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Cytotoxic constituents from Brazilian red propolis and their structure–activity relationship

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Abstract—Several classes of flavonoids [flavanoids (**1**–**10**), flavonol (**11**), isoflavones (**12**–**18**), isoflavanones (**19**–**22**), isoflavans (**23**–**26**), chalcones (**27**–**30**), auronol (**31**), pterocarpans (**32**–**37**), 2-arylbenzofuran (**38**), and neoflavonoid (**39**)] and lignans (**40**–**42**) isolated from the MeOH extract of Brazilian red propolis were investigated for their cytotoxic activity against a panel of six different cancer cell lines including murine colon 26-L5 carcinoma, murine B16-BL6 melanoma, murine Lewis lung carcinoma, human lung A549 adenocarcinoma, human cervix HeLa adenocarcinoma, and human HT-1080 fibrosarcoma cell lines. Based on the observed results, structure–activity relationships were discussed. Among the tested compounds, 7-hydroxy-6-methoxyflavanone (**3**) exhibited the most potent activity against B16-BL6 (IC_{50}, 6.66 μM), LLC (IC_{50}, 9.29 μM), A549 (IC_{50}, 8.63 μM), and HT-1080 (IC_{50}, 7.94 μM) cancer cell lines, and mucronulatol (**26**) against LLC (IC_{50}, 8.38 μM) and A549 (IC_{50}, 9.9 μM) cancer cell lines. These activity data were comparable to those of the clinically used anticancer drugs, 5-fluorouracil and doxorubicin, against the tested cell lines, suggesting that **3** and **26** are the good candidates for future anticancer drug development.

1. Introduction

Propolis, a natural resinous product collected by honeybees from the buds and exudates of various plant sources, has been used empirically as a traditional remedy in folk medicine for centuries.[1] It is well known for potential health benefit and is reported to possess valuable biological activities such as antioxidant,[2] antibacterial,[3,4] antiviral,[4] antifungal,[4,5] anti-inflammatory,[6] and anticancer[7,8] activities. Recently, propolis has been extensively marketed by the pharmaceutical industries as an alternative medicine and as the health-food in various parts of the world. Propolis has been claimed to improve the health and prevent diseases such as diabetes, heart diseases, and even cancer.[1,7] The chemical composition of propolis is complex and largely depends on the geographical origin and specific flora at the site of collection. More than 300 compounds have been reported so far from propolis.[9,10] Propolis has become a subject of increasing interest among the researchers owing to its versatile biological activities.[10–14] Artepillin C, a characteristic constituent found specifically in Brazilian propolis, has been reported to possess antitumor activity and apoptosis inducing activity.[13] A compound PM-3, 3-[2-dimethyl-8-(3-methyl-2-butenyl)benzopyran]-6-propenoic acid isolated from Brazilian propolis markedly inhibited the growth of MCF-7 human breast cancer cells.[14] A clerodane-type diterpene PMS-1, also isolated from Brazilian propolis, possessed the cytotoxicities against human hepatocellular carcinoma HuH13 cells, human lung carcinoma HLC-2, HeLa, KB, and rat W3Y cells.[1] As a part of our continued research in propolis,[8,10,15–20] we also found that a red-type propolis collected from the Paraiba state of Brazil displayed preferential cytotoxic activity against human pancreatic PANC-1 cancer cell line in nutrient deprived medium (NDM)[8] at the concentration of 10 μg/mL. Thus, we carried out a detailed phytochemical investigation. The work led to the isolation of 42 compounds. The isolated compounds comprised mainly flavonoids that could be categorized into flavanones (**1**–**10**), flavonol (**11**), isoflavones (**12**–**18**), isoflavanones (**19**–**22**), isoflavans (**23**–**26**), chalcones (**27**–**30**), auronol (**31**), pterocarpans (**32**–**37**), 2-arylbenzofuran (**38**), neoflavonoid (**39**), and lignans (**40**–**42**). In the present study, we further evaluated these constituents for their cytotoxicity against a panel of six different cancer cell lines including three murine cancer cell lines (colon 26-L5 carcinoma,[21] B16-BL6 mela-

Keywords: Cytotoxic activity; Brazilian red propolis; Flavonoids; Structure–activity relationship.
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noma,[22] Lewis lung carcinoma[23]) and three human cancer cell lines (lung A549 adenocarcinoma,[24] cervix HeLa adenocarcinoma,[25] HT-1080 fibrosarcoma[26]). In this paper, we report the cytotoxic activities of the constituents from Brazilian red propolis and their structure–activity relationships.

2. Results and discussion

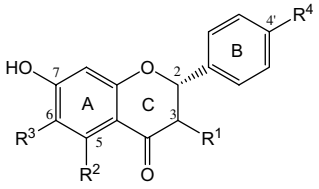
2.1. Chemicals

(2*S*)-7-Hydroxyflavanone (**1**), (2*S*)-liquiritigenin (**2**), (2*S*)-7-hydroxy-6-methoxyflavanone (**3**), (2*S*)-naringenin (**4**), (2*S*)-dihydrobaicalein (**5**), (2*S*)-dihydrooroxylin A (**6**), (2*R*,3*R*)-3,7-dihydroxyflavanone (**7**), garbanzol (**8**), (2*R*,3*R*)-3,7-dihydroxy-6-methoxyflavanone (**9**), alnustinol (**10**), alnusin (**11**), daidzein (**12**), formononetin (**13**), calycosin (**14**), xenognosin B (**15**), biochanin A (**16**), pratensein (**17**), 2′-hydroxybiochanin A (**18**), (3*S*)-vestitone (**19**), (3*S*)-violanone (**20**), (3*S*)-ferreirin (**21**), (3*R*)-4′-methoxy-2′,3,7-trihydroxyisoflavanone (**22**), (3*S*)-vestitol (**23**), (3*S*)-isovestitol (**24**), (3*S*)-7-*O*-methylvestitol (**25**), (3*S*)-mucronulatol (**26**), 2′,4′-dihydroxychalcone (**27**), isoliquiritigenin (**28**), 4,4′-dihydroxy-2′-methoxychalcone (**29**), (α*S*) − α,2′,4,4′-tetrahydroxydihydrochalcone (**30**), 2,6-dihydroxy-2-[(4-hydroxyphenyl)methyl]-3-benzofuranone (**31**), (6a*S*,11a*S*)-medicarpin (**32**), (6a*S*,11a*S*)-3,10-dihydroxy-9-methoxypterocarpan (**33**), (6a*R*,11a*R*)-3-hydroxy-8,9-dimethoxypterocarpan (**34**), (6a*S*,11a*S*)-6a-ethoxymedicarpin (**35**), (6a*R*,11a*R*)-3,4-dihydroxy-9-methoxypterocarpan (**36**), (6a*R*,11a*R*)-4-methoxymedicarpin (**37**), 2-(2′,4′-dihydroxyphenyl)-3-methyl-6-methoxybenzofuran (**38**), (7*S*)-dalbergiphenol (**39**), (+)-pinoresinol dimethyl ether (**40**), (+)-pinoresinol (**41**), and (+)-syringaresinol (**42**) were obtained from the MeOH extract of Brazilian red propolis.[8] The purity of these compounds was checked by TLC and ^{1}H NMR spectra, which did not show the presence of any impurity.

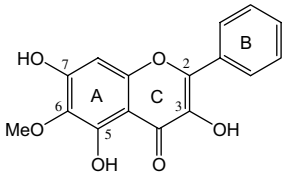
2.2. Cytotoxic activity

To investigate the cytotoxic activities of compounds **1**–**42** isolated from Brazilian red propolis, an in vitro assay was performed using three murine cancer cell lines [colon 26-L5 carcinoma (colon 26-L5),[21] B16-BL6 melanoma (B16-BL6),[22] Lewis lung carcinoma (LLC)[23]] and three human cancer cell lines [lung A549 adenocarcinoma (A549),[24] cervix HeLa adenocarcinoma (HeLa),[25] HT-1080 fibrosarcoma (HT-1080)[26]]. The results expressed as IC_{50} values are listed in Table 1–5. The conventional anticancer drugs in clinical use, 5-fluorouracil and doxorubicin, were used as positive controls in the present study. All the tested compounds exhibited different potency of cytotoxic activities in a concentration-dependent manner, although this effect varied among the cell types (see Table 1–5). Among them, a flavanone, (2*S*)-7-hydroxy-6-methoxyflavanone (**3**), showed the most potent activities against all the tested cancer cell lines (colon 26-L5, IC_{50} 5.85 μM; B16-BL6, IC_{50} 6.66 μM; LLC, IC_{50} 9.29 μM; A549, IC_{50} 8.63 μM; HeLa, IC_{50} 5.56 μM; HT-1080, IC_{50} 7.94 μM). The observed IC_{50} data were comparable to those of positive controls, 5-fluorouracil and doxorubicin, against B16-BL6, LLC, A549, and HT-1080 cell

Table 1. Structures and cytotoxic activity of flavanoids **1**–**10** and a flavonol **11**[a]



1 − 10

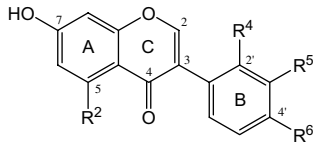


11

	Compound substitution				IC_{50} (μM)					
	R^1	R^2	R^3	R^4	Colon 26-L5	B16-BL6	LLC	A549	HeLa	HT-1080
1	H_2	H	H	H	51.9	99.9	>100	>100	>100	>100
2	H_2	H	H	OH	56.6	97.7	>100	>100	>100	>100
3	H_2	H	OMe	H	5.9	6.7	9.3	8.6	5.6	7.9
4	H_2	OH	H	OH	>100	>100	>100	>100	>100	>100
5	H_2	OH	OH	H	53.4	>100	>100	>100	>100	>100
6	H_2	OH	OMe	H	49.0	72.6	82.7	38.0	39.3	47.6
7	β-OH	H	H	H	>100	>100	>100	>100	>100	>100
8	β-OH	H	H	OH	>100	>100	>100	>100	>100	>100
9	β-OH	H	OMe	H	>100	>100	>100	>100	>100	>100
10	β-OH	OH	OMe	H	>100	>100	>100	>100	>100	>100
11					>100	>100	>100	>100	>100	>100
Doxorubicin[b]					0.33	8.05	8.04	7.88	0.68	9.32
5-Fluorouracil[b]					0.46	5.78	2.59	9.19	0.77	9.45

[a] Cytotoxic activities are expressed as IC_{50} values in micromolar units.
[b] Doxorubicin, 5-fluorouracil were used as positive control.

Table 2a. Structures and cytotoxic activity of isoflavones **12**–**18**[a]

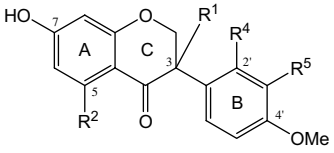


12 − 18

	Compound substitution				IC_{50} (μM)					
	R^2	R^4	R^5	R^6	Colon 26-L5	B16-BL6	LLC	A549	HeLa	HT-1080
12	H	H	H	OH	88.1	>100	>100	>100	>100	>100
13	H	H	H	OMe	83.6	>100	>100	>100	>100	>100
14	H	H	OH	OMe	98.0	>100	>100	>100	>100	>100
15	H	OH	H	OMe	49.3	34.1	>100	>100	69.8	>100
16	OH	H	H	OMe	98.0	>100	>100	>100	>100	>100
17	OH	H	OH	OMe	>100	>100	>100	>100	>100	>100
18	OH	OH	H	OMe	93.6	>100	>100	>100	>100	>100
Doxorubicin[b]					0.33	8.05	8.04	7.88	0.68	9.32
5-Fluorouracil[b]					0.46	5.78	2.59	9.19	0.77	9.45

[a] Cytotoxic activities are expressed as IC_{50} values in micromolar units.
[b] Doxorubicin, 5-fluorouracil were used as positive control.

Table 2b. Structures and cytotoxic activity of isoflavanones **19**–**22**[a]

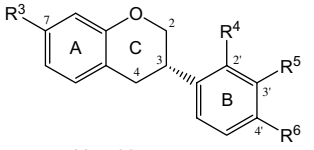


19 − 22

	Compound substitution				IC_{50} (μM)					
	R^1	R^2	R^4	R^5	Colon 26-L5	B16-BL6	LLC	A549	HeLa	HT-1080
19	β-H	H	OH	H	67.0	>100	>100	>100	>100	>100
20	β-H	H	OMe	OH	>100	>100	>100	>100	>100	>100
21	β-H	OH	OH	H	71.3	>100	>100	>100	>100	>100
22	α-OH	OH	OH	H	88.4	>100	>100	>100	>100	>100
Doxorubicin[b]					0.33	8.05	8.04	7.88	0.68	9.32
5-Fluorouracil[b]					0.46	5.78	2.59	9.19	0.77	9.45

[a] Cytotoxic activities are expressed as IC_{50} values in micromolar units.
[b] Doxorubicin, 5-fluorouracil were used as positive control.

Table 2c. Structures and cytotoxic activity of isoflavans **23**–**26**[a]

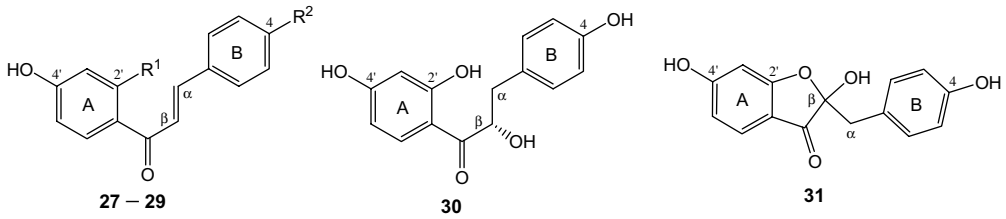


23 − 26

	Compound substitution				IC_{50} (μM)					
	R^3	R^4	R^5	R^6	Colon 26-L5	B16-BL6	LLC	A549	HeLa	HT-1080
23	OH	OH	H	OMe	67.0	57.4	92.3	>100	72.6	>100
24	OH	OMe	H	OH	49.8	33.6	>100	>100	77.1	75.3
25	OMe	OH	H	OMe	29.8	24.1	24.0	46.0	44.3	50.1
26	OH	OMe	OH	OMe	7.3	30.4	8.4	9.9	24.2	18.2
Doxorubicin[b]					0.33	8.05	8.04	7.88	0.68	9.32
5-Fluorouracil[b]					0.46	5.78	2.59	9.19	0.77	9.45

[a] Cytotoxic activities are expressed as IC_{50} values in micromolar units.
[b] Doxorubicin, 5-fluorouracil were used as positive control.

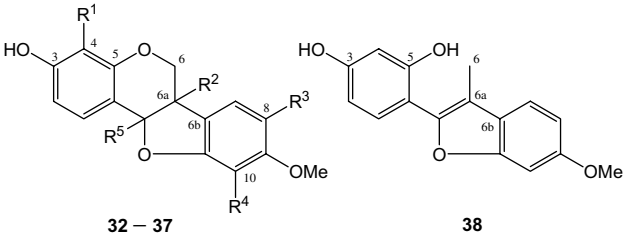
Table 3. Structures and cytotoxic activity of chalcones **27**–**30** and a auronol **31**[a]



	Compound substitution		IC_{50} (μM)					
	R^1	R^2	Colon 26-L5	B16-BL6	LLC	A549	HeLa	HT-1080
27	OH	H	7.4	44.3	39.5	41.6	23.1	34.0
28	OH	OH	21.8	80.5	84.0	98.9	82.6	76.7
29	OMe	OH	12.3	56.3	69.1	62.1	67.9	51.2
30			>100	>100	>100	>100	>100	>100
31			25.9	>100	>100	>100	>100	>100
Doxorubicin[b]			0.33	8.05	8.04	7.88	0.68	9.32
5-Fluorouracil[b]			0.46	5.78	2.59	9.19	0.77	9.45

[a] Cytotoxic activities are expressed as IC_{50} values in micromolar units.
[b] Doxorubicin, 5-fluorouracil were used as positive control.

Table 4. Structures and cytotoxic activity of pterocarpans **32**–**37** and a 2-arylbenzofuran **38**[a]



	Compound substitution					IC_{50} (μM)					
	R^1	R^2	R^3	R^4	R^5	Colon 26-L5	B16-BL6	LLC	A549	HeLa	HT-1080
32	H	β-H	H	H	β-H	11.3	42.4	17.2	39.5	23.4	28.4
33	H	β-H	H	OH	β-H	68.1	47.5	57.2	>100	61.2	>100
34	H	α-H	OMe	H	α-H	44.5	64.9	>100	>100	84.3	>100
35	H	α-OEt	H	H	α-H	>100	>100	>100	>100	>100	>100
36	OH	α-H	H	H	α-H	54.1	>100	>100	>100	>100	>100
37	OMe	α-H	H	H	α-H	29.4	>100	97.6	97.3	47.1	96.3
38						27.8	44.8	34.4	42.6	36.1	38.7
Doxorubicin[b]						0.33	8.05	8.04	7.88	0.68	9.32
5-Fluorouracil[b]						0.46	5.78	2.59	9.19	0.77	9.45

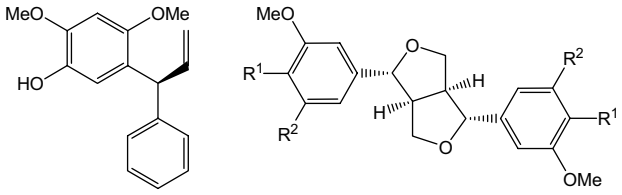
[a] Cytotoxic activities are expressed as IC_{50} values in micromolar units.
[b] Doxorubicin, 5-fluorouracil were used as positive control.

lines. Interestingly, in our previous work, compound **3** has induced 100% cell death, preferentially under the nutrient deprived condition at the concentration of 50 μM against human pancreatic PANC-1 cells.[8] In addition, an isoflavan-type flavonoid, (3*S*)-mucronulatol (**26**), also possessed the potent cytotoxic activities (Table 2c) against colon 26-L5 (IC_{50} 7.28 μM), LLC (IC_{50} 8.38 μM), and A549 (IC_{50} 9.9 μM) cells, while 2′,4′-dihydroxychalcone (**27**) displayed the potent activity against colon 26-L5 cells with an IC_{50} value of 7.43 μM (Table 3).

Upon careful inspection of the activity data (Table 1–5), good correlations between the structure and cytotoxic activity could be deduced for each class of compounds.

2.2.1. Flavanones and flavonol. Flavanones in general were selectively cytotoxic to colon 26-L5 carcinoma cell line (Table 1). Among the tested flavanones, 7-hydroxy-6-methoxyflavanone (**3**), and dihydrooroxylin A (**6**) were active against all the tested cancer cell lines. The most apparent structure–activity relationship in flavanones is the presence or absence of hydroxyl group at C-3. Flavanones possessing a hydroxyl group at C-3 (**7**– **10**) were found to be virtually inactive against all the tested cell lines with IC_{50} > 100 μM (Table 1). Alnusin (**11**), a flavonol possessing a hydroxyl group at C-3, was also

Table 5. Structures and cytotoxic activity of a neoflavonoid **39** and lignans **40–42**[a]



39 **40 − 42**

	Compound substitution		IC_{50} (μM)					
	R^1	R^2	Colon 26-L5	B16-BL6	LLC	A549	HeLa	HT-1080
39			60.3	53.8	>100	73.7	60.1	>100
40	OMe	H	>100	>100	>100	>100	>100	>100
41	OH	H	>100	>100	>100	>100	92.5	>100
42	OH	OMe	>100	>100	>100	>100	>100	>100
Doxorubicin[b]			0.33	8.05	8.04	7.88	0.68	9.32
5-Fluorouracil[b]			0.46	5.78	2.59	9.19	0.77	9.45

[a] Cytotoxic activities are expressed as IC_{50} values in micromolar units.
[b] Doxorubicin, 5-fluorouracil were used as positive control.

inactive against all the tested cell lines. Similarly, the presence of a 5-hydroxyl group in phenyl ring A on flavanones significantly decreased the activity (**2** > **4**; **3** > **6**). In contrast, the presence of a methoxyl group at C-6 is strongly favored for the activity (**3** > **1**, **6** > **5**). On the other hand, the presence of a 4′-hydroxyl group on ring B of flavanones did not show any significant difference in activity (**1** ≈ **2**). Based on these observations, the presence of a methoxyl group at C-6, without a hydroxyl group at C-3 and C-5, on flavanone skeleton is concluded to be the most essential criteria for the enhancement of activity.

2.2.2. Isoflavones, isoflavanones, and isoflavans . The isoflavones (**12**–**18**) displayed cytotoxicity selectively against human colon 26-L5 cancer cells (Table 2a). Isoflavones having a hydroxyl group at C-2′ were active (**15** > **13**), while the presence of a methoxyl group is favored than a hydroxyl group at C-4′ (**13** > **12**). However, hydroxylation at C-5 diminished the activity (**13** > **16**; **14** > **17**; **15** > **18**). Similarly, hydroxylation at C-3′ also reduced the activity (**13** > **14**). Based on these observations, it is concluded that the combination of 2′-hydroxyl and 4′-methoxyl groups, without hydroxyl group at C-5 and C-3′, on isoflavone skeleton seems to enhance the cytotoxic activity. Isoflavanones (**19**–**22**) also showed cytotoxicity selectively against the colon 26-L5 cells (Table 2b). As in isoflavones or in flavanones, the presence of a 2′-hydroxyl group is favorable, but hydroxylation at C-3, C-3′, or C-5 reduced the activity (**19** > **21** > **22**). Isoflavans (**23**–**26**) in general are active against all the tested cell lines (Table 2c). It is clearly observed that the increase in the number of the methoxyl group on the isoflavan skeleton enhances the activities against all the tested cancer cell lines (**25**, **26** > **23**, **24**). The different potency of cytotoxicity between isoflavans 7-*O*-methylvestitol (**25**) and mucronulatol (**26**) indicated that the methoxyl substituent is favored at C-2′ (**26** > **25**). Among the tested isoflavonoids, mucronulatol (**26**) displayed the most potent activity, especially against LLC (IC_{50} 8.38 μM) and A549 (IC_{50} 9.9 μM) cells with the comparable IC_{50} values to those of positive controls (Table 2c). However, its isoflavanone analogue **20** was found to be inactive against all the tested cell lines. The same trend was also observed in isoflavan **23** and its isoflavanone analogue **19**. Based on these observations, the absence of ketone carbonyl group in isoflavanoids strongly favors the cytotoxic activities against all the tested cell lines.

2.2.3. Chalcones. Chalcones also showed cytotoxic activities against all the tested cell lines with an exception of α,2′,4,4′-tetrahydroxydihydrochalcone **30** (Table 3). All the chalcones possessing a conjugated double bond showed strong cytotoxic activity. However, when the double bond is reduced to a single bond, the activity is totally diminished in all the tested cell lines (**27**, **28**, **29** > **30**). Further, the presence of a methoxyl group at C-2′ was found to be more favorable than that of a hydroxyl group for the cytotoxicity (**29** > **28**). As for ring B, chalcones without having any substitution preferred for activity increase (**27** > **28**). Interestingly, cyclization of dihydrochalcone (**30**) to auronol (**31**) showed slight improvement in the activity against colon 26-L5 cells (**31**, IC_{50} 25.85 μM; **30** , IC_{50} > 100 μM).

2.2.4. Pterocarpans. All the pterocarpans (**32**–**37**) in this study possessed 3-hydroxyl and 9-methoxyl substituents in their structures (Table 4). The observed activity data indicated that an increase in the number of the hydroxyl group results in the decrease in activity for the same parent structure (**33** < **32**). Conversely, the increase of the number of the methoxyl group at C-4 and C-8 on the parent structure seemed to enhance the activity (**34**, **37** > **36**). Moreover, the activity effects produced by the position of the additional methoxyl group were different among the cell types. 4-Methoxylated medicarpin (**37**) showed weaker activity than 8-methoxylated medicarpin (**34**) against B16-BL6 cells, but for the other

tested cancer cells **37** showed stronger activities than **34**. In addition, the presence of an ethoxyl group at C-6a in (6a*S*,11a*S*)-6a-ethoxymedicarpan (**35**) diminished the activities against all the tested cell lines (IC_{50} > 100 μM). The cleavage of a pyran ring in **32** to form 2-arylbenzofuran (**38**) also led to the slight loss in activity (**32** > **38**).

2.2.5. Neoflavonoid and lignans. (7*S*)-Dalbergiphenol (**39**), the only one neoflavonoid isolated from Brazilian red propolis, was moderately active for colon 26-L5, B16-BL6, A549, and HeLa cells, and inactive against LLC and HT-1080 cells (Table 5). The three lignans (**40**–**42**) did not show any cytotoxicities against all the six different tested cancer cell lines in this study (Table 5).

3. Conclusion

In the present study, the cytotoxic activities of 39 flavonoids and three lignans isolated from Brazilian red propolis were tested against six different cancer cell lines in vitro, and deduced their structure–activity relationships. All the tested compounds displayed the different potency of activity in a concentration-dependent manner. Flavanones, isoflavones and isoflavanones are selectively cytotoxic against colon 26-L5 carcinoma. The presence of the hydroxyl groups at C-3 and C-5 in flavanones, isoflavones, and isoflavanones reduces the activity. However, the presence of a methoxyl group at C-6 and without a hydroxyl group at C-3 in flavanones dramatically increased the activity not only in colon 26-L5, but also in B16-BL6, LLC, A549, HeLa, and HT-1080 cancer cell lines. Isoflavans, chalcones, and pterocarpans are active in all the tested cell lines, although their potency varies with regard to its substituents. Reduction of the C-4 carbonyl group in isoflavones and isoflavanones is favorable for inducing cytotoxic activity in B16-BL6, LLC, A549, HeLa, and HT-1080 cancer cell lines. Again, the presence of the methoxyl groups at C-7, C-2′/C-4′ in isoflavans dramatically enhances the cytotoxicity, comparable to those of positive controls, 5-fluorouracil and doxorubicin. In fact, the presence of methoxyl substituent has modulated the cytotoxicity of flavonoids.[27–29] In chalcones, the presence of a conjugated double bond plays an important role for their activity. Among the tested compounds, (2*S*)-7-hydroxy-6-methoxyflavanone (**3**), (3*S*)-mucronulatol (**26**), 2′,4′-dihydroxychalcone (**27**), and (6a*S*,11a*S*)-medicarpin (**32**) showed the most potent activities among the flavanones, isoflavoids, chalcones, and pterocarpans, respectively. The potency of 7-hydroxy-6-methoxyflavanone (**3**) against B16-BL6, LLC, A549, and HT-1080 cell lines is comparable to those of the clinically used anticancer drugs, doxorubicin and 5-flurouracil. Similarly, mucronulatol (**26**) also displayed potent activity against LLC, A549, and HT-1080 cell lines. These data suggest that 7-hydroxy-6-methoxyflavanone (**3**) and mucronulatol (**26**) are the good candidates for future anticancer drug development.

4. Experimental

4.1. Agents

5-Fluorouracil was purchased from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan) and doxorubicin HCl was from Kyowa Hakko Co. Ltd (Tokyo, Japan). Eagle's minimum essential medium (EMEM) and RPMI 1640 medium were purchased from Nissui Pharmaceutical Co., Ltd (Tokyo, Japan), while 25% glutaraldehyde solution and L-(+)-glutamine were obtained from Wako Pure Chemicals Ind., Ltd (Osaka, Japan), sodium bicarbonate was from Nacalai Tesque Inc. (Kyoto, Japan), and fetal bovine serum (FBS) from Gibco BRL Products (Gaithersburg, MD). Penicillin G and streptomycin sulfate were from Sigma Chemical Co. (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) and crystal violet were purchased from Nacalai Tesque. Cell culture flasks and 96-well plates were from Corning Inc. (Corning, NY, USA).

4.2. Cancer cell lines

Highly liver metastatic murine colon 26-L5 carcinoma cell line,[21] highly liver metastatic murine B16-BL6 melanoma cell line,[22] and highly lung metastatic murine Lewis lung carcinoma (LLC)[23] cell line were available and maintained in our laboratory. Highly metastatic human HT-1080 fibrosarcoma cell line (ATCC#CCL-121)[26] was obtained from American Type Culture Collection (Rockville, MD, USA). Human lung A549 adenocarcinoma (RCB0098)[24] and human cervix HeLa adenocarcinoma (RCB0007)[25] cell lines were purchased from Riken Cell Bank (Tsukuba, Japan).

All the cancer cell lines were maintained in EMEM, except for murine colon 26-L5 carcinoma cell line, which was maintained in RPMI 1640 medium. Both of these media were supplemented with 10% fetal bovine serum (FBS), and 0.1% sodium bicarbonate and 2 mM L-(+)-glutamine.

4.3. Cytotoxic activity assay

Cell viability other than LLC, in the presence or absence of tested compounds, was determined using the standard MTT assay[30] as described previously.[31] In brief, exponentially growing cells were harvested and 2×10^3 cells suspended in 100 μL of medium per well were plated in 96-well plate. After 24 h incubation at 37 °C under a humidified 5% CO_2 to allow cell attachment, the cells were treated with varying concentrations of test specimens in their respective medium (100 μL) and incubated for 72 h under the same conditions. After 2 h of the MTT (0.5 mg/mL, 100 μL) addition, the formazan formed was extracted with DMSO and its amount was measured spectrophotometrically at 550 nm with Perkin-Elmer HTS-7000 Bio Assay Reader (Norwalk, CT, USA). In the case of LLC cells, standard crystal violet staining assay was used in following the literature procedure.[32] In brief, exponentially growing cells were harvested and 1×10^3 cells suspended in 100 μL of medium per well were plated in 96-well plate.

After 24 h of incubation at 37 °C under a humidified 5% CO_2, 100 μL of medium containing various concentration of test specimen was added to each well and incubated for 72 h under the same conditions. After fixation with 25% glutaraldehyde solution (20 μL), the cells were stained with 0.5% crystal violet in 20% methanol/water for 30 min. After gentle rinsing with water, the retained crystal violet was extracted with 30% acetic acid and measured spectrophotometrically at 590 nm.

Each compound was dissolved by DMSO (1×10^4 μg/mL), and then diluted by the medium; final concentration of DMSO was less than 0.25%. 5-Fluorouracil and doxorubicin were used as positive controls, and IC_{50} values were calculated from the mean values of data from three wells.

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References and notes

1. Banskota, A. H.; Tezuka, Y.; Kadota, S. *Phytother. Res.* **2001**, *15*, 561–571.
2. Kumazawa, S.; Ueda, R.; Hamasaka, T.; Fukumoto, S.; Fujimoto, T.; Nakayama, T. *J. Agric. Food Chem.* **2007**, *55*, 7722–7725.
3. Scazzocchio, F.; D'Auria, F. D.; Alessandrini, D.; Pantanella, F. *Microbiol. Res.* **2006**, *161*, 327–333.
4. Kujumgiev, A.; Tsvetkova, I.; Serkedjieva, Y.; Bankova, V.; Christov, R.; Popov, S. *J. Ethnopharmacol.* **1999**, *64*, 235–240.
5. Silici, S.; Koc, N. A.; Ayangil, D.; Cankaya, S. *J. Pharmacol. Sci.* **2005**, *99*, 39–44.
6. Paulino, N.; Teixeira, C.; Martins, R.; Scremin, A.; Dirsch, V. M.; Vollmar, A. M.; Abreu, S. R.; de Castro, S. L.; Marcucci, M. C. *Planta Med.* **2006**, *72*, 899–906.
7. Tan-No, K.; Nakajima, K. T.; Shoii, T.; Nakagawasai, O.; Niijima, F.; Ishikawa, M.; Endo, Y.; Sato, T.; Satoh, S.; Tadano, K. *Biol. Pharm. Bull.* **2006**, *29*, 96–99.
8. Awale, S.; Li, F.; Onozuka, H.; Esumi, H.; Tezuka, Y.; Kadota, S. *Bioorg. Med. Chem.* **2008**, *16*, 181–189.
9. de Barros, M. P.; Sousa, J. P.; Bastos, J. K.; de Andrade, S. F. *J. Ethnopharmacol.* **2007**, *110*, 567–571.
10. Awale, S.; Shrestha, S. P.; Tezuka, Y.; Ueda, J.; Matsushige, K.; Kadota, S. *J. Nat. Prod.* **2005**, *68*, 858–864.
11. Alencar, S. M.; Oldoni, T. L.; Castro, M. L.; Cabral, I. S.; Costa-Neto, C. M.; Cury, J. A.; Rosalen, P. L.; Ikegaki, M. *J. Ethnopharmacol.* **2007**, *113*, 278–283.
12. Trusheva, B.; Popova, M.; Bankova, V.; Simova, S.; Marcucci, M. C.; Miorin, P. L.; da Rocha Pasin, F.; Tsvetkova, I. *Evid. Based Complement. Alternat. Med.* **2006**, *3*, 249–254.
13. Pisco, L.; Kordian, M.; Peseke, K.; Feist, H.; Michalik, D.; Estrada, E.; Carvalho, J.; Hamilton, G.; Rando, D.; Quincoces, J. *Eur. J. Med. Chem.* **2006**, *41*, 401–407.
14. Luo, J.; Soh, J. W.; Xing, W. Q.; Mao, Y.; Matsuno, T.; Weinstein, I. B. *Anticancer Res.* **2001**, *21*, 1665–1671.
15. Than, M. M.; Banskota, A. H.; Tezuka, Y.; Midorikawa, K.; Matsushige, K.; Kadota, S. *J. Trad. Med.* **2003**, *20*, 20–29.
16. Usia, T.; Banskota, A. H.; Tezuka, Y.; Midorikawa, K.; Matsushige, K.; Kadota, S. *J. Nat. Prod.* **2002**, *65*, 673–676.
17. Banskota, A. H.; Nagaoka, T.; Sumioka, L. Y.; Tezuka, Y.; Awale, S.; Midorikawa, K.; Matsushige, K.; Kadota, S. *J. Ethnopharmacol.* **2002**, *80*, 67–73.
18. Midorikawa, K.; Banskota, A. H.; Tezuka, Y.; Nagaoka, T.; Matsushige, K.; Message, D.; Huertas, A. A. G.; Kadota, S.. *Phytochem. Anal.* **2001**, *12*, 366–373.
19. Banskota, A. H.; Tezuka, Y.; Adnyana, I. K.; Midorikawa, K.; Matsushige, K.; Message, D.; Huertas, A. A. G.; Kadota, S. *J. Ethnopharmacol.* **2000**, *72*, 239–246.
20. Banskota, A. H.; Tezuka, Y.; Prasain, J. K.; Matsushige, K.; Saiki, I.; Kadota, S. *J. Nat. Prod.* **1998**, *61*, 896–900.
21. Ohnishi, Y.; Sakamoto, T.; Fujii, H.; Kimura, F.; Murata, J.; Tazawa, K.; Fujimaki, M.; Sato, Y.; Kondo, M.; Une, Y.; Uchino, J.; Saiki, I. *Tumor Biol.* **1997**, *18*, 113–122.
22. Hart, I. R. *Am. J. Path.* **1979**, *97*, 587–600.
23. Aptekman, P. M.; Lewis, M. R. *J. Immunol.* **1951**, *66*, 361–364.
24. Giard, J. D.; Aaronoson, S. A.; Todaro, G. J.; Arnstein, P.; Kersey, J. H.; Dosik, H.; Parks, W. P. *J. Natl. Cancer Inst.* **1973**, *51*, 1417–1423.
25. Gey, G. O.; Coffman, W. D. *Cancer Res.* **1952**, *12*, 264–265.
26. Rasheed, S.; Nelson-Rees, W. A.; Thot, E. M.; Arnstein, P.; Gardner, M. B. *Cancer* **1974**, *33*, 1027–1033.
27. Militão, G. C.; Dantas, I. N.; Pessoa, C.; Falcão, M. J.; Silveira, E. R.; Lima, M. A.; Curi, R.; Lima, T.; Moraes, M. O.; Costa-Lotufo, L. V. *Life Sci.* **2006**, *78*, 2409–2417.
28. Pouge, t C.; Lauthier, F.; Simon, A.; Fagnere, C.; Basly, J. P.; Delage, C.; Chulia, A. J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 3095–3097.
29. Ducki, S.; Forrest, R.; Hadfield, J. A.; kendall, A.; Lawrence, N. J.; Mcgown, A. T.; Rennison, D. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1051–1056.
30. Liu, Y.; Peterson, D. A.; Kimura, H.; Schubert, D. *J. Neurochem.* **1997**, *69*, 581–593.
31. Banskota, A. H.; Tezuka, Y.; Prasain, J. K.; Matsushige, K.; Saiki, I.; Kadota, S. *J. Nat. Prod.* **2005**, *61*, 896–900.
32. Saito, K.; Oku, T.; Ata, N.; Miyashiro, H.; Hattori, M.; Saiki, I. *Biol. Pharm. Bull.* **1997**, *20*, 345–348.