CYTOTOXICITY AND GENOTOXICITY OF *MAHANIMBINE AND GIRINIMBINE*, PURE COMPOUNDS FROM *MURRAYA KOENIGGII* ON *ACANTHAMOEBA* SPP. AND HUMAN CORNEAL EPITHELIAL CELL LINE

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Affectionately dedicated to

My loving children
Who bring joy and happiness throughout my life

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Mahanimbine (MH) and girinimbine (GR) are two carbazole alkaloids isolated from a local plant species, *Murraya koenigii* of the Rutaceae family. Both compounds have been shown to have insecticidal, antimicrobial, antifungal and anticancer properties. The aim of the present study was to assess the growth inhibitory mechanisms of MH and GR through cytotoxicity and genotoxicity approaches on four isolates of *Acanthamoeba*, causative agents for *Acanthamoeba* keratitis namely *A. castellanii* (IMR isolate), *A. polyphaga* (CCAP 1501/3A), *Acanthamoeba* sp. (HKL isolate) and *Acanthamoeba* sp. (SW isolate). The amoebae were treated with MH and GR at concentrations of 0.47 to 30 µg/ml for 72 h and the IC_{50} value was assessed based on viable cells by using eosin dye technique. The IC_{50} values obtained for MH against *A. castellanii* was 1.18 µg/ml (3.5 µM), *A. polyphaga* was 1.00 µg/ml (3.0 µM), *Acanthamoeba* sp. (HKL isolate) was 20.6 µg/ml (62.2 µM) and *Acanthamoeba* sp. (SW isolate) was 7.2 µg/ml (21.7 µM). The IC_{50} values for GR against A. *castellanii* was 3.04 µg/ml (11.1 µM), A. *polyphaga* was 2.00

µg/ml (7.6 µM), *Acanthamoeba* sp. (HKL isolate) was 6.8 µg/ml (25.8 µM) and *Acanthamoeba* sp. (SW isolate) was 4.2 µg/ml (15.9 µM). Light and scanning electron microscopy observation employed in this study displayed decreasing number of acanthapodia on the amoeba surface and most cells were in cystics appearance. Transmission electron microscopy observation revealed changes at the ultrastructural level mainly on nuclear, mitochondrial, lipid droplets and endoplasmic reticulum structure in compounds-treated *Acanthamoeba* cells. Fluorescence microscopy by acridine orange and propidium iodide (AO/PI) staining revealed the occurrence of condensed and fragmented *Acanthamoeba* chromatin proved the apoptosis mode of cell death, active in lysosomal activity due to acidification of the lysosomes (autophagy), as well as leakage of plasma membrane or necrosis after treatment with the compounds. In general, high percentage of apoptotic amoebae were observed in both compounds-treated *Acanthamoeba*. Total genomic DNA extraction on agarose gel electrophoresis confirmed the presence of fragmented *Acanthamoeba* DNA as indication of apoptosis mode of cell death in all isolates of *Acanthamoeba* treated with the compounds. Smeary pattern background signified the cell death due to necrosis however was also observed in compounds-treated *A. polyphaga* (CCAP 1501/3A) and *Acathamoeba* sp. (SW isolate). The genotoxic potential of MH and GR were also observed in all isolates of *Acanthamoeba* after 2 h treatment with the compounds at their IC_{25} values by alkaline comet assay. Results showed that 50 to 90 % of *Acanthamoeba* DNA were damaged (type 3 and 4) with different distributions of score in all treatments and all isolates of *Acanthamoeba* ($p< 0.05$). In order to establish a safety profile of MH and GR,

cytotoxicity (MTT, [4,5-dimethlthizol-2-yl]-,5-diphenyltetrazolium bromide] metabolic assay and AO/PI staining) and genotoxicity assessments on non-target cell, human cornea epithelial cells (HCEC) were also carried out. Cell proliferation assay confirmed the difference in degree of anti-proliferative effects of the two compounds after 24, 48 and 72 h observation. Observation by light microscopy after treatment with the compounds revealed distinctive effects of the compounds with apoptotic criteria cell morphology such as membrane blebbing and condensation of chromatin structure. Fluorescence microscopy after AO/PI staining observations clearly showed the early and late apoptosis in compounds-treated HCEC. Interestingly, the compounds exhibited no genotoxic potential on HCEC after 2h treatment although at *Acanthamoeba* spp.'s IC_{30} values where more than 95% intact DNA were observed. In conclusion, MH and GR have very potent anti-amoebic potential on *Acanthamoeba* spp. as shown by cytotoxicity and genotoxicity analyses. Both compounds however, also exhibited their cytotoxic potential but not genotoxic on non-target cell such as HCEC.

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SITOTOKSISITI DAN GENOTOKSISITI OLEH MAHANIMBINE DAN GIRINIMBINE, SEBATIAN ASLI DARI *MURRAYA KOENIGII* TERHADAP *ACANTHAMOEBA* SPP. DAN JUJUKAN SEL EPITELIUM KORNEA MANUSIA

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Mahanimbine (MH) dan girinimbine (GR) adalah dua alkaloid karbazol yang telah dipencilkan daripada tumbuhan tempatan iaitu *Murraya koenigii* dalam keluarga Rutacea. Kedua-dua sebatian ini mempunyai ciri-ciri untuk membunuh serangga, sebagai antimikrob, antikulat dan antikanser. Tujuan kajian ini dijalankan adalah untuk menentukan mekanisme asas terhadap rencatan pertumbuhan oleh MH dan GR melalui ujian sitotoksisiti dan genotoksisiti ke atas empat pencilan *Acanthamoeba,* agen penyebab keratitis *Acanthamoeba* iaitu *A. castellanii* (pencilan IMR), *A. polyphaga* (CCAP 1501/3A), *Acanthamoeba* sp. (pencilan HKL) dan *Acanthamoeba* sp. (pencilan SW). Keempat-empat pencilan tersebut telah dirawat dengan MH dan GR selama 72 jam pada kepekatan 0.47 hingga 30 µg/ml untuk mendapatkan nilai kepekatan 50% rencatan pertumbuhan (IC_{50}). Nilai IC_{50} yang diperolehi untuk MH melalui teknik pewarnaan eosin untuk menentukan sel viabel terhadap *A. castellanii* adalah 1.18 µg/ml (3.5 µM), 1.00 µg/ml (3.0 µM) terhadap *A. polyphaga*, 20.6 µg/ml (62.2 µM) terhadap *Acanthamoeba*

sp. (pencilan HKL) manakala 7.2 µg/ml (21.7 µM) terhadap *Acanthamoeba* sp. (pencilan SW). Nilai IC_{50} yang diperolehi apabila dirawat dengan MH terhadap *A. castellanii* (pencilan IMR) adalah 3.04 µg/ml (11.1 µM), 2.00 µg/ml (7.6 µM) terhadap *A. polyphaga* (CCAP 1501/3A), 6.8 µg/ml (25.8 µM) terhadap *Acanthamoeba* sp. (pencilan HKL) manakala 4.2 µg/ml (15.9 µM) terhadap *Acanthamoeba* sp. (pencilan SW). Pemerhatian melalui mikroskop cahaya dan mikroskop pengimbas elektron terhadap *Acanthamoeba* spp. yang dirawat dengan kedua-dua sebatian tersebut menunjukkan perubahan bilangan akantopodia pada permukaan amoeba dan kebanyakan amoeba berubah ke bentuk serupa sista. Mikroskop transmisi elektron pula menunjukkan perubahan selepas dirawat terutamanya ke atas struktur nukleus, mitokondria, titisan lipid dan struktur retikulum endoplasma. Pemerhatian melalui mikroskop pendaran dengan pewarnaan akridin oren dan propidium iodida (AO/PI) menunjukkan keadaan kromatin *Acanthamoeba* tersejat dan berbentuk fragmen membuktikan kematian amoeba secara apoptosis, aktiviti yang aktif dalam lisosom (proses autofagi) serta kebocoran membran plasma *Acanthamoeba* atau nekrosis, setelah dirawat dengan kedua-dua sebatian tersebut. Peratus amoeba yang mengalami apoptosis yang tinggi dapat dilihat dalam semua pencilan *Acanthamoeba* yang digunakan dalam kajian ini. Elektroforesis gel agarose menggunakan ekstrak keseluruhan DNA yang dijalankan untuk melihat kehadiran fragmentasi ke atas DNA *Acanthamoeba* spp. yang dirawat mengesahkan kematian *Acanthamoeba* secara apoptosis. Latar belakang yang kabut menunjukkan kematian secara nekrosis juga dapat dilihat ke atas *A. polyphaga* (CCAP 1501/3A) dan *Acanthamoeba* sp. (pencilan SW) yang

dirawat menggunakan sebatian. Potensi genotoksisiti oleh MH dan GR juga dilihat ke atas semua pencilan *Acanthamoeba* selepas 2 jam dirawat dengan sebatian-sebatian tersebut menggunakan nilai IC_{25} secara asai komet beralkali. Keputusan asai ini menunjukkan 50 hingga 90 % DNA *Acanthamoeba* mengalami kerosakan dengan taburan skor yang berbeza dalam setiap rawatan ke atas semua pencilan *Acanthamoeba*. Bagi menentukan profil yang selamat untuk MH dan GR, pendekatan sitotoksisiti (MTT dan AO/PI) dan genotoksisiti (asai komet beralkali) juga dijalankan ke atas sel epitelium kornea manusia (HCEC). Asai kebolehbiakan HCEC menunjukkan kedua-dua sebatian merencat pertumbuhan selepas 24, 48 dan 72 jam rawatan. Pemerhatian melalui mikroskop cahaya pula menunjukkan ciri-ciri sel HCEC yang mengalami apoptosis selepas dirawat dengan kedua-dua sebatian tersebut. Pemerhatian melalui mikroskop pendaran menyokong permerhatian sebelumnya apabila dapat dilihat dengan jelas kombinasi sel apoptosis peringkat awal dan akhir selepas rawatan. Walaubagaimanapun, kedua-dua sebatian tidak menunjukkan kesan genotoksik selepas dirawat selama 2 jam walaupun pada kepekatan IC_{30} sel *Acanthamoeba* dimana lebih 95 % DNA yang padat dapat dilihat. Ini disebabkan oleh pengkhususan sel HCEC adalah berbeza dengan *Acanthamoeba,* haiwan eukariot unisel. Kesimpulannya, MH dan GR adalah dua sebatian anti-amoeba yang sangat kuat aktivitinya ke atas sel *Acanthamoeba* berdasarkan analisis sitotoksisiti dan genotoksisiti. Kedua-dua sebatian juga menunjukkan kesan sitotoksisiti ke atas HCEC tetapi tiada kesan genotoksik.