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Constituents of Brazilian red propolis and their preferential cytotoxic activity against human pancreatic PANC-1 cancer cell line in nutrient-deprived condition

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Abstract—Human pancreatic cancer cells such as PANC-1 are known to exhibit marked tolerance to nutrition starvation that enables them to survive for prolonged period of time even under extremely nutrient-deprived conditions. Thus, elimination of this tolerance to nutrition starvation is regarded as a novel approach in anticancer drug development. In this study, the MeOH soluble extract of Brazilian red propolis was found to kill 100% PANC-1 cells preferentially in the nutrient-deprived condition at the concentration of 10 μg/mL. Further phytochemical investigation led to the isolation of 43 compounds including three new compounds, (6a*S*,11a*S*)-6a-ethoxymedicarpan (**1**), 2-(2′,4′-dihydroxyphenyl)-3-methyl-6-methoxybenzofuran (**2**), and 2,6-dihydroxy-2-[(4-hydroxyphenyl)methyl]-3-benzofuranone (**3**). Among them, (6a*R*,11a*R*)-3,8-dihydroxy-9-methoxypterocarpan (**21**, DMPC) displayed the most potent 100% preferential cytotoxicity (PC_{100}) at the concentration of 12.5 μM. Further study on the mode of cell death induced by DMPC against PANC-1 cells indicated that killing process was not accompanied by DNA fragmentation, rather through a nonapoptotic pathway accompanied by necrotic-type morphological changes.

1. Introduction

Pancreatic cancer is the most deadly of all malignancies associated with the lowest 5-year survival rates known for cancers.[1] It shows resistance to almost all known chemotherapeutic agents such as 5-fluorouracil, taxol, doxorubicin, cisplatin and campothecin, and still represents a challenging therapeutic problem at the start of the 21st century.[1,2] Currently, surgery is the only treatment modality that offers any prospect of potential cure.[2,3] Therefore, there is a dire need for searching new alternatives to improve the clinical outcome for patients diagnosed with pancreatic cancer. It has been estimated that more than two-thirds of human cancers could be prevented by modification of lifestyle, including dietary modification.[4]

Cancer cells, in general, proliferate extremely rapidly and the demand for nutrition as well as oxygen is always high. One of the ways that rapidly growing cancer cell obtains nutrition requirement is by randomly recruiting new blood vessels, a phenomenon commonly known as angiogenesis. However, newly formed blood vessels are poorly organized and despite continuous angiogenesis, large number of tumor cells are in hypoxic and anoxic states.[5–7] The cancer cells below 150 μm away from the blood capillary surface are in much stress for oxygen and nutrition due to the compromised and anisotropic blood supply. Thus the second way that cancer cells survive under such extreme state is by changing their energy metabolism and acquiring a tolerance against nutrition starvation. Among the different forms of cancer, pancreatic cancer cells have extremely high tolerance to nutrition starvation that enables them to survive for the prolonged period of time even in the complete absence of nutrition.[8] Thus, it has been hypothesized that the search of agents that can eliminate the tolerance of cancer cells starvation might be novel approach in anticancer drug discovery.[8–12] Accordingly, a novel strategy has been developed for screening of anticancer agents that

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preferentially kill the cancer cells under low nutrition state by eliminating their ability to tolerate the nutrition starvation, an approach termed as anti-austerity strategy for anticancer drug discovery.[9–12] Under this strategy, we screened 500 medicinal plants used in the Japanese Kampo medicine, and identified arctigenin[9] and a novel compound angelmarin[10] as the compounds having activity to eliminate the cancer cells, tolerance to nutrient starvation. In our continued study, we recently found that the methanol extract of red propolis collected from the Paraiba state of Brazil displayed 100% preferential cytotoxicity (PC_{100}) against human pancreatic PANC-1 cancer cell line in nutrient-deprived medium (NDM) at the concentration of 10 μg/mL. Thus, we carried out a detailed investigation to identify active constituents which led to the isolation of 43 compounds including three new compounds (Fig. 1). In this paper, we report the structures of these new compounds together with preferential cytotoxic activity and mode of cell death exerted by the active constituents against PANC-1 cells.

2. Results and discussion

2.1. Isolation and identification

The MeOH extract of Brazilian red propolis was subjected to a series of chromatographic separations that resulted in the isolation of three new compounds: (6a*S*,11a*S*)-6a-ethoxymedicarpan (**1**), 2-(2′,4′-dihydroxyphenyl)-3-methyl-6-methoxybenzofuran (**2**), 2,6-dihydroxy-2-[(4-hydroxyphenyl)methyl]-3-benzofuranone (**3**), together with 40 known compounds: (2*R*,3*R*)-3,7-dihydroxy-6-methoxyflavanone (**4**),[13] alnusin (**5**),[14] alnustinol (6),[14] (+)-pinoresinol dimethyl ether (**7**),[15] (2*S*)-dihydrooroxylin A (**8**),[16] (6a*S*,11a*S*)-medicarpan (**9**),[17] (6a*R*,11a*R*)-3,4-dihydroxy-9-methoxypterocarpan (**10**),[17] (2*S*)-dihydrobaicalein (**11**),[18] (6a*R*,11a*R*)-4-methoxymedicarpin (**12**),[19] (7*S*)-dalbergiphenol (**13**),[20] (2*S*)-7-hydroxy-6-methoxyflavanone (**14**),[21] (6a*R*,11a*R*)-3-hydroxy-8,9-dimethoxypterocarpan (**15**),[17] 2′,4′-dihydroxychalcone (**16**),[22] (3*S*)-7-*O*-methylvestitol (**17**),[17] (6a*S*,11a*S*)-3,10-dihydroxy-9-methoxypterocarpan (**18**),[23] (2*S*)-7-hydroxyflavanone (**19**),[24] (+)-pinoresinol (**20**),[25] (6a*R*,11a*R*)-3,8-dihydroxy-9-methoxypterocarpan (**21**),[17] (3*S*)-Mucronulatol (**22**),[26] (2*R*,3*R*)-3,7-dihydroxyflavanone (**23**),[27] biochanin A (**24**),[17] formononetin (**25**),[17] (3*S*)-ferreirin (**26**),[28] 2′-hydroxybiochanin A (**27**),[29] (3*S*)-violanone (**28**),[30] pratensein (**29**),[31] xenognosin B (**30**),[32] (+)-syringaresinol (**31**),[33] (3*S*)-vestitol (**32**),[17] isoliquiritigenin (**33**),[17] (3*S*)-vestitone (**34**),[30] (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone (**35**),[32] (2*S*)-liquiritigenin (**36**),[17] calycosin (**37**),[31] (2*S*)-naringenin (**38**),[28] garbanzol (**39**),[34] 4,4′-dihydroxy-2′-methoxychalcone (**40**),[35] (3*S*)-isovestitol (**41**),[17] (α*S*)-α,2′,4,4′-tetrahydroxydihydrochalcone (**42**),[36] and daidzein (**43**)[32] (Fig. 1). The structures of new compounds were eluci-

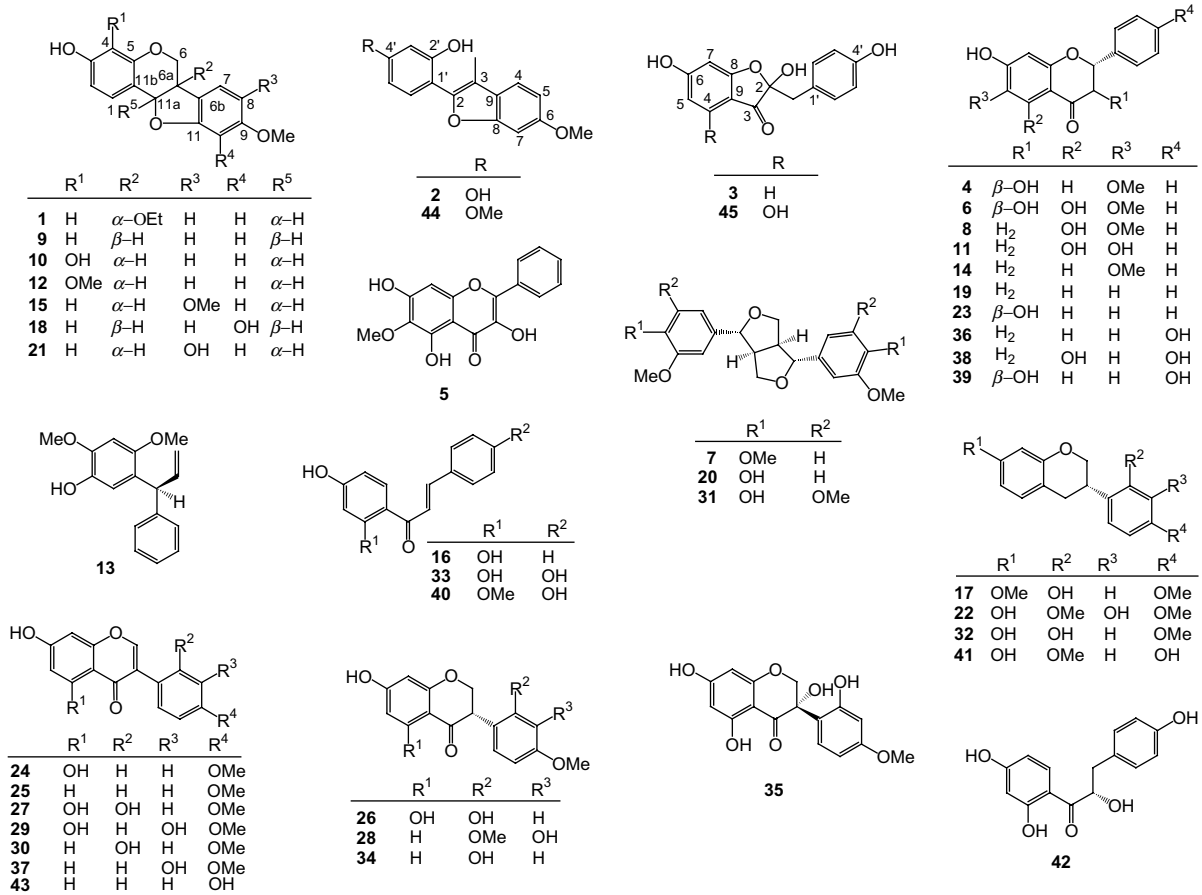

Figure 1. Structures of the compounds isolated from Brazilian red propolis.

dated by analysis of their spectroscopic data, while known compounds were identified on the basis of spectral data comparison with those published in the literatures.

Compound **1** was isolated as a yellow oily substance with α_D^{22} −31.5° (*c* 0.1, $CHCl_3$). Its molecular formula was deduced by HREIMS to be $C_{18}H_{18}O_5$ [*m/z* 314.1113 (M^+)]. The IR spectrum of **1** showed the absorptions due to hydroxyl (3515 cm^{-1}) group and aromatic ring (1610, 1495 cm^{-1}). The ^1H NMR spectrum of **1** exhibited signals due to two sets of three aromatic protons in ABX spin system (δ_H 7.34, d, J = 8.2; δ_H 6.55, dd, J = 8.2, 2.2; δ_H 6.35, d, J = 2.2 and δ_H 7.21, d, J = 8.2; δ_H 6.52, dd, J = 8.2, 2.2; δ_H 6.37, d, J = 2.2), an oxygenated methine (δ_H 5.52), two oxygenated methylenes (δ_H 4.23, 4.15; δ_H 3.47, 3.26), a methoxyl (δ_H 3.76), and a methyl group (δ_H 1.14) (Table 1). The ^{13}C NMR spectrum of **1**, on the other hand, displayed signals of 18 carbons, including twelve aromatic carbons, five oxygenated sp^3 carbons, and one methyl carbon (Table 1). These data closely resembled those of (6a*S*,11a*S*)-medicarpan (**9**),[17] isolated from the same extract, except for the disappearance of the signal due to a methine proton (δ_H 3.52) and the appearance of additional signals ascribable to an ethoxyl group ($\delta_{C\text{-}1'}$ 58.8; $\delta_{C\text{-}2'}$ 15.6) in **1**. The location of the ethoxyl group was deduced to be at C-6a based on HMBC correlations observed between oxymethylene protons at δ_H 3.47 and δ_H 3.26 oxygenated quaternary carbon at δ_C 81.7 (Fig. 2a). The absolute stereochemistry of **1** was assigned as 6a*S*, 11a*S* by comparison of CD spectral data ($[\theta]_{275}$ +8457; $[\theta]_{235}$ −20,662) with that of **9**.[17] Thus the structure of **1** was concluded as (6a*S*,11a*S*)-6a-ethoxymedicarpan.

Compound **2** was isolated as a brown oily substance and its molecular formula was determined by HREIMS to be $C_{16}H_{14}O_4$ [*m/z* 270.0911 (M^+)]. The IR spectrum of 2 indicated the presence of hydroxyl (3500 cm^{-1}) group, aromatic ring (1610, 1490 cm^{-1}). The ^1H NMR spectrum of **2** displayed signals due to two sets of three aromatic protons in ABX system (δ_H 7.41, 7.01, 6.91; δ_H 7.31, 6.54, 6.51), together with a methoxyl (δ_H 3.87) and a vinylic methyl group (δ_H 2.32). The ^{13}C NMR spectrum of **2**, on the other hand, exhibited the signals of 16 carbons, including twelve aromatic carbons, two olefinic carbons, a vinylic methyl carbon, and a methoxyl carbon (Table 1). These data were typical of arylbenzofuran derivative, and resemble close to 2-(2′-hydroxy-4′-methoxyphenyl)-3-methyl-6-methoxybenzofuran (**44**), previously reported from the *Indigofera micarocarpa* from Brazil.[37] However, **2** showed the presence of only one of methoxyl group instead of two in **44**. The location of methoxyl group was finally assigned to be at C-6 based on the HMBC correlations (Fig. 2b) between methoxyl protons at δ_H 3.87 and oxygenated aromatic carbon at δ_C 158.1 (C-6), together with the

Table 1. ^1H and ^{13}C NMR data for new compounds **1**–**3** (*J* values in parentheses)

Position	**1**		**2**		**3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	7.34 d (8.2)	132.1				
2	6.55 dd (8.2, 2.2)	110.1		147.9		106.0
3		156.9		111.8		195.8
4	6.35 d (2.2)	96.6	7.41 d (8.3)	123.7	7.26 d (8.3)	132.9
4a		156.3				
5			6.91 dd (8.3, 2.2)	111.7	6.38 dd (8.3, 1.7)	111.0
6	4.23 d (11.4)	69.5		158.1		172.1
	4.15 d (11.4)					
6a		81.7				
6b		116.7				
7	7.21 d (8.2)	124.8	7.01 d (2.2)	95.8	6.26 d (1.7)	97.6
8	6.52 dd (8.2, 2.2)	107.6		154.2		167.2
9		162.5		123.7		111.5
10	6.37 d (2.2)	103.7				
10a		161.9				
11a	5.52 s	81.7				
11b		113.8				
α					2.99 d (13.6)	40.6
					2.91 d (13.6)	
1′	3.47 m	58.8		109.9		123.8
	3.26 m					
2′	1.14 t (7.1)	15.6		155.3	6.89 d (7.3)	131.1
3′			6.54 d (2.4)	103.7	6.51 d (7.3)	114.5
4′				157.9		155.8
5′			6.51dd (8.3, 2.4)	107.9	6.51 d (7.3)	114.5
6′			7.31 d (8.3)	130.1	6.89 d (7.3)	131.1
2-OH					7.61 s	
3-CH_3			2.32 s	9.1		
4′-OH					9.11 s	
6-OCH_3			3.87 s	55.8		
9-OCH_3	3.76 s	55.4				

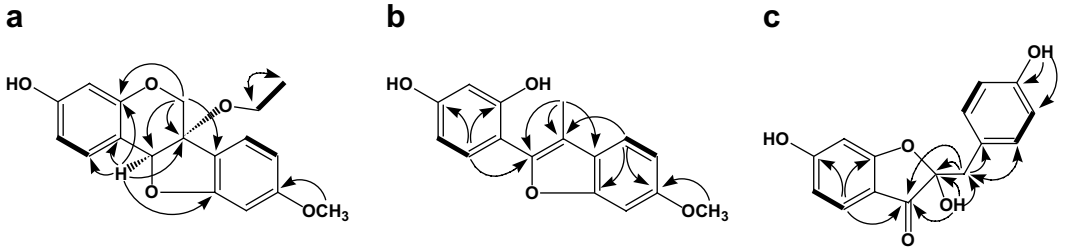

Figure 2. Connectivities (bold lines) deduced by the COSY spectrum and key HMBC correlations (arrows) observed for **1** (a), **2** (b), and **3** (c).

correlation between an ortho-coupled aromatic douplet at $\delta_{H\text{-}4}$ 7.41 (J = 8.3 Hz) with an olefinic carbon at δ_C 111.8 (C-3), C-6 and C-8. Thus the structure of compound **2** was concluded as 2-(2′,4′-dihydroxyphenyl)-3-methyl-6-methoxybenzofuran.

Compound **3** was isolated as red oily substance and its quasi-molecular ion peak at *m*/*z* 272.0656 (M^+) in HRE-IMS indicated its molecular formula to be $C_{15}H_{12}O_5$. The IR spectrum of **3** indicated the presence of hydroxyl (3200 cm^{-1}), carbonyl (1680 cm^{-1}) group, and aromatic ring (1600, 1510 cm^{-1}). The ^1H NMR spectrum of **3** displayed the signals due to three aromatic protons in the ABX spin system [δ_H 7.26, d, J = 8.3 Hz; δ_H 6.38, dd, J = 8.3, 1.7 Hz; δ_H 6.26, d, J = 1.7 Hz] and a set of symmetric pair of ortho-coupled aromatic protons at δ_H 6.89 (J = 7.3 Hz) and δ_H 6.51 (J = 7.3 Hz), together with geminal methylene protons at δ_H 2.99 (J = 13.6 Hz) and δ_H 2.91 (J = 13.6 Hz) (Table 1). Its ^{13}C NMR spectrum showed the signals of 15 carbons, including twelve aromatic carbons, a carbonyl carbon (δ_C 195.8), an acetal carbon (δ_C 106.0), and a methylene carbon (δ_C 40.6) (Table 1). These data closely resemble maesopsin (**45**), originally isolated from *Maesopsis eminii*.[38] However, **3** differs from **45** due to the presence of three aromatic protons in the ABX spin system instead of signal due to two meta coupled douplets at δ_H 5.76 (J = 1.7 Hz) and δ_H 5.73 (J = 1.7 Hz) in **45**. Thus, proton instead of hydroxyl substituent at C-4 in **3** was assumed, which was confirmed by HMBC correlations between aromatic douplet at δ_H 7.26 (J = 8.3 Hz, H-4) and ketone carbonyl at δ_C 195.8 (C-3) (Fig. 2c). Thus the structure of **3** was concluded as 4-dehydroxymaesopsin. Compound **3** exists as an enantiometric pair ($[\alpha]_D$ 0°) due to the reversible nature of hemiacetal at C-2.[38]

2.2. Chemical diversity

The isolated compounds comprised mainly flavonoids that include seven pterocarpans (**1**, **9**, **10**, **12**, **15**, **18**, **21**), four flavanonols (**4**, **6**, **23**, **39**), a neoflavonoid (**13**), an isoflavanonol (**35**), three isoflavanones (**26**, **28**, **34**), four chalcones (**16**, **33**, **40**, **42**), four isoflavans (**17**, **22**, **32**, **41**), seven isoflavones (**24**, **25**, **27**, **29**, **30**, **37**, **43**), six flavanones (**8**, **11**, **14**, **19**, **36**, **38**), and a flavonol (**5**), together with three lignans (**7**, **20**, **31**). This wide structural diversity among flavonoids in propolis have been observed for the first time in propolis, indicating the uniqueness of Brazilian red propolis among the propolis from other parts of the world.

The composition of propolis depends upon the local flora at the site of collection. Propolis from temperate zones, especially European propolis, contains predominantly cinnamic acid derivatives and flavonoids, and the poplar tree (*Populus nigra*) was considered as dominant plant source.[39] The polyprenylated benzophenones which were virtually absent in the temperate propolis were found to be the main constituents in the Cuban propolis (tropical sample) and the possible plant source was identified as genus *Clusia*.[40] Nepalese propolis which originates from the floral resin of genus *Dalbergia* mainly contains neoflavonoids, chalcones, and pterocarpans.[23] In present study, majority of the compounds have been reported from the plant belonging to genus *Dalbergia*. For example, four major compounds **4**, **17**, **25**, **36** together with **9**, **22**, **32**, **33**, and **43** were known to be present in *Dalbergia ecastophyllum*,[13] while **28**, **30**, **34** and **35** were reported from *D. odorifera*.[30,32] Similarly **13** and **24** were reported from *D. cultrate*[20] and *D. frutescens*,[41] respectively. Thus, the possible predominating plant source of Brazilian red propolis might be plants belonging to the genus *Dalbergia*.

2.3. In vitro biological evaluation

Propolis has been a subject of intense research, especially in the areas of anticancer research. A large number of compounds possessing anticancer activity have been reported. Phenethyl caffeate (CAPE) and its derivatives in Netherlands propolis possessed potent cytotoxicity against colon 26-L5 carcinoma.[42] A clerodane-type diterpene PMS-1 which was obtained from Brazilian propolis possessed cytotoxicity against human HuH13, HLC-2, HeLa, KB cancer cell lines.[43] A prenylated cinnamic acid derivative, artepillin C, from Brazilian propolis possessed potent colon cancer-preventing activity.[44] The prenylflavanones, propolin A–C, in Taiwanese propolis were reported for inducing apoptosis of human melanoma cells.[45,46] In our present study, we found that the methanol extract of red propolis showed strong cytotoxic activity against PANC-1 cancer cell line in NDM and isolated **43** compounds. All the isolated compounds (**1**–**43**) were tested for their preferential cytotoxic activity against PANC-1 cell line in NDM. PANC-1 cell is highly resistant to nutrient starvation, and can survive in NDM even after 48 h of starvation.[8–12] However, this tolerance to nutrient starvation was remarkably eliminated by the tested compounds in a concentration as well as time dependent manner. The tested compounds exhibited different potency of

Table 2. Preferential cytotoxic concentrations of the tested compounds in NDM

Compound	Preferential cytotoxic concentration[a] (μM)
1, **3**–**8**,**11**–**13**, **15**, **16**, **19**, **20**, **22**–**43**	>100
2, **9**, **14**, **18**	50
10, **17**	25
21	12.5
Taxol	>100
5-Fluorouracil	>100
Arctigenin[b]	1

[a] Concentration at which 100% cell death was observed in the cells cultured in nutrient-deprived medium (NDM).
[b] Positive control.

toxicity (Table 2). Among them, 3,8-hydroxy-9-methoxypterocarpan (DMPC, **21**) displayed the most potent preferential cytotoxicity at a concentration of 12.5 μM. Interestingly, conventional anticancer drug in clinical use, such as 5-fluorouracil, taxol was found to be virtually inactive (>100 μM). Arctigenin, an anti-austerity based anticancer agent, has been used as positive control in the present study that displayed total preferential cytotoxic activity at 1 μM.

Upon careful inspection of the activity of the tested compounds, it was observed that the compounds possessing medicarpan carbon framework with increasing number of hydroxyl group showed increased activity. Medicarpan possessing a hydroxyl group at C-4 or at C-8 was found to be stronger than that without (**21**, **10** > **9**). The position of hydroxyl group in the pterocarpan frame was also found to influence the activity. For example, **21** with a hydroxyl group at C-8 showed the most potent activity than that of 10 and 18 having a hydroxyl group at C-4 and at C-10, respectively (**21** > **10** > **18**). Thus, C-8 hydroxy group might play the most important role in medicarpan derivates for the enhancement of activity (**21** > **10** > **18**, **9** ≫ **1**, **12**, **15**).

2.4. Inhibition of Akt signaling is an unlikely mechanism

In our previous studies, we found that arctigenin, kigamicin D, pyrvinium pamoate, and troglitazone, that exhibited preferential cytotoxicity under the condition of nutrient deprivation, were shown to inhibit Akt activation.[9,11] Therefore, we examined whether DMPC, the most active constituent isolated in present study, also influences Akt activation in the cells under nutrient-deprived conditions. PANC-1 cells were incubated with DMPC for varying times. Cell lysates were subjected to immunoblotting analysis to detect Ser^{473} phosphorylation of Akt. Contrary to our previous findings, treatment of DMPC (100 μM) significantly increased Ser^{473} phosphorylation of Akt in PANC-1 cells in both NDM and DMEM (within 30 min), whereas total Akt protein level was not affected (Fig. 3). Interestingly, elevated Akt phosphorylation was sustained for up to 60 min in NDM. We conclude that DMPC (**21**) do not inhibit Akt signaling pathway in PANC-1 cells. Therefore, inhibition of Akt signaling is unlikely to be a major antitumor mechanism of DMPC in PANC-1 cells.

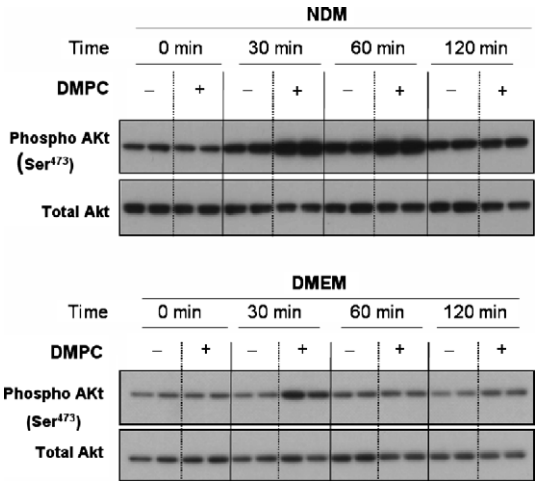

Figure 3. Effect of 3,8-dihydroxy-9-methoxypterocarpan (DMPC, **21**) on the activation of Akt as determined by Western blotting for the phosphorylated form (P-Akt Ser^{473}) in nutrient-deprived condition (NDM) and in DMEM.

2.5. Mode of PANC-1 cell death

In order to know the mode of PANC-1 cell death caused by DMPC in NDM, we then performed two distinct approaches: (1) annexin V/PI fluorescence detection and (2) DNA laddering.

2.5.1. Analysis of cell death by flow cytometry. Cell death was assessed by flow cytometry in cells treated with DMPC (**21**). Both adherent and floating cells were harvested by trypsin digestion and stained with annexin-V and propidium iodide (PI) according to the manufacturer's protocol. The *X* and *Y* axes indicate the fluorescence of annexin-V and PI, respectively. The early stages of apoptosis are characterized by the cells binding to annexin-V only (annexin-V^+/PI^-). Cells in late apoptosis are stained with annexin-V and PI (annexin-V^+/PI^+). Necrotic cells have lost the integrity of their plasma membrane and are predominantly stained with PI (annexin-V^-/PI^+). As shown in Figure 4, DMPC (**21**) triggered cell death (annexin-V^+/PI^+: combined necrosis, early and late apoptosis) in a time dependent manner.

2.5.2. DNA fragmentation and cell death detection. To investigate the precise mechanism of the cell death induced by DMPC under nutrient-deprived conditions, we examined the mode of cell death using the Hoechst 33342/propidium iodide staining method, as described previously.[9] As shown in Figure 5b, no nuclear fragmentation or chromatin condensations were observed, and the nuclear structures of PANC-1 cells were found to be maintained even after 24 h of treatment with 25 μM of DMPC. To further confirm these findings, we conducted the genomic DNA isolation from PANC-1 cells cultured in presence of DMPC (**21**) to examine whether it produced a typical ''ladder'' pattern when analyzed on an agarose gel. The DLD-1 cultured for 48 h in the ab-

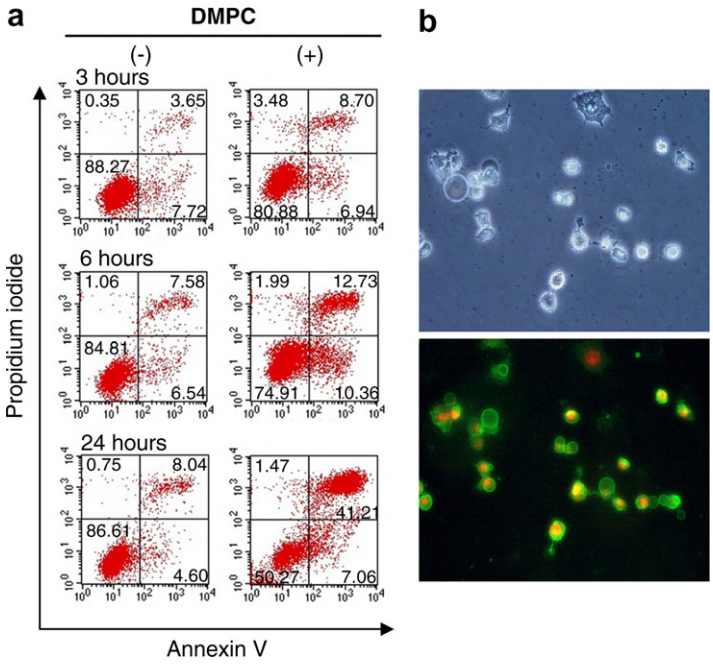

Figure 4. Effect of DMPC (**21**) on PANC-1. (a) After exposure to 25 μM of DMPC for indicated times, both floating cells and attached cells were stained with annexin V and propidium iodide. Stained cells were analyzed by flow cytometry. Annexin V–PI double positive cell population was increased in the time dependent manner. (b) Cells treated with 50 μM of DMPC for 24 h. After incubation, the cells were stained with annexin V (green) and propidium iodide (red), (upper) phase contrast microscopy, (lower) fluorescent microscopy. New compound-treated cells showed Annexin V–PI double positivity.

sence of amino acid treated with 1mM of 3-methyladenine is used as positive control (PC) in this study. As shown in Figure 5, DMPC did not induce typical DNA fragmentation. These results suggested that DMPC kills PANC-1 cells under NDM via nonapoptotic pathway, that does not lead to fragmentation of chromosomal DNA, but is accompanied by necrotic-type morphological changes of the cells.[47]

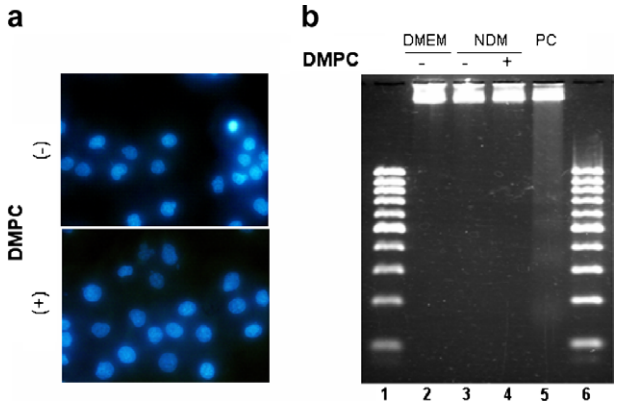

Figure 5. (a) PANC-1 were cultured for 24 h in nutrient-deprived media with or without 25 μM of DMPC. After incubation, the cells were stained with Hoechst 33342. Nuclear structure was maintained in the treated cells. (b) PANC-1 were cultured for 24 h in DMEM and nutrient-deprived media (NDM) with or without 25 μM of new compound. DNA ladder formation was analyzed with the genomic DNA of each treated cell. No ladder formation was observed in DMPC-treated cells. PC, positive control for apoptotic cells. (Lanes 1 and 6) Standard DNA ladder.

3. Conclusion

The methanol extract of red propolis collected from the Paraiba state of Brazil displayed 100% cytotoxicity, preferentially in nutrient-deprived medium (NDM) at the concentration of 10 μg/mL against PANC-1 cell line. Detailed phytochemical investigation led to the isolation of 43 compounds including three new compounds, (6a*S*,11a*S*)-6a-ethoxymedicarpan (**1**), 2-(2′,4′-dihydroxyphenyl)-3-methyl-6-methoxybenzofuran (**2**), and 2,6-dihydroxy-2-[(4-hydroxy-phenyl)methyl]-3-benzofuranone (**3**). All the isolated compounds were tested for their preferential cytotoxic activity against human pancreatic cancer PANC-1 cells under nutrient-deprived condition. Among them, (6a*R*,11a*R*)-3,8-dihydroxy-9-methoxypterocarpan (DMPC, **21**) displayed the most potent 100% preferential cytotoxicity at the concentration of 12.5 μM. DMPC triggered PANC-1 cell death in NDM in a time dependent manner via nonapoptotic pathway, that does not lead to fragmentation of DNA, but is accompanied by necrotic-type morphological changes.

4. Experimental

4.1. General methods

Optical rotations were measured on a JASCO DIP-140 digital polarimeter. IR spectra were measured with a Shimadzu IR-408 spectrophotometer in $CHCl_3$ solution or in KBr. NMR spectra were taken on a JEOL JNM-LA400 spectrometer with tetramethylsilane (TMS) as

an internal standard, and chemical shifts are expressed in δ values. HR-EI-MS measurements were performed on a JEOL JMS-700T spectrometer using a direct inlet system at the ionization voltage of 70 eV. CD spectra were measured in a JASCO J-805 spectropolarimeter. Medium pressure liquid chromatograph (MPLC) was performed with Yamazen MPLC system using a reversed-phase column (4.0 × 60 cm). Column chromatography was performed with BW-820MH silica gel (Fuji Silysia, Aichi, Japan) and LiChroprep RP-18 (Merck, Darmstadt, Germany). Analytical and preparative TLC was carried out on precoated silica gel $60F_{254}$ or RP-$18F_{254}$ plates (Merck, 0.25 or 0.50 mm thickness).

4.2. Biological material

Brazilian red propolis was collected from South coast of Paraiba State, Brazil, in 2005. A voucher specimen (TMPU 25226) is preserved in the Museum of Materia Medica, Research Center for Ethnomedicines, Institute of natural Medicine, University of Toyama, Japan.

4.3. Extraction and isolation

Brazalian propolis (49 g) was extracted with MeOH under sonication (500 mL × 1.5 h × 3 times) to give methanol extract (25 g). The MeOH extract was chromatographed on silica gel (8 × 45 cm) with a MeOH–$CHCl_3$ solvent system to give five fractions [1: MeOH–$CHCl_3$ (0:100) eluate, 6.3 g; 2: MeOH–$CHCl_3$ (2:98) eluate, 5.9 g; 3: MeOH–$CHCl_3$ (5:95) eluate, 3.5 g; 4: MeOH–$CHCl_3$ (10:90) eluate, 2.5 g; 5: MeOH–$CHCl_3$ (30:93) eluate, 3.9 g].

Fraction 2 (5.9 g) was rechromatographed on silica gel (3.5 × 36 cm) with hexane–EtOAc (10% → 60% EtOAc) to afford four subfractions (2-1, 1.2 g; 2-2, 498 mg; 2-3, 713 mg; 2-4, 275 mg). Subfraction 2-1 was further purified by normal-phase preparative TLC with EtOAc–benzene (1:7) to give **16** (3.0 mg), **17** (29.8 mg), and **18** (3.5 mg). Subfraction 2-3 was further rechromatographed on silica gel (2 × 40 cm) with 10% EtOAc–hexane to afford four subfractions (2-3-1, 315 mg; 2-3-2, 271 mg; 2-3-3, 198 mg; 2-3-4, 624 mg). Subfraction 2-3-1 was subjected to preparative TLC with 2% MeOH–CH_2Cl_2 to give **15** (8.9 mg). Subfractions 2-3-2 and 2-3-3 were separately subjected to preparative TLC with EtOAc–benzene (1:5) to give **4** (5.4 mg), **9** (5.0 mg), **11** (11.2 mg), and **12** (2.5 mg), **13** (12.6 mg), **14** (15.7 mg), and **15** (2.0 mg), respectively. Subfraction 2-3-4 was separated by reversed-phase preparative TLC with acetone–CH_3CN–H_2O (2:2:3) to yield **4** (11.4 mg), **7** (15.6 mg), and **8** (10.0 mg).

Fraction 3 (3.5 g) was rechromatographed on ODS (4 × 40 cm) with CH_3CN–MeOH–H_2O (1:1:2 → 1:1:1 → 1:2:1 → 2:2: 1) to afford four subfractions (3-1, 298 mg; 3-2, 872 mg; 3-3, 314 mg; 3-4, 1.24 g). Subfraction 3-1 was subjected to reversed-phase preparative TLC with CH_3CN–MeOH–H_2O (1:1:2) to give **28** (4.0 mg), **29** (2.0 mg), and 30 (2.5 mg). Subfraction 3-2 (872 mg) was rechromatographed on ODS (Yamazen ultra pack ODS-P-40C, 4 × 60 cm) using MPLC (Yamazen Prep. UV 254) with CH_3CN–MeOH–H_2O (2:2:3), followed by reversed-phase preparative TLC with MeOH–H_2O (1:1), to give **2** (9.0 mg), **22** (11.5 mg), **23** (2.1 mg), **24** (10.5 mg), **25** (116.5 mg), and **26** (1.5 mg). Subfraction 3-3 (314 mg) upon recrystallization with MeOH gave **25** (238 mg). Subfraction 3-4 (1.24 g) was rechromatographed on silica gel (2 × 40 cm) with 20% EtOAc–hexane, followed by normal-phase preparative TLC with 15% EtOAc–benzene or reversed-phase preparative TLC with CH_3CN–MeOH–H_2O (1:1:1), to give **17** (91 mg), **19** (7.2 mg), **20** (8.8 mg), **1** (1.5 mg), and **21** (14.0 mg), **22** (3.5 mg), and **25** (27.5 mg).

Fraction 4 (2.5 g) was rechromatographed on ODS (Yamazen ultra pack ODS-P-40C, 4 × 60 cm) using MPLC (Yamazen Prep. UV 254) with CH_3CN–MeOH–H_2O (1:1:2 → 1:1:1 → 1:2:1 → 2:2:1) to afford four subfractions (4-1, 561 mg; 4-2, 376 mg; 4-3, 301 mg; 4-4, 1.2 g). Subfraction 4-1 was subjected to reversed-phase preparative TLC with MeOH–H_2O (2:3), to give **37** (2.4 mg) and **38** (88 mg). Subfraction 4-2 was subjected to reversed-phase preparative TLC with acetone–H_2O (1:2) to give **34** (3.5 mg), **35** (3.6 mg), **36** (18.2 mg), **37** (2.1 mg), and **38** (20.5 mg). Subfraction 4-3 was rechromatographed on ODS (Yamazen ultra pack ODS-P-40C, 4 × 60 cm) using MPLC (Yamazen Prep. UV 254) with CH_3CN–MeOH–H_2O (2:2:3), to give **32** (5 mg) and **36** (34.5 mg). Subfraction 4-4 was subjected to normal-phase preparative TLC with 3% MeOH–$CHCl_3$ to give **31** (2.1 mg), **32** (5.5 mg), and **33** (2.5 mg).

Fraction 5 (3.9 g) was rechromatographed on ODS (4 × 40 cm) with CH_3CN–MeOH–H_2O (1:1:2 → 1:1:1 → 1:2:1 → 2:2:1) to afford four subfractions (5-1, 273 mg; 5-2, 254 mg; 5-3, 675 mg; 5-4, 2.17 g). Subfractions 5-1 and 5-2 were separately subjected to reversed-phase preparative TLC with MeOH–H_2O (1:3) and MeOH–H_2O (1:2) to give **42** (2.8 mg), **43** (7.6 mg), and **3** (4.2 mg) and **40** (7.2 mg), respectively. Subfraction 5-3 and 5-4 were separately rechromatographed on ODS using MPLC with MeOH–H_2O (2:3), to give **38** (13.5 mg), **39** (7.8 mg), **40** (2.3 mg), and **41** (20.7 mg).

4.3.1. (6a*S*,11a*S*)-6a-Ethoxymedicarpan (1). Yellow oily substance, α_D^{22} −31.5° (c 0.1, $CHCl_3$); CD λ_{max} (EtOH, 0.318 mM) nm: 275 ([θ] +8457), 235 ([θ] −20662); IR ν_{max} ($CHCl_3$) 3515, 3000, 2400, 1610, 1495, 1310, 1130, 1050, 1030, 1010 cm^{-1}; HREIMS 314.1113 [calcd for $C_{18}H_{18}O_5$ (M^+), 314.1154]. ^1H and ^{13}C NMR, see Table 2.

4.3.2. 2-(2′,4′-Dihydroxyphenyl)-3-methyl-6-methoxybenzofuran (2). Brown oily substance, IR ν_{max} ($CHCl_3$) 3500, 2750, 1610, 1490, 1460, 1440, 1270, 1150, 1110, 1070, 1045 cm^{-1}; HREIMS 270.0911 [calcd for $C_{16}H_{14}O_4$ (M^+), 270.0892]. ^1H and ^{13}C NMR, see Table 2.

4.3.3. 2,6-Dihydroxy-2-[(4-hydroxyphenyl)methyl]-3-benzofuranone (3). Red oily substance, α_D^{22} 0° (*c* 0.16, MeOH); IR ν_{max} (KBr) 3200, 1680, 1600, 1510, 1455, 1290, 1150 cm^{-1}; HREIMS 272.0656 [calcd for $C_{15}H_{12}O_5$ (M^+), 272.0685]. ^1H and ^{13}C NMR, see Table 2.

4.4. Preferential cytotoxicity under nutrient-deprived condition

PANC-1 cancer cell preferential cytotoxicity assay was done as described earlier. Briefly, PANC-1 cancer cells were seeded in 96-well plates (1×10^4/well) and incubated in fresh Dulbecco′s modified Eagle's medium (DMEM; Nissui Pharmaceuticals, Tokyo, Japan) at 37 °C under 5% CO_2/95% air for 24 h. The cells were then washed with PBS, and the medium was changed to either DMEM or nutrient-deprived medium (absence of glucose, amino acid, and serum), followed by immediate addition of serial dilutions of the test samples. After 24-h incubation, the cells were washed again with PBS, then 100 μl of DMEM with 10% WST-8 cell counting kit solution was added to the wells, and the plate was incubated for a further 2 h. Then, the absorbance of the wells at 450 nm was measured. Cell viability was calculated by the following equation:

$$\text{Cell viability}\ (\%) = [\text{Abs}_{(\text{test sample})} - \text{Abs}_{(\text{blank})}/\text{Abs}_{(\text{control})} - \text{Abs}_{(\text{blank})}] \times 100\%$$

4.5. Western blot analysis

The proteins were separated by gel electrophoresis on a polyacrylamide gel containing 0.1% SDS and then transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 2% BSA and 5% (w/v) skim milk, washed with PBS containing 0.3% Tween 20 (Sigma), then incubated overnight at room temperature with Akt antibody and the phosphospecific (Ser^{473}) Akt antibody (New England Biolabs, Ipswich, MA) diluted with PBS. After washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) as the second antibody.

4.6. Annexin V–PI staining

PANC-1 cells treated with DMPC (**21**) were stained with Annexin-V-fluos staining kit (Roche, Germany) according to the manufacturer's protocol. Stained cells were analyzed using FACS caliber (BD, Franklin Lakes, NJ) and observed using DM-IRB fluorescent microscope (Leica, Germany).

4.7. Nuclear staining and DNA ladder formation assay

PANC-1 cells were cultured for 24 h in DMEM and nutrient-deprived medium with or without 25 μM of DMPC (21). After incubation, the cells were stained with Hoechst 33342 for 5 min and examined using fluorescent microscope. For DNA ladder formation assay, the cells were washed twice with ice-cold PBS and collected by centrifugation. The cell pellets were resuspended in 500 μL of lysis buffer (20 mM Tris–HCl, 20 mM EDTA, 100 mM NaCl, 0.1% SDS) containing proteinase K (0.5 mg/mL) and incubated at 55 °C for 2 h. Genomic DNA was extracted using phenol/chloroform and ethanol precipitation. Extracted DNA was dissolved in TE buffer containing RNase A (0.02 mg/mL) and incubated at 37 °C for 30 min. DNA samples were separated on 2% agarose gel and stained with ethidium bromide. Stable 100 bp DNA ladder (Sigma Aldrich) was used as standard marker. The bands were detected with an enhanced chemiluminescence system (Amersham Biosciences UK Ltd., Buckinghamshire, United Kingdom).

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