Malaysian Journal of Analytical Sciences (MJAS)
Published by Malaysian Analytical Sciences Society

CYTOTOXICITY EFFECT OF NATURAL AND SYNTHETIC GIRINIMBINES AND THEIR DERIVATIVES AGAINST HUMAN LUNG CANCER CELL LINES A549

(Kesan Sitotoksik Girinimbin Semula Jadi dan Sintetik dan Terbitannya Terhadap Sel Garis Kanser Paru-Paru Manusia A549)

Fatin Nurul Atiqah Osman[1], Siti Mariam Mohd Nor[1]*, Mohd Azlan Nafiah[2]

[1]Department of Chemistry, Faculty of Science,
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Department of Chemistry, Faculty of Science and Mathematics,
Universiti Pendidikan Sultan Idris, 35900 Tanjung Malim, Perak, Malaysia

**Corresponding author: smariam@upm.edu.my*

Received: 8 September 2021; Accepted: 18 November 2021; Published: xx December 2021

Abstract

The present study was designed to evaluate the anticancer properties of girinimbine and its derivatives. Two different routes for the synthesis of girinimbine involving a two-step reaction or a one-pot reaction were studied. Girinimbine was synthesised through metal-catalysed heterocoupling, indole ring closure, ether formation, and Claisen cyclisation. Girinimbine derivatives were prepared by the semi-synthesis of isolated girinimbine from *Murraya koenigii* through alkylation or acylation reactions. Natural and synthetic girinimbines, five derivatives of *N*-substituted girinimbine, and three intermediates from the synthesis of girinimbine were evaluated for cytotoxicity activity against human lung cancer (A549) and normal lung (MRC-5) cell lines. The structures of all the synthesised compounds were confirmed by spectroscopic analysis and comparison with published data. The cytotoxicity assay showed that the natural girinimbine and nitrobiphenyl intermediate exhibited high toxicity (IC_{50} 6.2 and 17.0 µg/mL, respectively), whereas other compounds displayed moderate toxicity activity (IC_{50} 24.0–40.6 µg/mL) on A549 cells. All of the compounds demonstrated selectivity to A549 cancer cell lines with the SI values ranging from 2.70 to 4.68 (SI > 2), except for two *N*-alkylated girinimbines with the SI values of 0.93 and 1.70.

Keywords: girinimbine, derivative, *N*-alkylated, *N*-acylated, A549

Abstrak

Kajian ini telah dibentuk untuk menilai sifat antikanser girinimbin dan terbitannya. Dua laluan berbeza untuk mensintesis girinimbin melibatkan tindak balas dua-langkah atau tindak balas satu-bekas telah dikaji. Sintesis girinimbin telah dicapai melalui penggandingan hetero bermangkin logam, penutupan gelang indol, pembentukan eter dan pensiklikan Claisen. Terbitan girinimbin telah disediakan melalui sintesis-semi girinimbin terpencil daripada *Murraya koenigii* melalui tindak balas pengalkilan atau pengasilan. Girinimbin semula jadi dan sintetik, lima terbitan girinimbin tertukar ganti-*N* dan tiga bahan perantara daripada kerja sintesis telah dinilai untuk aktiviti sitotoksik terhadap sel garis kanser paru-paru manusia (A459) dan paru-paru normal (MRC-5). Kesemua struktur sebatian yang disintesis telah disahkan melalui analisis spektroskopik dan

perbandingan dengan data yang telah diterbitkan. Assai kesitotoksikan menunjukkan girinimbin semula jadi dan bahan perantara bifenilnitro telah memaparkan ketoksikan tinggi (IC_{50} 6.2 and 17.0 μg/mL) manakala sebatian yang lain menunjukkan aktiviti ketoksikan sederhana (IC_{50} 24.0-40.6 μg/mL) ke atas sel A549. Kesemua sebatian menunjukkan kepilihan kepada sel garis kanser A459 dengan nilai SI dalam julat daripada 2.70-4.68 (SI > 2) kecuali dua girinimbin teralkil-*N* dengan nilai SI 0.93 dan 1.70.

Kata kunci: girinimbin, terbitan, teralkil-*N*, terasil-*N*, A549

Introduction

Cancer is one of the major diseases that cause many deaths worldwide with an estimated 19.29 million new cases and 9.96 million deaths in 2020. Lung cancer alone contributed to 11.4% of new cases and 18% of deaths [1]. Lung cancer is the third most common cancer in Malaysia with 19.8% of deaths. Males are the largest proportion to suffer from lung cancer and 92% is attributed to the smoking habit, which is the main factor for lung cancer. The increasing incidence of cancers in the country is due to increasing ageing population, exposure to cancer risk, and unhealthy lifestyle. The increasing trend is also due to late diagnosis and limited treatment availability. New cases of lung cancer are comparatively higher in the age group of 45 years old [2]. According to the Malaysian Study on Cancer Survival (MySCan) 2018, lung cancer recorded the lowest survival rate among all cancers due to being diagnosed at an advanced stage. Moreover, lung cancer patients also recorded the lowest survival rate compared to other types of cancers (11.0% for 1-year and 35.5% for 5-year) [3].

This evidence shows that cancer is an urgent matter that requires much attention, especially better prevention, early detection, easy access and available treatment, and cancer care services. In terms of cancer treatment, advancements in research and drug development are a few approaches to developing a potential drug and creating a variety of anticancer drugs. A new approach of anticancer discovery has been taken over by natural products due to their structural diversity, such as varied substituent groups from simple alkyls, rings, or attachment of terpene of various complexity and length, and also the presence of various functional groups that can be further modified to enhance their activity.

In the last few decades, the medicinal role of natural and semi-synthetic carbazoles has expanded significantly. Carbazoles, which is an attractive compound to be introduced in the pharmaceutical industry as a candidate for drug development, have a broad scope of biological actions. Girinimbine (**8**) is a carbazole mainly found in *Murraya koenigii* or locally known as curry tree. This plant is native to Malaysia and can be used as herbs, spices, and condiments (food and flavour), to treat illness in the traditional way (medicinal) and make soaps or insect repellents (source for materials). Girinimbine (**8**) has drawn the attention of researchers due to its wide range of pharmacological effects, such as anti-inflammatory [4], anticancer [5-9], antiplatelet [10], antioxidant [4,11], and exhibited strong activity against *Aedes aegypti* [12]. Several syntheses of 8 have been reported involving reaction with ylide [13], base-induced cyclisation [14], oxidative intramolecular allylation [15, 16], Buchwald-Hartwig amination [17], Ti-mediated annulation [18], decarboxylative allylation/Claisen rearrangement [19], and Grignard addition and ring-closing metathesis [20].

To further explore the potential anticancer properties of carbazole compounds, the cytotoxicity of five girinimbine derivatives (**9**-**13**) was evaluated on human lung cancer (A549) and normal lung (MRC-5) cell lines. All *N*-alkylated (**9**-**11**) and *N*-acylated derivatives (**12** and **13**) were prepared *via* semi-synthetic modification of girinimbine (**8i**) isolated from *M. koenigii*, as previously reported by our group [21]. Currently, there is no data on the effect of all these derivatives (**9**-**13**) on A549 and MRC-5 cell lines. A comparative study between the isolated girinimbine (**8i)** and synthesised girinimbine (**8s**) would also be an interesting field to explore. In this study, **8s** was synthesised *via* two different routes using established

protocols, including indole ring closure, aryl coupling, and thermal cyclisation. The cytotoxicity effects were also compared with all intermediates produced in the synthesis of girinimbine. The compounds **8i** and **8s** would act as the references for the cytotoxicity properties of the derivatives.

Materials and Methods

General

All chemicals and solvents used in this study are commercially available. All reactions were monitored using thin-layer chromatography (TLC) and silica gel 60 F245 (Merck KGaA) precoated aluminium-backed plates and visualised under ultraviolet light (UVP, UV Lamp UVGL-58) prior to dipping in H_2SO_4. The solvents were removed under vacuo by an EYELA rotary evaporator N-100 or a Buchi=poiuuu9 rotavapor R-215. The organic phases were dried under Na_2SO_4. Column chromatography was performed with 100–150 mesh of silica gel 60 (0.040–0.063 mm) as the stationary phase. The digital melting point equipment (Electrothermal IA9000 Series) was used to measure the melting point and the measurement was repeated at least three times for each compound. The IR spectra were obtained by Perkin-Elmer FT-IR Model Spectrum 100 series spectrophotometer using UATR techniques. The MS spectra were obtained from a Shimadzu model QP5050A spectrometer. 1D and 2D NMR spectra were recorded with a JEOL FT-NMR 500 MHz spectrophotometer using $CDCl_3$. The chemical shifts (δ) were recorded in ppm relative to the TMS signal described with an appropriate abbreviation for multiplicities as s (singlet), d (doublet), t (triplet), and m (multiplet), whereas the coupling constants J are given in Hz.

Isolated natural girinimbine (**8i**) from the bark of *M. koenigii* was obtained from the laboratory of the co-researchers in the Department of Chemistry, Universiti Pendidikan Sultan Idris, Malaysia for data comparison and the semi-synthesis of girinimbine derivatives (**9**-**13**) as previously reported [21].

Synthesis of Girinimbine (8s): 3-Methyl-2'-nitrobiphenyl-4-ol (3)

A mixture of $^{i}Pr_2NEt$ (0.43 mmol), $Pd(OAc)_2$ (0.43 mmol), nBu_4NBr (0.43 mmol), 1-bromo-2-nitrobenzene (**1**) (1.71 mmol), and 4-iodo-2-methylphenol (**2**) (0.43 mmol) was refluxed in *p*-xylene (10 mL) at 130°C for 3 days. The mixture was then allowed to warm to room temperature before the addition of water (10 mL) and ether (10 mL). The separated organic phase was washed with water (10 mL), dried, and evaporated. The product was purified using column chromatography (hexane-ethyl acetate, 9:1).

Compound **3**: Yellow oil (81 mg, 83%); IR (UATR) 3479, 3063, 2928, 1609, 1518, 1354, 1275, 1211 cm^{-1}; δ_H (500 MHz, $CDCl_3$) 7.77 (1H, d, J 8.0 Hz, H-aromatic), 7.55 (1H, t, J 8.0 Hz, H-aromatic), 7.41 (1H, t, J 8.0 Hz, H-aromatic), 7.07 (1H, s, H-aromatic), 7.01 (1H, d, J 8.0 Hz, H-aromatic), 7.00 (1H, d, J 8.0 Hz, H-aromatic), 6.78 (1H, d, J 8.0 Hz, H-aromatic), 5.00 (1H, br. s, OH), 2.25 (3H, s, CH_3); δ_C (125 MHz, $CDCl_3$) 154.3, 149.6, 136.2, 132.3, 132.1, 130.8, 129.8, 127.8, 126.9, 124.6, 124.2, 115.5, 16.0; m/z (EI) 229 (M^+, $C_{13}H_{11}NO_3$ requires 229).

2-Hydroxy-3-methylcarbazole (6)

Route 1: 3-Methyl-2'-nitrobiphenyl-4-ol (**3**) (0.35 mmol), $SnCl_2{\cdot}2H_2O$ (2.18 mmol), and EtOH (3 mL) were refluxed at 60°C for 6 h. The mixture was poured into cold water (10 mL) and $NaHCO_3$ was added to reach pH 7. The precipitate was filtered and the aqueous layer was extracted with ethyl acetate (3 × 10 mL). The combined organic phase was dried and evaporated. The crude product was purified using column chromatography (chloroform-ethyl acetate, 9.5:0.5).

Route 2: 1,2-Dibromobenzene (**4**) (0.81 mmol) and 5-amino-2-methylphenol (**5**) (0.81 mmol) were added into toluene (3 mL). Then, CuBr (0.41 mmol), DMEDA (0.2 mmol), K_2CO_3 (0.81 mmol), $Pd(dppf)Cl_2$ (0.41 mmol), and KOAc (1.22 mmol) were added to the solution. The reaction mixture was refluxed under a nitrogen atmosphere at 120°C for 3 days. After 3 days, the reaction mixture was cooled to room temperature

and washed with ethyl acetate (3 × 10 mL), dried, and filtered. The combined organic layer was concentrated using rotary evaporation and the residue was purified by column chromatography (hexane-ethyl acetate, 7:2).

Compound **6**: Brown solid (Route 1: 62 mg, 90%; Route 2: 77 mg, 48%); mp 242–243°C (lit. 245°C) [22]; IR (UATR) 3375, 2923, 2857, 1606, 1493, 1268, 753 cm^{-1}; δ_H (500 MHz, $CDCl_3$) 6.97 (1H, br. s, NH), 6.92 (1H, t, J 9.2 Hz, H-aromatic), 6.87 (1H, d, J 9.2 Hz, H-aromatic), 6.71 (1H, s, H-aromatic), 6.69 (1H, s, H-aromatic), 6.67 (1H, d, J 8.0 Hz, H-aromatic), 6.60 (1H, d, J 8.0 Hz, H-aromatic), 2.10 (3H, s, CH_3); δ_C (125 MHz, $CDCl_3$) 155.7, 145.1, 132.3, 131.9, 131.1, 129.6, 128.6, 128.2, 125.9, 119.5, 116.9, 115.8, 16.2; m/z (EI) 197 (M^+, $C_{13}H_{11}NO$ requires 197).

3-Methyl-2-[(-methylbut-3-yn-2-yl)oxy]-9H-carbazole (7)

2-Hydroxy-3-methylcarbazole (**6**) (0.57 mmol) was added to anhydrous acetonitrile (4 mL) under nitrogen atmosphere and cooled at 0°C before the addition of DBU (0.74 mmol), $CuCl_2 \cdot 2H_2O$ (0.01 mmol), and 3-chloro-3-methyl-1-butyne (0.74 mmol). The reaction mixture was stirred at 0°C for 5 h. The residue was partitioned between water (10 mL) and toluene (20 mL). The organic fraction was washed with 1 N HCl (10 mL), 1 N NaOH (10 mL), 1 N $NaHCO_3$ (10 mL), and brine (10 mL). The organic layer was dried, filtered, and concentrated. The crude product was purified by column chromatography (hexane-ethyl acetate, 9:1).

Compound **7**: Brown solid (96 mg, 64%); mp 121–123°C (lit. 121–124°C) [18]; IR (UATR) 3409, 3361, 2924, 2856, 1510, 1447, 1377, 1132, 754 cm^{-1}; δ_H (500 MHz, $CDCl_3$) 7.49 (1H, d, J 8.0 Hz, H-aromatic), 7.19 (1H, br. s, NH), 7.17 (1H, s, H-aromatic), 7.15 (1H, s, H-aromatic), 7.11 (1H, t, J 8.0 Hz, H-aromatic), 6.78 (1H, t, J 8.0 Hz, H-aromatic), 6.73 (1H, d, J 8.0 Hz, H-aromatic), 3.75 (1H, s, C≡CH), 2.33 (3H, s, CH_3), 1.68 (6H, s, 2 × CH_3); δ_C (125 MHz, $CDCl_3$) 153.7, 143.8, 133.0, 131.5, 130.6, 128.3, 126.8, 119.5, 118.8, 115.6, 97.8, 91.9, 79.9, 73.0, 30.0, 20.2, 17.2; m/z (EI) 263 (M^+, $C_{18}H_{17}NO$ requires 263).

Girinimbine (8s)

3-Methyl-2-[(-methylbut-3-yn-2-yl)oxy]-9H-carbazole (**7**) (0.76 mmol) was refluxed in toluene (2 mL) for 29 h. The reaction mixture was concentrated to dryness and purified by column chromatography (hexane-ethyl acetate, 9:1).

Compound **8s**: White solid (10 mg, 50%); mp 175–176°C (lit. 175–177°C) [23]; IR (UATR) 3420, 3313, 2967, 1638, 1449, 1315, 1149, 744 cm^{-1}; δ_H (500 MHz, $CDCl_3$) 7.89 (1H, d, J 6.9 Hz, H-aromatic), 7.87 (1H, br. s, NH), 7.65 (1H, s, H-aromatic), 7.36 (1H, d, J 8.0 Hz, H-aromatic), 7.28 (1H, t, J 8.0 Hz, H-aromatic), 7.15 (1H, t, J 6.9 Hz, H-aromatic), 6.61 (1H, d, J 10.3 Hz, HC=CH), 5.68 (1H, d, J 10.3 Hz, HC=CH), 2.31 (3H, s, CH_3), 1.47 (6H, s, 2 × CH_3); δ_C (125 MHz, $CDCl_3$) 150.0, 139.7, 135.0, 129.6, 124.5, 124.1, 121.4, 119.7, 119.5, 118.8, 117.5, 117.0, 110.6, 104.7, 76.1, 27.8, 16.3; m/z (EI) 263 (M^+, $C_{18}H_{17}NO$ requires 263).

Cytotoxicity: Cell culture

The lung cancer (A549) and normal lung (MRC-5) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cell lines were cultured in RPMI 1640 media supplemented with 10% foetal calf serum (FCS) and 1% penicillin-streptomycin. The cells were maintained and incubated at 37°C in a 5% CO_2 atmosphere. Cell viability was determined by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and compared with the untreated controls.

MTT assay

A stock solution of 30 mg/mL of the compounds was prepared by dissolving the stock solution with DMSO. Subsequently, different concentrations of the compound were prepared by dissolving the stock solution with a culture medium. DMSO only (0.5%, v/v) was used as a control in a complete culture medium. Cells were seeded at 5 × 10^3 cells/well in 96-well plates (100 µL/well). After 24 h of incubation, the cells were treated with different concentrations of the compounds (100, 50, 25, 12.5, 6.25, 3.125, and 1.56 µg/mL) and incubated for 72 h. MTT (5 mg/mL in PBS) was added to each well in the plate and incubated

for 4 h. The supernatants were aspirated and DMSO (100 µL) was added to each well to solubilise the insoluble formazan blue crystals. The absorbance was measured at 570 nm using a Thermo Labsystems Opsys MR microplate spectrometer. All samples were presented as mean ± standard deviation for three measurements. The percentage of cell viability was calculated using the equation (1).

The IC_{50} was obtained by plotting the cell viability (%) versus concentration (µg/mL) of each compound. The value range of the cytotoxicity is based on the U.S. National Cancer Institute (NCI) and GERAN protocol, in which IC_{50} < 21 µg/mL = highly cytotoxic, IC_{50} 21–200 µg/mL = moderately cytotoxic, IC_{50} 201-500 µg/mL = weakly cytotoxic, and IC_{50} > 500 µg/mL = no cytotoxicity [24-27].

One-way analysis of variance (ANOVA) with Tukey's honest significance test was used to assess the significance of differences, with *p*-values of less than 0.05 considered statistically significant.

$$\text{Cell viability} = \frac{\text{Average absorbances of triplicate treated cells}}{\text{Average absorbances of control cells}} \times 100\% \quad (1)$$

Results and Discussion

Synthesis of girinimbine

2-Hydroxy-3-methylcarbazole (**6**) was synthesised either *via* a two-step reaction (Figure 1, Route 1) or a one-pot reaction (Figure 1, Route 2). In Route 1, the synthesis approach involved a biaryl coupling reaction between **1** and **2** to produce an intermediate nitrobiphenyl **3**, followed by a reduction with $SnCl_2 \cdot 2H_2O$ to produce an indole **6** (90%). In Route 2, indole **6** was directly obtained (48%) from the coupling of 1,2-dibromobenzene (**4**) and 5-amino-2-methylphenol (**5**) *via* the formation of a C-N bond and a C-C bond by Pd-catalysed and Cu-catalysed reactions, respectively. The IR spectra showed a broad absorption at 3479 and 3375 cm^{-1}, indicating the OH and the overlapping of OH and NH stretching for **3** and **6,** respectively, whereas the strong absorption of conjugated N-O in **3** was displayed at 1518 cm^{-1}. The NMR spectra showed a singlet of aromatic protons for **3** and **6** at δ 7.07, 6.71, and 6.69 ppm, whereas quaternary carbons of **3** and **6** were confirmed at δ 115.5–154.3 ppm and δ 115.8–155.7 ppm.

Indole **6** was then treated with 3-chloro-3-methyl-1-butyne using $CuCl_2 \cdot 2H_2O$ as a catalyst in the presence of a base, DBU, to produce ether **7**. The absorption of C-H of alkyne was shown at 3361 cm^{-1}. According to the ^{1}H-NMR spectrum, the singlet of the alkyne proton and the methyl protons of aromatic were observed at δ 3.75 and 2.23 ppm, respectively. In addition, the broad peak of NH appeared at δ 7.19 ppm. The ^{13}C-NMR spectrum of **7** showed seven quaternary carbons in the range of δ 153.7–73.0 ppm. The alkyne carbons were assigned to the signals at δ 30.0 and 79.9 ppm, while the signal of methyl carbons was observed at δ 20.2 and 17.2 ppm.

The synthesis of girinimbine (**8s**) was then accomplished by refluxing **7** in toluene to form a pyran ring (Figure 1). Girinimbine (**8s**) was obtained as a white solid with a melting point of 175–176 °C (lit. 175–177 °C) [16]. The IR spectrum showed absorption at 3420 cm^{-1}, which indicated the presence of a secondary amine. Two absorptions of aromatic C=C could be observed at 1638 and 1449 cm^{-1}, while C-O absorption appeared at 1149 cm^{-1}. It gave a molecular ion peak at m/z 263 in the MS spectrum, thus reflecting the molecular formula of $C_{18}H_{17}NO$. According to the ^{1}H-NMR spectrum, a broad singlet of NH appeared at δ 7.87 ppm, whereas a singlet of aromatic proton was observed at δ 7.65 ppm. Two doublet signals of *cis* protons of an alkene appeared at δ 6.61 and 5.68 ppm with a coupling constant of 10.3 Hz. The ^{13}C-NMR spectrum showed the presence of eight quaternary carbons at δ 150.0, 139.7, 135.0, 124.1, 118.8, 117.0, 104.7, and 76.1 ppm. Two signals of alkene carbon could be observed at δ 117.5 and 129.6 ppm, while the

other three signals of the methyl groups appeared in the upfield region, δ 27.8 ppm, and δ 16.3 ppm.

The NMR data of the synthesised girinimbine (**8s**) is in agreement with the isolated girinimbine (**8i**) and the literature (Table 1). The ^{1}H-NMR of **8s** shows a signal of NH at δ 7.87 ppm, H4 at δ 7.65 ppm, and H8 at δ 7.36 ppm, which were comparable with **8i** (NH at δ 7.84 ppm, H4 at δ 7.67 ppm, and H8 at δ 7.35 ppm), and Sukari's data (NH at δ 7.84 ppm, H4 at δ 7.66 ppm, and H8 at δ 7.36 ppm) [23]. Similar ^{13}C-NMR data were also observed, such as C-1a at δ 139.7 ppm (**8s**), δ 139.7 ppm (**8i**), and δ 139.6 ppm [23]. The signal for C-8a of **8s** was resonance at δ 135.0 ppm, which is in the range of **8i** and the reference compound at δ 139.4–135.0 ppm.

N-substituted girinimbine derivatives (**9**-**13**) were synthesised and characterised as previously described by our group [21]. All of the intermediates (**3**, **6** and **7**), natural and synthesised girinimbines (**8i** and **8s**), and its *N*-alkylated and *N*-acylated derivatives (**9**-**13**) were subjected to a cytotoxicity assay that was cultured with lung cancer (A549) and normal lung (MRC-5) cell lines.

Cytotoxicity assay of girinimbine and its derivatives

The cytotoxicity effect of natural girinimbine (**8i**) and all of the synthesised compounds (**3**, **6**, **7**, **8s**, **9**-**13**) (Figures 1 and 2) was evaluated using human lung cancer cell lines (A549) and normal lung cell lines (MRC-5) using the MTT method to examine their potential as therapeutic alternatives in cancer treatment. The data for MTT assay after 72 h of exposure were plotted in the graph of the percentage of viable cells versus concentration of the compounds (μg/mL) to identify their IC_{50} values (Figure 3). All compounds were found to inhibit the growth and reduce the viability of the A549 and MRC-5 cell lines in a concentration-dependent manner. In the absence of the compounds (0 μg/mL), the cells represent 100% viability. The plot demonstrates that all semi-synthesised derivatives **9**-**13** showed less inhibition compared to their parent compound **8i**.

All of the synthesised compounds, including naturally isolated girinimbine (**8i**), exhibited toxic activities with IC_{50} values in the range of 6.2–40.6 μg/mL against lung cancer cell lines (Table 2). Both **8i** and the intermediate nitrobiphenyl **3** with IC_{50} 17.0 and 6.2 μg/mL showed highly cytotoxic and potent compounds. Based on these experimental data, the IC_{50} values of both compounds were comparable with doxorubicin (13.1 μg/mL) and the reported **8i** (8.33 μg/mL) [5]. The presence of the nitro group in **3** seemed to increase the activity of the compound against A549 cell lines [28]. It was observed that the intermediates **3**, **6**, and **7** exhibited a slightly higher cytotoxic activity against A549 cells compared to the synthesised girinimbine (**8s**). However, all girinimbine derivatives (**9**-**13**) showed no significant cytotoxicity effect compared to the synthesised girinimbine (**8s**) (IC_{50} 33.0 μg/mL) against A549 cells, except *N*-acylated girinimbine **12** (IC_{50} 25.5 μg/mL). *N*-alkylated girinimbine **11** and *N*-acylated girinimbine **13** showed less potent activity towards A549 cell lines compared to other derivatives within the group due to the branched alkyl substituent. Alkyl and benzyl substituents have been proven to enhance cytotoxic activities, as seen in derivatives **9** and **10** [29, 30]. In comparison, derivative **12** showed stronger cytotoxicity than derivative **10** probably due to the presence of a carbonyl group in the structure [31, 32]. It was observed that the synthesised girinimbine (**8s**) exhibited a 5-fold higher IC_{50} value and was less toxic compared to the isolated girinimbine (**8i**) in A549 cells with the concentration of 33.0 and 6.2 μg/mL, respectively. Due to the limited amount of **8s**, the cytotoxicity of this compound was unrepeated. Factors that could be considered for the lower toxicity of **8s** were probably the presence of the trace of impurity from the synthesis work or the solvent used for purification. Besides, the systematic errors in IC_{50} measurement could mislead the effects due to uneven proliferation [33].

The normal lung cell lines (MRC-5) were used to measure the selectivity index (SI). The SI values can be used to identify whether the compounds are specifically cytotoxic to target cancer cells without displaying toxicity or very minimal toxicity towards

normal cells. The range of the SI values are SI < 2 = non-selective (toxic) and SI > 2 = selective (non-toxic) [34,35]. The larger the SI values, the more toxic the compounds towards cancer cells and safer towards normal cells. From the results, the SI values of all compounds exceeded 2, indicating that the compounds were selective towards A549 cancer cells with the SI values ranging from 2.70 to 4.68, except *N*-alkylated girinimbines **9** and **11** (Table 2). The isolated girinimbine (**8i**) and intermediate nitrobiphenyl **3** with the SI values of 4.68 and 4.56, respectively, are the most toxic compounds compared to other compounds. This indicates that their cytotoxicity activity is selective for the lung cancer cells A549 and can act as an anticancer drug without causing excessive damage to the normal cells. These compounds can be further investigated for lead therapeutic agents. However, derivatives **9** and **11** displayed no selectivity between cancer and normal cells due to the low SI values of 1.70 and 0.93, respectively.

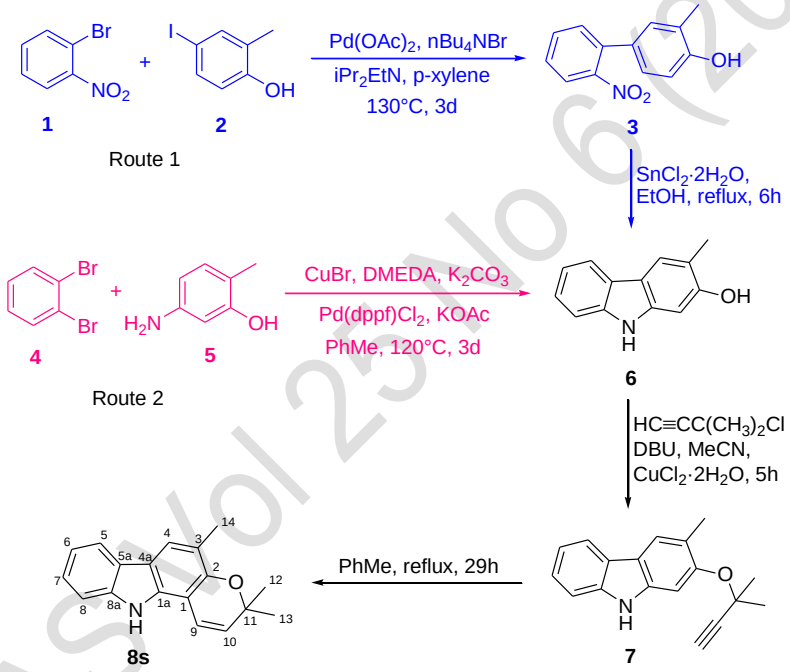

Figure 1. Synthesis of girinimbine (**8s**). Two different synthetic approaches to produce an intermediate indole **6** were applied *via* a two-step reaction (Route 1) or a one-pot reaction (Route 2)

Table 1. Comparison of ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) data for girinimbines

C/H	δ_H ($CDCl_3$, ppm, J in Hz)			δ_C ($CDCl_3$, ppm)		
	8s	**8i**[a]	**Lit.**[b]	**8s**	**8i**[a]	**Lit.**[b]
1	-	-	-	104.7	104.6	104.6
1a	-	-	-	139.7	139.7	139.6
2	-	-	-	150.0	150.0	149.8
3	-	-	-	124.1	124.1	123.9
4	7.65 (1H, s)	7.67 (1H, s)	7.66 (1H, s)	121.4	121.3	121.3
4a	-	-	-	117.0	116.9	116.9
5	7.89 (1H, d, J 6.9)	7.92 (1H, d, J 6.9)	7.92 (1H, d, J 8.3)	119.5	119.5	119.4
5a	-	-	-	118.8	118.8	118.6
6	7.15 (1H, t, J 6.9)	7.18 (1H, t, J 6.9)	7.19 (1H, t, J 8.8)	119.7	119.7	119.6
7	7.28 (1H, t, J 8.0)	7.31 (1H, t, J 8.0)	7.31 (1H, t, J 8.2)	124.5	124.5	124.4
8	7.36 (1H, d, J 8.0)	7.35 (1H, d, J 8.0)	7.36 (1H, d, J 8.3)	110.6	110.6	110.3
8a	-	-	-	135.0	135.0	134.9
9	6.61 (1H, d, J 10.3)	6.58 (1H, d, J 9.8)	6.60 (1H, d, J 9.2)	117.5	117.4	117.3
10	5.68 (1H, d, J 10.3)	5.67 (1H, d, J 9.8)	5.69 (1H, d, J 9.2)	129.6	129.6	129.5
11	-	-	-	76.1	76.1	75.6
12	1.47 (3H, s)	1.49 (3H, s)	1.47 (3H, s)	27.8	27.8	27.4
13	1.47 (3H, s)	1.49 (3H, s)	1.47 (3H, s)	27.8	27.8	27.4
14	2.31 (3H, s)	2.34 (3H, s)	2.33 (3H, s)	16.3	16.3	16.1
NH	7.87 (1H, br. s)	7.84 (1H, br. s)	7.84 (1H, br. s)	-	-	-

[a] Girinimbine (**8i**) was isolated from the bark of *M. koenigii* for this study

[b] Sukari et al. [23]

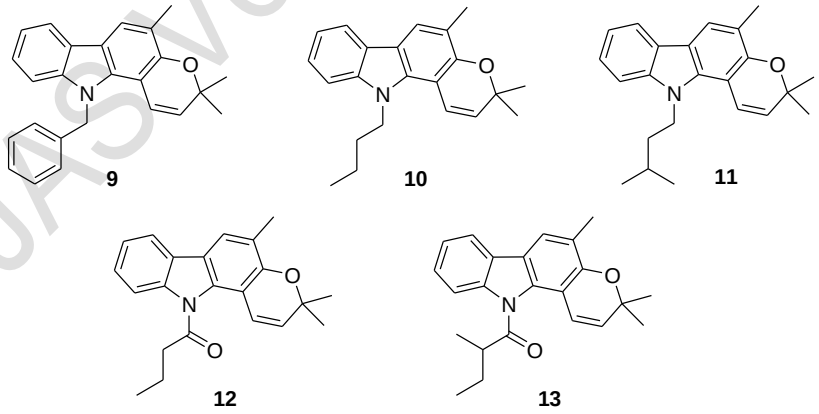

Figure 2. *N*-Alkylated and *N*-acylated girinimbines for cytotoxicity assay

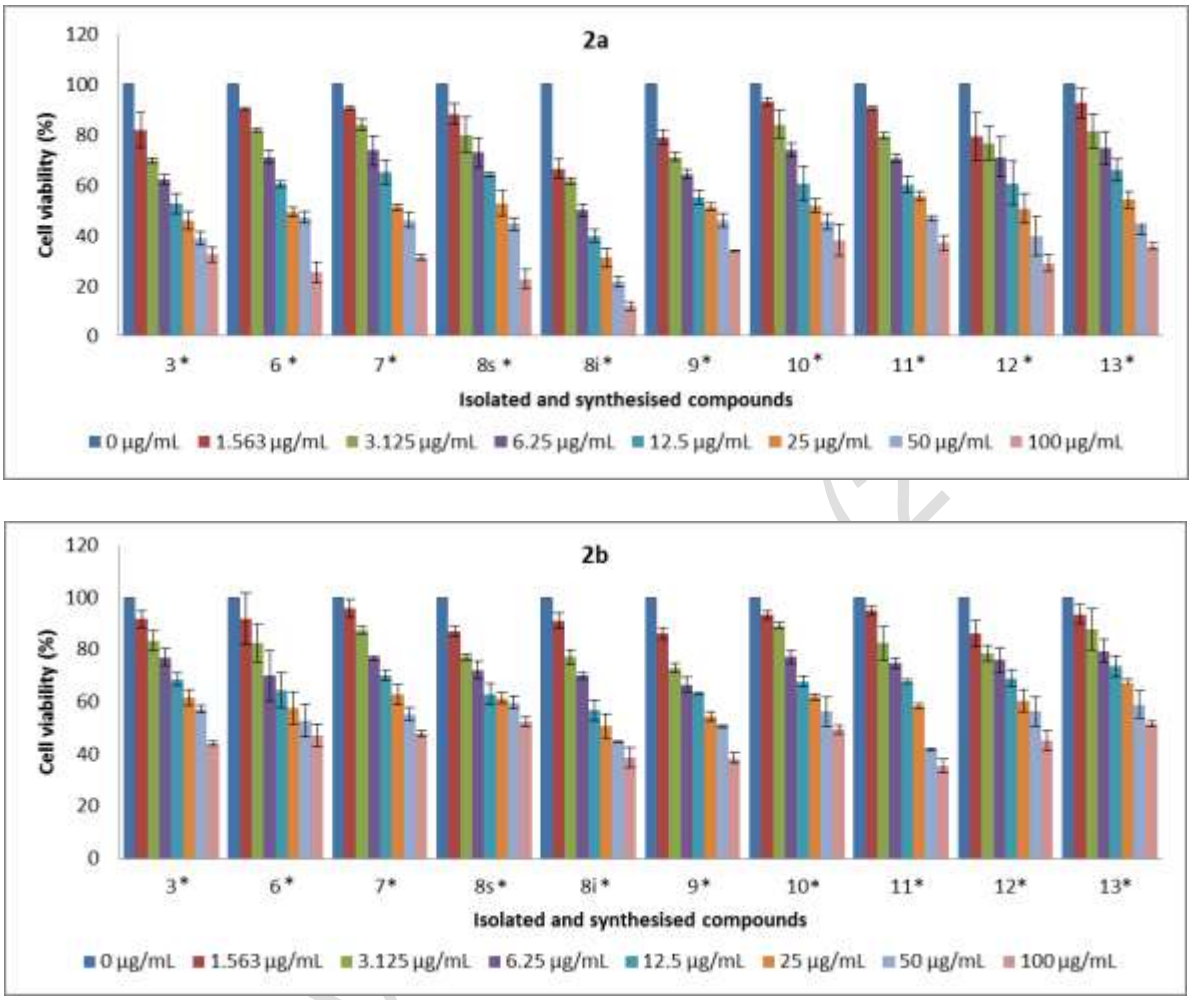

Figure 3. Cytotoxicity effect of isolated and synthesised compounds against lung cancer (A549) cell lines (2a) and normal lung (MRC-5) cell lines (2b). Results are presented as mean ± SD (n = 3). * $p < 0.05$ compared with the non-treated sample (0 µg/mL). All treated samples with isolated and synthesised compounds at all concentrations were statistically significant

Table 2. Cytotoxicity activity of the compounds against lung cancer (A549) and normal lung (MRC-5) cell lines

Compound		IC_{50} (µg/mL)		Selectivity Index (SI)[b]
		Lung Cancer (A549)	Normal Lung (MRC-5)	
Synthesised Intermediates	3	17.0 *	77.6	4.56
	6	24.0 *	71.0	2.96
	7	31.8 *	86.0	2.70
Girinimbines	8s	33.0 *	> 100	> 3.03
	8i[a]	6.2	29.0	4.68
N-Alkylated Girinimbines	9	31.0 *	52.7	1.70
	10	31.2 *	96.1	3.08
	11	40.6 *	37.7	0.93
N-Acylated Girinimbines	12	25.5 *	77.3	3.03
	13	35.7 *	> 100	> 2.80
Doxorubicin	-	13.1	Not Tested[c]	-

[a] Girinimbine (**8i**) was isolated from the bark of *M. koenigii* for this study

[b] SI = IC_{50} value for MRC-5/ IC_{50} value for A549. * Significant difference between structurally modified/synthesised and parent compound 8**i** ($p < 0.05$)

[c] There were several issues regarding the purchasing of doxorubicin during the COVID-19 pandemic, where the test was done without the normal cells

Conclusion

Girinimbine (**8s**) was successfully synthesised *via* two different routes and characterised. All of the synthesised intermediates (**3**, **6**, and **7**), synthesised and natural girinimbines (**8i** and **8s**), *N*-alkylated girinimbines (**9**-**11**), and *N*-acylated girinimbines (**12** and **13**) were evaluated for cytotoxicity assay against lung cancer (A549) and normal lung (MRC-5) cell lines. All of the compounds reduced the viability of A549 and MRC-5 cells in a dose-dependent manner. All compounds exhibited moderate to high toxicity against A549 cells. The intermediate nitrobiphenyl **3** and natural girinimbine (**8i**) displayed the highest toxicity and were comparable with doxorubicin. All compounds showed selectivity against A549 cells with SI > 2, with isolated girinimbine (**8i**) and intermediate **3** as the most toxic compounds (SI values of 4.68 and 4.56, respectively). However, *N*-alkylated girinimbines **9** and **11** exhibited no selectivity towards A549 cells.

Acknowledgement

The authors sincerely thank the financial support provided by Universiti Putra Malaysia (Geran GP-IPS: 9436717). The authors also thank Dr. Mohd Azlan Nafiah and the late Dr. Kartini Ahmad for providing the isolated girinimbine for the NMR data comparison and for the semi-synthesis of girinimbine derivatives.

References

1. International Agency for Research on Cancer (2020). The global cancer observatory (Globocan 2020). Access from https://gco.iarc.fr/today/home. [Access online 15 February 2021].
2. Ministry of Health Malaysia (2017). National Strategic Plan for Cancer Control Programme 2016-2020. Access from https://www.moh.gov.my/index.php/dl/554756755a584a6961585268626938794d4445334c314a31616e567259573476546d4630615739755957786655335279595852 6c5a326c6a5831427359573566516d39766131396d6157356862454 1794e564e46554651794d4445334c6e426b5a673d3d. [Access online 23 January 2021].
3. Ministry of Health Malaysia (2018). Malaysian study on cancer survival (MyScan). Access from https://www.moh.gov.my/moh/resources/Penerbitan/Laporan/Umum/Malaysian_Study_on_Cancer_Survival_MySCan_2018.pdf. [Access online 23 January 2021].

4. Shah, A. S., Wakade, A. S. and Juvekar, A. R. (2008). Immunomodulatory activity of methanolic extract of *Murraya koenigii* (L) Spreng leaves. *Indian Journal of Experimental Biology*, 46(7): 505-509.
5. Mohan, S., Abdelwahab, S. I., Cheah, S. C., Sukari, M. A., Syam, S., Shamsuddin, N. and Rais Mustafa, M. (2013). Apoptosis effect of girinimbine isolated from *Murraya koenigii* on lung cancer cells *in vitro*. *Evidence-Based Complementary and Alternative Medicine: eCAM*, 2013: 689865.
6. Iman, V., Mohan, S., Abdelwahab, S. I., Karimian, H., Nordin, N., Fadaeinasab, M., Noordin, M. I. and Noor, S. M. (2016). Anticancer and anti-inflammatory activities of girinimbine isolated from *Murraya koenigii*. *Drug Design, Development and Therapy*, 11: 103-121.
7. Syam S., Abdul, A. B., Sukari, M. A., Mohan, S., Abdelwahab, S. I. and Wah, T. S. (2011). The growth suppressing effects of girinimbine on Hepg2 involve induction of apoptosis and cell cycle arrest. *Molecules*, 16(8): 7155-7170.
8. Mahamadhanif, S. S., Neeta, T., Rajshekhar, K., Rajesh, A. R., Mahes, B. P., Faya, A. M., Harun, M. P., Wesam, S. A., Kavita, J. and Girish, A. H. (2015). Current perspective of natural alkaloid carbazole and its derivatives as antitumor agents. *Anti-Cancer Agents in Medicinal Chemistry*, 15(8): 1049-1065.
9. Wang, S. L., Cai, B., Cui, C. B., Yan, S. Y. and Wu, C. F. (2007). Induction of apoptosis by girinimbine in K562 cell. *Chinese Traditional and Herbal Drugs*, 38(11): 1677-1681.
10. Ko, F. N., Lee, Y. S., Wu, T. S. and Teng, C. M. (1994). Inhibition of cyclooxygenase activity and increase in platelet cyclic AMP by girinimbine, isolated from *Murraya euchrestifolia*. *Biochemical Pharmacology*, 48(2): 353-360.
11. Kok, Y. Y., Mooi, L. Y., Ahmad, K., Sukari, M. A., Mat, N., Rahmani, M. and Ali, A. M. (2012). Anti-tumour promoting activity and antioxidant properties of girinimbine isolated from the stem bark of *Murraya koenigii* S. *Molecules*, 17(4): 4651-4660.
12. Sukari, M. A., Noor, H. M., Bakar, N. A., Ismail, I. S., Rahmani, M. and Abdul, A. B. (2013). Larvicidal carbazole alkaloids from *Murraya koenigii* against dengue fever mosquito *Aedes aegypti* Linnaeus. *Asian Journal of Chemistry*, 25(14): 7719-7721.
13. Joshi, B. S., Kamat, V. N. and Gawad, D. H. (1970). On the structures of girinimbine, mahanimbine, isomahanimbine, koenimbidine and murrayacine. *Tetrahedron*, 26(5): 1475-1482.
14. Bergman, J. and Carlss, R. (1978). Conversion of diindolyl methanes to 3-vinylindoles. A simple synthesis of the indole alkaloid olivacine. *Tetrahedron Letters*, 19(42): 4055-4058.
15. Knölker, H. J. and Hofmann, C. (1996). Transition-metal complexes in organic-synthesis. 33. Molybdenum-mediated total synthesis of girinimbine, murrayacine and dihydroxygirinimbine. *Tetrahedron Letters*, 37(44): 7947-7950.
16. Gruner, K. K., Hopfmann, T., Natsumoto, K., Jager, A., Katsuki, T. and Knölker, H. J. (2011). Efficient iron-mediated approach to pyrano[3,2-*a*]carbazole alkaloids - first total syntheses of *O*-methylmurrayamine A and 7-methoxymurrayacine, first asymmetric synthesis and assignment of the absolute configuration of (-)-*trans*-dihydroxygirinimbine. *Organic and Biomolecular Chemistry*, 9(7): 2057-2061.
17. Knölker, H. J. (2009). Synthesis of biologically active carbazole alkaloids using selective transition-metal-catalyzed coupling reactions. *Chemistry Letters*, 38(1): 8-13.
18. Hesse, R., Gruner, K. K., Kataeva, O., Schmidt, A. W. and Knölker, H. J. (2013). Efficient construction of pyrano[3,2-*a*]carbazoles: application to a biomimetic total synthesis of cyclized monoterpenoid pyrano[3,2-*a*]carbazole alkaloids. *Chemistry (Weinheim an der Bergstrasse, Germany)*, 19(42): 14098-14111.
19. Dai, J., Ma, D., Fu, C. and Ma, S. (2015). Gram scale total synthesis of 2-hydroxy-3-methylcarbazole, pyrano[3,2-a]carbazole and prenylcarbazole alkaloids. *European Journal of Organic Chemistry*, 2015 (25): 5655-5662.
20. Mandal, T., Chakraborti, G., Karmakar, S. and Dash, J. (2018). Divergent and orthogonal approach to carbazoles and pyridoindoles from oxindoles *via* indole intermediates. *Organic Letters*, 20(16): 4759-4763.
21. Nor, S. M. M., Osman, F. N. A., Ahmad, K. and Nafiah, M. A. (2017). *Synthesis of N-alkylated and N-acylated derivatives of girinimbine*. *Asian Journal of Chemistry*, 29 (4): 801-804.
22. Bhattacharyya, P., Jash, S. S. and Chowdhury, B. K. (1986). A biogenetically important carbazole alkaloid from *Murraya koenigii* Spreng. *Chemistry and Industry*, 7: 246.

23. Abu Bakar, N. H., Sukari, M. A., Rahmani, M., Md. Sharif, A., Khalid, K. and Yusuf, U. K. (2007). Chemical constituents from stem barks and roots of *Murraya koenigii* (Rutaceae). *Malaysian Journal of Analytical Sciences*, 11(1): 173-176.
24. Nguyen, N. H., Ta, Q. T. H., Pham, Q. T., Luong, T. N. H., Phung, V. T., Duong, T. H. and Vo, V. G. (2020). Anticancer activity of novel plant extracts and compounds from *Adenosma bracteosum* (Bonati) in human lung and liver cancer cells. *Molecules, 25 (12):* 2912-2928.
25. Kuete, V., Ango, P. Y., Yeboah, S. O., Mbaveng, A. T., Mapitse, R., Kapche, G. D., Ngadjui, B. T. and Efferth, T. (2014). Cytotoxicity of four Aframomum species (*A. arundinaceum*, *A. alboviolaceum*, *A. kayserianum* and *A. polyanthum*) towards multi-factorial drug resistant cancer cell lines. *BMC Complementary and Alternative Medicine*, 14: 340.
26. Goldin, A., Venditti, J. M., Macdonald, J. S., Muggia, F. M., Henney, J. E. and Devita Jr, V. T. (1981). Current results of the screening program at the Division of Cancer Treatment, National Cancer Institute. *European Journal of Cancer*, 17 (2): 129-142.
27. Geran, R. I., Greenberg, N. H., Macdonald, M. M., Shumacher, A. M. and Abbott, B. J. (1972). Protocols for screening chemical agents and natural products against animal tumors and other biological systems. *Cancer Chemotherapy Reports, Part III*, 3: 1-103.
28. Nepali, K., Lee, H. Y. and Liou, J. P. (2019). Nitro-group-containing drugs. *Journal Medicine Chemistry*, 62: 2851-2893.
29. Bashir, M., Bano, A., Ijaz, A. S. and Chaudhary, B. A. (2015). Recent developments and biological activities of N-substituted carbazole derivatives: a review. *Molecules*, 20 (8): 13496-13517.
30. Liu, K., Zang, Y., Shen, C., Li, C., Ma, J., Yang, J., Sun, X., Chen, X., Wang, N. and Zhang, D. (2021). Synthesis and biological evaluation of pyranocarbazole derivatives as anti-tumor agents. *Bioorganic and Medicinal Chemistry Letters*, 33: 127739.
31. Kolli, S. K., Prasad, B., Babu, P. V., Ashfaq, M. A., Ehtesham, N. Z., Raju, R. R. and Pal, M. (2014). TFAA/H_3PO_4 mediated unprecedented *N*-acylation of carbazoles leading to small molecules possessing anti-proliferative activities against cancer cells. *Organic and Bimolecular Chemistry*, 12 (32): 6080-6084.
32. Kaushik, K., Kumar, N. and Pathak, D. (2012). Synthesis of some newer carbazole derivatives and evaluation for their pharmacological activity. *Der Pharmacia Sinica*, 3(4): 470-478.
33. He, Y., Zhu, Q., Chen, M., Huang, Q., Wang, W., Li, Q., Huang, Y. and Di, W. (2016). The changing 50% inhibitory concentration (IC_{50}) of cisplatin: a pilot study on the artifacts of the MTT assay and the precise measurement of density-dependent chemoresistance in ovarian cancer. *Oncotarget*, 7(43): 70803-70821.
34. Badisa, R. B., Darling-reed, S. F., Joseph, P., Cooperwood, J. S., Latinwo, L. M. and Goodman, C. B. (2009). Selective cytotoxic activities of two novel synthetic drugs on human breast carcinoma MCF-7 Cells. *Anticancer Research*, 29(8): 2993-2996.
35. Chipoline, I. C., da Fonseca, A.C.C., da Costa, G. R. M., de Souza, M. P., Rabelo, V. W. H., de Queiroz, L. N., de Souza, T. L. F., de Almeida, E. C. P., Abreu, P. A., Pontes, B., Ferreira, V. F., de Carvalho da Silva, F. and Robbs, B. K. (2020). Molecular mechanism of action of new 1,4-naphthoquinones tethered to 1,2,3-1H-triazoles with cytotoxic and selective effect against oral squamous cell carcinoma. *Bioorganic Chemistry*, 101: 103984.