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BIOAUTOGRAPHY, COMBINATION EFFECTS AND PHOTO-ACTIVATED ENZYMATIC RESTRICTION INHIBITORY ACTIVITY OF ANTIMICROBIAL ALKALOIDS FROM GLYCOSMIS PENTAPHYLLA (RETZ.) DC.

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Abstract: *Glycosmis pentaphylla* (Retz.) DC., locally known as nerapan, has long been used in Asian countries as a traditional remedy for ailments attributed to microbial infections. This study aims to isolate antimicrobial alkaloids from *G. pentaphylla*, to determine their combination effects with selected antimicrobial agents and to screen for their photoactivated enzymatic restriction inhibitory activity. Bioautography-guided isolation of antimicrobial alkaloids was performed by using column chromatography with *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* as the indicator microbes. The antimicrobial effects of the alkaloids combined with selected antimicrobial agents, namely, ciprofloxacin, erythromycin, vancomycin, and ketoconazole, were determined by using a checkerboard assay. Photoactivated enzymatic restriction inhibitory activity was assessed by using agarose gel electrophoresis. Two antimicrobial active alkaloids were isolated and identified as arborinine and arborine. The antimicrobial activity of arborinine and arborine was determined to be in the range of 250 µg/ml and 1000 µg/ml. Partial synergy was observed for all arborine-antibiotics and arborinine-ketoconazole interactions against *S. aureus* and *C. albicans*, respectively. Arborine was relatively the strongest photoactivated enzymatic restriction inhibitor, particularly against EcoRI, PstI, and SalI. The results obtained are promising and encourage further research on the alkaloids as potential antimicrobial-enhancing agents.

Keywords: *Alkaloids, antimicrobial, bioautography, Glycosmis pentaphylla, photo-activated enzymatic restriction inhibition*

1. Introduction

The emergence of microbial resistance has been a threat in therapies for microbial infections. This has led to an interest in plant-based antimicrobial agents and synergistic antimicrobial enhancers, as plants are great sources of bioactive compounds (Cheesman et al., 2017; Khameneh et al., 2019). The antimicrobial combination is a promising strategy aimed at increasing treatment efficacy and controlling resistance prevalence. The subsequent reduction in the side effects of highly toxic antimicrobial agents is expected through efficacy enhancement, allowing the use of a drug in low dosages (van Vuuren & Viljoen, 2011; Bollenbach, 2015). Although plant-based antimicrobials are considered milder in terms of effectiveness than conventional antimicrobial agents, their various mechanisms of action render microbes less likely to develop resistance (Bhardwaj et al., 2016). Alkaloids possessing acridone and quinazolinone nuclei are among the active scaffolds that have inspired researchers in the search for new antimicrobial agents due to their promising activity (Asif, 2014; He et al., 2017). Naturally, these compounds exist abundantly in the species of the Rutaceae family (da Silva et al., 2017).

Glycosmis pentaphylla (Retz.) DC. is a shrub or small tree of the Rutaceae family known by its common name orangeberry, or nerapan in Malay. It is also called a toothbrush plant for the use of its mild astringent and bitter fibrous stems as a toothbrush (Hazarika & Dutta, 2013). The species is wildly found in secondary thickets of low and medium altitudes from India to Sri Lanka, South East Asia including Peninsular Malaysia, Sumatra and Java, Southern China, and north eastern Australia, and is also grown elsewhere (Chua & van Valkenberg, 2001; Wang et al., 2006). *G. pentaphylla* has long been widely used in various traditional medicine preparations including Ayurvedic medicine to cure diarrhea, coughs, rheumatism, anemia, and jaundice. The leaf and root are used in remedies for treating fever, liver complaints, bronchitis, eczema, skin affections, inflammation, stomach pain, bilious attacks, and intestinal trouble and are also taken as an appetizer during confinement (Chua & van Valkenberg, 2001; Aye et al., 2019; Mat Salleh & Latiff, 2002; Sreejith et al., 2012).

Numerous phytochemicals are present in different parts of *G. pentaphylla* including alkaloids, amides, imides, terpenoids, coumarins, and flavonoids, and their pharmacological properties have been reviewed (Sreejith et al., 2012). The species is characterized by the accumulation of anthranilate-derived alkaloids, namely quinazolines, furoquinolines, quinolones, and acridones (da Silva et al., 2013). Arborine, a quinazolin-4-one alkaloid is present abundantly in the leaf, whereas arborinine, an acridone, and

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skimmianine, a furoquinoline, are distributed in almost all parts of the plant (Sreejith et al., 2012; Yang et al., 2012). Different types of carbazole alkaloids such as glycosmisine, glycozoline, glycozolidine, glycozilinine, and biscarbalexin are also found mainly in the stems and roots (Yang et al., 2012; Chen et al., 2015; Kumar et al., 2018).

The antimicrobial properties of the crude extracts of *G. pentaphylla* have been demonstrated in many studies. Chloroform, ethyl acetate, and methanol extracts with moderate to high polarity exhibited a broad spectrum of activity particularly against the most susceptible microbes, namely *S. aureus*, *E. coli*, *Salmonella paratyphi*, and *C. albicans* (Howlander et al., 2011; Bulbul & Jahan, 2016; Ali et al., 2011; Murugan & Natarajan, 2016). The reported antimicrobial active alkaloids of *G. pentaphylla* include arborinine (Das & Deka, 2017), glycozolidol (Battacharyya et al., 1985), skimmianine, kokusaginine, haplopine, and flindersine (Hanawa et al., 2004). A few carbazole alkaloids namely glycoborinine, glycobomine, and carbalexin A are the antimicrobial active compounds identified in the stem extract. In addition, glycoborinine was determined as a photoactivated alkaloid when tested against *S. aureus* and *B. subtilis* (Yu et al., 2012). Therefore, this study was performed to isolate antimicrobial alkaloids from *G. pentaphylla*, to assess their combination effects with selected antimicrobial agents and to screen for their photoactivated enzymatic restriction inhibitory activity.

2. Materials and Methods

2.1 Chemicals

All solvents and chromatographic materials were from the Merck brand (Merck KGaA, Darmstadt, Germany). Vancomycin, erythromycin, ketoconazole, iodonitrotetrazolium chloride, and thiazolyl blue tetrazolium bromide were purchased from Sigma-Aldrich Chemicals (Sigma-Aldrich Inc., St. Louis, USA), whereas ciprofloxacin was purchased from BioChemika-Fluka (Buchs, Switzerland). Broth and agar media (Meuller Hinton and Sabauraud Dextrose) were from the BD Difco brand (Becton, Dickinson, and Co., Maryland, USA). A plasmid, pNEB 193, the DNA marker (1 kb ladder), and the restriction enzymes used in this study were purchased from Thermo Fisher Scientific (Fermentas, Vilnius, Lithuania).

2.2 Instrumentations

Visualization of chromatograms from thin-layer chromatography (TLC) and spectral recording of melting point (uncorrected), UV, infrared, ^{1}H NMR, ^{13}C NMR, and mass of the isolated compounds were performed by using the respective instrumentations as previously reported (Mohd Kamal et al., 2018). The electrophoresis gel images were captured using AlphaImager DE500 (Genetic Technologies Inc., Australia).

2.3 Plant Materials

G. pentaphylla (Retz.) DC. was collected at Pasir Putih, Kelantan, Malaysia. The species verification was performed by Dr. Shamsul Khamis, the botanist of the National University of Malaysia Herbarium (UKMB). A specimen with voucher number PIIUM 0001-1 was kept at the Herbarium, Kulliyyah of Pharmacy, International Islamic University Malaysia. The air-dried leaves were ground to powder form and left in the 4 °C cold room before further investigation.

2.4 Alkaloid Extraction

Acid-base extraction method as previously described (Mohd Kamal et al., 2018) was employed to furnish crude alkaloidal extracts of the leaf (130.68 g).

2.5 Alkaloid Chromatography

The crude alkaloidal extract (0.5 g) was chromatographed through silica gel packed column (40 g, 60-120 mesh) (glass column, 2 x 83 cm). The column was gradient eluted with 200 ml of each of the following: hexane (100%), hexane/$CHCl_3$ (9:1 - 0:1, 10% increment of $CHCl_3$) and $CHCl_3$/MeOH (99:1 - 9:1, 1% increment of MeOH). Twenty ml of eluents was collected while fractionation was monitored by TLC analysis to obtain eight fractions (GP1 to GP8). The fractions, GP3 and GP6 showed antimicrobial active spots labelled GP3-3 and GP6-3, respectively, as revealed by TLC agar overlay bioautographic screening. To elute GP3-3 (222 mg, 60%), fraction GP3 (370 mg) was re-chromatographed on silica gel (40 g) (column 2 x 40 cm) with 50 ml each of hexane (100%), hexane/$CHCl_3$ (1:1 - 1:9, 10% increment of $CHCl_3$), and 250 ml of $CHCl_3$ (100%). Fraction GP6 (370 mg) was further chromatographed using 100 ml of $CHCl_3$ (100%), followed by 200 ml each of $CHCl_3$/MeOH (99:1 - 99:3, 1% increment of MeOH), to elute GP6-3 with $CHCl_3$/MeOH (98:2) (148 mg, 40%).

2.6 Antimicrobial activity

2.6.1 Microbial strains

The lyophilized strains of the American Type Culture Collection, namely *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Candida albicans* ATCC 90028 were used. The bacterial and yeast cultures were grown and maintained on Mueller Hinton and Sabouraud Dextrose media, respectively.

2.6.2 Microbial inocula

The microbial inocula were prepared from bacterial cultures incubated at 37 °C for 18 h and yeast cultures incubated at 30°C for 48 h (Rahalison et al., 1991). The concentration of bacterial and yeast inocula was adjusted to an absorbance of 0.11 to 0.12 and 0.1 to 0.2 at 600 nm, respectively, using UV Spectrophotometer. The absorbance corresponded to 10^6 to 10^7 colony forming units (CFU)/ml for bacteria, and 10^7 to 10^8 CFU/ml for yeast.

2.6.3 TLC Agar Overlay Bioautographic Assay

The procedure used was adapted from Rahalison et al. (1991) with some modifications by Mohd Kamal et al. (2018). Ten mg/ml of each alkaloid fraction was

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chromatographed on a sterile 4 cm x 10 cm silica gel G60 F_{254} aluminum sheet TLC plates with a layer thickness of 0.2 mm. The TLC of each fraction was developed in duplicate under sterile conditions. One chromatogram was used as a reference for alkaloid characterizations on TLC under UV lights at 254 nm and 366 nm, and with Dragendorff's reagent staining. Molten agar at 37 ℃ was mixed with microbial inocula at a ratio of 9:1. The second chromatogram was placed in a sterile square petri dish lined with moist filter paper. Twenty ml of the inoculated agar was rapidly distributed onto the chromatograms and left to solidify. The chromatograms with bacteria and yeast or bioautograms were incubated at 37 °C for 18 h and 30 °C for 48 h, respectively. The bioautograms were then stained with 0.5% iodonitrotetrozolium chloride (INT) before being incubated for another four hours. The antimicrobial activity of the alkaloids was determined through the formation of a growth inhibitory area observed as a stainless zone, surrounded by pink surroundings of living microbes.

2.6.4 Checkerboard assay

The alkaloid-antimicrobial agent combination effects were studied against *S. aureus*, *E. coli*, and *C. albicans* following CLSI (2006) guidelines with minor modifications. The serial dilution of each alkaloid (2000 µg/ml to 0.977 µg/ml) was prepared in the 96-well plate in a vertical dimension, whereas the antimicrobial agent (1000 µg/ml to 7.8125 µg/ml) was serially diluted in the horizontal dimension. Twenty µl of the compound was prepared for each test concentration for an activity assay, whereas the combination of 10 µl of alkaloid and 10 µl of the antimicrobial agent was tested for the combination study. A total of 180 µl of inoculated broth containing 10^4 to 10^5 CFU/ml of bacteria or 10^5 to 10^6 CFU/ml of yeast was added to the designated wells. The bacterial and yeast plates were incubated at 37 °C for 18 h and 30 °C for 48 h, respectively. All samples were tested in triplicate. The minimum inhibitory concentration (MIC) of the test sample against the respective microbe was determined as the lowest concentration that did not cause turbidity and a colour change from yellow to blue after 30 minutes of subsequent incubation with 10 µl of 0.25% thiazolyl blue tetrazolium bromide in each well. The antimicrobial combination effect was determined using the fractional inhibitory concentration index (FICI) (Choi et al., 2009).

2.7 Photoactivated Enzymatic Restriction Inhibitory Activity

2.7.1 DNA amplification

A DNA fragment was synthesized by Polymerase Chain Reaction (PCR) using pNEB 193 as a template. The selected primers used were forward 5'-TCGCGCGTTTCGGTGATGAC and reverse 5'-AGCGTCAGACCCCGTAGAAAAGATC. PCR was performed in 0.2 ml PCR tubes based on the protocol provided by the SensiMixPlus SYBR Kit. Standard PCR reactions were carried out in a total volume of 50 µl of the following mixture: 25 µl of SensiMixPlus SYBR Kit, 2 µl of 200 nM forward and reverse primer, 10 µl of 100 ng DNA template, and 2 µl of 5 µM magnesium chloride with added sterile distilled water up to 50 µl. PCR cycling conditions were conducted using the BioradTM; IQ5 multicolor real-time PCR detection system as follows: 94 °C for 2 minutes; 25 cycles of 94 °C for 15 seconds; 64 °C for 30 seconds; 72 °C for one minute and 72 °C for 10 minutes (Hanawa et al., 2004). The resulted 1.5-kb DNA fragment was analyzed by 0.8% agarose gel electrophoresis.

2.7.2 Photoactivated enzymatic restriction inhibitory assay

The assay was carried out as described by Hanawa et al., (2004) for the antimicrobial active alkaloids and antibiotic, ciprofloxacin. A total of 100 ng of 1.5-kb DNA fragment was mixed with one nmol of the compounds (2 µl from 500 µM in 2.5% DMSO solution) in a 1.5-ml microcentrifuge tube. The total volume was adjusted to 8 µl with sterile distilled water. The mixtures were irradiated with UVA (24.1 kJ/m2) on ice. After irradiation, 10 units of restriction enzymes (1 µl, respectively, depending on the supplied concentrations of the enzymes) and 1 µl of 10X buffers for the respective enzymes were added to the mixtures. The restriction enzymes used in this study are BamHI, EcoRI, KpnI, SacI, BfuI, MssI, HindIII, PstI, XbaI, SmaI, SgsI, PacI, DraI, and SalI. The mixtures were incubated for 1 h at 37 ℃. A mixture of 10 µl of sample and 1 µl of gel loading dye was loaded into each well of the 0.8% agarose gel chamber and 1.5 µl of DNA ladder was loaded in the first lane. The samples were electrophoresed at 100 volts or 400 mA for 35 minutes. The gel was stained for an hour in a staining tray containing 200 ml of distilled water and 2 drops of ethidium bromide. Then, it was destained with 200 ml of distilled water for 20 minutes. The image of the gel was captured and analyzed.

3. Results and Discussion

3.1 Bioautography-guided isolation of antimicrobial alkaloids

In this study, alkaloidal fractions of *G. pentaphylla* were initially screened for their antimicrobial active alkaloids against the indicator microbes, namely *S. aureus*, *E. coli*, and *C. albicans*. The TLC agar overlay bioautography screening allows the detection of bioactive compounds in an extract and facilitates their isolation through a bioassay-guided approach (Dewanjee et al., 2015). Two antimicrobial active alkaloids were detected, labelled as GP3-3 and GP6-3 from GP3 and GP6 fractions (Figure 1), respectively. The alkaloids were successfully isolated by using column chromatography and identified based on their spectroscopic data and comparison with that of literature. GP3-3 was assigned to 2,3-dimethoxy-1-hydroxy-10-methylacridone or arborinine (Figure 2a) (Mawardi et al., 2010), with characteristics identical to that of previously reported from *Ruta angustifolia* (Mohd Kamal et al., 2018). GP6-3 was confirmed as 2-benzyl-1-methylquinazolin-4(1H)-one with the trivial name arborine (Figure 2b) (Farediah et al., 1996).

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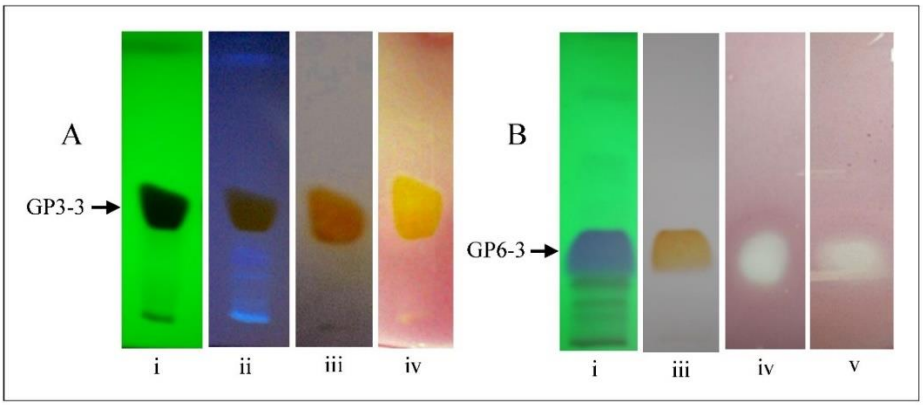

Figure 1. Bioautography of *G. pentaphylla* leaf fractions showing GP3-3 and GP6-3 as the respective antimicrobial active alkaloids. A: fraction GP3, B: fraction GP6, visualized chromatograms with i: UV254 light, ii: UV366 light, iii: Dragendorff's reagent, and the bioautograms iv: *S. aureus*, v: *E. coli*.

GP6-3: 2-Benzyl-1-methylquinazol-4-one [Arborine]; $C_{16}H_{14}N_2O$; MW: g/mol; colorless rhombohedral plates; MP: 156-156ºC, Rf: 24.7 ($CHCl_3$:EtOAc, 9:1); IR ($CHCl_3$) cm^{-1}: 1641, 1598, 1527, 707; UV/Vis λ_{max} (MeOH) nm: 203, 229, 276, 304; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.39 (*dd*, 1.0, 8.0, H-5), 7.72 (*t*, 8.5, H-7), 7.50 (*t*, 8.0, H-6), 7.35-7.39 (*m,* H-8), 7.28-7.39 (*m,* H-2", H-3", H-4", H-5", H-6"), 4.35 (2H, *s*, H-C1'), 3.65 (3H, *s*, *N*-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 169.04 (C-4), 162.25 (C-8a), 141.57 (C-2), 133.90 (C-7), 134.58 (C-1"), 129.20 (C-6" & C-2"), 128.32 (C-3", C-4" & C-5"), 127.50 (C-5), 126.05 (C-6), 120.14 (C-4a), 114.54 (C-8), 43.50 (C-1'), 34.93 (*N*-CH_3). MS (EI, 70 eV): *m/z* (%) = 285 [M^+] (52), 270 (100), 256 (15), 242 (85), 227 (5), 212 (12), 199 (59), 171 (12), 143 (9), 115 (10).

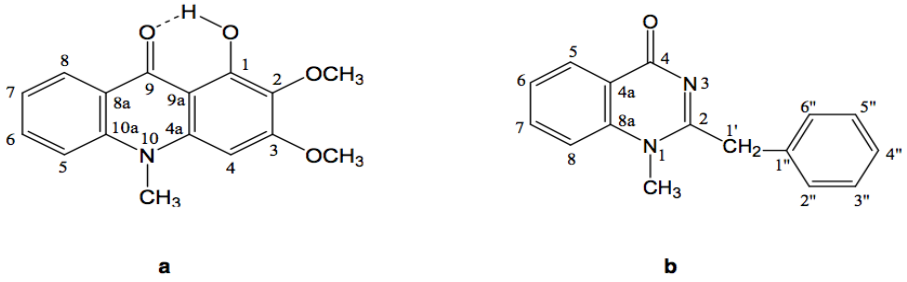

Figure 2. Antimicrobial alkaloids of *G. pentaphylla*. a: Arborinine b: Arborine.

3.2 Antimicrobial activity and combination effects

Arborinine and arborine exhibited a different degree of antimicrobial activity at MIC in the range of 250 µg/ml to 1000 µg/ml against *S. aureus*, *E. coli*, and *C. albicans*. A dual combination of antimicrobial agents is a multi-targeted strategy expected to enhance the actions or counter any possible adverse side effects of both agents (Monte et al., 2014). Arborine was combined with selected antimicrobial agents of different mechanisms of action including ciprofloxacin as a DNA gyrase inhibitor, erythromycin as a protein synthesis inhibitor, and vancomycin as a bacterial cell wall synthesis inhibitor (Khameneh et al., 2019). Considering the activity against *C. albicans*, both arborine and arborinine have been evaluated for their antifungal combined effects with ketoconazole, which disrupt the cell membrane permeability by blocking ergosterol synthesis (Cui et al., 2015). The antimicrobial combinations have resulted in different effects as interpreted from their FICI values (Table 1).

The most effective combination was arborine-ciprofloxacin partial synergism interaction, FICI (0.25) with the respective eight- and two-fold antibacterial activity enhancement against *S. aureus*. In comparison, the two-fold enhancement in the activity of ciprofloxacin in a dual antimicrobial agent combination was achieved with either erythromycin or vancomycin, but with an additive effect (FICI, 1.00) (Mohd Kamal et al., 2018). The antimicrobial combination effects are expected from the simultaneous

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action of both agents on different cellular targets that enhance each other's activity. As arborine is a quinazolinone that is more favorable to interact with the cell wall and DNA structures of Gram-positive bacteria (Mohamed et al., 2013), ciprofloxacin molecules would go into the cell and target the DNA gyrase, inhibiting DNA synthesis, which results in bacterial death (Khameneh et al., 2019). Meanwhile, partial synergy (FICI, 0.75) was observed in arborine-vancomycin and arborine-erythromycin interactions, resulting in either four- or two-fold enhancement in their antimicrobial activity. Vancomycin acts by targeting the peptidoglycan wall of the Gram-positive microbe while erythromycin acts as an inhibitor to protein synthesis (Khameneh et al., 2019), which combination may contribute to the dual mechanism of action, although it may not be up to a synergistic level. Therefore, it is suggested that arborine-ciprofloxacin is a potent combination in achieving combined effects in the treatment against *S. aureus*.

Table 1. Antimicrobial combination effects of alkaloids from *G. pentaphylla* and selected antimicrobial agents.

Microorganism	Combination	MIC (µg/ml)		FIC	FICI	Combination effect
		Alone	Combined			
S. aureus ATCC 25923	Arborine Ciprofloxacin	1000 0.195	125 0.098	0.125 0.500	0.625	Partial synergy
	Arborine Erythromycin	1000 1.098	500 0.275	0.500 0.250	0.750	Partial synergy
	Arborine Vancomycin	1000 2.500	250 1.250	0.250 0.500	0.750	Partial synergy
	Ciprofloxacin Erythromycin	0.195 1.098	0.098 0.549	0.500 0.500	1.000	Additive*
	Ciprofloxacin Vancomycin	0.195 2.500	0.098 1.250	0.500 0.500	1.000	Additive*
E. coli ATCC 25922	Arborine Ciprofloxacin	500 0.039	500 0.156	1.000 4.000	5.000	Antagonism
	Arborine Erythromycin	500 50	500 6.25	1.000 0.125	1.125	Indifference
	Arborine Vancomycin	500 250	250 125	0.500 0.500	1.000	Additive
	Ciprofloxacin Erythromycin	0.039 50	0.019 1.562	0.500 0.031	0.531	Additive*
	Ciprofloxacin Vancomycin	0.039 250	0.002 7.813	0.050 0.031	0.081	Synergy*
C. albicans ATCC 90028	Arborine Ketoconazole	500 1.562	500 0.781	1.000 0.500	1.50	Indifference
	Arborinine Ketoconazole	250 1.562	125 0.390	0.500 0.250	0.750	Partial synergy
	Ketoconazole Miconazole	1.562 5.000	1.562 2.500	1.000 0.500	1.500	Indifference

*: Previously reported by Mohd Kamal et al., 2018.

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Arborine-antimicrobial agent combination against *E. coli* exhibited only slight enhancement in the antibacterial activity of both agents. Interestingly, combination with vancomycin resulted in a two-fold enhancement in the antimicrobial activity of both agents despite the low susceptibility of Gram-negative bacteria toward vancomycin due to the presence of an outer membrane barrier against large glycopeptide (Zhou et al., 2015). This finding demonstrated an achievable broader spectrum of activity of vancomycin against Gram-negative bacteria when used in combination with other compounds as demonstrated in previous studies (Leite et al., 2015). Nevertheless, the combination was not as effective as that of ciprofloxacin-vancomycin, which exhibited a strong synergy at a FICI of 0.081 (Mohd Kamal et al., 2018). Thus, arborine and ciprofloxacin are suggested as suitable combination agents for vancomycin against Gram-negative bacteria, which potential application in treating infections by multidrug-resistant pathogens may be further investigated (Zhou et al., 2015). Meanwhile, the combination of arborine and ciprofloxacin produced antagonistic effects, by which arborine hindered the effect of ciprofloxacin four-fold.

Arborinine is the best combination agent for ketoconazole against *C. albicans* with two- and four-fold enhancement in antimicrobial activity based on partial synergism (FICI, 0.625), compared with that of arborine or miconazole combinations. The planar structure of arborinine allows it to function as a DNA intercalator with the potential to synergize with azole antifungals. The disrupting activity of azoles against fungal cell plays an important role in increasing the intracellular concentration of DNA intercalators to bind with DNA, exerting cell cycle arrest and DNA damage (Li et al., 2013). Meanwhile, the arborine-ketoconazole combination produced an insignificant effect, which is evident in the two-fold enhancement in the antifungal activity of ketoconazole at the MIC of arborine. Additionally, the ketoconazole-miconazole insignificant interaction was observed in the two-fold increase in miconazole activity. The enhancement effect of miconazole was most probably due to its dual actions in inhibiting peroxidases, which allows accumulation of intracellular peroxide, resulting in cell death, and the shared ergosterol synthesis inhibitory property with ketoconazole (Fothergill et al., 2014).

3.3 Photo-activated enzymatic restriction inhibitory activity

The bioactive alkaloids, particularly acridones, quinolines, and quinazolines have gained considerable interest in the field of antimicrobial, and anticancer drug development. These compounds partly rely on the ability of their polyheteroaromatic structures to interact with DNA or the systems involved in the mechanisms controlling cellular topology, repair, and replication (Tillequin, 2007; Kamel et al., 2016). In this study, arborinine and arborine exhibited the ability to inhibit the activity of the restriction enzymes, resulting in uncleaved DNA fragment that can be observed on the electrophoresis gel. Both alkaloids are photoactivated compounds, which inhibited the enzymatic activity of restriction endonucleases to varying degrees under UVA irradiation but were inactive inhibitors in the dark (Figure 3).

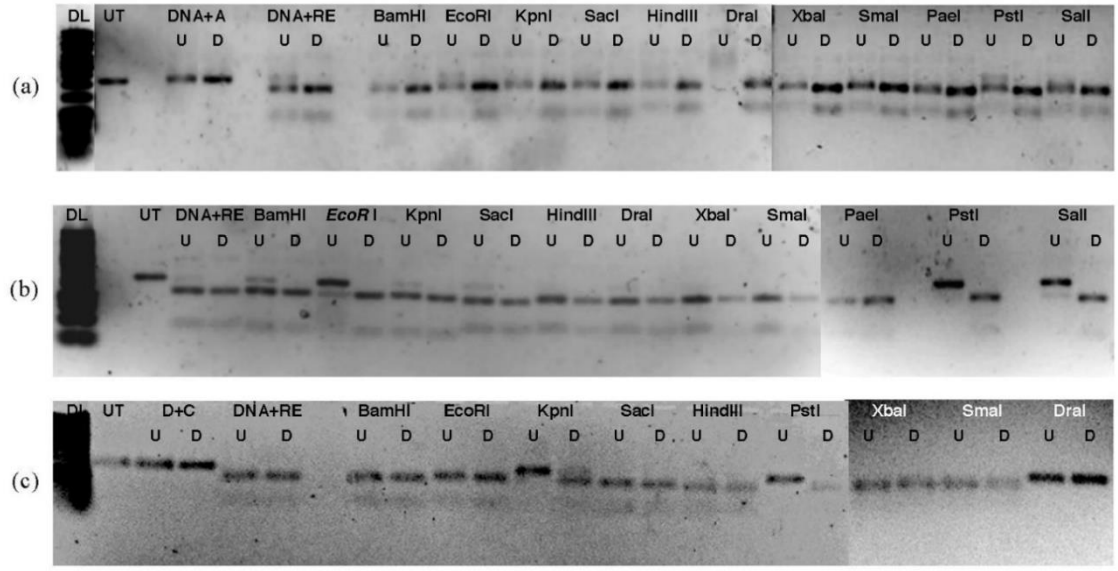

Figure 3. Effects of test compounds on the cleavage of the 1.5 kb DNA fragment by the selected restriction enzymes which have restriction sites on the fragment. A: arborinine, B: arborine, C: ciprofloxacin, DL: DNA ladder, RE: restriction enzyme, U: UVA light irradiation, D: Dark, UT: untreated 1.5 kb DNA fragment.

Both alkaloids inhibited EcoRI, a Type 2A enzyme with restriction sites recognizing a 5'ApT sequence, and PstI, a Type 2B restriction enzyme that recognizes at least one A (or T) in the recognition site, which does not have either 5'ApT or 5'TpA sequence. Arborinine showed relatively the strongest inhibitory activity against DraI, a Type I enzyme recognizing a 5'-TpA sequence in the restriction site, and moderate inhibition against EcoRI and PstI. The inhibition of

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the cleaving activity of DraI may be partly attributed to the resistance of its recognition sequence against UV irradiation. Meanwhile, an insignificant effect was observed in the cleaving activity of other enzymes with restriction sites lacking the 5'-TpA sequence (Hanawa et al. 2001). Arborine was found to be the most active inhibitor, with relatively strong activity against EcoRI, PstI, and SalI, whereas moderate activity against BamHI, a Type 2A enzyme. The strong sequence-specific binding affinity of arborine in the 5'-GAATTC-3' restriction site of EcoRI demonstrates its potential therapeutic property as the sequence has also been observed in BRCA 2 breast cancer 2, an early onset oncogene (Hassan et al., 2014). The positive control, ciprofloxacin, exhibited inhibition activity against DraI both in the dark and under UVA irradiation. It is a strong photoactivated inhibitor against KpnI, which restriction site contains a 5'ApT sequence, and PstI. Therefore, in descending order of photo-activated enzymatic restriction inhibitory activity, the alkaloids are ranked as arborine > ciprofloxacin > arborinine.

4. Conclusion

Among the tested combinations, arborine-ciprofloxacin is the most potent combination agent against S. aureus, whereas arborinine interacts well with ketoconazole against C. albicans. The findings support the proposed combinations of quinazolones-macrolides and acridones-azoles for potential combined antimicrobial effects against S. aureus and C. albicans, respectively. This study has also identified these alkaloids as photoactivated enzymatic restriction inhibitors. These results warrant further investigation of antimicrobial alkaloids in *G. pentaphylla* for the development of new antimicrobial-enhancing agents

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6. References

Ali, M. A., Abu Sayeed, M., Syaifuddin, A., Sarmina, Y., Mst. Astaq, M. K. & Abu Hanif, M. (2011). An evaluation of antimicrobial activities of *Glycosmis pentaphylla. Research Journal of Agriculture and Biological Sciences* 7(2): 328-331.

Asif, M. (2014). Chemical characteristics, synthetic methods, and biological potential of quinazoline and quinazolinone derivatives. *International Journal of Medicinal Chemistry* 395637. doi: 10.1155/2014/395637

Aye, M. M., Aung, H. T., Sein, M. M. & Armijos, C. (2019). A Review on the phytochemistry, medicinal properties and pharmacological activities of 15 selected Myanmar medicinal plants. *Molecules* 24: 293. doi:10.3390/molecules24020293

Battacharyya, P., Cakrabartty, P. K. & Chowdury, B. K. (1985). Glycozolidol, an antibacterial carbazole alkaloid from *Glycosmis pentaphylla*. *Phytochemistry* 24(4): 882-883.

Bhardwaj, M., Singh, B. R., Sinha, D. K., Kumar, V., Prasanna Vadhana, O. R., Varan Singh, S., Nirupama, K. R., Pruthvishree, Archana Saraf, B. S. (2016). Potential of herbal drug and antibiotic combination therapy: A new approach to treat multidrug resistant bacteria. *Pharmaceutica Analytica Acta* 7(11): 1-14. doi: 10.4172/2153-2435.1000523

Bollenbach, T. (2015). Antimicrobial interactions: mechanisms and implications for drug discovery and resistance evolution. *Current Opinion in Microbiology* 27: 1-9. doi: 10.1016/j.mib.2015.05.008.

Bulbul, I. J. & Jahan, N. (2016). Study on antioxidant and antimicrobial activities of methanolic leaf extract of *Glycosmis pentaphylla* against various microbial strains. *Journal of Pharmacognosy and Phytochemistry* 5(4): 53-57.

Cheesman, M. J., Ilanko, A., Blonk, B. & Cock, I. E. (2017). Developing new antimicrobial therapies: Are synergistic combinations of plant extracts/compounds with conventional antibiotics the solution? *Pharmacognosy Review* 11: 57-72.

Chen, Y., Tang, C., Wu, Y., Mo, S., Wang, S., Yang, G. & Mei, Z. (2015). Glycosmisines A and B: isolation of two new carbazole–indole-type dimeric alkaloids from *Glycosmis pentaphylla* and an evaluation of their antiproliferative activities. *Organic & Biomolecular Chemistry* 13(24): 6773-6781.

Choi, J. G., Kang, O. H., Lee, Y. S., Oh, Y. C., Chae, H. S., Jang, H. J., Shin, D. W. & Kwon, D. Y. (2009). Antibacterial activity of methyl gallate isolated from *Galla rhois* or carvacrol combined with nalidixic acid against nalidixic acid resistant bacteria. *Molecules* 14(5): 1773-1780. doi: 10.3390/molecules14051773

Chua, L. S. L. & van Valkenberg, J. L. C. H. (2001). *Glycosmis pentaphylla* (Retz.) A.D.C. In: van Valkenberg,, J. L. C. H. & Bunyapraphatsara, N. (editors). Plant Resources of South East Asia 12(2): Medicinal and Poisonous Plants 2, pp. 275-278, Leiden: Backhuys Publishers.

CLSI (2006). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, Approved Standard Seventh Edition, Document M7-A7 [ISBN 1-56238-587-9]. Wayne: Clinical and Laboratory Standards Institute.

DOI:https//doi.org/10.22452/mjs.vol41no3.1
Malaysian Journal of Science 41(3): 1-9 (October 2022)

Cui, J., Ren, B., Tong, Y., Dai, H. & Zhang, L. (2015). Synergistic combinations of antifungals and anti-virulence agents to fight against *Candida albicans*. *Virulence* 6(4): 362-371.

Das, M. M. & Deka, D. C. (2017). Evaluation of anticancer and antimicrobial activity of arborinine from *Glycosmis pentaphylla. Journal of Biologically Active Products from Nature* 7(2): 131-139.

da Silva, M. F. G. F., Fernandes, J. B., Forim, M. R., Vieira, P. C. & de Sa´, I. C. G. (2013). Alkaloids derived from anthranilic acid: Quinoline, acridone and quinazoline. In: K. G. Ramawat, and J. M. Me´rillon (eds.). K. G. Ramawat, and J. M. Me´rillon (eds.). Natural Products, pp. 715-859, Berlin Heidelberg: Springer-Verlag.

Dewanjee, S., Gangopadhyay, M., Bhattacharya, N., Khanra, R. & Dua, T. K. (2015). Bioautography and its scope in the field of natural product chemistry. *Journal of Pharmacceutical Analysis 5*(2): 75–84.

Farediah, A., Hazar Bebe, M. I. & Mawardi, R. (1996). Arborine, a larval growth inhibitor from *Glycosmis pentaphylla*. *Pertanika Journal of Science and Technology* 4(1): 11-15.

Fothergill, A.W. (2014). Miconazole: a historical perspective. *Expert Review of Anti-infective Therapy* 4(2): 171-175.

Hanawa, F., Fokialakis, N. & Skaltsounis, A-L. (2004). Photo activated DNA binding and antimocrobial activities of furoquinoline and pyranoquinolone alkaloids from Rutaceae. *Planta Medica* 70*:* 531-535.

Hanawa, F., Okamoto, M. & Towers, G. H. N. (2001). Inhibition of restriction enzyme's DNA sequence recognition by PUVA treatment. *Photochemistry Photobiology* 74(2): 269-273.

Hassan, S. A., Barthwal, R., Padmadeo, S. R. & Barukab, O. M. (2014). Restriction inhibition assay: A qualitative and quantitative method to screen sequence specific DNA binder from herbal plants. *Tropical Journal of Pharm*aceutical *Research* 13(2): 267-273.

Hazarika, P. & Dutta, D. (2013). Traditional knowledge for using plant resources as tooth brushing stick (datun) by the indigenous communities of Assam, India. *International Journal of Herbal Medicinal* 6(6): 22-34.

He, D., Wang, M., Zhao, S., Shu, Y., Zeng, H., Xiao, C., Lu, C. & Liu, Y. (2017). Pharmaceutical prospects of naturally occurring quinazolinone and its derivatives. *Fitoterapia* 119:136-149.

Howlader, M. A., Farhana, R., Shapna, S., Mohammad, R. R., Shams-Ud-Doha, K.M., Rumana, M. & Apurba, S. A. (2011). Antimicrobial, antioxidant, and cytotoxic effects of methanolic extracts of leaves and stems of *Glycosmis pentaphylla* (Retz.). *Journal of Applied Pharmaceutical Science* 1(8):137-140.

Kamel, M. M., Zaghary, W. A., Al-Wabli, R. I. & Anwar, M. M. (2016). Synthetic approaches and potential bioactivity of different functionalized quinazoline and quinazolinone scaffolds. *Egyptian Pharmaceutical Journal* 15(3): 98-131.

Khameneh, B., Iranshahy, M., Soheili, V. & Bazzaz, B. S. F. (2019). Review on plant antimicrobials: a mechanistic viewpoint. *Antimicrobial Resistence and Infection Control* 8: 118. doi: 10.1186/s13756-019-0559-6

Kumar, A., Banerjee, N., Singamaneni, V., Dokuparthi, S. K., Chakrabarti, T. & Mukhopadhyay, S. (2018). Phytochemical investigations and evaluation of antimutagenic activity of the alcoholic extract of *Glycosmis pentaphylla* and *Tabernaemontana coronaria. Natural Products Research* 32(5), 582-587.

Leite, G. C., Neto, L. V. P., Gaudereto, J. J., de Maio Carrilho, C. M. D., Ross, F., Anna Sara Levin A. S. & Costa, S. F. (2015). Effect of antibiotics combination and comparison of methods of synergism in multiresistant Gram negative bacteria. *Journal of Infectious Diseases and Therapy* 3: 207. doi: 10.4172/2332-0877.1000207

Li, D-D., Xu, Y., Zhang, D-Z., Quan, H., Mylonakis, E., Hu, D-D., Li, M-B., Zhao, L-Z., Zhu, L-H., Wang, Y. & Jiang, Y. Y. (2013). Fluconazole assists berberine to kill fluconazole-fesistant *Candida albicans*. *Antimicrobial Agents and Chemotherapy* 57(12): 6016-6027.

Mat Salleh, K. & Latiff A. (2002). Tumbuhan Ubatan Malaysia. pp. 473, Bangi: Pusat Pengurusan Penyelidikan Universiti Kebangsaan Malaysia.

Mawardi, R., Rosmiati, M. S., Najihah, M. H., Mohd Aspollah, S., Gwendoline, C. L. E., Abdul Manaf, A. & Hazar Bebe, M. I. (2010). Alkaloids and sulphur-containing amides from *Glycosmis citrifolia* and *Glycosmis elongata*. *Sains Malaysiana* 39(3): 445-451.

Mohamed, M. A., Ghanem, H. M., Abd El-Ghaffar, N. F. & Mohamed, S. S. (2013). Biological evaluation and molecular docking of substituted quinazolinones as antimicrobial agents. *Australian Journal of Basic and Applied Sciences* 7: 263–274.

DOI:https//doi.org/10.22452/mjs.vol41no3.1
Malaysian Journal of Science 41(3): 1-9 (October 2022)

Mohd Kamal, L. Z., Mohd Hassan, N., Md Taib, N. & Soe, M. K. (2018). Graveoline from *Ruta angustifolia* (L.) Pers. and its antimicrobial. synergistic potential in erythromycin or vancomycin combinations. *Sains Malaysiana* 47(10): 2429-2435.

Monte, J., Abreu, A. C., Borges, A., Simões, L. C. & Simões, M. (2014). Antimicrobial activity of selected phytochemicals against *Escherichia coli* and *Staphylococcus aureus* and their biofilms. *Pathogens* 3(2): 473-498. doi:10.3390/pathogens3020473

Murugan, N. & Natarajan, D. (2016). Phytochemical, antioxidant and antibacterial activities of *Glycosmis pentaphylla* (Rutaceae) leaf extracts against selected multi-drug resistant bacteria. *Journal of Chemical and Pharmaceutical Research* 8(1): 737-744.

Rahalison, L., Hamburger, M., Hostettmann, K., Monod, M. & Frenk, E. (1991). Bioautographic agar overlay method for the detection of antifungal compounds from higher plants. *Phytochemical Analysis* 2:199-203.

Sreejith, P. S., Praseeja, R. J. & Asha, V. V. (2012). A review on the pharmacology and phytochemistry of traditional medicinal plant, *Glycosmis pentaphylla* (Retz.) Correa. *Journal of Pharmacy Research 5*(5): 2723-2728.

Tillequin F. (2007). Rutaceous alkaloids as models for the design of novel antitumor drugs. *Phytochemistry Review* 6: 65-79.

van Vuuren, S. & Viljoen, A. (2011). Plant-based antimicrobial studies - Methods and approaches to study the interactions between natural products. *Planta Medica* 77: 1168-1182.

Wang, J., Di, Y., Yang, X., Li, S., Wang, Y. & Hao, X. (2006). Hydroquinone diglycoside acyl esters from the stems of *Glycosmis pentaphylla*. *Phytochemistry* 67(5): 486-491.

Yang, G.-Z., Wu, Y. & Chen, Y. (2012). Alkaloids from the stems of *Glycosmis pentaphylla*. *Helvetica Chimica Acta* 95: 1449–1451.

Yu., C., Bo, Y., Jing, X., Tong, Z., Hua, F. & Guang-zhong, Y. (2012). Photo-activated DNA binding and antimicrobial activities of alkaloids from *Glycosmis pentaphylla. Acta Pharmaceutica Sinica* 47(12): 1646−1652.

Zhou, A., Kang, T. M., Yuan, J., Beppler, C., Nguyen, C., Mao, Z., Nguyen, M. Q., Yeh, P. & Miller, J. H. (2015). Synergistic interactions of vancomycin with different antibiotics against *Escherichia coli*: Trimethoprim and nitrofurantoin display strong synergies with vancomycin against wild-type *E. coli*. *Antimicrobial Agents and Chemotherapy* 59(1): 276-281.

DOI:https//doi.org/10.22452/mjs.vol41no3.1
Malaysian Journal of Science 41(3): 1-9 (October 2022)