APPENDX A

List of chemical and materials used in this study

Name of chemicals	Company
Absolute ethanol	E. Merk, Germany
Alpha tocopherol	Sigma-Aldrich Co. LLC.
Ascorbic acid	Sigma-Aldrich Co. LLC.
2,2′-Azino-bis(3-ethylbenzothiazoline -6-sulfonic acid	Sigma-Aldrich Co. LLC.
Bovine serum albumin	Sigma Chemical Co., Dosert, UK
Carbonate	Sigma Chemical Co., Dosert, UK
2,2-Diphenyl-1-picrylhdrazyl	Sigma-Aldrich Co. LLC.
DMEM	GIBCO-BRL, Grand Island, Japan
Ethydium bromide	Wako Pure Chemical Industries, Japan
Ethylene diamine-N,N,N’N, -tetraacetic acid (EDTA)	Fisher Scientific, UK
Fetal bovine serum (FBS)	Biochrom AG, Germany
Filter paper No.1	Whaman International Ltd.,
Folin-ciocalteu	Folin-ciocalteu
Gallic acid	E. Merk, Germany
6-Hydroxy-2,5,7,8-tetramethylchroman -2-carboxylic Acid (Trolox)	Sigma-Aldrich Co. LLC.

Name of chemicals	Company
Iodonitrotetrazolium chloride	Sigma-Aldrich Co. LLC.
Malondialdehyde	Sigma-Aldrich Co. LLC.
Methylene blue	Sigma Chemical Co., USA
Minimal essential medium (MEM)	GIBCOBRL, NY, USA
MTT ([3,[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl-tetrazolium bromide)	Sigma Chemical Co., Dorsert,UK
MTT dry	USB Corporation, USA
Nitrocellulose membrane	Amersham, Japan
Normal saline	E. Merk, Germany
Penicillin	General Drug House Co., Ltd
Phosphate buffer solution	Sigma-Aldrich Co. LLC.
Phosphomolybdic acid	Sigma-Aldrich Co. LLC.
Phosphotungtic acid	Sigma-Aldrich Co. LLC.
Potassium chloride	Wako Pure Chemical Industries, Japan
Potassium dihydrogen phosphate	Sigma-Aldrich Co. LLC.
Potassium peroxodisulphate	Sigma-Aldrich Co. LLC.
Potassium persulfate	E. Merk, Germany
Propidium iodide	Wako Pure Chemical Industries, Japan
Proteinase K	Wako Pure Chemical Industries, Japan
Sodium bicarbonate	Wako Pure Chemical Industries, Japan
Sodium carbonate	Sigma-Aldrich Co. LLC.
Sodium carbonate decahydrate	Sigma-Aldrich Co. LLC.

Name of chemicals	Company
Sodium chloride	Wako Pure Chemical Industries, Japan
Sodium dodecyl sulfate	Wako Pure Chemical Industries, Japan
Sodium hydrogen carbonate	Sigma-Aldrich Co. LLC.
Sodium hydroxide	Wako Pure Chemical Industries, Japan
Sodium hydroxide pellets	Sigma-Aldrich Co. LLC.
Streptomycin sulfate	Wako Pure Chemical Industries, Japan SW-
Superodide dismutase from bovine live	Sigma-Aldrich Co. LLC.
TEMED	Amtesco, USA
Thiobarbituric acid	E. Merk, Germany
Trichloroacetic acid	E. Merk, Germany
Tris	Sigma-Aldrich Co. LLC.
Triton X-100	Wako Pure Chemical Industries, Japan
Trypan blue	Wako Pure Chemical Industries, Japan
Xanthine	Sigma-Aldrich Co. LLC.
Xanthine oxidase from bovine live	Sigma-Aldrich Co. LLC.

APPENDIX B

Preparation of some reagents and buffer

1. Human colon cancer cell culture

1.1 Incomplete Dulbecco's modified Eagle (DMEM)

DMEM	1 package (13.5 g.)	
HEPES	3.9	g.
$NaHCO_3$	3.7	g.
Penicillin G	0.0625	g.
Streptomycin	0.1	g.
DW	1	L.

Sterilize with 0.22 µM filter and store at 2-8 ºC (pH 7.4)

1.2 Complete DMEM medium

DMEM	90	ml.
Fetal bovine serum	10	ml.

1.3 MEM medium

MEM powder	10.1	g.
$NaHCO_3$	1.5	g.
Sodium pyruvate	0.11	g.
Penicillin G	0.0625	g.
Streptomycin sulfate	0.1	g.
DW	1	L.

Sterilize with 0.22 µM filter and store at 2-8 ºC (pH 7.4)

1.4 Complete MEM

MEM	90	ml.
Fetal bovine serum	10	ml.

1.5 Phosphate buffered saline (PBS pH 7.4)

NaCl	8	g.
KCl	0.2	g.
Na_2HPO_4	1.44	g.
KH_2PO_4	0.24	g.
DW	800	ml.

Adjust pH 7.4 using 1 N HCl then adjust volume to 1 liter

1.6 0.025% EDTA

EDTA.2 Na	0.025	g.
PBS (-)	100	ml.

Sterilize by autoclave 121^{O} C, 15 min

1.7 0.05% trypsin - 0.25% EDTA

0.25% trypsin	20	ml.
0.025% EDAT	80	ml.

1.8 0.2% trypan blue

Trypan blue	0.2	g.
PBS (-)	100	ml.

2. MTT solution

[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]	0.25	g
PBS 1X	50	ml

The solution was filtered with 0.22 μM filter and kept in dark at 4 °C

2.1 0.1% Crystal violet in 1% ethanol

Crystal violet powder	1	g
95% Ethanol	10	ml
Deionized water	990	ml

2.2 0.1% acetic acid in 50% ethanol

Gracial acetic acid	50	ml
100% ethanol	250	ml
Deionized water	200	ml

2.3 0.5% w/v crystal violet solution

Crystal violet powder	0.2	g
100% Ethanol	80	ml
Deionized water	320	ml

2.4 0.2% Trypan blue

Trypan blue powder	0.2	g
PBS(1X)	100	ml

APPENDIX C

List of instrument used in this study

Instrument	Company
Analytical balance	Sartorius, Germany
Autoclave S4-240	Tomy Seiko Co. Ltd., Tokyo, Japan
CO_2 incubator (BL-1600)	ASTEC Co.Ltd., Fukuoka, Japan
Light microscope	Nikon DIAPHOT 300, Tokyo, Japan
Microplate reader spectrofluorometer	Corona Electric, Japant (MTP-32 microplate reader)
Minigel electrophoresis system-Mupid-2	CosmoBio, Tokyo, Japan
pH meter	Horiba, Japan
Phase contrast microscope	Olympus, Tokyo, Japan
Refrigerated centrifuge	Tomy Seiko Co., Ltd., Japan
-80°C refrigerator	Sanyo, Thailand
-20°C refrigerator	Foma Scientific
Shaker water bath	Ikemoto, Japan
Sonicator-250 sonifer	Branson Sonic Power, USA
Speed vacuum concentrator	Tomy Seiko, Tokyo, Japan
Spectrophotometer- Genesys 20	Tecan Trading AG, Switzerland
Vacuum rotary evaporator	Sigma-Aldrich Co. LLC.
Water bath incubator	Yamato, Japan

APPENDIX D

AMES TEST

Ames test is an *in vitro* test by mixing a specific bacterium *Salmonella typhimurium* (TA98) with the tested substance and enzyme (S9 mix). This bacterium does not form visible colony when cultured in the medium without histidine since it requires histidine for growth (His-). It could not produce the essential enzyme for histidine synthesis. When the bacterium is grown in the medium containing a mutagen, this mutagen is capable of altering the base sequence in the DNA to become abnormal and the bacterium is able to synthesize histidine independent of the medium (His+). The bacterium could then form the colony or mutant colony. With this criterion, when testing any mutagen is required, the bacterium will be cultured in the medium containing such substance. If the bacterium is able to grow until the colony is formed, it is an indication that the substance is mutagenic.

1. The S9 mix preparation

S9 mix was freshly prepared each day and usually kept in ice. The components of 1 ml the standard S9 mix were

0.2 M Sodium phosphate buffer. pH 7.4	0.5	ml.
0.4 M Mgcl-1.65 M KCl	0.02	ml.
1 M Glucose-6-phosphate	0.005	ml.
0.1 M NADPH	0.04	ml.
0.1 M NADH	0.295	ml.
Sterile distilled H_2O	2.295	ml.
Rat liver S9 fraction	0.1	ml.

The ingredients should be added in the order and should be chilled. Any left S9 fraction or S9 mix was discarded.

2. Preparation of some reagents

Preparation of minimal glucose agar plate

The components of 1,000 ml minimal glucose agar medium were

Bacto-Difco agar	15	g.
Distilled water	850	ml.
10x Vogel-Bonner medium E	100	ml.
40% Glucose	50	ml.

The ingredients should be autoclaved separately. When the solution has cooled slightly, added together, Mixed well and poured 30 ml. into each plate.

The components of 1,000 ml the Vogel-Bonner medium E (ten-fold solution) are

$MgSO_4.7H_2O$	2	g.
Citric acid. H_2O	20	g.
K_2HPO_4	100	g.
$NaNH_4\ HPO_4.\ 4H_2O$	35	g.

Preparation of top agar containing histidine and biotin

A: The components of 10 ml top agar

Bacto-Difco agar	0.6	g.
NaCl	0.5	g.
Distilled H_2O	100	ml.

The solution was sterile by autoclave at 1 Ib, 121°C. 20 min.

B: The components of 100 ml. 0.5 mM histidine/biotin

Ingredient	per liter	
D-Biotin	124	mg.
L-Histidine HCl H_2O	105	mg.
Distilled H_2O	100	ml.

Dissolve histidine and biotin by stirring in water. Sterilize by filteration through membrane filter (0.22 µm pore size)

Depending on numbers of plate for each test, calculate a total volume of top agar required (according to the amount of top agar used = 2 ml per plate) . The Histidine/biotin solution will be added to the Top agar before starting the mutagenic experiment by the ratio of 10 ml of solution B : 100 ml solution A.

APPENDIX E

COLON CANCER CELL LINES

Table 8 Characteristics of colon cancer cell lines (derived from www.atcc.org)

Cell line	Growth properties	Tumor stage	Oncogene	Disease
HCT-15	Adherent	Dukes' type C	p53 mutation (Ser-241 to Phemutation)	Colorectal adenocarcinoma
SW 48	Adherent	Dukes' type C, grade IV	Wild-type p53	Colorectal adenocarcinoma
SW 480	Adherent	Dukes' type B	p53 mutation (Arg-273 to His and Pro-309 to Ser)	Colorectal adenocarcinoma

HCT-15

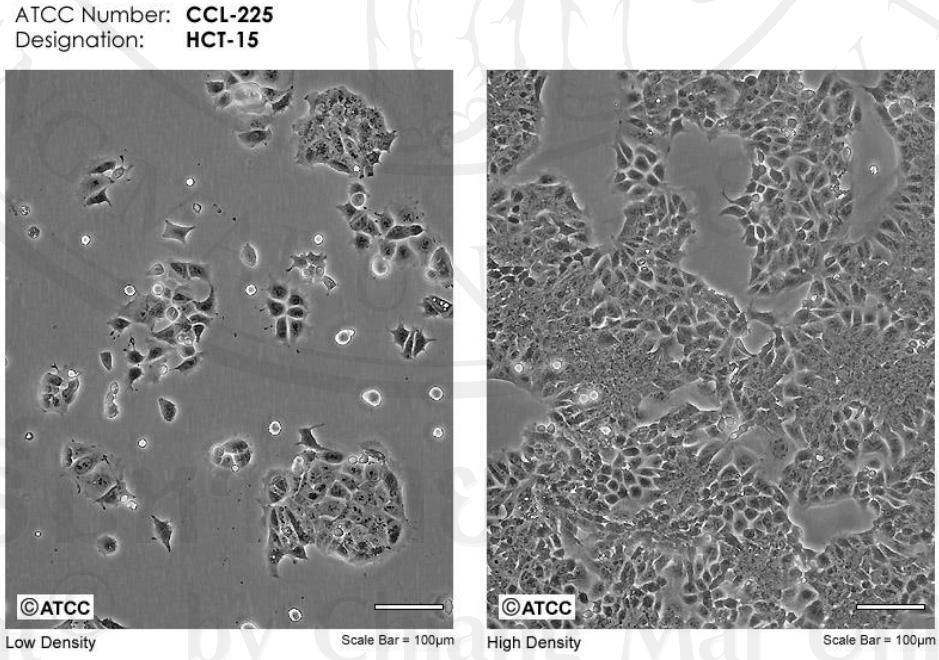
Subculturing

Protocol:

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:2 to 1:10 is recommended

Medium Renewal: 2 to 3 times per week



http://www.atcc.org/products/all/CCL-225.aspx

SW48

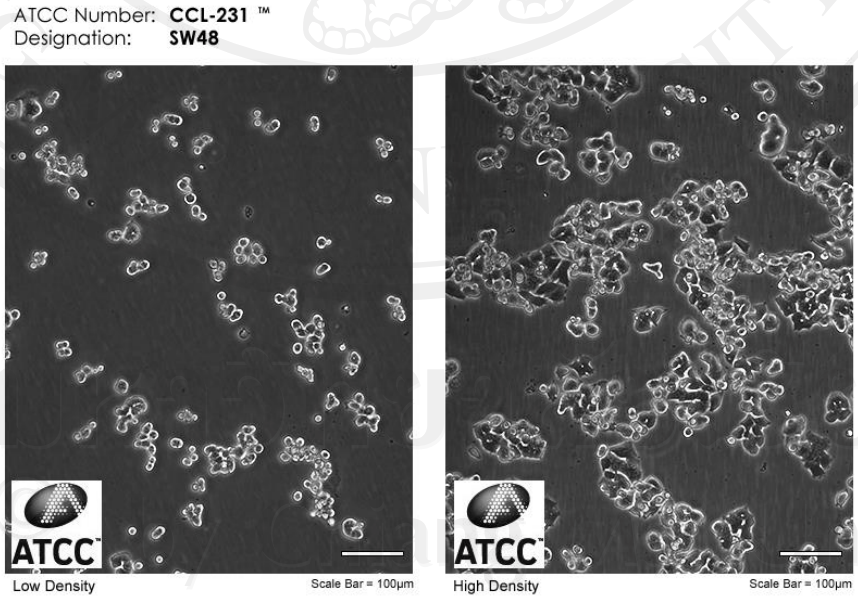
Subculturing

Protocol:

1. Culture never becomes 100% confluent. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. To remove trypsin-EDTA solution, transfer cell suspension to a centrifuge tube and spin at approximately 125 x g for 5 to10 minutes. Discard supernatant and resuspend cells in fresh growth medium. Add appropriate aliquots of cell suspension to new culture vessels. .
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:2 to 1:6 is recommended

Medium Renewal: 1 to 2 times per week



http://www.atcc.org/Products/All/CCL-231.aspx

SW480

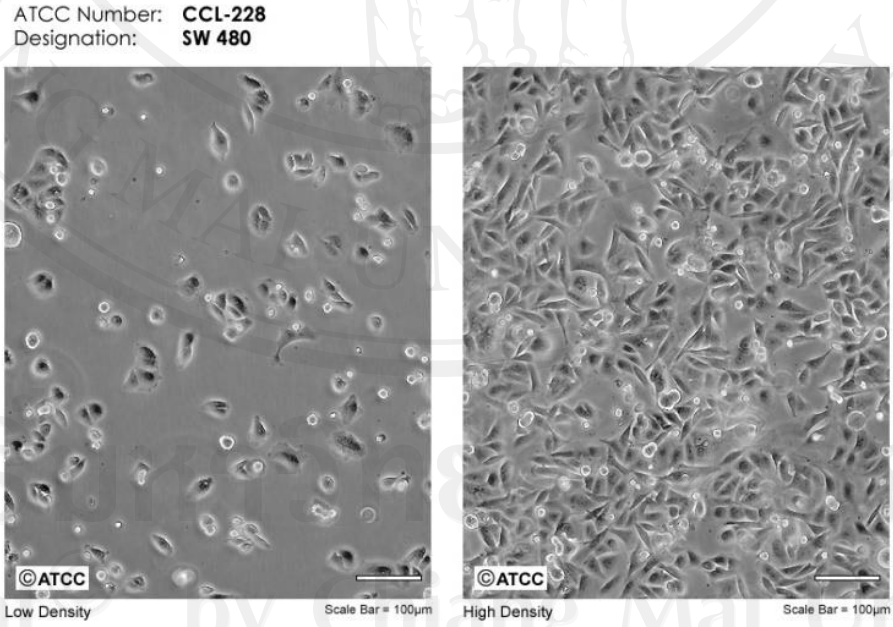
Subculturing

Protocol:

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum which contains trypsin inhibitor.
3. Add 2.0 to 3.0 mL of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 mL of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C. without CO_2.

Subcultivation Ratio: A subcultivation ratio of 1:2 to 1:8 is recommended

Medium Renewal: 1 to 2 times per week



http://www.atcc.org/Products/All/CCL-228.aspx

APPENDX F

ABTS RADICAL SCAVENGING ACTIVITY (ABTS ASSY)

1. Stock solution

7 mM ABTS

ABTS [2.2´-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] 0.0384 g

Dissolved in 10 ml of 95% ethanol

140 mM potassium persulfate ($K_2S_2O_8$)

Potassium persulfate 0.3784 g

Dissolved in 10 ml of deionized water

Working solution(ABTS radical cation solution)

Mixed 5 ml of 7mM ABTS and 140 mM $K_2S_2O_8$ 88 µl

Stored in dark at room temperature

2. Trolox standard curve and inhibition (%)

2.1 The standard trolox was prepared at concentration 0.0005, 0.0010, 0.0015, 0.0020, 0.0025, 0.0030, 0.0035, 0.0040, 0.0045 and 0.0050 mg/mL

2.2 The stock ABTS radical cation were diluted in deionized water to reach an absorbance of 0.70 (±0.02) at 734 nm. 10 µl of each trolox solution at various concentrations was mixed in 1.0 ml of working ABTS radical cation. Then, the mixed solution was measured by spectrophotometer at 734 nm.

2.3 Inhibition (%) was calculated by the equation:

$$\%\text{inhibition} = \frac{\text{Initial Absorbance-Final Absorbance X 100}}{\text{Initial Absorbance}}$$

$$\text{The trolox equivalent antioxidant (TEAC)} = \frac{IC_{50}\ \text{Trolox}}{IC_{50}\ \text{Sample}}$$

Table 9 Trolox concentration (%) of ABTS assay

Trolox (mg/ml)	Average %inhibition	SD %inhibition
0.0005	9.32	2.41
0.0010	18.37	2.83
0.0015	25.72	1.38
0.0020	31.79	2.52
0.0025	41.40	1.40
0.0030	47.86	2.57
0.0035	55.10	3.20
0.0040	61.87	4.48
0.0045	72.02	1.62
0.0050	81.31	1.93

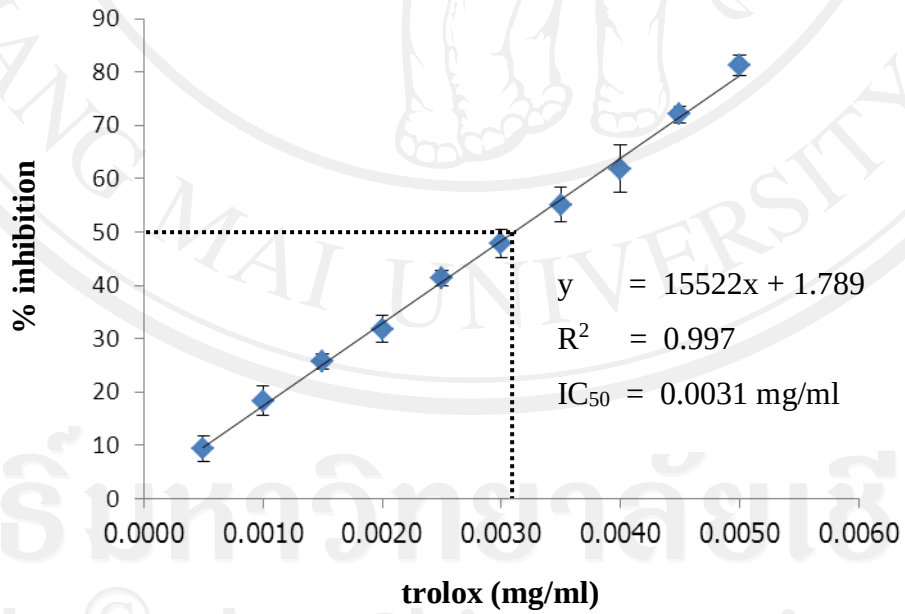


Figure 13 A standard curves between trolox and inhibition (%) of ABTS assay

APPENDX G

DPPH RADICAL SCAVENGING ACTIVITY (DPPH ASSY)

1. Stock solution

1.1 130 µM 2,2-diphenyl-1-picrylhydrazyl (DPPH) methanol solution (MW. 394.323) DPPH (Singma) 0.0051 g. was be dissolved in 100 mL methanol.

1.2 1 M Tris-HCl (pH 7.9) (MW. 12.12)

Tris-base 12.112 g. was dissolved in 80 mL deionized water (DI). After that, pH was adjusted to 7.9 by 1 M HCl, then DI was added to final volume 100 mL.

2. Gallic acid standard curve and inhibition (%)

2.1 The standard gallic acid was prepared at concentration 0.001, 0.002, 0.003, 0.004 and 0.005 mg/ml

2.2 0.8 ml of gallic acid was mixed with 0.067 ml of 1 M Tris-HCl buffer (pH=7.9) and 0.8 mL of 130 µM DPPH in methanol.

2.3 After leave in shading for 20 min the absorption spectrum at 517 nm was determined.

2.4 Inhibition (%) was calculated by the equation:

$$\%\text{inhibition} = \frac{\text{Initial Absorbance-Final Absorbance X 100}}{\text{Initial Absorbance}}$$

Gallic acid concentration was expressed as gallic acid equivalent (GAE, µg/mg extract)

$$\text{The gallic acid equivalent antioxidant (GAE)} = \frac{IC_{50}\ \text{Trolox}}{IC_{50}\ \text{Sample}}$$

2.5 A standard curve between % inhibition and gallic acid concentration (Fig. 14) was plotted from data of % inhibition of each gallic acid concentration show in Table 9

Table 1O Gallic acid concentration (%) of DPPH assay

Gallic acid (mg/ml)	A_{517}			Inhibition (%)			average %inhibition	SD %inhibition
	1	2	3	1	2	3		
0.001	0.583	0.592	0.589	18.23	16.91	17.12	17.42	0.64
0.002	0.485	0.462	0.471	31.94	35.20	33.70	33.61	1.63
0.003	0.345	0.373	0.361	51.61	47.64	49.22	49.48	1.97
0.004	0.235	0.267	0.261	67.04	62.55	63.27	64.28	2.39
0.005	0.181	0.195	0.195	74.61	72.65	72.57	73.27	1.14

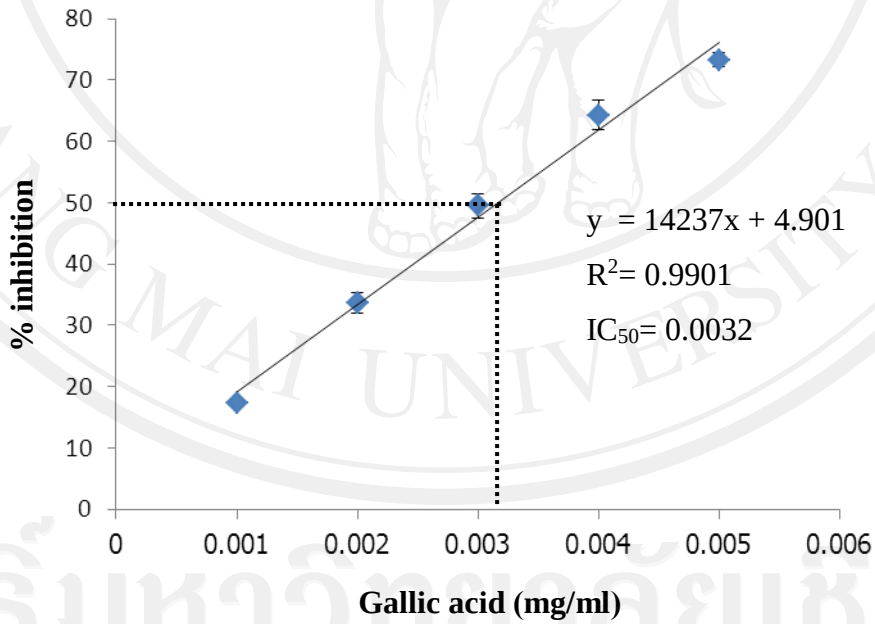


Figure 14 A standard curves between gallic acid and inhibition (%) of DPPH assay

APPENDX H

ASCORBIC ACID ASSAY

1. Stock solution

1.1 Dissolve 100 mg

1.2 ascorbic acid in 100 ml of 4% oxalic acid solution in a standard flask (1 mg/ml)

2. Working standard

Dilute 10 ml of the stock solution to 100 ml with 4% oxalic acid. The concentration of working standard is 100 µg/ml.

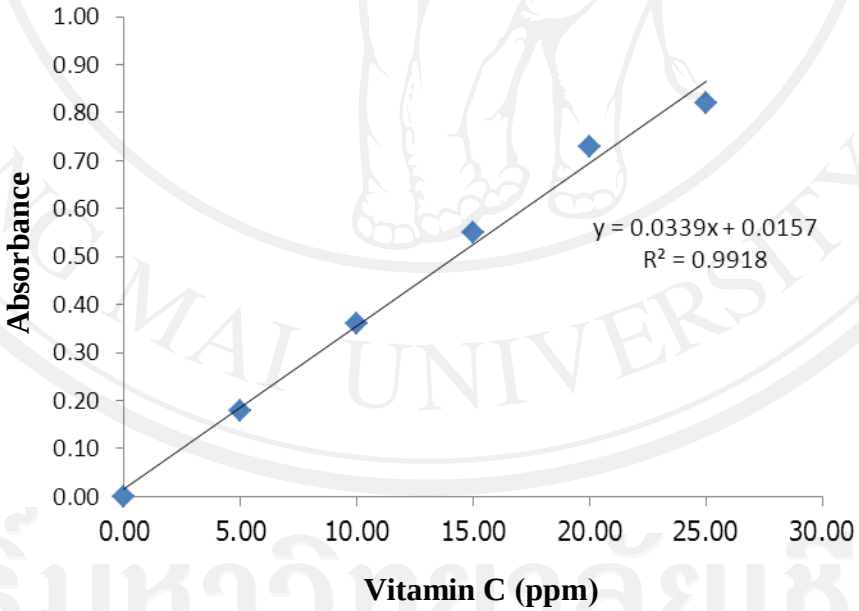


Figure 15 A standard curve vitamin C concentration

APPENDX I

TOTAL PHENOLIC ASSAY

1. Stock solution

1.1 50% Folin-Ciocalteu reagent (Labscan)

Folin-Ciocalteu 50 mL was diluted with 50 mL deionized water

1.1 5% Na_2CO_3

Na_2CO_3 5 g was dissolved in 100 mL deionized water.

2. Gallic acid standard curve and phenolic compound content

2.1 The standard gallic acid was prepared at concentration 0.01, 0.04, 0.08, 0.12, 0.16, 0.20 and 0.24 mg/mL

2.2 0.25 mL of gallic acid was mixed with 0.025 mL of 95% ethanol, 1.25 mL of deionized water and 0.125 mL of 50% Folin-Ciocalteu reagent

2.3 After leave at ambient temperature for 5 min. 25 mL of 5% Na_2CO_3 was added and then further leave in shading for 2 hr.

2.4 The absorption spectrum at 750 nm was determined.

2.5 A standard curve between A_{750} nm and gallic acid concentration (Fig. 16) was plotted from data of A_{750} nm of each gallic acid concentration shown in Table 10.

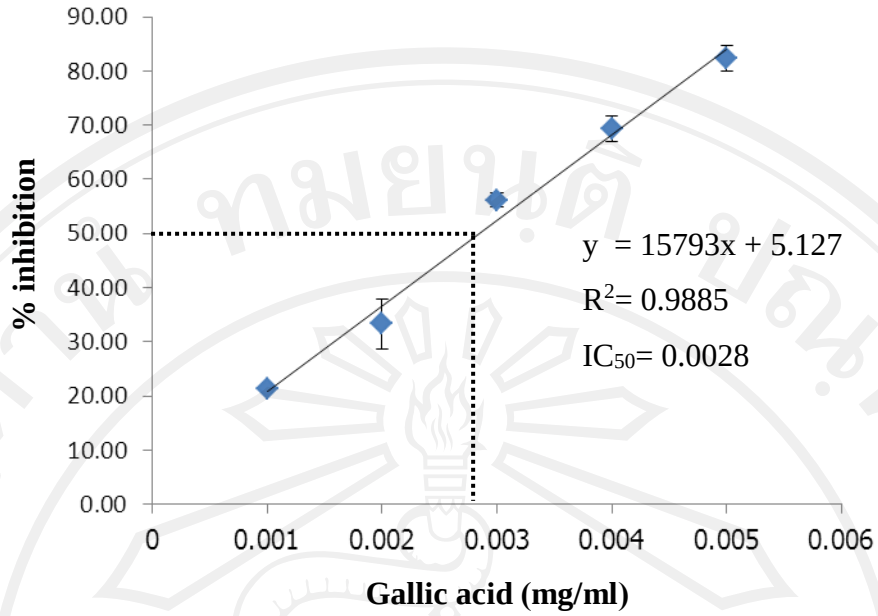


Figure 16 A standard curve between gallic acid and inhibition (%) of DPPH assay

APPENDX J

Calculation of the doses of the extracts used in the experiments

***Moringa oleifera* Lam.**

One bowl of fresh leaves of *M. oleifera* is 10 grams

Normally, one bowl is consumed at each meal (equivalent to 3 bowls of fresh leaves) altogether 30 gram

If 3 meals a day, 90 grams of *M. oleifera* are eaten

8 grams of the extract is obtained from 100 grams of fresh leaves

Consuming 90 grams of leaves will get about 7.2 grams of the substance

A person of 60 kg will get 7.2 grams of the substance

Thus a person of 1 kg receives 0.12 gram of the substance or 120 mg/kg BW

Therefore 60, 120, 180 and 240 mg/kg BW concentrations were used

***Pseuderanthemum platiferum* (Nees)Radlk.)**

One fresh leaf is 1 gram

Normally 5 leaves are consumed each time, so 5 grams are taken

One hundred grams of fresh leaves give 6 grams of the extract

If 10 leaves/day (morning and evening8) are taken, about 0.6 gram of the extract is consumed

A man of 60 kg BW will receive 0.6 gram of the extract

So, a man of 1 kg BW will receive 0.01 gram of the extract or 10 mg/kg BW

Therefore 5, 10, 15 and 20 mg/kg BW concentrations were used.

Curriculum Vitae

Name Miss. Supaporn Pamok

Date of Birth July 14, 1983

Education Background

2005	Bachelor of Science (Animal Production Technology), Mahasarakham University
2007	Master of Science (Animal Production Technology), Mahasarakham University
2014	Doctor of Philosophy (Biodiversity and Ethnobiology), Chiang Mai University

Scholarships The Office of the Higher Education Commission

Publications

1. Pamok, S., Saenphet, K. and Vinitketkumnuen, U. 2009. Antiproliferative effects on colon cancer cell lines by aqueous extracts from the leaves of *Moringa oleifera* Lam.4th Global summit on medicinal and aromatic plants, December 1-5, Kuching, Sarawak, Malaysia, 281 pp. (Poster)

2. Pamok, S., Saenphet, K. and Vinitketkumnuen, U. 2010. Antiproliferative effects on colon cancer cell lines by extracts from the leaves of *Moringa oleifera* Lam.and *Pseuderanthemum palatiferum* (Nees) Radlk. Program&Abstracts. Commission on higher education congress III university staff development consortium. September 9-11, Royal cliff grand hotel and spa. Chon Buri, Thailand. 280 pp. (Poster)

3. Pamok, S., Saenphet, K., and Saenphet, S. 2011. Total phenols and ascorbic acid in *Moringa oleifera* Lam. and *Pseuderanthemum palatiferum* (Nees) Radlk. and their antioxidant activities. The international symposium on medicinal and aromatic plants. December 15-18, The empress hotel, Chiang Mai, Thailand. 118 pp. (Poster)

4. Pamok, S., Vinitketkumnuen, U., Saenphet, S. and Saenphet, K. 2011. Antiproliferative effect of *Moringa oleifera* Lam. and *Pseuderanthemum palatiferum* (Nees) Radlk extracts on the colon cancer cells. *Journal of Medicinal Plants Research.* 6(1): 139-145.