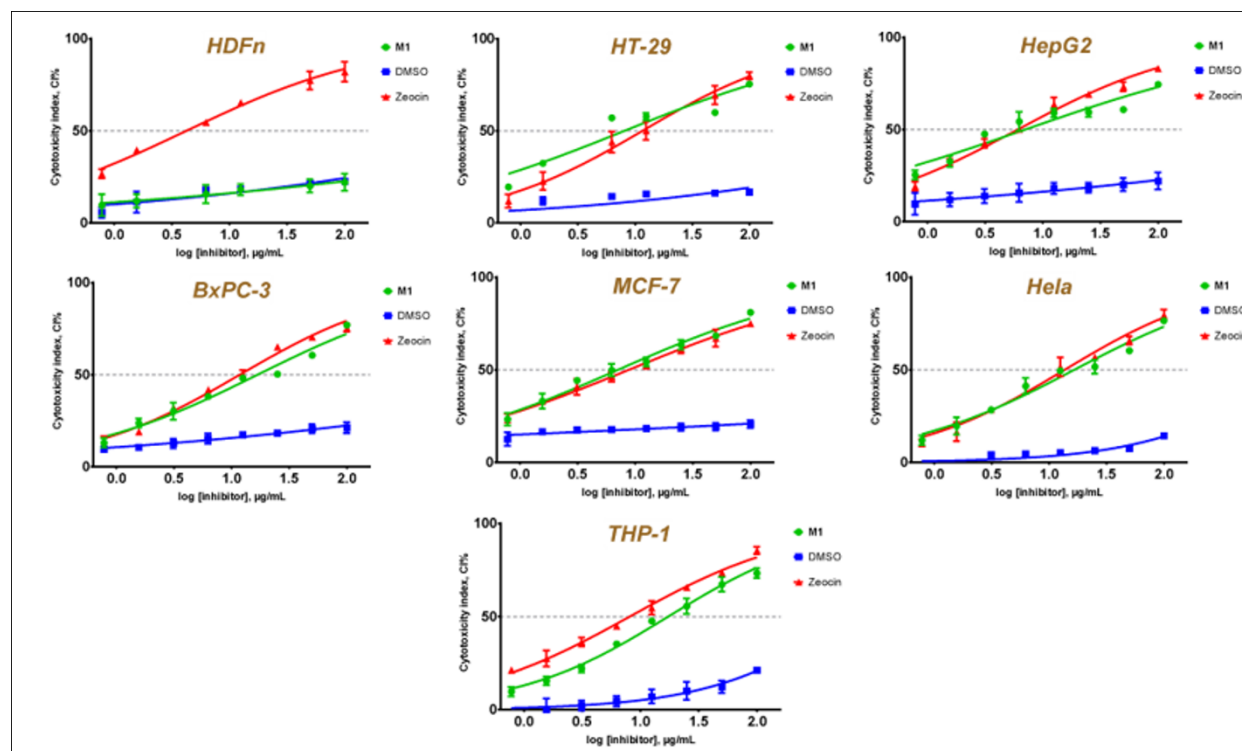
Tocopherols have been communicated to affect human mastocytoma (HMC-1), MCF-7, U937 myeloid leukemia, and HT-29 cell lines. Vitamin E inhibits the proliferation of U937 by inhibiting the formation of oxLDL-induced macrophage foam cells by affecting the two expressions, NF-κB pathway and P-selection [24]. Among all tocopherols, delta-tocopherol induced apoptosis to HMC-1 cells at high concentrations. The proinflammatory response of reactive oxygen species (ROS) and stress-activated expressions, Nrf2 and NF-κB, are enhanced by delta-tocopherol. Delta-tocopherol also prevented lipid accumulation and antiangiogenic effects. Some studies imparted evidence that vitamin E could inhibit the proliferation of vascular smooth muscle cells by preventing PKC phosphorylation and stimulating platelet-derived growth factors. In contrast, vitamin E with tocopherol associated proteins (TAP) and albumin, have molecular functions such as modulating gene expression by acting as a transporter of ligands to enzymes and regulating enzymes ligand transfer. These functions are usually observed for antioxidants [24]. In a separate study, vitamin E was found to function as a free radical scavenger which prevents the reduction of DNA oxidative damage as verified by the decline of urinary excretion of 8-oxodeoxyguanosine (a product of DNA oxidation) [24,25] The accompaniment of chronic inflammation with oxidative stress was hypothesized to provide a suitable environment for cancer development. By addressing the reduction of biochemical markers affiliated to cancer development, the selection of M1 in its potential herbal and pharmaceutical effects proves

promising [2,26].

Table 2. Cytotoxic Activities (IC_{50}) of Inhibitors Against HDFn, HT-29, HEPG2, BxPC-3, MCF-7, HELA and THP-1

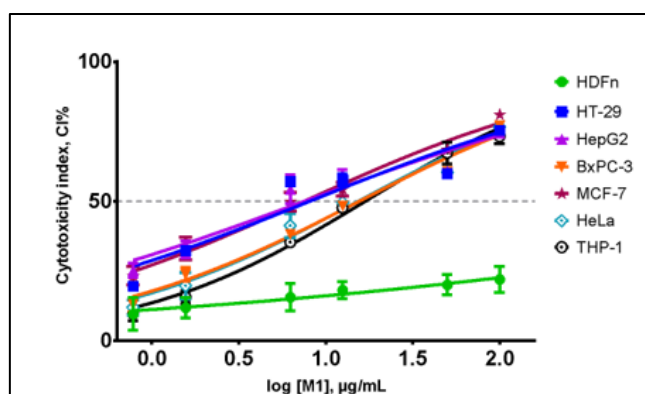
Cell Line	IC_{50}^* ($\mu\text{g/mL}$)		
	M1	DMSO	Zeocin
HDFn	>100	>100	4.40
HT-29	7.98	>100	11.60
HepG2	7.09	>100	6.11
BxPC-3	16.89	>100	11.83
MCF-7	7.12	>100	8.85
HeLa	16.63	>100	13.71
THP-1	17.43	>100	8.15

* IC_{50} values were extrapolated from dose-response curves calculated from nonlinear regression analysis using GraphPad Prism 8.02. One-way ANOVA was the treatment employed for each cell line to evaluate the significant variances among the data groups. The statistical analyses are as follows: HDFn, F (2, 21) = 32.06, $P < 0.0001$; HT-29, F (2, 21) = 11.65, $P = 0.0004$; HepG2, F (2, 21) = 14.23, $P = 0.0001$; BxPC-3, F (2, 21) = 6.586 $P = 0.0060$; MCF-7, F (2, 21) = 13.28, $P = 0.0002$, HeLa, F (2, 21) = 11.17, $P = 0.0005$, THP-1, F (2, 21) = 11.20, $P = 0.0005$.



Data are shown as mean $\pm$ SEM. GraphPad Prism 8.02 was used to perform extra sum-of-squares F-test to (A) evaluate the significance of the best-fit-parameter (half-maximal inhibitory concentration) among different treatments, and to (B) determine the differences among the dose-response curve fits. HDFn F(Dfn, DFd) = (A) F (2, 21) = 9.964, $P = 0.0009$ and (B) F (4, 66) = 616.9, $p < 0.0001$; HT-29 (A) F (2, 21) = 3.436, $P = 0.0512$ and (B) F (4, 66) = 149.6, $p < 0.0001$; HepG2 (A) F (2, 21) = 5.731, $P = 0.0103$ and (B) F (4, 63) = 370.9, $p < 0.0001$; BxPC-3 (A) F (2, 21) = 6.638, $P = 0.0058$ and (B) F (4, 66) = 480.9, $p < 0.0001$; MCF-7 (A) F (2, 21) = 6.602, $p = 0.0060$ and (B) F (4, 66) = 640.8, $p < 0.0001$; HeLa (A) F (2, 21) = 5.135, $P = 0.0153$ and (B) F (4, 66) = 468.8, $p < 0.0001$; THP-1 (A) F (2, 21) = 6.140, $P = 0.0080$ and (B) F (4, 66) = 699.3, $p < 0.0001$.

Figure 3. Dose-response curves showing the cytotoxic a of M1, DMSO, and Zeocin on the cell viability of HDFn, HT-29, HepG2, BxPC-3, MCF-7, HeLa, and THP-1. Each plot displays the effect of **M1** and controls against each cell line



Each plot displays the effect of **M1** against each cell line. Data are shown as mean $\pm$ SEM. GraphPad Prism version 8.02 was used to perform extra sum-of-squares F-test to (A) evaluate the significance of the best-fit-parameter (half maximal inhibitory concentration) among different treatments, and to (B) determine the differences among the dose-response curve fits. The results are: **M1**, (A) $F(6, 49) = 1.978$, $P = 0.0869$ (B) $F(12, 154) = 121.4$, $p < 0.0001$.

Figure 4. Dose-response curves showed the cytotoxic activities of M1 on the cell viability of HDFn, HT-29, HepG2, BxPC-3, MCF-7, HeLa, and THP-1

Conclusion

M1 was found to be rich in phytosterols and tocopherols, which have been reported to be anticancer agents. This thereby presents *M. rubra* bark as a potential raw material for such applications. The most responsive cancer cells to **M1** were HepG2, MCF-7, and HT-29. HeLa, BxPC-3, and THP-1 were moderately responsive to **M1**. Normal HDFn trials treated with **M1** were not affected by the inhibitor, whereas Zeocin was highly cytotoxic to wild type HDFn cells. Due to the low toxicity of **M1** to normal human dermal fibroblasts, we the researchers recommend that further bioassays be performed to reveal the signaling pathways that could be modulated in aberrant cells and that *in vivo* assays also be done to substantiate *Morus rubra* as a possible anticancer agent.

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