# Antimutagens in the Brazilian Folk Medicinal Plant Carqueja (*Baccharis trimera* Less.)

Toru Nakasugi* and Koichiro Komai†

Research Laboratory of Medicinal Products of Plant Origin, Inabata Koryo Company Ltd., Tagawa 3-5-20, Yodogawa-ku, Osaka 532 Japan

Antimutagens from carqueja (*Baccharis trimera* Less., Compositae), a Brazilian folk medicinal plant used to treat liver disease and rheumatism, were examined. The methanol extract prepared from aerial parts of this plant strongly reduced the mutagenicity of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]-indole, when *Salmonella typhimurium* TA98 was used in the presence of rat liver microsomal fraction. The antimutagens were purified with chromatography. Antimutagenic activities were detected with a modified Ames test. Four active substances were identified in the methanol extract, 5,4′-dihydroxy-7-methoxyflavone (genkwanin), 5,4′-dihydroxy-6,7-dimethoxyflavone (cirsimaritin), 5,7,4′-trihydroxy-6-methoxyflavone (hispidulin), and 5,7,4′-trihydroxyflavone (apigenin). It is thought that these flavones are major active substances in carqueja. As for the structure−activity relationships, a decreasing tendency of the antimutagenic activity was found when the moiety of the hydroxy group of flavones was replaced by the methoxy group, except for cirsimaritin.

## INTRODUCTION

In recent years, interest in cancer chemoprevention is increasing, and antimutagens and antitumor promotors are being sought. Regarding antimutagens, strong antimutagenicity has been reported in extracts of European herbs (Natake et al., 1989), Chinese medicinal plants (Niikawa et al., 1995), vegetables and fruits (Morita et al., 1978), teas (Kojima et al., 1989), and spices (Watanabe et al., 1989) and their components. We have also examined the antimutagenicity of many edible plants with a modified Ames test in a search for cancer-preventing substances. In previous papers, we have reported that the aloe-emodin in *Aloe arborescens* Mill. (Nakasugi and Komai, 1994), cirsimaritin, and salvigenin in *Rosmarinus officinalis* L. (Nakasugi and Komai, 1996) strongly reduced the mutagenicity of 3-amino-1-methyl-5-*H*-pyrido[4,3-*b*]indole (Trp-P-2). Trp-P-2, which is one of the heterocyclic amines, is well-known for its strong mutagenicity and specific carcinogenicity in the mammalian liver (Wakabayashi et al., 1982; Sugimura, 1985; Ohgaki and Wakabayashi, 1989).

Heterocyclic amines are the pyrolysates that are formed during cooking from amino acids or proteins present in meat or fish. Humans are often exposed to the risk of consuming these carcinogens through food.

Although it is desirable to avoid the intake of heterocyclic amines, it is difficult to do so because of the prevalence of cooking in daily life. The intake of antimutagens, which strongly reduce the mutagenicity of heterocyclic amines, is therefore desired, and the extensive study of such antimutagens is needed. We recently found that the methanol extract prepared from the leaves of *Baccharis trimera* Less. more strongly reduced the mutagenicity of Trp-P-2 than did extracts of aloe and of rosemary. *B. trimera* Less. is identical to *B. genistelloides* Pers. and known locally as carqueja in Brazil. This plant is used there to treat liver diseases and rheumatism (Gene et al., 1992; Hashimoto, 1996) and is also known to have an antiinflammatory effect and antihepatotoxic effect (Gene et al., 1992; Soicke and Leng-Peschlow, 1987). As for the constituents of carqueja, it has been reported that carqueja has many phytochemicals such as an essential oil, clerodane type diterpenes, saponins and their glycosides, and several flavones related to cirsimaritin (Chialva and Doglia, 1989; Herz et al., 1977; Soicke and Leng-Peschlow, 1987). However, the antimutagens of carqueja against Trp-P-2 have not been revealed until now, and the active substances involved in the methanol extract of carqueja were unknown.

It has been expected that understanding the unknown antimutagens involved in antimutagenic plants would be very imformative in the search for effective chemopreventive agents against cancer. We have therefore been interested in identifying the antimutagens involved in the methanol extract of carqueja. In this paper, the isolation of the antimutagens in carqueja and their antimutagenicity against Trp-P-2 are described.

## MATERIALS AND METHODS

**Materials.** Aerial parts of *Baccharis trimera* Less. (carqueja), which were dried and chopped, were purchased from Laboratorio Centroflora Ltda. (Sao Paulo, Brazil). Bacto agar and nutrient broth were purchased from Difco (Detroit, MI). S-9 (which was prepared from the livers of 7-week-old male Sprague−Dawley rats treated with phenobarbital and 5,6-benzoflavone), and cofactor I, used for the activation of Trp-P-2, was purchased from Oriental Yeast Co. (Tokyo, Japan). Trp-P-2 as the acetate form was purchased from Wako Pure Chemicals (Osaka, Japan). Silica gel (Silicagel 60 No. 9385, 230-240 mesh) for chromatography was purchased from Merck (Darmstadt, Germany). Sephadex LH-20 for gel chromatography was purchased from Pharmacia Biotech (Uppsala, Sweden). Acacetin, apigenin, eupatorin, eupatorin-5-methyl ether, genkwanin, and luteorin were obtained from Funakoshi

† Present address: Laboratory of Pesticide Chemistry, Department of Agricultural Chemistry, Kinki University, Nara 631, Japan.

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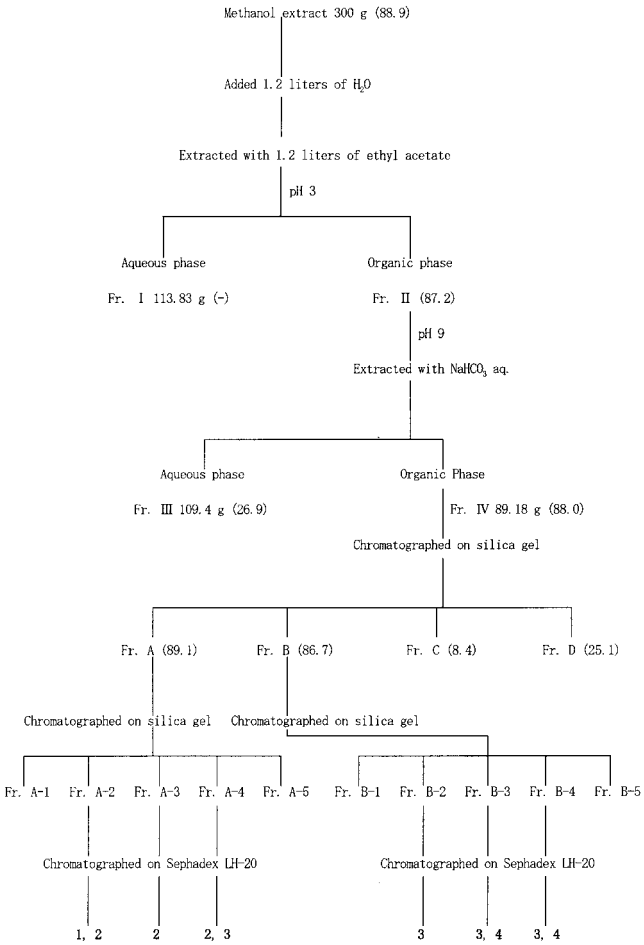


**Figure 1.** Isolation of compounds **1**−**4**. The figures in parentheses are antimutagenic activities (inhibition %) at the amount of 0.15 $\mu$g of Trp-P-2/plate when each fractionated sample was used in the antimutagenicity test at the dose of 0.5 mg/plate. A minus sign in parentheses indicates a lack of activity.

Co. (Tokyo, Japan) (made at Extrasynthese Co., Genay, France). Salvigenin was obtained from rosemary as described in a previous study (Nakasugi and Komai, 1996).

**Purification and Isolation of Active Substances.** First, 3.5 kg of the plant material was extracted for 2 h with 19 L of boiled methanol. The methanol extract was filtered with no. 2 filter paper (Advantec Toyo, Tokyo, Japan). The residue was similarly extracted with 12.7 L of boiled methanol, and the methanol extract was filtered. The filtrates (13.75 and 12.85 Ls) were combined, and the solution was concentrated under reduced pressure to 346.82 g. A total of 46.82 g of this concentrate was used for the measurement of the antimutagenic activity. The other 300 g of this concentrate was used for the purification of the active substances to monitor the antimutagenic activities as shown in Figure 1. Three hundred grams of the concentrate from carqueja was added to 1.2 L of $H_2O$ and stirred. The aqueous solution was brought to pH 3 with 5% hydrochloric acid and was partitioned with 1.2 L of ethyl acetate three times. The aqueous phase was neutralized and concentrated to 113.83 g (fraction I). A 1/8 volume of the organic phase was conccentrated to 24.69 g for the antimutagenicity test (fraction II). The residual 7/8 volume of the organic phase (3.16 L) was conccentrated to 2.0 L, and subsequently the solution was repartitioned with 2 L of 5% $NaHCO_3$ three times. The $NaHCO_3$ phase was neutralized, the salts were removed, and the solution was concentrated to109.4 g (fraction III). Subsequently, the organic phase washed with $NaHCO_3$ was concentrated to 89.18 g (fraction IV). Fraction IV that exhibited the strong antimutagenicity was further purified as follows: 80 g of the concentrate was chromatographed on silica gel (glass column with 7.5 cm × 50 cm length) with chloroform−methanol with increasing methanol content and was fractionated into fractions A−D (yields: 24.42, 23.27, 17.83, and 8.72 g, respectively). Since fractions A and B showed strong antimutagenic activities, they were rechromatographed on silica gel (glass column with 4.5 cm × 60 cm length) with chloroform−methanol with increasing methanol content. Chromatography yielded the following subfractions: A-1 (3.06 g), A-2 (11.83 g), A-3 (2.96 g), A-4 (3.17 g), A-5 (720 mg), B-1 (1.87 g), B-2 (7.69 g), B-3 (4.18 g), B-4 (3.36 g), and B-5 (1.88 g) (Figure 1). Subfraction A-2 was chromatographed on Sephadex LH-20 (glass column with 2.5 cm × 45 cm length) with methanol as eluent and gave **1** and **2**. They were recrystalized from methanol (yields: 4 mg and 144 mg, respectively). Subfraction A-3 was chromatographed on Sephadex LH-20 (glass column with 2.5 × 45 cm length) with methanol as eluent and gave **2**. Subfraction A-4 was chromatographed on Sephadex LH-20 (glass column with 2.5 cm × 45 cm length) with methanol as eluent and gave **2** and **3**. They were recrystallized from methanol (yields: 42 and 141 mg, respectively). Subfraction B-2 was chromatographed on Sephadex LH-20 (glass column with 2.5 cm × 45 cm length) with methanol as eluent and gave **3** (Figure 1). This compound was recrystallized from methanol (yield: 302 mg). Subfraction B-3 was chromatographed on Sephadex LH-20 (glass column with 2.5 cm × 45 cm length) with methanol as eluent and gave **3** and **4**. They were recrystalized from methanol (yields: 313 and 67 mg, respectively). Subfraction B-4 was chromatographed on Sephadex LH-20 (glass column with 2.5 cm × 45 cm length) with methanol as eluent and gave **3** and **4**. They were recrystallized from methanol (yields: 103 and 24 mg, respectively).

**Instrumental Analysis of the Isolated Compounds.** The compounds isolated with chromatography were analyzed with the following instruments: The melting point (mp) was obtained with a melting point measurement instrument (Yanagimoto MP, Yanaco, Suita, Japan). The ultraviolet spectra (UV) and infrared spectra (IR) were determined with a UV-240 spectrometer (Shimadzu, Kyoto, Japan) and FT-IR 8020D spectrometer (Shimadzu), respectively. Electron impact mass spectra (EI/MS) were obtained on a mass spectrometer (Shimadzu GC-MS 9100MK). Proton nuclear magnetic resonance ($^1$H NMR) spectra and carbon nuclear magnetic resonance ($^{13}$C NMR) spectra were recorded at 270 MHz on a JNM-EX 270 spectrometer (JEOL, Japan).

**Bacterial Assay for the Measurement of the Antimutagenic Activities against Trp-P-2.** The antimutagenic activities of the test samples against Trp-P-2 were measured by the method of Ames et al. (1975) with some minor modifications and using *S. typhimurium* TA98 in the presence of S-9 mix. The composition of the media and the method for preparation of suspended culture of *S. typhimurium* TA98 and of the S-9 mix were described previous by Nakasugi et al. (1996). Trp-P-2, used as the mutagen, was diluted with distilled water at the concentration of 1.5 $\mu$g/mL, followed by sterilization through a membrane filter (cellulose acetate, 0.45 $\mu$m, Advantec Toyo, Dismic 25CS). The samples for the antimutagenicity test were dissolved in dimethyl sulfoxide at each concentration, followed by sterilization through a membrane filter (PTFE, 0.2 $\mu$m, Sartorius, Minisart SRP15, Göttingen, Germany). The dimethyl sulfoxide used as the control solution was sterilized in a similar manner. The details of the antimutagenicity test were as follows: 0.1 mL of sample solution, 0.1 mL of the bacterial suspension, 0.1 mL of Trp-P-2 (1.5 $\mu$g/mL) solution, and 0.5 mL of S-9 mix were added to 3 mL of molten soft agar. The reagent mix was briefly shaken by hand and was poured onto an MBB agar plate. This plate was incubated at 37 °C for 3 days, and then the number of revertant colonies was counted. The number of surviving cells was also counted along with the mutagenicity assay for cytotoxicity of the test samples against *S. typhimurium* TA98 as follows: 0.1 mL of the bacterial suspension diluted to $10^5$-fold with phosphate buffer and 0.1 mL of sample solution or 0.1 mL of dimethyl sulfoxide was added to 3 mL of molten soft agar, and then the mixed solution was poured onto an

MBB agar plate. After incubation at 37 °C for 3 days, the number of surviving cells was counted.

**Calculation of the Antimutagenic Activities.** The reversion and cell toxicity counts were done in triplicate. The antimutagenic activity (inhibition %) = $\{(C - N) - (S - A)\} \times 100/(C - N)$ where $C$ is the number of $His^+$ revertant colonies of the control solution in the presence of Trp-P-2, $S$ is the number of $His^+$ revertant colonies of each sample solution in the presence of Trp-P-2, $N$ is the number of spontaneous $His^+$ revertant colonies of the control solution, and $A$ is the number of $His^+$ revertant colonies of each sample solution in the absence of Trp-P-2. $C$, $N$, $S$, and survival were the mean of three plate counts.

## RESULTS

**Identification of the Isolated Compounds.** **1** was a pale yellow crystal with a mp of 296−298 °C. The UV spectrum in methanol gave $\lambda_{max}$ 266, 290 (sh), and 333 nm. The addition of aluminum chloride shifted these values to 275, 300, 346, and 380 nm. The addition of sodium methylate shifted band I to 386 nm, and the addition of sodium acetate shifted band I to 347, 380−390 nm (sh); however, it did not shift band II. The IR spectrum in KBr showed absorbances at 3250−3500 (br), 1664, 1606, 1591, 1568, and 1502 $cm^{-1}$. The EI/MS spectrum gave a molecular ion peak at $m/z$ 284 ($M^+$) and fragment ion peaks at $m/z$ 255 and 241. The spectrum of $^1H$ NMR in dimethyl sulfoxide-$d_6$ gave signals at $\delta$ 3.86 (3H, s, $OCH_3$), 6.36 (1H, d, $J$ = 2.0 Hz, 6-H), 6.74 (1H, d, $J$ = 2.0 Hz, 8-H), 6.80 (1H, s, 3-H), 6.92 (2H, d, $J$ = 9.0 Hz, 3′-H and 5′-H), 7.93 (2H, d, $J$ = 9.0 Hz, 2′-H and 6′-H), and 12.92 (1H, s, 5-OH). The spectrum of $^{13}C$ NMR in dimethyl sulfoxide-$d_6$ gave signals at $\delta$ 181.8 (C-4),165.0 (C-7), 164.0 (C-2), 161.2 (C-4′), 161.1 (C-5), 157.1 (C-9), 128.4 (C-2′ and C-6′), 120.9 (C-1′), 115.9 (C-3′ and C-5′), 104.6 (C-10), 102.9 (C-3), 97.8 (C-6), 92.6 (C-8), and 55.9 ($OCH_3$-7). **1** was identified as genkwanin, because these instrumental analysis data corresponded with reference data (Kuroyanagi et al., 1985; Mesquita et al., 1986). **2** was a pale yellow crystal with the mp of 255−256 °C. The UV spectrum in methanol gave $\lambda_{max}$ 273 and 330 nm. The addition of aluminum chloride shifted these values to 288 (sh), 299, and 357 nm. The addition of sodium methylate shifted band I to 388 nm, and the addition of sodium acetate shifted band I to 336 and 386 nm (sh), but did not shift band II. The IR spectrum in KBr showed absorbances at 3200−3400 (br), 1654, 1598, 1572, and 1503 $cm^{-1}$. The EI/MS spectrum gave a molecular ion peak at $m/z$ 314 ($M^+$) and fragment ion peaks at $m/z$ 299, 285, 284, 271, 255, 241, and 181. The spectrum of $^1H$ NMR in dimethyl sulfoxide-$d_6$ gave signals at $\delta$ 3.74 (3H, s, $OCH_3$), 3.92 (3H, s, $OCH_3$), 6.82 (1H, s, H-8), 6.90 (1H, s, H-3), 6.92 (2H, d, $J$ = 9.6 Hz, H-3′ and H-5′), 7.94 (2H, d, $J$ = 9.6 Hz, H-2′ and H-6′), and 12.83 (1H, s, 5-OH). The spectrum of $^{13}C$ NMR in dimethyl sulfoxide-$d_6$ gave signals at $\delta$ 182.2 (C-4), 164.0 (C-2), 161.3 (C-4′), 158.6 (C-7), 152.6 (C-5), 152.1 (C-9), 131.9 (C-6), 128.5 (C-2′ and C-6′), 121.1 (C-1′), 116.0 (C-3′ and C-5′), 105.1 (C-10), 102.7 (C-3), 91.5 (C-8), 60.0 ($OCH_3$-6), and 56.4 ($OCH_3$-7). **2** was identified as cirsimaritin, because these instrumental analysis data corresponded with reference data (Liu et al., 1992; Mesquita et al., 1986). **3** was a pale yellow crystal with a mp of 290−292 °C. The UV spectrum in methanol gave $\lambda_{max}$ 272 and 331 nm. The addition of aluminum chloride shifted these values to 286 (sh), 299, and 360 nm. The addition of sodium methylate shifted band I to 390 nm but did not shift band II, and the addition of sodium acetate shifted bands II and I to 295 (sh) and 367 nm, respectively. The infrared spectrum in KBr showed absorbances at 3300−3500 (br), 1653, 1602, 1560, 1494, and 1464 $cm^{-1}$. The EI/MS spectrum gave a molecular ion peak at $m/z$ 300 ($M^+$) and fragment ion peaks at $m/z$ 285, 282, and 257. The spectrum of $^1H$ NMR in dimethyl sulfoxide-$d_6$ gave signals at $\delta$ 3.74 (3H, s, $OCH_3$-6), 6.57 (1H, s, H-8), 6.76 (1H, s, H-3), 6.91 (2H, d, $J$ = 9.5 Hz, H-3′ and H-5′), 7.91 (2H, d, $J$ = 9.5 Hz, H-2′and H-6′), and 13.06 (1H, s, OH-5). The spectrum of $^{13}C$ NMR in dimethyl sulfoxide-$d_6$ gave signals at $\delta$ 182.0 (C-4), 163.7 (C-2), 161.2 (C-4′), 157.8 (C-7), 152.7 (C-5), 152.5 (C-9), 131.4 (C-6), 128.4 (C-2′ and C-6′), 121.2 (C-1′), 115.9 (C-3′ and C-5′), 103.8 (C-10), 102.3 (C-3), 94.3 (C-8), and 59.9 ($OCH_3$-6). **3** was identified as hispidulin, because the above instrumental analysis data corresponded with reference data (Liu et al., 1992; Hertz and Sumi, 1964). **4** was a pale yellow crystal with a mp of >300 °C. The UV spectrum in methanol gave $\lambda_{max}$ 267 and 334 nm. The addition of aluminum chloride exhibited the four bands at 274, 301, 324, and 390 nm. The addition of sodium methylate shifted the values of UV spectrum in methanol to 276, 300, 342, and 382 nm, and the addition of sodium acetate shifted the values of UV spectrum in methanol to 274, 298 (sh), and 372 nm. The IR spectrum in KBr showed absorbances at 3400−3250, 3100−2600, 1651, 1608, 1587, 1556, and 1500 $cm^{-1}$. The EI/MS spectrum gave a molecular ion peak at $m/z$ 270 ($M^+$) and fragment ion peaks at $m/z$ 269, 242, 153, 152, 124, 121, and 118. The spectrum of $^1H$ NMR in dimethyl sulfoxide-$d_6$ gave signals at $\delta$ 6.20 (1H, d, $J$ = 2.0 Hz, 6-H), 6.43 (1H, d, $J$ = 2.0 Hz, 8-H), 6.70 (1H, s, 3-H), 6.95 (2H, d, $J$ = 9.0 Hz, 3′-H and 5′-H), 7.90 (2H, d, $J$ = 9 Hz, 2′-H and 6′-H), and 12.87 (1H, s, 5-OH). The spectrum of $^{13}C$ NMR in dimethyl sulfoxide-$d_6$ gave signals at $\delta$ 181.6 (C-4), 164.1 (C-7), 163.7 (C-2), 161.4 (C-4′), 161.1 (C-5), 157.2 (C-9), 128.3 (C-2′ and C-6′), 121.1 (C-1′), 115.9 (C-3′ and C-5′), 103.6 (C-10), 102.8 (C-3), 98.7 (C-6), and 93.9 (C-8). **4** was identified as apigenin, because the above instrumental analysis data coincided with reference data and the analysis data of authentic sample (Kuroyanagi et al., 1992). The structure of the four flavones identified in the methanol extract and of four flavones used for comparison are shown in Figure 2.

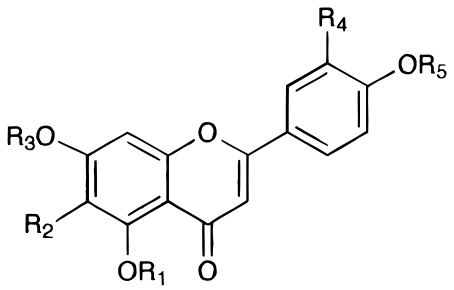


**Figure 2.** Structures of flavones used for the antimutagenicity test. **1**, $R_1 = R_2 = R_4 = R_5 = H$, $R_3 = CH_3$, genkwanin; **2**, $R_1 = R_4 = R_5 = H$, $R_2 = OCH_3$, $R_3 = CH_3$, cirsimaritin; **3**, $R_1 = R_3 = R_4 = R_5 = H$, $R_2 = OCH_3$, hispidulin; **4**, $R_1 = R_2 = R_3 = R_4 = R_5 = H$, apigenin; **5**, $R_1 = R_4 = H$, $R_2 = OCH_3$, $R_3 = R_5 = CH_3$ salvigenin; **6**, $R_1 = R_4 = H$, $R_2 = OCH_3$, $R_3 = R_5 = CH_3$, eupatorin; **7**, $R_3 = H$, $R_2 = R_4 = OCH_3$, $R_1 = R_5 = CH_3$ eupatorin-5-methyl ether; **8**, $R_1 = R_2 = R_3 = R_4 = H$, $R_5 = CH_3$, acacetin; **9**, $R_1 = R_2 = R_3 = R_5 = H$, $R_4 = OH$ luteorin.

**Antimutagenicity of Each Fraction and the Isolated Flavones.** The methanol extract (46.82 g of the concentrate) from carqueja showed very strong

**Table 1. Effects of Five Subfractions of Fraction A on Frameshift Mutation Caused by Trp-P-2 (3-Amino-1-methyl-5*H*-pyrido[4,3*b*]indole) in *S. typhimurium* TA98[a]**

| sample | dose (μg/plate) | no. of revertant colonies/plate | antimutagenic activity (inhibition %) | no. of surviving cells/plate |
|---|---|---|---|---|
| control | 0 | 2680 | 0 | 233 |
| fraction A-1 | 500 | 2892 | inactive | 169 |
| | 100 | 3132 | inactive | 260 |
| fraction A-2 | 500 | 309 | 88.5 | 261 |
| | 100 | 2116 | 21.0 | 274 |
| fraction A-3 | 500 | 85 | 96.8 | 262 |
| | 100 | 290 | 89.2 | 281 |
| fraction A-4 | 500 | 200 | 92.5 | 280 |
| | 100 | 484 | 81.9 | 278 |
| fraction A-5 | 500 | 1091 | 59.3 | 149 |
| | 100 | 2899 | inactive | 267 |

[a] Dimethyl sulfoxide as a control solution was used in the presence of 0.15 μg of Trp-P-2 and S-9 mix. The mean number of spontaneous mutation colonies was 31/plate. The number of revertants, with spontaneous mutation colonies and surviving cells are the means of three plates.

**Table 2. Effects of Five Subfractions of Fraction B on Frameshift Mutation Caused by Trp-P-2 (3-Amino-1-methyl-5*H*-pyrido[4,3*b*]indole) in *S. typhimuriu*m TA98**

| sample | dose (μg/plate) | no. of revertant colonies/plate | antimutagenic activity (inhibition %) | no. of surviving cells/plate |
|---|---|---|---|---|
| control | 0 | 2795 | 0 | 268 |
| fraction B-1 | 500 | 453 | 83.8 | 229 |
| | 100 | 1548 | 44.6 | 278 |
| fraction B-2 | 500 | 299 | 89.3 | 264 |
| | 100 | 871 | 68.8 | 266 |
| fraction B-3 | 500 | 335 | 88.0 | 240 |
| | 100 | 1173 | 58.0 | 263 |
| fraction B-4 | 500 | 344 | 87.7 | 243 |
| | 100 | 1315 | 53.0 | 259 |
| fraction B-5 | 500 | 768 | 72.5 | 216 |
| | 100 | 2875 | inactive | 279 |

[a] Dimethyl sulfoxide as a control solution was used in the presence of 0.15 μg of Trp-P-2 and S-9 mix. The mean number of spontaneous mutation colonies was 31/plate. The number of revertants, with spontaneous mutation colonies, and surviving cells are the means of three plates.

antimutagenic activity (88.9% at the dose of 0.5 mg/plate) (Figure 1). As a result of partition by ethyl acetate and $NaHCO_3$, the antimutagenic activities were found in fractions II and IV. Silica gel column chromatography of fraction IV yielded active fractions A and B. The results of the antimutagenicity tests of subfractions A-1, A-2, A-3, A-4, and A-5 are shown in Table 1. Subfractions A-3 and A-4 exhibited strong antimutagenic activities at the dose of 100 μg/plate, while the activity of subfraction A-2 was weak. Activities of subfractions A-1 and A-5 were not detected at the same dose. The results of the antimutagenicity tests of subfractions B-1, B-2, B-3, B-4, and B-5 are shown in Table 2. Subfraction B-2 exhibited strong antimutagenic activity at a dose of 100 μg/plate. Subfractions B-1, B-3, and B-4 exhibited some antimutagenic activity, whereas no activity was found in subfraction B-5. Stronger antimutagenic activities were present at the 500 μg/plate doses. The antimutagenic activities of the four flavones (**1**−**4**) isolated from carqueja are shown in Table 3, as are the results of the antimutagenicity test of the flavones (**5**−**9**) used for comparison. Cirsimaritin and hispidulin showed very strong antimutagenicity. Their antimutagenic activities were 81.0% and 77.2% at the dose of 25 μg/plate, respectively. In addition, apigenin exhibited even stronger antimutagenic activity than the two flavones above (85.2%). Luteorin, which is known as a strong desmutagen (Samejima et al., 1996), showed the strongest activity (90.9%) at the dose of 50 μg/plate among the tested flavones, while eupatorin was weaker (70.7%).

**Table 3. Effects of the Four Isolated Flavones and Four Related Flavones on the Frameshift Mutation Caused by Trp-P-2 (3-Amino-1-methyl-5*H*-pyrido[4,3*b*]indole) in *S. typhimurium* TA98[a]**

| compds. (in Figure 2) | dose (μg/plate) | no. of revertant colonies/plate | antimutagenic activity (inhibition %) | no. of surviving cells/plate |
|---|---|---|---|---|
| control | 0 | 1571 | 0 | 245 |
| acacetin (**8**) | 50 | 169 | 89.2 | 229 |
| | 25 | 185 | 88.2 | 224 |
| apigenin (**4**) | 50 | 106 | 93.3 | 233 |
| | 25 | 197 | 87.5 | 232 |
| cirsimaritin (**2**) | 50 | 153 | 90.3 | 222 |
| | 25 | 265 | 83.1 | 227 |
| eupatorin (**6**) | 50 | 417 | 73.5 | 232 |
| | 25 | 770 | 51.0 | 217 |
| eupatorin | 50 | 954 | 39.3 | 221 |
| 5-methyl ether (**7**) | 25 | 1075 | 31.6 | 224 |
| genkwanin (**1**) | 50 | 759 | 51.7 | 233 |
| | 25 | 446 | 71.6 | 250 |
| hispidulin (**3**) | 50 | 211 | 86.6 | 223 |
| | 25 | 332 | 78.9 | 237 |
| luteorin (**9**) | 50 | 87 | 94.5 | 233 |
| | 25 | 225 | 85.7 | 237 |
| salvigenin (**5**) | 50 | 345 | 78.0 | 214 |
| | 25 | 462 | 70.6 | 200 |

[a] Dimethyl sulfoxide as a control solution was used in the presence of 0.15 μg of Trp-P-2 and S-9 mix. The mean number of spontaneous mutation colonies was 44/plate. The number of revertants, with spontaneous mutation colonies, and surviving cells are the means of three plates.

## DISCUSSION

This study demonstrated that the antimutagens in the Brazilian folk medicinal plant carqueja are four flavones: genkwanin, cirsimaritin, hispidulin, and apigenin. The methanol extract from carqueja strongly reduced the mutagenicity of Trp-P-2 at low toxicity. After partitioning, the antimutagenic activities existed in the ethyl acetate phase (fraction IV). As a result of the purification with chromatography, four active flavones were obtained from this fraction. The total amounts (calculated) of each flavone obtained from 3.5 kg of carqueja leaves were 134 mg of apigenin, 6 mg of genkwanin, 862 mg of cirsimaritin, and 1.264 g of hispidulin. Although all of the isolated flavones exhibited strong antimutagenic activity, judging from the obtained amounts, it is thought that cirsimaritin and hispidulin are the main contributors to the antimutagenicity of this plant. These flavones may be expected as the chemopreventive agents to cancers because of the strong antimutagenic activities. However, these flavones may cannot exhibit the sufficient effects by the absorption, bioabsorption, and metabolism after they were absorbed in human body. The assay in vivo is therefore desired. In terms of structure−activity relationships, a decreasing antimutagenic activity was found when the moiety of hydroxy group of flavones was replaced by the methoxyl group: Apigenin exhibited decreased antimutagenic activity when the 7-hydroxy group or 4′-hydroxy group was replaced by the methoxyl group. Similary, hispidulin exhibited decreased antimutagenic activity when the 7,4′-dihydroxy groups were replaced by the dimethoxyl group. Their tendency was similar to that described on the antimutagenic effects

of flavonoids by Edenharder et al. (1993). However, there was an exception to the above tendencies; e.g., cirsimaritin exhibited higher activity than hispidulin in spite of the fact that the 7-hydroxy moiety of hispidulin was replaced by methoxyl group. This exception means that the increase in the number of the methoxyl group does not necessarily cause a decrease in the antimutagenic activities. In general, the antimutagens are divided into desmutagens and bio-antimutagens according to differences in their modes of action. Desmutagens are antimutagens that inactivate the mutagens before the mutagens are incorporated into the bacterial cells and include antimutagens that act directly on mutagens or on activated mutagens. Other desmutagens inhibit the action of P-450 enzymes in the metabolic activation of mutagens. Bio-antimutagens are antimutagens that act on the DNA repair system after the mutagen is incorporated into DNA. Although we did not determine whether the four flavones (**1**−**4**) isolated in the present study are desmutagens or bio-antimutagens, we suspect that the four flavones (**1**−**4**) are desmutagens because Samejima et al. (1995) reported that luteorin was a strong desmutagen against Trp-P-2 but not a bio-antimutagen. As for the desmutagenic mechanism, as Liu et al. (1992) reported that cirsimaritin, chrysoeriol, and hispidulin inhibited the metabolic activation of benzo[*a*]pyrene in hamster embryo cells, we speculate that one of the desmutagenic mechanisms of the cirsimaritin, hispidulin, apigenin, and genkwanin examined here may be inhibition of the metabolic activation of Trp-P-2: the inhibition of P-450 enzymes mediated N-oxidation of Trp-P-2, the inhibition of NADPH reductase, or the competition for the bacterial *N*,*O*-acetyltransferase.

However, because these flavones may also exhibit the activity by the other desmutagenic mechanism such as directly interaction with the reactive electrophilic metabolite of Trp-P-2, the detailed experiments with pre- or post-incubation assays is need. Further studies to determine whether these four flavones (**1**−**4**) are desmutagens or bio-antimutagens and to identify their antimutagenic mechanisms are underway in our laboratory.

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