Antitumor Agents from the Cashew (*Anacardium occidentale*) Apple Juice

Isao Kubo,* Masamitsu Ochi, Paulo C. Vieira, and Sakae Komatsu

Division of Entomology and Parasitology, College of Natural Resources, University of California, Berkeley, California 94720

Three anacardic acids (1-3) have been isolated as cytotoxic agents against BT-20 breast carcinoma cells from the cashew *Anacardium occidentale* (Anacardiaceae) apple juice. In addition to these anacardic acids, the cytotoxicity of their 13 congeners (4-16) isolated from the cashew nut and nut shell oil has also been examined. Anacardic acids (1-4), cardols (5-8), and methylcardols (9-12) have been found to exhibit moderate cytotoxic activity against both BT-20 breast and HeLa epithelioid cervix carcinoma cells.

INTRODUCTION

We now control many tumors with available antitumor agents. However, the need for new antitumor agents still exists, especially against solid tumors. The unacceptable and undesired side effects of many antitumor agents are a major problem that needs to be improved, due to the need for the antitumor agents to be used for long durations. It seems that edible plants may be a good source of new antitumor agents. With this in mind, we have screened for new antitumor agents from various tropical fruits and vegetables that have been continuously consumed by many people for many years.

In recent years the cashew (*Anacardium occidentale* L.) (Anacardiaceae) apple has increased in value, especially in the countries where it is grown, such as Brazil. There is no doubt that the nut is the most important product of the cashew tree. However, this tree also yields the pear-shaped "apple" to which the nut is attached. The cashew apple is very sour and astringent until fully ripe, when it becomes edible. In contrast to the nut, the apple was neglected until recently, although it is available in far greater tonnage. A number of processes have now been developed for converting the cashew apple into various products such as juice, jam, syrup, chutney, and beverage (Winterhalter, 1991). Cashew apple juice is, in fact, one of the most popular juices in Brazil today.

In our continuing search for prostaglandin synthetase inhibitors from tropical plants (Kubo et al., 1987) we began to test their antitumor activity, since many prostaglandin-related compounds exhibited potent antitumor activity (Zenser et al., 1980; Powles et al., 1982). Our preliminary screening has found the cashew (*A. occidentale*) apple juice to show significant (ED_{50} <20 μg/mL) in vitro cytotoxicity against BT-20 breast carcinoma cells. The active principles from a regularly imbibed beverage such as cashew apple juice may be superior as antitumor agents as compared to many nonnatural products. This paper deals with the identification of cytotoxic principles of cashew apple juice.

MATERIALS AND METHODS

General. UV spectra were recorded on a Hitachi 100-80 spectrophotometer in ethanol. IR spectra were acquired on a Perkin-Elmer 1310 IR in KBr. NMR spectra were recorded on a JEOL GX-400 (400 MHz for ^1H and 100 MHz for ^{13}C) in $CDCl_3$. EIMS were acquired on a JEOL DX-303HF by direct inlet 70 eV. Recycle HPLC (R-HPLC) was performed on a LC-09 (Japan Analytical Industry, Tokyo, Japan) (Kubo and Nakatsu, 1991).

Chemicals. The phenolic compounds 1-16 (Figure 1) used for bioassay were from our previous study (Kubo et al., 1986). However, the repurification of some of them was achieved by R-HPLC. Anethole was isolated from the seeds of *Pimpinella anisum* (Umbelliferae) as previously described (Kubo and Himejima, 1991). Indole, butylated hydroxyanisole (BHA), vitamin C, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO).

Plant Materials. Suco de Caju, a commercial cashew apple juice (Maguary and Milani) was purchased at market places in São Paulo, São Carlos, and Rio de Janeiro, Brazil.

Cell Lines and Media. The cell lines BT-20 ATCC HTB 19, breast carcinoma isolated from human, and HeLa ATCC CCL 219, epithelioid cervix carcinoma cell from human, used for the assay were purchased from American Type Culture Collection (Rockville, MD). Both cell lines were maintained on minimum essential medium (Eagle) with nonessential amino acids supplemented with 10% (v/v) heat-inactivated fetal calf serum and incubated in a humidified atmosphere of 5% CO_2-95% air at 37 °C. The human monolayer culture were subcultured with a 0.25% trypsin/EDTA solution.

Cytotoxic Assay. The cytotoxic assays were performed according to a microculture tetrazolium (MTT) method (Carmichael et al., 1987). Briefly, cells were harvested and inoculated into 96-well microtiter plates at 4000-6000 cells/well, with various concentrations of the samples. After incubation, 50 μg/mL MTT (3 mg/mL in PBS, pH 7.2) was added. The formazan dye was solubilized by adding 100 μg/mL DMSO to each well, followed by gentle shaking. The extinction coefficient was measured for each well using a Uniskan I Photometer Labsystem, at a wavelength of 620 nm.

Combination Study. The combination data were obtained according to the checkerboard method (Norden et al., 1979). Thus, a series of the 2-fold dilutions of anacardic acid (1) was tested in combination with 2-fold dilutions of the other (anethole, indole, BHA, and vitamin C) (Figure 2). Each compound was tested at least twice.

RESULTS AND DISCUSSION

The cytotoxicity-guided fractionation using BT-20 breast carcinoma cells has led to the isolation of three active principles from the cashew (*A. occidentale*) apple by R-HPLC using an ODS C_{18} column. The active compounds were identified by means of spectroscopic methods as anacardic acids, more specifically, 6-[8(*Z*),-11(*Z*),14-pentadecatrienyl]salicylic acid (1), 6-[8(*Z*),11-(*Z*)-pentadecadienyl]salicylic acid (2), and 6-[8(*Z*)-pentadecenyl]salicylic acid (3). These nonisoprenoid alkyl side chain phenolic compounds were previously isolated from the cashew (*A. occidentale*) nut shell oil together

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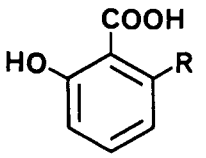
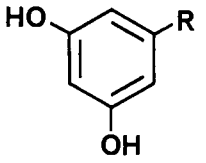


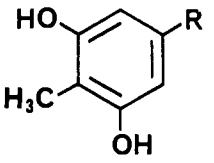
R

1: $C_{15:3}$, 6-[8(Z),11(Z),14-pentadecatrienyl]salicylic acid
2: $C_{15:2}$, 6-[8(Z),11(Z)-pentadecadienyl]salicylic acid
3: $C_{15:1}$, 6-[8(Z)-pentadecenyl]salicylic acid
4: $C_{15:0}$, 6-pentadecylsalicylic acid
17: H Salicylic acid



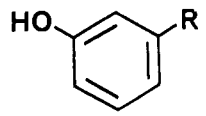
R

5: $C_{15:3}$, 5-[8(Z),11(Z),14-pentadecatrienyl]resorcinol
6: $C_{15:2}$, 5-[8(Z),11(Z)-pentadecadienyl]resorcinol
7: $C_{15:1}$, 5-[8(Z)-pentadecenyl]resorcinol
8: $C_{15:0}$, 5-pentadecylresorcinol
18: H, Resorcinol



R

9: $C_{15:3}$, 2-methyl-5-[8(Z),11(Z),14-pentadecatrienyl]resorcinol
10: $C_{15:2}$, 2-methyl-5-[8(Z),11(Z)-pentadecadienyl]resorcinol
11: $C_{15:1}$, 2-methyl-5-[8(Z)-pentadecenyl]resorcinol
12: $C_{15:0}$, 2-methyl-5-pentadecylresorcinol



R

13: $C_{15:3}$, 3-[8(Z),11(Z),14-pentadecatrienyl]phenol
14: $C_{15:2}$, 3-[8(Z),11(Z)-pentadecadienyl]phenol
15: $C_{15:1}$, 3-[8(Z)-pentadecenyl]phenol
16: $C_{15:0}$, 3-pentadecylphenol

Figure 1. Structures of 16 phenolic compounds isolated from the cashew (apple, nut, and nut shell oil), salicylic acid, (17), and resorcinol (18).

with other similar phenolic compounds such as cardols, methylcardol, and cardanols, with properties as molluscicides (Kubo et al., 1986), prostaglandin synthetase inhibitors (Kubo et al., 1987; Grazzini et al., 1991), and antimicrobial agents (Gellerman et al., 1969; Himejima and Kubo, 1991). Similar nonisoprenoid long-chain phenols including anacardic acid (3) were isolated as antitumor agents against Sarcoma ascites in mice from the sarcotesta of *Ginkgo biloba* L. (Ginkgoaceae) (Itokawa et al., 1987).

In addition to the 3 above-mentioned anacardic acids (1–3) isolated from the cashew apple, 13 additional phenolic compounds (4–16) isolated from the cashew nut and cashew nut shell oil (Tyman, 1979) were also examined for comparison purposes. They were first assayed against BT-20 breast carcinoma cells at 10 μg/mL. As a result, anacardic acids (1–4), cardols (5–8), and methylcardol (9–12) exhibited significant cytotoxicity. Interestingly, cardanols (13–16) did not demonstrate significant activity against BT-20 breast carcinoma cells, although 5-[8(Z),-11(Z),14-pentadecatrienyl]resorcinol (5) was previously reported to exhibit potent antitumor activity against Sarcoma 180 ascites in mice (Itokawa et al., 1987).

The ED_{50} values of the phenolic compounds (1–12) were first obtained against BT-20 breast carcinoma cells (Table I). Then, only the active compounds (ED_{50} < 10 μg/mL) were tested against HeLa epitheloidid cervix carcinoma cells. The most effective cytotoxicity was observed for the cardols, followed by the anacardic acids and then the

Anacardic acid (1) μg/mL	BHA 20	10	5	2.5	0 μg/mL
5	88	80	76	72	71
2.5	81	75	72	70	62
1.25	78	69	45	55	53
0.625	68	68	33	26	20
0.313	65	48	25	8	0
0	55	35	18	8	0

Figure 2. Combination study of anacardic acid (1) with BHA by the checkerboard method against BT-20 breast carcinoma cells.

Table I. Cytotoxicity of the Phenolic Compounds (1–16) of the Cashew (*A. occidentale*) and Salicylic Acid (17) and Resorcinol (18) against BT-20 Breast and HeLa Epithelioid Cervix Carcinoma Cells

phenolic tested	ED_{50}, μg/mL BT-20	HeLa
1	3.23	3.84
2	3.08	3.91
3	4.02	2.69
4	7.42	4.94
5	2.08	2.56
6	2.63	3.01
7	1.72	2.74
8	6.25	4.02
9	3.73	5.05
10	2.64	7.38
11	6.23	6.06
12	7.38	>10
13	>10	–[a]
14	>10	–
15	>10	–
16	>10	–
17	>10	–
18	>10	–

[a] Not tested.

methylcardols. In addition to the natural phenolic compounds (1–16), salicylic acid (17) and resorcinol (18) were also tested. Neither salicylic acid nor resorcinol exhibited any activity against these two carcinoma cells at this concentration. By comparison of salicylic acid (17) and resorcinol (18) with the corresponding anacardic acids (1–4) and cardols (5–8), it seems that the addition of a C_{15} alkyl side chain plays an important role in increasing the cytotoxicity. In fact, log P (as the hydrophobic parameter) of long-chain phenols was found to be one of the most important factors for the ED_{50} values against Chinese hamster V-79 cells (Itokawa et al., 1989).

Moreover, all of the phenolic compounds isolated from the cashew (nut, apple, and nut shell oil) have a C_{15} alkyl side chain with up to three double bonds. In the previous investigation of their molluscicidal and antimicrobial activities, an increase in the number of double bonds in the side chain, in general, increases the biological activity (Gellerman et al., 1969; Himejima and Kubo, 1991; Kubo et al., 1986). However, this seems not to be the case in the cytotoxic activity, although the phenolics possessing a C_{15} saturated alkyl side chain were found to be somehow less cytotoxic than the ones with a C_{15} unsaturated alkyl group.

Although the cytotoxicity of these phenolics may not be potent enough to use them as a leading compound for drug design, they are worthy of further investigation as natural products isolated from a daily beverage. Therefore, the enhancement of their cytotoxicity by combining them with other substances was studied. The procedure for selecting these "other substances" is, however, still in a preliminary stage. The initial selection of other substances was based largely on our previous combination studies of antimicrobial agents. For example, the antifungal activity of polygodial against a filamentous fungus *Candida albicans* was enhanced 32-fold by combining it with sublethal amounts of anethole (Kubo and Himejima, 1991). Similarly, the antibacterial activity of crinitol against a cariogenic bacterium *Streptococcus mutans* was increased 64-fold by BHT (Kubo et al., 1992a), and that of δ-cadinene was enhanced 128-fold by indole (Kubo et al., 1992b). Vitamin C was also added for the combination study because of its high content in the cashew apple. Thus, the ascorbic acid (vitamin C) content in the cashew apple is reported to be greater than 6 times that for oranges (Cecchi and Rodriguez-Amaya, 1981). Moreover, vitamin C (antioxidant) was found to increase the antifungal activity of both polygodial and warburganal 16-fold against a dermatomycotic fungus *Pityrosporum ovale* (Kubo and Himejima, 1992). Since these four compounds have been used as food additives, we can assume that their safety has been established.

For the combination study, 6-[8(*Z*),11(*Z*),14-pentadecatrienyl]salicylic acid (1), otherwise known as anacardic acid, was selected for the following reasons: (1) it has been isolated from the edible cashew apple; (2) its ED_{50} values against the two human solid tumor (BT-20 breast and HeLa epitheloidid cervix) cells are less than 4 μg/mL, that is, it satisfies the NCI requirement to warrant further investigation; and (3) most importantly for conducting this experiment, it is available in quantity.

Anacardic acid (1) was, therefore, combined with anethole, BHA, indole, and vitamin C, respectively. The combination study was carried out against BT-20 breast carcinoma cells by the checkerboard method (Norden et al., 1979). In contrast to the antimicrobial activity, none of these four compounds dramatically increased the cytotoxicity of anacardic acid (1). Their effect was only additive. As an example, the combination study of anacardic acid (1) with BHA by the checkerboard method is shown in Figure 2.

The total yield of the antitumor anacardic acids (1–3) in the freeze-dried cashew apple juice was reported at about 0.05% (Kubo et al., 1986). In addition to these anacardic acids, the high content of vitamin C in the cashew apple is described above (Cecchi and Rodriguez-Amaya, 1981). Together with previous papers (Kubo et al., 1987; Itokawa et al., 1987), these data suggest that consuming the cahsew apple and/or its products continuously for long durations may be advantageous in controlling tumors.

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