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Bioactivities of Anastasia Black (Russian Sweet Pepper)

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Abstract. *Anastasia Black (Russian sweet pepper) of Capsicum annuum L. var. angulosum Mill. (Solanaceae) was successively extracted with hexane, acetone, methanol and 70% methanol, and the extracts were further separated into a total of twenty-three fractions by silica gel or octadecylsilane (ODS; C18) column chromatography. These extracts and fractions were investigated for their cytotoxicity, anti-human immunodeficiency virus (HIV), anti-Helicobacter pylori (H. pylori), urease inhibition and multidrug resistance (MDR) reversal activity. Some fractions of hexane and acetone extracts showed higher cytotoxic activity against three human oral tumor cell lines (squamous cell carcinoma HSC-2, HSC-3, submandibular gland carcinoma HSG) than against three normal human oral cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF), suggesting a tumor-specific cytotoxic activity. No fractions displayed anti-HIV activity, but some hydrophobic fractions showed higher anti-H. pylori activity, urease inhibition activity and MDR reversal activity. The higher MDR activity of these fractions against MDR gene-transfected L5178 mouse lymphoma T cells may possibly be due to their higher content of carotene or polyphenol. These data suggest that Anastasia Black should be further investigated as a potent supplement for cancer chemotherapy.*

Sweet pepper (*Capsicum annuum* L.) is a traditional medicine and, especially in tropical and subtropical areas, sweet pepper has actually been used for the prevention and treatment of typhus, intermittent fevers, dropsy, gout, dyspepsia, cholera, sore throat, scarlatina, hoarseness, yellow fever, diarrhea and as a rubefacient drug (1, 2). Capsiate of the capsaicin analog in sweet pepper prevented carcinogenesis in mice, induced apoptosis of Jurkat tumor cells (3) and inhibited nuclear factor-kappaB (NF-Î B) activation (4).

Sweet pepper contains five times more vitamin C, as high as 52.8 mg/100 g juice, than lemon (10.5 mg/100 g) (5). Vitamin C affects the immune system (6) and displays therapeutic activity in cardiovascular disease (7), high blood pressure (8), cancer (9, 10), cataract (11), diabetes (12) and other diseases (13, 14).

Red and green sweet peppers (*Capsicum annuum*) also contain α-tocopherol (vitamin E) levels as high as 15.54 and 8.70 mg/100 g in the fresh edible part, respectively (2). α-Tocopherol is effective against age-related diseases such as cancer (15), cardiovascular disease and myocardial infarction (16-18). α-Tocopherol can alleviate damage to the cell membrane and DNA, and reduce the risk of lens opacities in cataract caused by high cholesterol in the plasma (19, 20), skin disorders such as acne, dermatitis, eczema and psoriasis (21, 22), and Parkinson's disease (23), caused by free radicals or various reactive oxygen species. However, in contrast to the red and green sweet pepper, little information is available about Anastasia Black. This work was, therefore, undertaken to validate the medicinal claims made for this vegetable by measuring the cytotoxicity, anti-HIV activity, anti-*H. pylori* activity, urease inhibition activity and MDR reversal activity.

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Materials and Methods

Plant material. Dried powder of Anastasia Black, a variety of sweet pepper which is the unripe fruit of *Capsicum annuum* L. var. *angulosum* Mill. (Solanaceae), cultivated in a heated greenhouse in the suburbs of Moscow and/or St. Petersburg in Russia, was kindly donated by Field Co. Ltd. (Shin-Beppu machi, Miyazaki 880-0834,

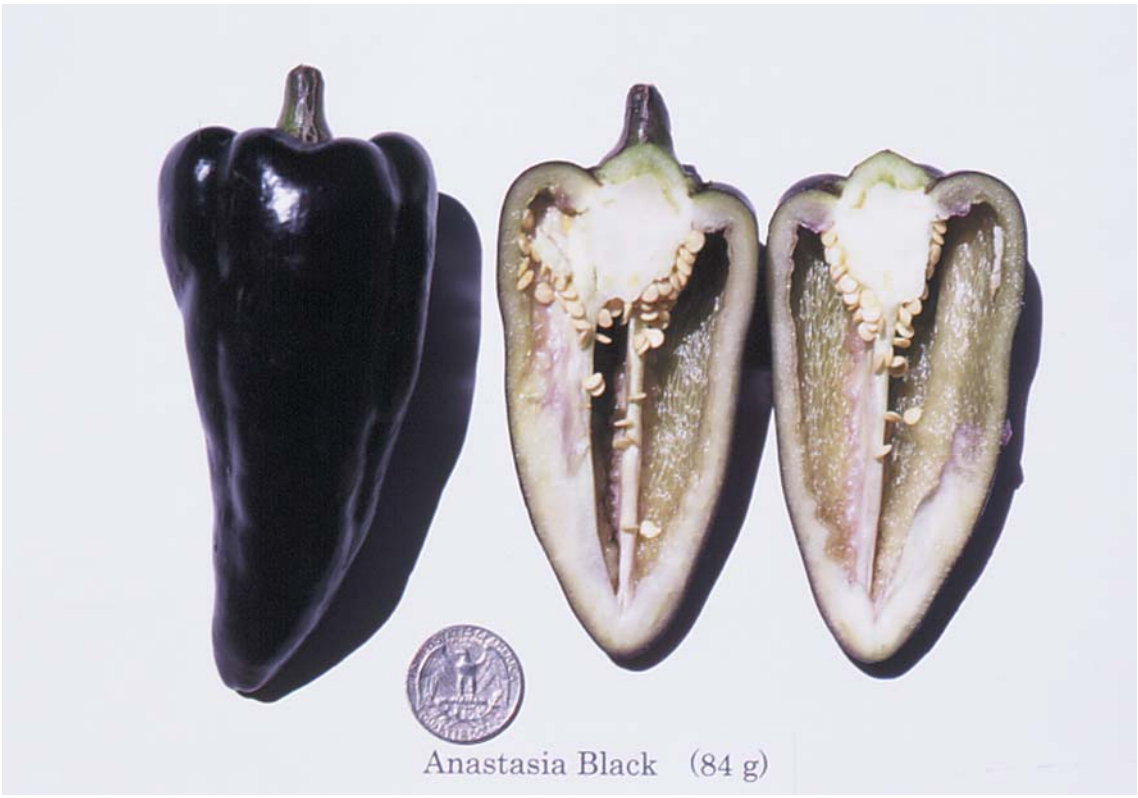

Figure 1. *Russian black sweet pepper (Anastasia Black)(84 g, fresh).*

Japan). This is also cultivated in a greenhouse in the laboratory farm of Field Co. Ltd. and a voucher specimen (P-37) has been deposited in the Herbarium of Josai University, Japan (Figure 1).

Chemicals. The following chemicals and reagents were obtained from the indicated companies: horse serum, RPMI1640 medium, Dulbecco's modified Eagle's medium (DMEM), McCoy's 5A medium (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), metronidazole, erythromycin (Wako Pure Chem. Ind., Ltd., Osaka, Japan); rhodamine 123, (±)-verapamil, 3'-azido-2',3'-dideoxythymidine (AZT), amphotericin B (Sigma Chem. Co., St. Louis, MO, USA); dextran sulfate (DS)(8 kD) (Kowa Chem. Co., Tokyo, Japan); curdlan sulfate (CRDS) (Ajinomoto Co., Tokyo, Japan). Clarithromycin was donated by Taisho Pharmaceutical Co. (Tokyo, Japan). A strain of *H. pylori* (ATCC43504) was purchased from the American Type Culture Collection (Rockville, MD, USA). Jack bean urease was obtained from Sigma-Aldrich Co.

Extraction procedure. Dry powdered Anastasia Black (400 g) was successively extracted with hexane, acetone, MeOH and 70% MeOH at room temperature. After evaporation of the solvent *in vacuo*, the hexane extract **[H0]** (5.75 g), acetone extract **[A0]** (7.27 g), MeOH extract **[M0]** (69.3 g) and 70% MeOH extract **[70M0]** (49.1 g), respectively, were obtained (Figure 2).

Initially, an aliquot of the hexane extract **[H0]** (4.5 g) was applied to silica gel column chromatography, which was eluted stepwise with hexane and hexane-acetone (9:1) **[H1]** (3.03 g), hexane-acetone (4:1) **[H2]** (0.66 g), hexane-acetone (3:2) **[H3]** (0.19 g) and hexane-acetone (2:3) **[H4]** (0.06 g) (Figure 2).

Next, the acetone extract **[A0]** (4.8 g) was applied to silica gel column chromatography, which was then eluted stepwise with benzene **[A1]** (0.30 g), benzene-EtOAc (9:1) **[A2]** (0.11 g), benzene-EtOAc (4:1) **[A3]** (0.13 g), benzene-EtOAc (3:2) **[A4]** (1.37 g), benzene-EtOAc (2:3) **[A5]** (1.36 g) and EtOAc **[A6]** (1.23 g) (Figure 2).

Then, the MeOH extract **[M0]** (10 g) was applied to silica gel column chromatography, which was then eluted stepwise with $CHCl_3$ and $CHCl_3$-MeOH (49:1) **[M1]** (0.05 g), $CHCl_3$-MeOH (24:1) **[M2]** (0.09 g), $CHCl_3$-MeOH (9:1) **[M3]** (0.43 g), $CHCl_3$-MeOH (4:1) **[M4]** (1.58 g), $CHCl_3$-MeOH (3:2) **[M5]** (0.23 g) and MeOH **[M6]** (6.48 g) (Figure 2).

Finally, the 70% MeOH extract **[70M0]** (10 g) was applied to octadecylsilane (ODS; C18) column chromatography, which was then eluted stepwise with MeOH-H_2O (1:4) **[70M1]** (6.22 g), MeOH-H_2O (2:7) **[70M2]** (1.36 g), MeOH-H_2O (1:3) **[70M3]** (0.21 g), MeOH-H_2O (1:2) **[70M4]** (0.24 g), MeOH-H_2O (1:1) **[70M5]** (0.49 g), MeOH-H_2O (2:1) **[70M6]** (0.41 g) and MeOH **[70M7]** (0.93 g) (Figure 2).

Assay for cytotoxic activity. Three human oral tumor cell lines [squamous cell carcinoma (HSC-2, HSC-3), submandibular gland

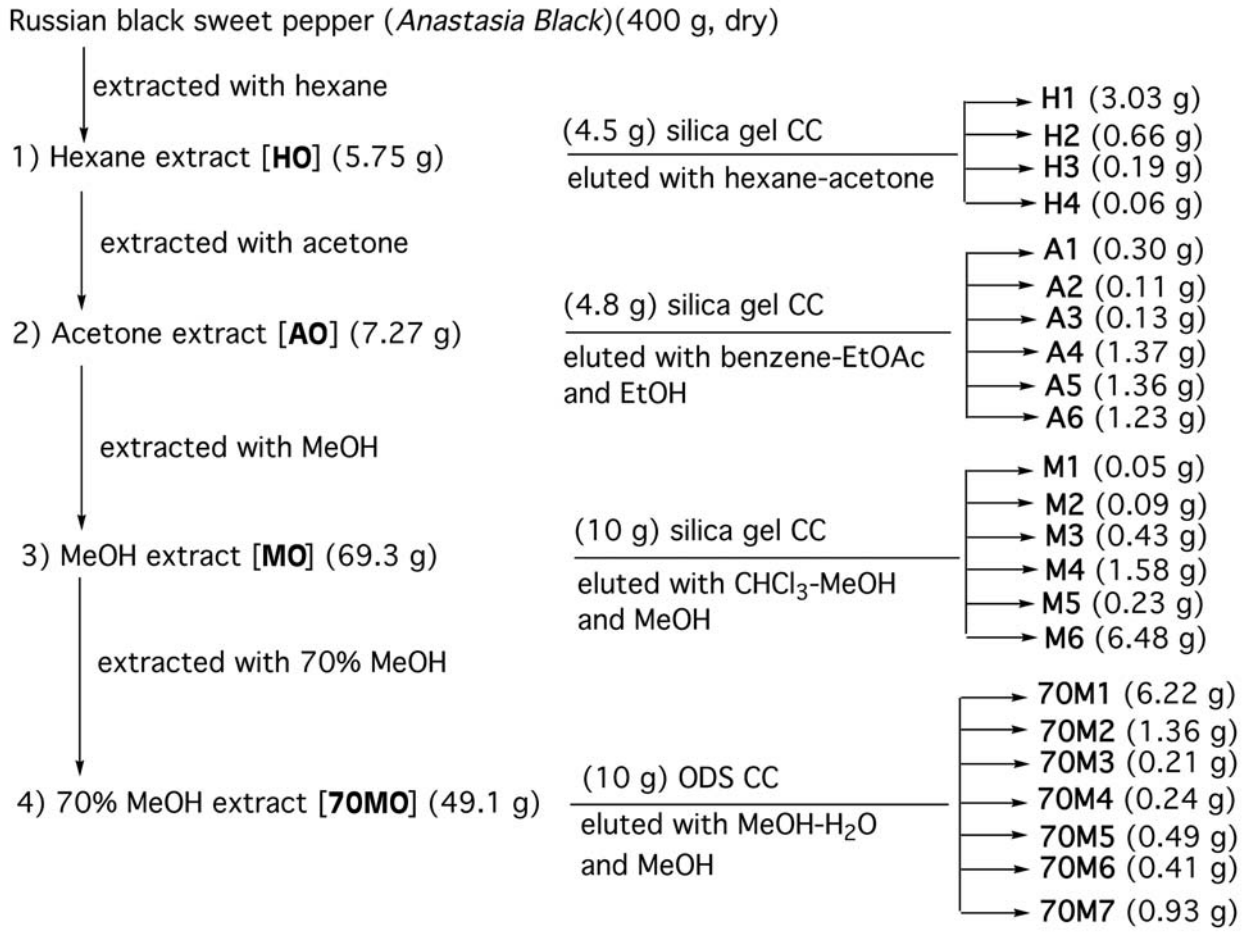

Figure 2. *Fractional separation of Anastasia Black extracts. CC: column chromatography.*

carcinoma (HSG)], and three human oral normal cells [gingival fibroblasts (HGF), pulp cell (HPC), periodontal ligament fibroblast (HPLF)] (5-7 population doubling levels) were cultured in DMEM medium supplemented with 10% heat-inactivated FBS.

The MTT assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) by mitochondrial dehydrogenase of metabolically active cells to a blue formazan which can be measured spectrophotometrically.

These cells were incubated for 24 h with the indicated concentrations of the test extracts or fractions. The relative viable cell number was then determined by the MTT assay. In brief, cells were washed once with Ca, Mg-free phosphate-buffered saline (PBS), and incubated for 4 h with 0.2 mg/mL MTT. After removal of the medium, the cells were dissolved in 100 μL of DMSO. The absorbance at 540 nm, which reflects the relative viable cell number, was then determined. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve (Table I) (24).

Assay for human immunodeficiency virus (HIV) inhibition activity. Human T cell leukemia virus 1 (HTLV1)-bearing CD4-positive human T cell lines, MT-4 cells, were infected with HIV-1_{IIIB} at a multiplicity of infection (m.o.i.) of 0.01. HIV- or MOCK-infected MT-4 cells (1.5 x 10^5/mL, 200 μL) were placed into 96-well microtiter plates and incubated in the presence of varying concentrations of each extract or fraction in RPMI1640 medium supplemented with 10% FBS. After incubation for 5 days at 37˚C in a 5% CO_2 incubator, cell viability was quantified by MTT assay. In brief, 20 μL of a 7.5 mg/mL MTT solution in PBS was added to each well, using the Titertek multidrop. After incubation for 1 h at 37˚C, a fixed volume of 150 μL medium was removed from each well. Solubilization of the formazan crystals was achieved by addition of 100 μL 10% (v/v) Triton X-100 in acidified isopropanol containing 0.4% (v/v) of concentrated HCl. Finally, the optimal density of each well was measured using an automatic plate reader with a test wavelength of 540 nm and a reference wavelength of 690 nm. Blank wells were prepared with all reagents except MT-4 cells and Anastasia Black extracts. All data represent the mean values of triplicate measurements. The CC_{50} was defined as the concentraton of the extracts, fractions and chemical compounds that reduces the absorbance (OD_{540}) of the mock-infected control sample by 50%. The percent protection achieved by the test compound in HIV-infected cells was calculated by the following formula:

$$\frac{(OD_t)_{HIV} - (OD_c)_{HIV}}{(OD_c)_{mock} - (OD_c)_{HIV}} \times 100 \text{ (expressed in \%)}$$

where $(OD_t)_{HIV}$ is the optical density measured with a given concentration of the test extracts or fractions in HIV-infected cells $(OD_t)_{HIV}$ is the optical density measured for the control untreated HIV-infected cells; and $(OD_c)_{mock}$ is the optical density measured for control untreated mock-infected cells. The concentration achieving 50% protection according to the formula above was defined as the 50% effective concentration (EC_{50}). The selectivity index was defined as CC_{50}/EC_{50} (25-27).

Assay for anti-H. pylori activity. Cell cultures: *H. pylori* strains (ATCC 43504) were inoculated on a modified BHM agar plate containing 10% horse serum, and cultured at 37˚C for 5 days in a jar conditioned with Anaero pack (Mitsubishi Gas Chemical, Tokyo, Japan). The colonies were inoculated in Brain Heart Infusion containing 10% fetal bovine serum and cultured in micro aerophil for 48 h.

Assay: The bacterial suspension was diluted to about 1 x 10^7 colony-forming units (CFU)/mL with 0.9% saline. The extracts were dissolved in DMSO and then diluted with culture medium. A 96-well assay plate was used for the susceptibility of *H. pylori*. To the solution of the extracts, bacterial suspension was added to a density of 10^6 CFU/100 μL/well. The mixture was incubated in micro aerophil at 37˚C for 48 h. The 50% inhibitory concentration (MIC_{50}) of each extract or fraction was calculated from the dose-response curve (28).

Assay for urease inhibition. Jack bean urease was used for evaluating enzyme inhibition by Anastasia Black extracts and fractions, as previously described (29, 30). Briefly, each assay mixture with 25 μL (4U) of Jack bean urease and 25 μL (100 μg) of each extract or fraction was preincubated for 0.5 and 3 h at room temperature in a 96-well assay plate, respectively. After preincubation, 0.2 mL of 100 mM phosphate buffer, pH 6.8, with 500 mM urea and 0.002% phenol red was added and incubated at room temperature. The time required for raising the pH of a phosphate buffer from 6.8 to 7.7 by ammonium carbonate, produced during enzyme reactions, was measured by microplate reader (570 nm).

Assay for MDR reversal activity. The experiments of MDR reversal were performed with freshly prepared samples, since the storage in DMSO at even –20˚C caused significant loss of activity.

The L5178 mouse T cell lymphoma cell line was transfected with a human MDR1/A-containing retrovirus, as previously described (31). MDR1-expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain the expression of the MDR phenotype. The L5178 MDR cell line and the L5178Y parent cell line were grown in McCoy's 5A medium with 10% heat-inactivated horse serum, L-glutamine and antibiotics (penicillin: 100 unit/mL; gentamycin: 100 μg/mL).

The cells were adjusted to a density of 2 x 10^6/mL, resuspended in serum-free McCoy's 5A medium and 0.5 mL aliquots of the cells were distributed into Eppendorf centrifuge tubes. Two to 20.0 μL of 1.0 mg/mL of each extract or fraction was added and incubated for 10 min at room temperature. Rhodamine 123 (R123) indicator (10 μL; 5.2 mM final concentration) was added and incubated for a further 20 min at 37˚C. After washing twice and resuspending in 0.5 mL PBS, the fluorescence of the cell population was measured by flow cytometry, using a Beckton-Dickinson FACScan instrument (cell sorter). (±)-Verapamil was used as a positive control in the R123 accumulation experiments (32). The ratio of R123 accumulation was calculated from the fluorescence intensity of the samples. The percentage of control of untreated mean fluorescence intensity was measured for parental and MDR cell lines and compared to the fluorescence intensity of treated cells. The MDR reversal activity was calculated by the following equation (32, 33) (Table III):

$$\text{MDR reversal activity} = \frac{\text{(MDR-treated / MDR control)}}{\text{(parental-treated / parental control)}}$$

Results

Cytotoxic activity. The hexane extract **[H0]** showed some cytotoxic activity, whereas other extracts **[A0, M0, 70M0]** showed much lower cytotoxic activity (Table I).

Following column chromatography, the cytotoxic activity in the fractions of the hexane extract **[H2, H3, H4],** acetone extracts **[A2, A3, A4, A5],** and the methanol extract **[M2]** was significantly increased.

Normal human oral cells (HGF, HPC, HPLF) showed higher resistance to all these fractions, as compared to oral tumor cell lines (HSC-2, HSC-3, HSG), resulting in the elevation of the tumor specificity index (TS) of some fractions **[H2, H3, H4, A2, A3, A4, A5, M2]** (Table I).

Anti-human immunodeficiency virus (HIV) activity. No extracts or fractions showed any anti-HIV activity (SI<1), in contrast to the positive controls, dextran sulfate (DS) (SI=207), curdlan sulfate (CRDS) (SI>1487), AZT (SI=2840) and ddC (>1237) (Table II).

Anti-H. pylori activity. H. pylori is responsible for chronic gastritis, peptic ulcer and risk of development of stomach cancer. Today, control of this organism is an important goal of medicinal science. Therefore, many plants used in traditional medicine have been screened for antimicrobial activity against *H. pylori* (34). Several plants and their principles have been shown to be effective agents for the treatment of symptomatic and asymptomatic forms of *H. pylori* infections. The anti-*H. pylori* activity of the hexane fraction (**[H3]** (MIC_{50}=7.6 μg/mL) was three times that of metronidazole (MIC_{50}=30 μg/mL), although clarithromycin (MIC_{50}=1.2 x 10^{-3} μg/mL) and amoxicillin (MIC_{50}=1.7 x 10^{-2} μg/mL), used as clinically standard antibacterial agents, were the most potent (Table II).

Urease inhibition activity. Urease is a nickel-containig enzyme that catalyzes the hydrolysis of urea. The enzymatic hydrolysis of urea causes an abrupt increase in overall pH,

Table I. *Cytotoxic activity of Anastasia Black extracts and fractions.*

Extract and fraction	Cytotoxic activity[1] (CC_{50}: μg/mL) Human oral cell line								TS[2] $\left[\frac{B}{A}\right]$
	Tumor cells				Normal cells				
	HSC-2	HSC-3	HSG	Total (A)	HGF	HPC	HPLF	Total (B)	
H0	141	160	151	452	150	164	190	504	1.1
H1	160	184	191	535	>200	>200	>200	>600	>1.1
H2	77	137	103	317	160	164	173	497	1.6
H3	103	128	126	357	146	158	160	464	1.3
H4	78	146	90	314	168	>200	>200	>568	>1.8
A0	>200	>200	>200	>600	172	>200	>200	>572	><1.0
A1	185	>200	200	>585	>200	>200	>200	>600	><1.0
A2	138	154	161	453	171	181	198	550	1.2
A3	38	133	95	266	160	157	166	483	1.8
A4	79	139	174	392	184	182	187	553	1.4
A5	64	140	131	335	153	155	162	470	1.4
A6	143	>200	>200	>543	158	>200	>200	>558	><1.0
M0	>200	>200	>200	>600	>200	>200	>200	>600	><1.0
M1	>200	>200	>200	>600	>200	>200	>200	>600	><1.0
M2	130	137	155	422	152	169	171	492	1.2
M3	>200	>200	>200	>600	165	>200	>200	>565	><0.9
M4	>200	>200	>200	>600	>200	>200	>200	>600	><1.0
M5	>200	>200	>200	>600	>200	>200	>200	>600	><1.0
M6	>200	>200	>200	>600	>200	>200	>200	>600	><1.00
70M0	100	>200	>200	>500	>200	>200	>200	>600	><1.2
70M1	>200	>200	>200	>600	>200	>200	>200	>600	><1.0
70M2	>200	>200	>200	>600	>200	>200	>200	>600	><1.0
70M3	>200	>200	>200	>600	>200	>200	>200	>600	><1.0
70M4	>200	>200	>200	>600	>200	>200	>200	>600	><1.0
70M5	>200	>200	>200	>600	>200	>200	>200	>600	><1.0
70M6	>200	>200	>200	>600	>200	>200	>200	>600	><1.0
70M7	>200	>200	>200	>600	>200	>200	>200	>600	><1.0
Gallic acid	22	59	35	116	71	74	61	206	1.8
Quercetin	43	164	153	360	>200	>200	>200	>600	>1.7
A_{540}	0.856	1.000	0.243		0.321	0.342	0.312		

[1] Near confluent tumor cell lines (HSC-2, HSC-3, HSG cells) and normal cells (HGF, HPC, HP2F) were incubated for 24 h with various concentrations of Anastasia Black extracts or fractions, and the relative viable cell number (A_{540}) was determined by MTT method. 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve. Each value represents the mean from duplicate determinations. Control A_{540} values of HSC-2, HSC-3, HSG cells and HGF, HPC, HPLF at the time of MTT assay were 0.856, 0.701, 1.000, 0.243 and 0.321, 0.342 and 0.312, respectively.

[2] $$TS = \frac{\Sigma\, CC_{50}\ (\text{normal cells})\ (B)}{\Sigma\, CC_{50}\ (\text{tumor cells})\ (A)} = \frac{CC_{50}(HGF) + CC_{50}(HPC) + CC_{50}(HPLF)}{CC_{50}(HSC\text{-}2) + CC_{50}(HSC\text{-}3) + CC_{50}(HSG)}$$

which negatively influences human health and agriculture (35). Especially, urease activity constitutes a virulence factor in human infections of the urinary and gastrointestinal tracts. *H. pylori* produces a large amount of urease, which is believed to play an essential role in facilitating its survival. Urease inhibitors have been regarded as targets for new anti-ulcer drugs (36). A new diterpene ester, isolated from *Euphorbia decipiens,* showed inhibitory activity against urease enzyme (IC_{50} = 81 mM *vs* . thiourea: IC = 21 mM) (37). This is the first natural urease inhibitor.

Table II. *Anti-HIV, anti-H. pylori and jack bean urease inhibitory activity of Anastasia Black extracts and fractions.*

Extract and fraction	Anti-HIV activity		SI $\left[\frac{CC_{50}}{EC_{50}}\right]$	Anti- H. pylori activity	Inhibition (%) against Jack bean urease(400 μg/mL) Preincubation time	
	CC_{50} (μg/mL)	EC_{50} (μg/mL)		MIC_{50} (μg/mL)	0.5 h	3 h
H0	≠ 75.07	> 200	< 1	-	3.3	18.0
H1	≠ 76.12	> 200	< 1	-	3.2	21.0
H2	≠ 43.54	> 200	< 1	50.0	2.8	31.2
H3	≠ 7.77	> 8	< 1	7.6	2.7	31.1
H4	≠ 20.88	40	< 1	78.0	3.3	18.1
A0	≠ 79.55	> 200	< 1	78.0	1.9	51.4
A1	≠ 90.82	> 200	< 1	72.0	2.9	27.5
A2	≠ 67.04	> 200	< 1	38.0	3.2	19.6
A3	≠ 12.47	40	< 1	49.0	2.3	43.3
A4	≠ 81.8	> 200	< 1	-	1.9	51.5
A5	≠ 23.75	> 40	< 1	38.0	3.5	12.0
A6	≠ 94.1	> 200	< 1	80.0	3.7	8.8
M0	≠ 530.87	> 1000	< 1	-	3.7	8.2
M1	≠ 374.1	> 1000	< 1	44.0	3.5	12.9
M2	≠ 88.01	> 200	< 1	40.0	2.8	30.7
M3	≠ 93.85	> 200	< 1	45.0	3.9	2.9
M4	≠ 442.11	> 1000	< 1	-	3.8	5.2
M5	≠ 346.96	> 1000	< 1	-	3.9	3.0
M6	≠ 755.8	> 1000	< 1	-	3.8	4.6
70M0	≠ 489.03	> 1000	< 1	-	3.7	6.8
70M1	> 1000	> 1000	>< 1	-	3.7	7.9
70M2	609.19	> 1000	< 1	-	3.7	7.3
70M3	≠ 438.37	> 1000	< 1	-	3.6	11.3
70M4	≠ 160.11	> 200	< 1	-	3.4	14.3
70M5	≠ 101.99	> 200	< 1	-	3.5	12.9
70M6	≠ 455.58	> 1000	< 1	-	3.7	7.3
70M7	≠ 523.3	> 1000	< 1	-	3.7	8.2
Gallic acid	≠ 3.37	> 8	< 1	-	1.5	63.3
Quercetin	≠ 5.59	> 8	< 1	-	3.5	12.6
DS (μg/mL)[1)]	≠ 633.91	≠ 3.056	≠ 207			
CRDS (μg/mL)[2)]	>1000		> 1487			
AZT (μM)	≠ 57.59	≠ 0.02	≠ 2840			
ddC (μM)	>5000	≠ 4.042	> 1237			
Metronidazole				30.0		
Amoxicillin				1.7×10^{-4}		
Clarithromycin				1.2×10^{-3}		

[1)] DS: dexatran sulfate. [2)] CRDS: curdlan sulfate.

Here, the urease inhibitory activity of a total of 27 extracts and fractions of Anastasia Black (400 μg/mL) was measured after 3-h preincubation. The acetone fraction (**[A4]** (51.5%), acetone extract (**[A0]** (51.4%) and acetone fraction (**[A3]** (43.3%) showed slightly lower urease inhibitory activity than gallic acid (63.3%), but higher activity than quercetin (12.6%).

Assay for MDR reversal activity. The expression of the MDR1/A gene in the L5178 mouse T-cell lymphoma cell line resulted in MDR, as reflected by the reduced intracellular accumulation of R123. Addition of (±)-verapamil as control reversed the MDR, as reflected by the increase in R123 accumulation (fluorescence activity intensity = 7.86) (data not shown) (Table III).

Table III. *MDR reversal activity of Anastasia Black extracts and fractions (4 and 40 μg/mL) in mouse lymphoma-5178 cells.*

Extract and fraction	Fluorescence activity ratio[a,b]	
	4 μg/mL in 25% DMSO	40 μg/mL in 25% DMSO
Par(control)[c]		
MDR+R123(mean)[d]		
(±)-verapamil (positive control) 10 μg/mL	1.00	1.00
25%DMSO (control)	0.92	0.92
gallic acid	0.31	0.28
H0	5.52	26.21
H1	3.73	4.03
H2	12.45	57.95
H3	7.24	20.94
H4	14.72	81.74
A0	2.84	7.05
A1	1.26	1.44
A2	22.94	15.83
A3	3.72	8.08
A4	2.22	12.65
A5	6.72	13.89
A6	2.46	21.68
M0	0.66	0.64
M1	0.70	1.65
M2	2.08	2.49
M3	4.25	7.82
M4	0.47	0.46
M5	0.46	0.45
M6	0.44	0.42
70M0	0.79	0.54
70M1	0.72	0.46
70M2	0.46	0.41
70M3	0.42	0.33
70M4	0.36	0.33
70M5	0.32	0.31
70M6	0.28	0.33
70M7	0.32	0.42

[a] refs. 32, 33.

[b] The R-123 accumulation was calculated from fluorescence of one height value using the following equation:

$$\log(y)=\log_{10}\frac{x}{256}$$

then the fluorescence activity ratios were calculated according to the formula given below:

$$\text{MDR reversal activity} = \frac{\text{(MDR-treated/MDR control)}}{\text{(parental-treated/parental control)}}$$

[c] Par: a parental cell without MDR-1 gene.

[d] MDR: a parental cell with MDR-1 gene.

The hexane extract and fractions **[H0, H1-4],** acetone extract and fractions **[A0, A1-6]** and some of the methanol extracts and fractions **[M2, M3]** showed higher MDR reversal activity than (±)-verapamil. Other fractions were much less active (Table III).

The data are consistent with the report of Schelz (personal communication) that the fractions eluted with polar solvents were ineffective. The MDR reversal effect possibly depends on the lipophilic carotenoid content of the fractions, as seen in saffron derivatives (38) and isolated carotenes from different plants (39).

Discussion

The present study demonstrates that Anastasia Black was more cytotoxic against three oral tumor cell lines when compared to normal cell lines (Table I). Vitamin C has an antioxidant action, but a pro-oxidant action at high dose may inhibit the growth of tumor cells *in vitro*. Low-dose vitamin C shows antioxidant action, protecting the cells from free radicals or reactive oxygen species (40). Vitamin C also was effective in preventing carcinogenesis from pre-cancerous stages to colon cancer. Additionally, antioxidant vitamins (A, C, E) were effective in reducing pre-cancerous abnormalities (41). Vitamin E protects against damage to the cell membrane and chromosomes, which might lead to cancerous changes, vitamin E treatment resulting in a 32% decrease in the incidence of prostate cancer and a 41% decrease in prostate cancer deaths (42).

A high intake of vitamin C through vitamin C-rich diets could also inhibit ulcer caused by *H. pylori* and stomach cancer. However, vitamin E could not inhibit the growth of *H. pylori*. This means that vitamin C exerts the protective effects against cancer or ulcers *via* a biochemical mechanism (43). On the other hand, high vitamin E intake by HIV-positive patients resulted in a 35% decrease in risk of progression to AIDS, when compared to those men with low vitamin E intake (44).

Capsaicinoid, a capsaicin analog, induces apoptosis in Jurkat tumor cells by methylation of the phenolic hydroxyl group of nor-dihydrocapsiate. This property of capsaicinoid suggests its ability to scavenge the highly reactive hydroxyl radical (45). Capsaicin and nordihydrocapsiate inhibited the early and late stages of T cell activation mediated by CD69 antigen (CD69), cell surface expression of Tac antigen (CD25) and intercellular adhesion molecule-1 (ICAM-1), progression to the S-phase of the cell cycle and proliferation in response to the co-engagement of T cell receptor (TCR) and CD28 antigen (CD28). Moreover, capsaicin and nordihydrocapsiate also inhibit nuclear factor-kappa B (NF-Î B) activation by cytokines such as tumor necrosis factor (TNF-α) (4).

Among three Anastasia sweet peppers, the cytotoxic activity of Black was higher than that of Anastasia Green (46) and Red

(47). Anti-HIV activity and anti-*H. pylori* activity was not found for the three Anastasia sweet peppers. Among twenty-seven Anastasia Black extracts and column fractions, the hexane fraction **[H3]** (MIC_{50}= 7.6 μg/mL) showed the highest anti-*H. pylori* activity. The hexane and acetone extracts and fractions of the three Anastasia sweet peppers showed higher MDR reversal activity, when compared to a control (±)-verapamil.

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