CYTOTOXIC ISOFLAVONOIDS OF *PACHYRRHIZUS EROSUS* SEEDS

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ABSTRACT

By bioactivity-directed fractionation, phytochemical and cytotoxic studies of the seeds of ***Pachyrrhizus erosus*** *L. resulted in the isolation of isoflavonoid-based compounds, one novel compound and eight known compounds, comprising the novel coumaronochromene, pachyrrhisomene {1}, the known pterocarpan, neodulin {2}, the known 3-arylcoumarin, pachyrrhizin {3}, the known isoflavonoid, dehydroneotenone {4}, five known rotenoids, rotenone {5}, 12a-hydroxyrotenone {6}, 12a-hydroxypachyrrhizone {7}, 12a-hydroxyerosone {8}, and 12a-hydroxymunduserone {9}. The identities of these compounds were elucidated or confirmed using combination of modern one- and two- dimensional NMR techniques, such as ^1H-^1H COSY, CSCM-1D, ^1H-^1H NOESY, and selective INEPT, as well as by comparison with published spectroscopic data. It is likely that the novel compound, pachyrrhisomene {1} is derived from the same biosynthetic intermediate as the pterocarpan, neodulin {2}. All of these compounds were evaluated for their anticancer potential in a battery of tumour cell lines, comprishing P-388 lymphocytic leukemia, KB-carcinoma of the nasopharynx, a multi-drug resistant variant of KB, KB-VI, and a number of human cancer cell lines derived from a variety of tumour types, namely fibrosarcoma, lung, colon, melanoma, and breast. Two compounds, rotenone {5} and 12a-hydroxyrotenone {6} were observed to exhibit potent but nonspecific activity.*

INTISARI

*Dari studi fitokimia dan sitotoksitas biji bangkuang (****Pachyrrhizus erosus*** *L) dengan fraksinasi bioaktivitas-terarah, telah diisolasi senyawa-senyawa berbasis isoflavonoida, sebuah senyawa baru dan delapan buah senyawa yang telah diketahui, yang terdiri dari suatu senyawa baru kumaronokhromene, pachyrrhisomene {1}, senyawa pterokarpan neodulin {2}, suatu 3-aril kumarin pachyrrhizin {3}, suatu isoflavonoida, dehydroneotenone {4}, dan lima senyawa rotenoida, rotenone {5} 12a-hydroxyrotenone {6}, 12a-hydroxypachyrrhizone {7}, 12a-hydroxyerosone {8}, dan 12a-hydroxymunduserone {9}. Identitas senyawa-senyawa ini ditentukan dan dikonfirmasi menggunakan teknik modern NMR satu- dan dua- dimensi, seperti ^1H-^1H COSY, CSCM-1D, ^1H-^1H NOESY, dan selective INEPT serta dengan membandingkan data spektroskopi dari pustaka. Nampaknya senyawa baru, pachyrrhisomene {1} diturunkan dari senyawa antara biosintesa yang sama dengan senyawa pterokarpan, neodulin {2}. Semua senyawa ini dievaluasi untuk potensi anti-kanker menggunakan beberapa sel kanker yang terdiri dari P-388 leukemia lymphocytic, karsinoma nasopharynx KB, suatu varian KB, yang resistant terhadap banyak obat, KB VI, dan sejumlah sel kanker manusia dari beberapa macam jenis tumor, seperti fibrosarkoma, paru-paru, kolon, melanoma dan payudara. Dua senyawa, rotenone {5} dan 12a-hydroxyrotenone {6} terlihat menunjukkan aktivitas yang kuat, tetapi bersifat tidak spesifik.*

INTRODUCTION

In the continuation of a collaborative study on Indonesian medicinal plants, we have evaluated the seeds of *Pachyrrhizus erosus* L. (Leguminosae) for potential antitumour activity. The plant is cultivated in Indonesia for its edible tubers. This plant is not to be used as fodder, as the leaves are poisonous to ruminants. In Java, the pulverized seeds mixed with sulphur are applied to heal a type of skin eruption which spread quickly by scratching, and seeds are used as fish poison (1). This plant is well studied phytochemically and rotenone and its derivatives have been found to be the constituents responsible for its insecticidal activity (2, 3, 4), however, to best our knowledge, this plant has not been studied for its potential antitumour activity.

MATERIALS AND METHODS

General Procedures

Melting points were measured on a Kofler hotstage apparatus and uncorrected. Optical rotations were taken at room temperature with a Perkin Elmer 241 polarimeter. The UV spectra were obtained on Beckman DU-7 specrometer and IR spectra were measured on Nicolet MX-FT-IR (KBr) interferometer. ^1H- and ^{13}C-NMR spectra were recorded in a Varian XL-300 or NMC-360 instruments. Low-resolution mass spectra were obtained with a Varian MAT 112S instrument operating at 70eV.

Cytotoxic Assay

Human fibrosarcoma (HT-1080), KB and P-388 cell lines were purchased from the ATCC. The other cell lines,

derived from a number of human cancers [breast(UISO-BCA-1], colon (UISO-COL-1), lung (UISO-LUC-1), and melanoma (UISO-MEL-2) were established from primary human tumours in the Division of Surgical Oncology, University of Illinois at Chicago. A multidrug-resistant KB cell line (KB-VI), supplied by Dr. Igor B. Roninson was developed by treating KB cells with sublethal dose of Vinblastine over an extended period of time (5). The P-388 cells were cultured in Fisher's medium supplemented with 10% heat-inactivated (56 °C for 30 minutes) FBS (fetal bovine serum). The KB cells were maintained in D-MEM contained 10% FBS. The HT-1080 and LUC-1 cell lines were cultured in Eagle's Minimum Essential amino acids (NAA) and 10% FBS. The COL-2 and BCA-1 cell lines were maintained in MEME with 1% NAA and 15% FBS, and the MEL-2 cell line was grown in Eagle's Minimum Essential Medium with Hank's salts (MEMH) with 10% FBS. The KB-VI cell line was maintained in Dublecco's modified Eagle's Minimum Essential Medium (D-MEM) contained 10% FBS and 1 µg/ml of vinblastin. All the cell lines except MEL-2 were cultured at 37 °C in a humidified atmosphere comprised of 5% CO_2. The MEL-2 was maintained in a closed culture vessel at 37 °C.

Extracts, fractions, and compounds were evaluated for cytotoxic potential essentially by established procedures (6, 7). In brief, the cells (at log growth phase) were treated with different concentrations of test compounds for 48 hours (HT-1080, LUC-1, and P-388) or 72 hours (BCA-1, COL-1, KB and MEL-2). In the case of the KB-VI cell line, the cytotoxicity assay was performed in vinblastine-free D-MEM medium supplemented with 10% FBS and incubation period of 72 hours was used. All treatments were performed in duplicate. At the end of incubation period, the growth rate was determined either by directly counting the cell numbers (P-388) or by protein determination. ED_{50} values (µg/ml) were defined as the concentration of a compound that inhibited cell growth by 50%.

Plant Materials

Dried mature seeds (6 kg) of *P.erosus* were collected in Padalarang, West Java, Indonesia, and identified by one of us (K.P.). A voucher specimen has been deposited in the herbarium of the Department of Biology, Bandung Institute of Technology, Bandung, Indonesia.

Extraction and Isolation

Finely powdered dried seeds of *P.erosus* (6 kg) was defatted with pet. ether (3 x 8 l) and the marc extracted with MeOH (3 x 8 l) to afford an initial MeOH extract (400 g) on removal of solvent in vacuo. A portion of the dried MeOH extract was partitioned between $CHCl_3$ and H_2O (6 l of each), which yielded on drying, 120 g of $CHCl_3$-soluble extract (P-388, ED_{50} = 1 µg/ml) and 280 g of an H_2O-soluble extract (P-388, ED_5 > 50 µg/ml). A portion of chloroform-soluble extract (100 g) was subjected to grafity column chromatography over silica gel (2.0 kg), using $CHCl_3$ and $CHCl_3$-MeOH mixture of increasing polarity as solvent. A total of 12 combined fractions was collected, with cytotoxic activity was concentrated in F01 (P-388, ED_{50} 0.2 µg/ml) and in F06 (P-388, ED_{50} 0.1 µg/ml). Further purification of F01 (2400 mg) through silica gel column chromatography several times with petroleum ether-chloroform (1:1) and mixtures of increasing polarity as solvent, afforded pachyrrhisomene {1}, neoduline {2}, pachyrrhizin {3}, dehydroneotenone {4} and rotenone {5}.

Pachyrrhisomene {1}, light yellow powder (28 mg, yield 0.00047% w/w), m.p.= 238-240 °C, $[\alpha]_D$= +67° (c 0.1, $CHCl_3$); UV, λ_{max} (log ε) ($CHCl_3$) 251 (3.80), 272.5 (3.50), 374.5 (4.00), 393.0 (4.20) nm; IR, ν_{max} (KBr) 1600, 1510, 1476, 1340, 1289, 1104, 1035, 1020, 834 cm^{-1}; ^{1}H-NMR, ($CDCl_3$) δ 8.14 (s, H-8), 7.81 (d, J= 2Hz, H-2''), 7.48 (s, H-5), 7.12 (s, H-6'), 6.82 (d, J= 2Hz, H-3"), 6.79 (d, J= 1.8Hz, H-4), 6.71 (s, H-3'), 6.35 (d, J= 1.8Hz, H-2), 6.04 (s) and 6.02 (s, 2H, H-2'''); ^{13}C-NMR, ($CDCl_3$) δ 156.50 (C-7), 154.03 (C-8a), 149.40 (C-2'), 146.13 (C-4'), 145.79 (C-2''), 142.98 (C-5'), 130.83 (C-3), 122.73 (C-6), 120.31 (C-1'), 118.97 (C-5), 114.53 (C-4a), 112.24 (C-4), 106.41 (C-3''), 104.32 (C-6'), 101.63 (C-2'''), 101.37 (C-2), 99.82 (C-8) and 93.69 (C-3'). EIMS, (70eV) m/z $[M]^+$ 306 (100 %), 277 (6), 195 (17), 153 (17). FABMS, m/z $[M+1]^+$ = 307.

Neodulin {2}, white needle crystals, (20 mg, yield 0.00034% w/w), m.p.= 225-227 °C; $[\alpha]_D$ -265° (c 0.1, $CHCl_3$); UV, λ_{max} (log ε) ($CHCl_3$) 250 (4.14), 256.5 (4.09), 305 (4.10) nm; IR, ν_{max} (KBr) 1645, 1639, 1608, 1540, 1511, 1040, 935 cm^{-1}; ^{1}H-NMR, ($CDCl_3$), δ 7.72 (s, H-1), 7.56 (d, J= 2Hz, H-2''), 7.09 (s, H-4), 6.74 (s, H-7), 6.72 (d, J= 2Hz, H-3''), 6.44 (s, H-10), 5.92 & 5.90 (2H, s, H-2'''), 5.68 (d, J= 7Hz, H-11a), 4.28 (dd, J= 11, 6Hz, H-6α), 3.72 (dd, J= 11, 10Hz, H-6ß), 3.60 (ddd, J= 7, 5, 11Hz, H-6a). EIMS (70eV) m/z $[M]^+$ 308 (100%), 291 (2), 221 (6), 195 (11), 162 (34), 158 (24).

Pachyrrhizin {3}, light yellow needle crystals (212 mg, yield 0.0035% w/w), m.p.: 204-206 °C. UV, λ_{max} (log ε) ($CHCl_3$) 252 (4.20), 291.5 (3.80), 351 (3.40) nm. IR ν_{max} (KBr) 1728, 1608, 1510, 1035, 935 cm^{-1}; ^{1}H-NMR, ($CDCl_3$) δ 7.88 (s, H-4), 7.77 (d, J= 2Hz, J-2''), 7.75 (s, H-5), 6.90 (s, H-6'), 6.86 (d, J= 2Hz, H-3''), 6.67 (s, H-3'), 5.98 (s, H-2'''), 3.78 (s, OCH_3). EIMS, (70eV) m/z $[M]^+$ 336 (100%), 321 (7), 293 (36), 265 (10), 199 (6).

Dehydroneotenone {4}, white needle crystals (24 mg, yield 0.0004% w/w), m.p.: 240-242 °C. UV, λ_{max} (log ε). 244 (4.20), 310 (3.6) nm; IR, ν_{max} (KBr) 1700, 1650, 1504,

1041, 935, 877 cm^{-1}; ^{1}H-NMR, ($CDCl_3$) δ 8.54 (s, H-5), 799 (s, H-2), 7.73 (d, J= 2Hz, H-2''), 7.58 (s, H-8), 6.91 (d, J= 2Hz, H-3''), 6.86 (s, H-6'), 6.64 (s, H-3'), 5.97 (2H, s, H-2'''), 3.74 (3H, s, OCH_3); EIMS (70eV) m/z 336 $[M]^+$ 336 (100), 305 (72), 291 (12), 263 (12), 235 (9), 211 (10), 175 (33), 161 (71).

Rotenone {5}, light yellow powder (92 mg, yield 0.00153% w/w), m.p.: 165-166 °C; $[\alpha]_D$ -230° (0.1, $CHCl_3$); UV, λ_{max} (log ε) ($CHCl_3$) 240.5 (4.10), 294.5 nm (4.20); IR, ν_{max} (KBr) 1674, 1610, 1511, 909 cm^{-1}; ^{1}H-NMR, $CDCl_3$ δ 7.78 (d, J= 8.5Hz, H-11), 6.73 (s, H-1), 6.45 (d, J= 8.5Hz, H-10), 6.41 (s, H-4), 5.20 (t, J= 9Hz, H-5'), 5.01 & 4.89 (s, H-7'), 4.86 (bs, H-6a), 4.60 (dd, J= 12, 2.5Hz, H-6α), 4.16 (d, J= 12Hz, H-6ß), 3.76 (d, J= 3.5Hz, H-12a), 3.73 (s, OCH_3), 3.70 (s, OCH_3), 3.30 (dd, J=16, 8Hz, H-4') & 2.92 (dd, J= 16, 8Hz, H-4'), 1.70 (s, H-8); EIMS, (70eV) m/z $[M]^+$ 394 (12%), 192 (100), 177 (18), 159 (1).

Further purification of F06 (2800 mg), through silica gel cc. several times with EtOAc:MeOH (95:5) and mixtures of increasing polarity as solvent afforded 12aOH-rotenone {6}, 12aOH-pachyrrhizone {7}, 12aOH-erosone {8} and 12aOH-munduserone {9}.

12a-Hydroxyrotenone {6}, gummy solid (92 mg, yield 0.000153% w/w), m.p.: 88-90 °C. $[\alpha]_D$ +137° (c 0.1, $CHCl_3$); UV, λ_{max} (log (log ε) (MeOH) 237 (4.50), 276 (2.80), 304 (3.70), 338 (3.60) nm; IR, ν_{max} (KBr) 3450, 1670, 1605, 1505, 1330, 1155, 1085, 815 cm^{-1}. ^{1}H-NMR, ($CDCl_3$), δ 7.78 (d, J= 8.5Hz, H-11), 6.58 (s, H-1), 6.49 (d, J= 8, 5Hz, H-10), 6.45 (s, H-4), 5.20 (t, J= 9Hz, H-5'), 5.00 (s, H-7') & 4.90 (s, H-7'), 4.60 (s, -OH), 4.54 (m, H-6a), 4.48 (m, H-6), 3.79 (s, $-OCH_3$), 3.69 (s, $-OCH_3$), 3.30 (dd, J= 15, 8Hz, H-4'), 2.90 (dd, J= 15, 8Hz, H-4'), 1.73 (s, H-8'). EIMS, (70eV) m/z $[M]^+$ 410 (7%), 280 (100), 207 (37), 161 (4).

12a-Hydroxypachyrrhizone {7}, white needle crystals (216 mg, yield 0.0036% w/w), m.p.: 216-218 °C. $[\alpha]_D$ +126° (c 0.1, $CHCl_3$); UV, λ_{max} (log ε) ($CHCl_3$) 249 (4.50), 288.5 (3.80), 354 (3.50) nm; IR, ν_{max} (KBr) 3450, 1681, 1623, 1500, 1035, 935, 877 cm^{-1}; ^{1}H-NMR, (DMSO-d5), δ 7.99 (d, J= 2Hz, H-2'), 7.87 (s, H-11), 7.00 (d, J= 2Hz, H-3'), 6.69 (s, -OH), 6.59 (s, H-1), 6.50 (s, H-4), 5.95 & 5.87 (s, H-2''), 4.80 (bs, H-6a), 4.62 (dd, J= 12, 2Hz, H-6), 4.00 (s, $-OCH_3$). EIMS, (70eV) m/z $[M]^+$ 382 (20%), 363 (1), 208 (12), 191 (100), 165 (9), 147 (5).

12a-Hydroxyerosone {8}, gummy solid, (36 mg, 0.0006% w/w), m.p.: 282-284 °C. $[\alpha]_D$ +170° (c 0.1, $CHCl_3$); UV, λ_{max} (log ε) ($CHCl_3$) 237 (3.80), 341 (3.40) nm; IR, ν_{max} (KBr) 3450, 1670, 1620, 1500, 1030, 945, 872 cm^{-1}; ^{1}H-NMR, ($CDCl_3$), δ 8.17 (s, H-11), 7.52 (d, J= 2Hz, H-2'), 6.99 (s, H-8), 6.70 (d, J= 2Hz, H-3'), 6.44 (bs, H-6a), 6.52 (s, H-1), 6.48 (s, H-4), 4.60 (dd, J= 12, 2Hz, H-6), 4.46 (dd, J= 12, 2Hz, H-6), 3.77 (s, $-OCH_3$), 3.68 (s, $-OCH_3$). EIMS, (70eV) m/z $[M]^+$ 368 (22%), 208 (100), 207 (38), 193 (6), 165 (5), 133 (14).

12a-Hydroxymunduserone {9}, gummy solid, (54 mg, 0.0009% w/w), m.p.: 312-314° (dec). $[\alpha]_D$ +34° (c 0.1, $CHCl_3$); UV, λ_{max} (log ε) ($CHCl_3$) 241 (4.25), 281.5 (4.50), 316 (3.80) nm; IR, ν_{max} (KBr) 3450, 1670, 1620, 1490, 1440, 1250, 1090, 905 cm^{-1}. ^{1}H-NMR, ($CDCl_3$), δ 7.81 (d, J= 9Hz, H-11), 6.67 (s, H-1), 6.62 (dd, J= 9, 3Hz, H-10), 6.47 (s, H-4), 6.34 (d, J= 3Hz, H-8), 4.77 (bs, H-6a), 4.62 (dd, J= 12, 2Hz, H-6) & 4.58 (dd, J= 12, 2Hz, H-6), 3.78 (s, $-OCH_3$), 3.73 (s, $-OCH_3$), 3.67 (s, $-OCH_3$). EIMS, (70eV) m/z $[M]^+$ 358 (2%), 208 (100), 207 (55), 193 (11), 165 (22). The structures of the isolated compounds are shown in **Figure 1.**

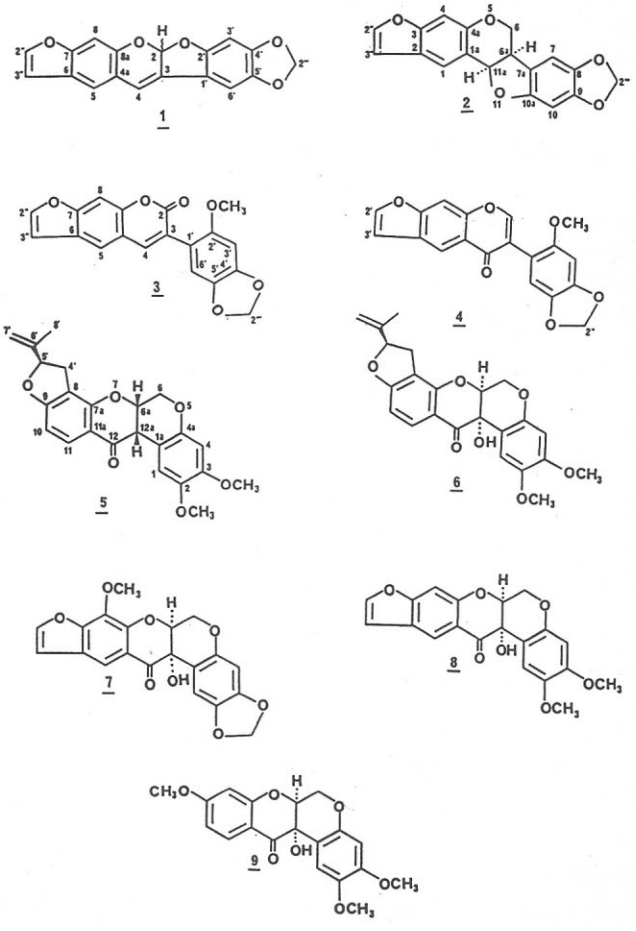

Fig. 1: Isolated Isoflavonoids 1-9

RESULTS AND DISCUSSION

The isolated compounds in this study were all isoflavonoid based compounds. These isolates have been previously isolated from leguminous plant species except for pachyrrhisomene {1} (8). The physical and spectral data of the isolated known compounds {2-9} were comparable with those of published values (m.p., $[\alpha]_D$, UV, IR, ^{1}H-, ^{13}C-NMR) (2-4, 9, 10). Pachyrrhisomene {1} is a novel compound elucidated by means of its physical and spectral data. This compound {1} is a coumaronochromone derivative that has been isolated from leguminous plants (8, 11). The molecular formula of compound 1 was analyzed as $C_{18}H_{10}O_{15}$ based on molecular ion peak of EIMS [M]$^+$306, and its positive ion FABMS [M+1]$^+$307. Considerable conjugation was infered from the UV spectrum of compound 1, but no bathochromic or hypsochromic effects were observed upon addition of flavonoid shift-reagents. No carbonyl or hydroxy groups were evident in compound 1 from its IR and ^{13}C-NMR spectra. The ^{1}H- and ^{13}C-NMR spectra showed that compound 1 is closely related to pachyrrhizin {3} but has a lack of carbonyl and metoxy groups. This compound is also similar to neodulin {2}, but all the ^{1}H- and ^{13}C-resonances are in the aromatic region. No resonances in the aliphatic region were observed. The acetal carbon and proton were observed at δ 101.21 of the ^{13}C-NMR and δ 6.35 of the ^{1}H-NMR spectra. In ^{1}H-^{1}H COSY spectrum, the acetal proton has an allylic coupling (J= 1.8Hz) with H-4. This correlation was also confirmed by a selective INEPT NMR experiment ($^3J_{CH}$= 8Hz). Compound 1 clearly has both furan (δ 7.81, d, J= 2Hz, H-2''; δ145.79, C-2'' and δ 6.82, J= 2Hz, H-3''; δ 106.32, C-3'') and methylene dioxy, δ 6.04 & 6.02, 2H, s, H-2''', δ 101.63, C-2''') functionalities. The configuration of the stereocentre at C-2 was not able to be assigned by ^{1}H-^{1}H NOESY NMR experiment. Apparently both aromatic rings of this compound are planar, and from the ^{1}H-^{1}H NOESY experiment, the acetal proton (H-2) does not have correlations with any protons of these rings. Most probably compound 1 is derived in the plant from the same biosynthetic intermediate as neodulin {2}. Our proposed biogenetic inter-relationships of these two compounds are shown in Figure 2. Cytotoxic data evaluation are shown in Table 1. Two of the known compounds, rotenone {5} and 12a-hydroxyrotenone {6} were observed to exhibit potent but non-specific cytotoxic activity. As compared with KB cells, the KB-VI cell line was equally sensitive to these compounds. The other isolates {1-4; 7-9} did not show any significant activity. This results clearly demonstrate the importance of A-ring substituents in the mediation of rotenoid toxicity. It appears that exo-ethylidene furan functionality on the A-ring is necessary for the presence of cytotoxic activity.

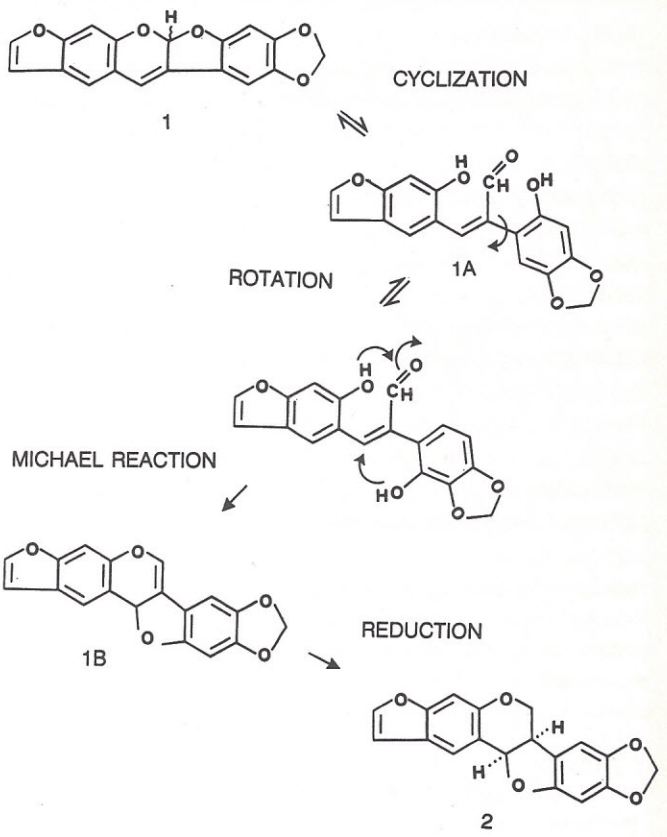

Fig. 2: Proposed Biogenetic Interrelationships of Compounds 1 and 2.

Table 1. The Cytotoxic Potential of Isolates 1-9[a]

Sample	A	B	C	D	E	F	G	H
Pachyrrhisomene (1)	12	16	24	15	18	16	11	25
Neodulin (2)	18	12	32	18	24	32	18	18
Pachyrrhizin (3)	50	50	50	50	50	50	50	50
Dehydroneotenone (4)	18	12	20	16	24	18	20	24
Rotenone (5)	.01	.08	.10	.05	.05	.15	.10	.06
12a OH-Rotenone (6)	.01	.05	.09	.10	.10	.30	.30	.09
12a OH-Pachyrrhizone (7)	12	18	28	26	24	14	24	18
12a OH-Erosone (8)	16	20	18	12	20	30	20	30
12a OH-Munduserone (9)	18	28	24	16	19	28	18	32

[a]Results are expressed as ED_{50} values (μg/ml)
Compounds considered active when $ED_{50} \leq 4$ μg/ml.

[b]Key : A. P-388 B. KB
C. KB-VI D. HT-1080
E. Lung Cancer F. Colon Cancer
G. Melanoma H. Breast Cancer

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