

Full Length Research Paper

Antioxidant, antibacterial and antiviral properties of *Goniothalamus umbrosus* leaves methanolic extract

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The antioxidant, antibacterial, and antiviral effects of crude methanol extract from the leaves of *Goniothalamus umbrosus* were studied. Good free radical scavenging activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was shown by the extract at EC_{50} of 0.263 mg/ml compared to 0.01 mg/ml for trolox as the positive control. The extract showed greater antibacterial activity against Gram-positive bacteria than Gram-negative with methicillin resistant *Staphylococcus aureus* (MRSA) isolates were more sensitive than methicillin sensitive *S. aureus*. Cytotoxicity testing of the leaf extract and goniothalamine a styrylpyrone derivative (SPD) as a lead compound found mostly in the genus *Goniothalamus* was performed on Vero cells. From the test, the CC_{50} value for the extract was 125 µg/ml and for SPD 31.25 µg/mL (1.563×10^{-7} µM). Antiviral test was carried out against Herpes Simplex virus type 1 infected to Vero cells. The results showed that *G. umbrosus* leaf methanol extract was effective in showing the antiviral activity in all three treatments but SPD is effective only in the first treatment; 1) (C+V)+E, extract and SPD were able to control virus post-infection 2) (C+E)+V: the extract has prophylactic activity implied by treatment of cells with the extract for one day before viral infection and C+(V+E) refers to the ability of extract to control viral infectivity when extract and virus was added simultaneously to cell. This study showed the potential of non-cytotoxic *G. umbrosus* leaf methanol extract as herbal alternative against MRSA and herpes simplex virus (HSV) -1.

Key words: *Goniothalamus umbrosus*, leaf, methanol extract, styrylpyrone derivative, antioxidant, antibacterial, cytotoxicity, antiviral.

INTRODUCTION

Goniothalamus umbrosus J. Sinclair or locally known as kenerak is widely utilized by locals in Malaysia for post-partum healthcare, general well-being, male libido and for

abortion (Mat-Salleh and Latiff, 2002). Studies related to *G. umbrosus* includes the chemical composition (Ahmad and Din, 2002; Abbdewahab et al., 2009; Abdelwahab et al., 2009), antioxidant, antibacterial activity (Abdel-Wahab et al., 2009a,b) and genotoxicity in Chinese hamster ovary (CHO) cell line (Umar-Tsafe et al., 2004). Different *Goniothalamus* species have the ability to elaborate series of acetogenins and styryl lactones which are cytotoxic against a broad array of cancer cells including breast, colon, kidney and pancreatic carcinoma cells (reviewed in Wiart, 2007; Yang et al., 2000).

The antiviral activity of *G. umbrosus* or in other species of the same genus is not known. The emergence of viral resistant variants after prolonged treatment in immunocompromised patients has been the main reasons for the continuous search of novel anti herpetic agents. It is

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Abbreviations: CHO, Chinese hamster ovary; MRSA, methicillin resistant *Staphylococcus aureus*; SPD, styrylpyrone derivative; DPPH, 1,1-diphenyl -2-picrylhydrazyl; HSVs, herpes simplex viruses; MEM, minimal essential medium; NRU, neutral red uptake; E, extract; V, viral; C, cells; MSSA, methicillin sensitive *S. aureus*; VISA, vancomycin intermediate-resistant *Staphylococcus aureus*; VRSA, vancomycin resistant *Staphylococcus aureus*; OD, optical density.

known that the herpes simplex viruses (HSVs) have evolved mechanisms to inhibit host cell death, or apoptosis (Jerome et al., 1998). On the other hand, styryl lactones from *Goniothalamus* sps have been known to activate apoptosis in mammalian and cancer cells (Inayat-Hussain et al., 1999; Lee et al., 2003).

Thus, in this study we would like to test on a hypothesis that the *G. umbrosus* leaf extract which may contain styryl lactones has the capability in promoting apoptosis of HSV-1 infected cells resulting in the inhibition of virus replication. In this study, we tested goniothalamine, a styrylpyrone derivative to provide an insight as the potential compound that may exhibit the antiviral property. The objectives of this study include determination of the antioxidant, antibacterial activity towards clinically important bacteria and cytotoxicity to normal cells of crude methanol extract from *G. umbrosus* leaves.

MATERIALS AND METHODS

Plant collection and what

The plant collected near Sungai Kelantan, Kelantan, Malaysia was identified by Kamarudin Mat-Salleh with a voucher specimen deposited in the UKM Herbarium (Voucher No: NZS 3148). Air-dried *G. umbrosus* leaves (200 g) were finely powdered before extracted with methanol using a Soxhlet apparatus. The crude methanol extract were concentrated with a rotary evaporator (Laborata 4000, Heidolph, Schwabach, Germany) and dried. Goniothalamine or SPD from *G. umbrosus* prepared by the method of Jewers et al. (1972) was tested in the cytotoxicity and antiviral study.

Free radical scavenging activity of DPPH

The assay was used to measure the free radical scavenging capacity of the methanol extract according to Mothana et al. (2008). Various concentrations of the extract at 500 µl were added to 500 µl of 0.1 mM 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, country) in methanol solution. After 30 min incubation at 25°C, the absorbance was measured at =517 nm using a Shimadzu 1601 UV/Vis spectrophotometer. Percentage of free radical scavenging activity was calculated using the equation: $(Abs_{control} - Abs_{sample}) / Abs_{control} \times 100$. Antioxidant activity was expressed as EC₅₀ (effective concentration in mg/ml of samples or positive controls that reduces the absorbance of DPPH by 50% compared to the negative control) which was estimated from dose-response curves.

Synthetic antioxidant reagent 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) (Sigma-Aldrich, country) was used as the positive control and all tests were carried out in triplicate.

Antibacterial test

Assay was done by disc diffusion method (Bauer et al., 1966) on Mueller Hinton agar plates plated with test organisms listed in Table 1. Bacteria were obtained from the stock culture collection Microbiology Laboratory, School of Biosciences and Biotechnology, Universiti Kebangsaan Malaysia. Sterile filter paper disks (6 mm diameter) soaked with 50 l of extract with concentrations of 100,

50, 25, 10 or 5 mg/ml and placed onto the plated agar. Streptomycin (10 g/disc) were used as a positive standard against Gram negative bacteria, Penicillin (10 g/disc) for Gram positive bacteria and vancomycin (10 g/disc) for methicillin resistant *Staphylococcus aureus* (MRSA). Negative control was performed using paper discs loaded with 50 µl of methanol. The plates were incubated at 37°C for 24 h after which zones of inhibition were measured. The experiment was carried out in duplicate.

Cytotoxicity test

The methanol extract (1 mg) was first reconstituted in 50 l methanol and then further diluted with 950 l minimal essential medium (MEM) with 5% fetal bovine serum (GIBCO)-MEM/FCS to obtain a 1 mg/ml stock solution. Confluent Vero cells (African green monkey kidney cells) grown in 96-well microtitre plate were treated with the extract that was diluted to 1, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.016 and 0.008 mg/ml for 48 h incubation. We included SPD for initial screening of cytotoxicity and antiviral activity in this study.

Cytotoxic activity was determined by the protocol for neutral red uptake (NRU) assay (Fotakis and Timbrell, 2005). Absorbance of released dye was recorded by using microplate reader (Labsystems Multiscan Multisoft) at =550 nm. Readout from each well was normalized against the absorbance from empty wells and data was presented as the percentage of survived cells compared to control cells. The 50% cytotoxic concentration (CC₅₀) value was determined.

Antiviral assay

The viral dose that kills cell 50% of cell population (TCID₅₀) was first determined using the NRU assay (Danve et al., 2002). Screening for antiviral activity was performed using 3 different treatments according to Nurul-Aini et al. (2006); 1) To evaluate the effect of extract (E) after viral (V) adsorption, cells (C) were inoculated with virus for two hours after which the extract/SPD were added or simply (C+V)+E; 2) To evaluate the effect of compounds on cells before virus inoculation, cells were treated for one day with extract before virus was added, (C+E)+V and 3) to evaluate the ability of extract to control viral infectivity thus effecting viral replication cycle; the virus and extract were inoculated at the same time to cells, C+(V+E) and incubated for 48 hours. For the antiviral tests, the extracts were diluted to 1.0 CC₅₀, 0.5 CC₅₀ and 0.25CC₅₀ with SPD at 0 CC₅₀ (31.25 µg/ml) and 0.5 CC₅₀ (15.63 µg/ml).

The viral concentration used for cell inoculations was fixed at 10TCID₅₀. Commercial antiviral drug Acyclovir (Sigma-Aldrich) was used as a positive control. Optical density (OD) was related directly to the percentage of viable cells that was inversely proportional to the CPE. The viral replication was measured by the difference between the OD of the cell control and the OD of the virus control OD: viral replication = [1-OD virus control]-[1-OD cell control]. The viral replication was then evaluated in presence of E: viral replication E = [1-OD (virus+E)]-[1-OD cell control]. The percentage of reduction of the viral replication in the presence of E was calculated by the formula: Percentage of reduction (% of reduction) = [1- (viral replication E/viral replication)]×100. When the percentage of reduction of the viral replication was found to be below 10% the extract was considered as not having antiviral activity (-). When the percentage of reduction of the viral replication was above 50% in duplicates, the extract was scored as having antiviral activity (+). With a percentage of reduction of the viral replication between 10 and 50%, the extract was classified undetermined antiviral activity (±).

Table 1. Antibacterial activity of methanol extract of *G. umbrosus* leaves in Gram-positive and Gram-negative bacteria. Note: Average zone of inhibition (in mm) of duplicate including the diameter of the filter paper disc (6 mm). S=streptomycin (10 g/disc) P=penicillin(10 g/disc) V=vancomycin (10 g/disc). NT= not tested.

Test organism	Extract concentration (mg/ml)					Antibiotic control		
	100	50	25	10	5	S	P	V
Gram-positive bacteria								
<i>Staphylococcus aureus</i> ATCC 11632	9	8	8	7	8	NT	25.0	NT
<i>Streptococcus epidermidis</i> ATCC 12228	8	8	6	6	9	NT	20.0	NT
<i>Bacillus thuringiensis</i> ATCC 10792	8	6	6	6	6	NT	12.0	NT
MRSA 11 (Clinical isolate)	12	10	8	9	9	NT	NT	20.0
MRSA 12 (Clinical isolate)	9	8	7	6	6	NT	NT	20.0
MRSA 13 (Clinical isolate)	18	8	9	11	6	NT	NT	21.0
MRSA 14 (Clinical isolate)	20	9	7	6	6	NT	NT	20.0
MRSA 15 (Clinical isolate)	11	10	9	6	6	NT	NT	17.0
MRSA 17 (Clinical isolate)	8	6	6	6	6	6	6	16
Gram-negative bacteria								
<i>Pseudomonas aeruginosa</i> ATCC 10145	13	10	9	8	8	19	NT	NT
<i>Vibrio cholera</i>	10	9	8	6	6	14	NT	NT
<i>Vibrio parahaemolyticus</i> ATCC 17802	9	8	7	6	6	11	NT	NT
<i>Salmonella typhi</i>	8	9	7	9	9	9	NT	NT
<i>Salmonella dysenteriae</i>	10	6	6	6	6	26	NT	NT
<i>Serratia marcescens</i> ATCC 13880	8	8	6	6	6	12	NT	NT

Table 2. The free radical scavenging activity percentage and 50% effective concentration (EC₅₀) of crude methanol extract of *G. umbrosus* leaves and trolox as control.

Test sample	Free radical scavenging activity (%)							EC ₅₀ mg/ml
	Concentration (mg/ml)							
	0.005	0.025	0.05	0.195	0.391	0.781	1.563	
<i>G. umbrosus</i>	-	-	-	26	65	75	84	0.263
Trolox	39	86	88	91	92	91	93	0.01

RESULT AND DISCUSSION

Antioxidant activity

The free radical scavenging effect of crude methanol extract of *G. umbrosus* leaves and trolox with their EC₅₀ values are shown in Table 2. The extract showed maximum inhibition of about 84% and promising antioxidant activity with an EC₅₀ of 0.263 mg/ml. Since EC₅₀ is a measure of inhibitory concentration, a lower EC₅₀ value would reflect greater antioxidant activity of the sample (Yamaguchi et al., 1998).

Abdel-Wahab et al. (2009a) reported that the dichloromethane extract of *G. umbrosus* leaf has low antioxidant activity due to lack of phenolic content. However, a study on the antioxidant activity of *G. macrophyllus* root methanol extract showed IC₅₀ value of 0.96 mg/ml (Batubara et al., 2010). The antioxidant activity in plants has always been investigated for their capacity to scavenge free radicals. However, in

Annonaceous plant species this activity have not been reported widely even though anticancer agents like acetogenins and phenolic compounds styryl-lactones which are cytotoxic against a broad range of cancer types (Wiat, 2007; McLaughlin, 2008) are available. The anticancer activity has not been associated with the direct antioxidant activity but to the inhibition of superoxide dismutase activity in malignant cells that leads to free radical mediated damage to mitochondrial membranes, release of cytochrome c, and entry of cells into apoptosis (Huang et al., 2000). The antioxidant activity of phenolics have always been associated with their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and a metal chelation potential (Rice-Evans et al., 1995).

Antibacterial activity

The zone of inhibition of the crude methanol extract of

G. umbrosus leaves against test bacteria is shown in Table 1. Broad spectrum antibacterial activity was exhibited by the methanol extract against both Gram-positive and Gram-negative bacteria. What is more interesting from this study is that MRSA isolates were more sensitive towards the test substance compared to the non-MRSA or termed Methicillin sensitive *S. aureus* (MSSA). *S. aureus* may occur as a commensal on skin and in the nose frequently (Whitt and Salyers, 2002) and throat less commonly. The selective activity of the test substance towards more virulent organism such as MRSA shown in this study is very interesting and should be looked into more closely for the specific target or selectivity and mechanism of action.

The results from this study is very important for the development of novel antibiotic for new evolutions of the MRSA bacterium that have been dubbed Vancomycin intermediate-resistant *Staphylococcus aureus* (VISA) and Vancomycin Resistant *Staphylococcus aureus* (VRSA) (Schito, 2006).

The antibacterial activity in this study is consistent with the findings of Abdelwahab et al. (2009) that demonstrated a broad spectrum of antibacterial activity against both Gram positive and Gram negative organisms at different extent. The methanol extract used in their study however was extracted first in dichloromethane which most probably causing lipophilic secondary metabolites such as acetogenins and styryl-lactones to be removed. This study however is in agreement with the study reported by Wiart (2007) that showed methanol extract of *G. scortechinii* inhibited the growth of several nosocomial bacteria with higher antibacterial activity against Gram-positive bacteria than Gram-negative bacteria. He hypothesized a new antibacterial pathway that would encompass a 'bacterial apoptosis' due to the fact that mitochondria in eukaryotic cells take their origin in pro-bacterial ancestors from which they inherited NADH:ubiquinone oxidoreductase (Gabaldon et al., 2005).

MRSA strains were different from MSSA mainly due to the ability to the production of different penicillin-binding protein, PBP2A or PBP2', which allows the synthesis of the peptidoglycan layer and cell wall synthesis in the presence of β -lactams (Appelbaum, 2007). Another important virulence factor associated overall in 77% of MRSA strains is the Pantan-Valentine leukocidin (PVL) (Stevens et al., 2007) which at lower concentrations induces the apoptosis of leukocytes (Genestier et al., 2005). Further studies should be done to determine if any of these factors becomes the target of antibacterial property from the methanol extract of *G. umbrosus* leaves.

Cytotoxicity of *G. umbrosus* leaf methanol extract and SPD

The CC₅₀ value of leaves crude methanol extract of *G.*

umbrosus was 125 g/ml and SPD was 31.25 μ g/mL (1.563×10^{-7} μ M). These values can be considered as low in cytotoxicity as suggested by Shier (1991). Compounds which demonstrated the CC₅₀ value of more than 10-25 g/mL can be considered as weak cytotoxicity while compounds with the CC₅₀ value of less than 5.0 g/ml were considered very active. Thus the crude methanol extract and SPD can be further evaluated for development as antibacterial and antiviral drugs.

Evidence on goniotalamin showed its principal advantage in showing great cytotoxic activity against colon and breast cancer cells and less toxic against normal cells (Wattanapiromsakul et al., 2005). In this study we chose the neutral red assay which is based on the accumulation of the neutral red dye in the lysosomes of viable and uninjured cells (Fotakis and Timbrell, 2005) over the MTT assay which is mainly based on the enzymatic conversion of MTT in the mitochondria. This is to avoid contradicting results that might rise from the direct mitochondrial activities of acetogenins and styryl-lactones such as goniotalamin which might be available in the extract.

Antiviral activity

In the antiviral study, the extract showed the ability to reduce viral replication by all means of treatment (Table 3). There is an agreement in the activities shown by the extract and SPD in treatment (C+V)+E but not in others. The result demonstrated that the extract and SPD were capable to control viral infection after 2 h post-infection which according to Honess and Roizman (1974) is the early stage of replication particularly during viral attachment. This implies that both *G. umbrosus* leaf methanol extract and SPD were effective in controlling virus post-infection

Cells that were treated for one day with the extract before virus was added, (C+E)+V at 1CC₅₀ and 0.5CC₅₀ showed control to viral replication implying the ability to act as a prophylactic substance against viral infection (Table 3). The extract was also effective in protecting cells against viral infection when added together to cells in C+(V+E). This refers to the ability of extract to control viral infectivity during the early replication phase. In this study, acyclovir which is a nucleoside analogue showed the ability to protect cell from viral infection in treatment (C+E)+V and when added simultaneously with virus to cell, C+(V+E). The methanol extract have not been purified and may contain more than one antiviral compound that could inhibit the replication of HSV by different mechanism. This explains the data showing that the methanol extract has antiviral activity in all the treatments compared to SPD which is only effective in post-infection treatment. Further purification of the extract will allow the determination of compounds able to inhibit HSV replication.

Herpesviruses possess Bcl-2 homologues that block

Table 3. The antiviral effect of crude methanol extract of *G. umbrosus* leaves, goniiothalamine and acyclovir (E) in different treatments.

Test substance	CC ₅₀ equivalent	Concentration (µg/ml)	(C+V)+E	(C+E)+V	C+(V+E)
<i>G. umbrosus</i>	2	250	+	±	+
	1	125	+	+	+
	0.5	63	+	+	+
Goniothalamine	1	31.25	+	±	-
	0.5	15.63	+	-	-
Acyclovir		125			

Note: The antiviral activity of the extract or goniiothalamine (E) was evaluated based on the percentage of reduction in the viral replication (% of reduction). Degree of antiviral activity: - = no antiviral activity (% of reduction is below 10%); + = having antiviral activity (% of reduction was above 50% in duplicates); (±) = undetermined (% of reduction between 10 and 50%). Acyclovir as positive control.

apoptosis at the mitochondria before activation of the caspases (Cheng et al., 1997; Marshall et al., 1999; Nava et al., 1997). Restoring the ability of an infected cell to progress through apoptosis is an attractive means of treating viral infections. On the other hand, the apoptosis-inducing effect of styryl-lactones from *G. cheliensis*, on human promyelocytic leukemia HL-60 showed the activation of caspase-3, reduced the expression of the anti-apoptotic gene Bcl-2, and increased the expression of the pro-apoptotic gene Bax via cAMP dependent protein kinase mechanism (Wang et al., 2005). Thus, further studies on understanding the mechanism of action for methanol extract of *G. umbrosus* leaves and SPD in inducing apoptosis in virus infected cells is currently being undertaken.

Conclusion

This study demonstrates the ability of methanol extract of *G. umbrosus* leaves as an antioxidant with broad spectrum antibacterial activity and pronounced activity towards MRSA. The extract and SPD were active in inhibiting viral replication after virus was infected to cells. The extract was effective as a prophylaxis and when available during virus infection.

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