CHAPTER II

LITERATURE REVIEW

M. citrifolia

M. citrifolia Linn. belongs to family Rubiaceae (subfamily Rubioideae). This small tree is distributed widely throughout the warm region of Pacific and tropical countries including Thailand. It has many different names in different countries such as "Yor-bann" or "Mataasuea" in Thailand, "Indian Mulberry" in India, "Ba-ji-tian" in China, "Nono" in Tahiti and "Noni" in Hawaii etc. (Wang and Su, 2001). This plant grows as an evergreen shrub or small crooked tree that grows 3 to 8 meters in height. It has large oblong and shiny green leaves. It's white flowers are tubular, with cone-like heads. The fruit is yellow-white in color, oval in shape, about the size of a potato and has a "bumpy" surface. The ripen fruit has a characteristic of cheese-like, offensive odor. Each fruit contain 4 seeds, 3 mm in length (Figure 2.1). *M. citrifolia* is one of the sixty-six medicinal plants selected for the primary health care in Thailand. This plant is regarded as safe to be used to relieve the symptoms of nausea and vomiting that are not severe.

Various parts of this plant have been used in traditional medicine. They were used either alone or in combination with other medicinal plants (Table 2.1, 2.2 and 2.3).

Several previous studies demonstrated that the fruit of this plant possessed various compounds (Table 2.4).

Figure 2.1 *M. citrifolia.* A; twig with leaves, cluster of flowers and fruit. B; flower,lateral view C; longitudinal section of flower D; stamen. and E; fruit (Jayaweera, 1982)

Table 2.1 Uses of *M.citrifolia* in traditional Hawaiian medicine. The plant portions were used either alone or in combination with other plant material (Hirazumi, 1997)

Indication	Plant portion	Reference
Asthma	fruit	3
	bark	3
Backache	fruit	11
Blood purifier	fruit	3
	root	3
Boils/poultice	fruit	1, 2, 4, 6, 9, 10, 11, 12, 14, 15, 16
	leaf	1, 5, 6, 16
Broken bones	fruit	1, 6, 8, 9, 16
	leaf	10, 11, 16
Bruises/sprains	fruit	3, 8 11
	leaf	1, 3, 5, 6
Concussions	fruit	1, 6
Constipation/enema/purgative/	fruit	3, 5, 6
suppository	bark	5
	flower	5, 7
Cuts/deep cuts	fruit	5, 8, 9, 10, 13, 16
	leaf	3
	bark	1, 6
	seed	6
Fever	leaf	6
Gout	fruit	5
Gynecological problems	fruit	3, 5, 6
	flower	3

Table 2.1 *(continued)* Uses of *M.citrifolia* in traditional Hawaiian medicine. The plant portions were used either alone or in combination with other plant material (Hirazumi, 1997)

Indication	Plant portion	Reference
Heart problems/hypertension	fruit	5, 6, 8, 11
Kidney problems/diabetes	fruit flower	5, 6, 10, 11, 16 5, 10
Lice infection/insecticide	fruit	4, 5, 8, 15
Rheumatic joints	leaf	5
Skin eruptions	root	1, 6
Stomach pain/internal hemorrhage	fruit	3
Thrush	fruit flower	3 3
Tonic	fruit leaf bark	4, 16 4, 12 4, 12
Tuberculosis	fruit bark	3, 4, 5, 15 3
Weakness/loss of appetite, thirst or perspiration	fruit root	3, 6, 16 3
Worms	fruit	3, 5

1 = Abbott, 1992; 2 = Brigham, 1911; 3 = Chun, 1994; 4 = Degener, 1973;

5 = Gutmanis, 1994; 6 = Handy *et al.*, 1934; 7 = Kailkainahaole, 1968;

8 = Kondo Corum, 1985; 9 = Krauss, 1981; 10 = Krauss, 1993;

11 = McBride, 1975; 12 = Mitchell, 1992; 13 = Nagata, 1971;

14 = Rock, 1913; 15 = Wagner *et al.*, 1990; 16 = Whistler, 1992

Table 2.2 Medicinal uses of *M.citrifolia* in several Pacific Islands. The plant portions were used either alone or in combination with other plant material (Hirazumi, 1997)

Location	Plant portion	Use	Reference
Austral Islands	leaf root	medicine medicine	Brown,1935
Cook Islands	fruit root	urinary tract ailments, abdominal swelling, diaphramic hernia stonefish stings	Whistler,1992
Fiji	fruit leaf bark root flower stem	ringworms, bad breath and a raspy voice, mouth ulcers, hemorrhoids diarrhea, problems with menstruation, fever fever, ringworms, scabies, itch, acute rheumatic pain, rheumatism, stiffness, inflammation, boils, gastric ulcers, ulcers, infected ears with pus, hemorrhoids, pregnancy pain, pain caused by barbs of poisonous fish, removal of splinters sinusitis, wounds with pus malnutrition ulcers swollen testicles, hernia	Cambie and Ash,1994 Rock,1913 Cambie and Ash,1994
Fiji (Indians)	leaf bark root	broken bones and sprains urinary disorders urinary disorders	Singh,1986

Table 2.2 *(continued)* Medicinal uses of *M.citrifolia* in several Pacific Islands. The plant portions were used either alone or in combination with other plant material (Hirazumi, 1997)

Location	Plant portion	Use	Reference
Futuna	fruit	infections of the mouth and gums, sore throat., toothaches	Whistler,1992
		ulcerated mouth	Biggs,1973
Gilbert Islands	fruit	medicine	Luomala,1953
		stomachache, diarrhea, bloody stool	Cambie and Ash,1994
	leaf	deodorant	
	root	scabies, skin eruptions, boils, ulcers coral cuts	
Makatea	fruit	medicine	Wilder,1934
	flower	medicine	
Marqueses	fruit	tonic	Whistler,1992
	leaf	inflammation of the breasts	Handy,1923
		inflammation	Brown,1935
	root	medicine	
Micronesia	fruit	ulcerated sores on the feet diabetes, tuberculosis	Weiner,1970
	leaf	injured eyes, eye infections	
	root	boils, stonefish and sting ray wounds, small-pox ointment	
New Caledonia	leaf	fever	Holdsworth, 1974
	unspecified	abscesses	
Niue	leaf	styes	Whistler,1992
		medicine	Yuncker,1943
	bark	medicine	
	flower	medicine	
Ponape	fruit	"heart attack" pain	Glassman,1952
	leaf	rheumatism	

Table 2.2 *(continued)* Medicinal uses of *M.citrifolia* in several Pacific Islands. The plant portions were used either alone or in combination with other plant material (Hirazumi, 1997)

Location	Plant portion	Use	Reference
	bark	hemostatic in menstruation	
	root	hemostatic in menstruation	
	flower	pain after childbirth	
	stipule	scorpion fish wounds	
	terminal bud	abscesses	
Rarotonga	leaf	medicine	Brown,1935
Rurutu	fruit	medicine	Brown,1935
Samoa	fruit	infections of the mouth and gums, sore throat, toothaches	Whistler,1992
		diarrhea, intestinal worms, worms, cough, tuberculosis, eye complaints, fever with vomiting, thrush abscesses, "spreading dark spots on the skin"	Dittmar,1993
	leaf	infant cold/fever, fever, inflammations, inflamed gums, inflammation of the breasts, filarial inflammation, boils, sore throat, pharyngitis thrush, abscesses, styes, centipede bites, elephantiasis, swellings, septicemia, wounds, severe constipation, rheumatism	
		ague, tetanus, diuretic inflammation of the limbs	Uhe,1974
		swelling of the joints	Cambie and Ash,1994
	Bark	mouth infections	Whistler,1992
		infant diarrhea, stomach complaints, worms, sore throat, cough, abscesses, "spreading dark spots on the skin"	Dittmar,1993

Table 2.2 *(continued)* Medicinal uses of *M.citrifolia* in several Pacific Islands. The plant portions were used either alone or in combination with other plant material (Hirazumi, 1997)

Location	Plant portion	Use	Reference
	root	sore throat, cough jaundice, toothache	Uhe,1974
	flower	cough, sore throat with cough, conjunctivitis, irritated red eyes, sore eyes, styes, abscesses, "spreading dark spots on the skin"	Dittmar,1993
	unspecified	infections of the mouth and gums, sore throat, toothaches	Whistler,1992
Tahiti	fruit	boils, diabetes, variety of ailments	Whistler,1992
	leaf	boils, diabetes, variety of ailments	
		inflammation	Brown,1935
	unspecified	diabetes, fish poisoning, reef fish stings, tonsillitis, abdominal swelling, burns, ranula, whitlows	Whistler,1992
		abscesses	Holdsworth,1974
Tunga	fruit	infections of the mouth and gums, sore throat, toothaches	Whistler,1992
		throat infections	Biggs,1973
		sore gums	Cambie and Ash,1994
	leaf	aching joints, aching muscles, tonic	
		boils	Yuncker,1959
		stomachaches, styes	Whistler,1992
		breast carcinoma, induration of the breast with pain and redness or with redness only, dysuria, postpartum discharge, secondary amenorrhea, severe bleeding in early pregnancy, vaginal bleeding	Singh *et al.*,1984

Table 2.2 *(continued)* Medicinal uses of *M.citrifolia* in several Pacific Islands. The plant portions were used either alone or in combination with other plant material (Hirazumi, 1997)

Location	Plant portion	Use	Reference
	bark	stomachaches	Whistler,1992
		for afterbirth, infertility, menorrhagia, postpartum, hemorrhage, secondary amenorrhea, vaginal bleeding	Singh *et al.*,1984
	petiole	styes	Whistler, 1992
	unspecified	infections of the mouth and gums, sore throat, toothaches	
		infant diarrhea	Cambie and Ash, 1994

Table 2.3 Medicinal uses of *M. citrifolia* in other regions of the world. The plant portions were used either alone or in combination with other plant material (Hirazumi, 1997)

Location	Plant portion	Use	Reference
Africa			
- Africa	leaf	purgative	Altachul,1973
- Congo	bark	febrifuge	Quisumbing, 1978
America			
- Dominica	fruit	sores, inflammation	Ayensu, 1981
Martinique, St. John, Virgin Islands Tobago Trinidad	leaf	rheumatic joints, headache, pain	
- British Guiana	leaf	pain	Little and Wadsworth, 1964
- Caribbean	leaf	wounds, rheumatic joints, headache, pain	Morton, 1992
- El Salvador	root	GI and liver ailments	Morton, 1981
- Puerto Rico, Virgin Island	leaf	wounds headache, head colds, neuralgia	Little and Wadsworth, 1964
- Virgin Islands	unspecified	heart trouble	Morton 1981

Table 2.3 *(continued)* Medicinal uses of *M. citrifolia* in other regions of the world. The plant portions were used either alone or in combination with other plant materials (Hirazumi, 1997)

Location	Plant portion	Use	Reference
Asia			
- China	root	febrifuge, tonic roborant	Perry, 1980
- India	fruit	spongy gums, throat complaints, dysentery, leucorrhea, sapremia	Anonymous, 1962
		emmenagogue, deobstruent, sore throat	Quisumbing, 1978
	leaf	infant diarrhea, dysentery, tonic, fever, wounds, ulcers, gout	
	root	cathartic, febrifuge, gout	Anonymous, 1962
- Indonesia	fruit	laxative	Quisumbing, 1978
		cough, bilious fevers, emetic, enlarged spleen, difficult urination, diabetes, antihelmintic, emmenagogue, wound cleanser	Perry, 1980
		liver diseases, beri beri, hemorrhage, coughs	Burkill, 1966
	bark	astringent against bowel complaints, wound cleanser	Perry, 1980
	root	wound cleanser	
- Japan	root	febrifuge, tonic roborant	
- Malay Peninsula	fruit	emmenagogue, leucorrhea, aspreamia	Burkill, 1968
	leaf	fever, small-pox ointment cough, nausea enlarged spleen, colic	Perry, 1980
	bark	ague	

Table 2.3 *(continued)* Medicinal uses of *M. citrifolia* in other regions of the world. The plant portions were used either alone or in combination with other plant materials (Hirazumi, 1997)

Location	Plant portion	Use	Reference
- New guinea	leaf	stomachache, sores, boils, severe fever, pneumonia	Holdsworth,1974
		sores of leprosy, headache, dysentery	Weiner,1970
	bark	skin disorders, childbirth	
	root	fever, centipede bite antiseptic	Perry,1980
	unspecified	sores on the feet	Weiner,1970
- Philippines	fruit	emmenagogue	Quisumbing,1978
	leaf	ulcers, arthritis	
	seed	purgative, vermifuge	Morton,1992
- Solomon Islands	leaf	dysentery	
	bark	childbirth	
- Southeast Asia	fruit	sore throat	Morton, 1992
- Taiwan	leaf	ulcers, knife wounds	Perry, 1980
	root	dysentery	
- Vietnam	fruit	deobstruent, emmenagogue	Lassak,1983
		stomachache, aperient, dysentery, uterine hemorrhage, metrorrhea, cough, coryza, edema, neuralgia	WHO, 1990
	leaf	fever, dysentery, diarrhea, furunculosis	

Table 2.3 *(continued)* Medicinal uses of *M. citrifolia* in other regions of the world. The plant portions were used either alone or in combination with other plant materials (Hirazumi, 1997)

Location	Plant portion	Use	Reference
- Vietnam, Laos, Cambodia	root	hypertension, osteodynia, lumbago	Perry, 1980
	fruit	lumbago, asthma, dysentery, emollient, deobstruent, emmenagogue	
	leaf	emollient, deobstruent, emmenagogue, febrifuge, tonic, dysentery	
	root	stiffness, tetanus, vermifuge, arterial tension	
- Australia	fruit	asthma, coughs, cold, sore throat, respiratory infections	Barr *et al.*, 1990
	root	antiseptic	Lassak,1983

Table 2.4 Chemical constituents found in *M. citrifolia* fruit (Modified from Jeffrey, 1995 and Hirazumi, 1997)

Compounds	Documented effected of compounds
Carbohydrates and lipids : Monosaccharides - glucose	: It is a main source of energy for living organisms. It is used in hypoglycemia, in ketosis to counteract hepatotoxins, to reduce cerebrospinal pressure and cerebral oedema, and as a sclerosing agent in the treatment of varicose veins.
: Polysaccharides - glucuronic acid - galactose - arabinose - rhamose - glycosides - trisaccharide fatty acid ester	: Immunostimulatory, immunomodulatory, antibacterial, antitumour and anticancer.
: Organic acids - acetic acid	: Bacteriocidal activity begins above 5% concentration.
- ascorbic acid	: Essential dietary requirement in humans, and used to treat scurvy. It is employed as an antimicrobial and antioxidant in foodstuffs.
- butyric aicid - isovaleric acid	- -
- valeric acid	: Low toxicity (LD_{50} intravenously in mice 1290 $mgkg^{-1}$).

Table 2.4 *(continued)* Chemical constituents found in *M. citrifolia* fruit (Modified from Jeffrey, 1995 and Hirazumi, 1997)

Compounds	Documented effected of compounds
: Fatty acids and lipids - linoleic acid	: It is a nutrient and an essential fatty acid component of vitamin E. Also, It is regarded as a beneficial dietary component for men who may be prone to coronary heart disease.
- octanoic acid	: Antifungal activity against dermatophytes and *Candida* spp.
- decanoic acid - hexanoic acid - lauric acid - otanoic acid - oleic acid - palmitic acid	- - - - - -
Phenolics : Coumarins - scopoletin	: Hypotensive activity in animals. It also exhibits spasmolytic, antibacterial and antifungal activities. In plants, it acts as a bud growth inhibitor of *Pisum sativum* and stimulator of germination in *Striga asiatica*.
: Phenols and phenolic acids - benzoic acid	: Antifungal and choleretic activities.

Table 2.4 *(continued)* Chemical constituents found in *M. citrifolia* fruit (Modified from Jeffrey, 1995 and Hirazumi, 1997)

Compounds	Documented effected of compounds
: Phenylpropanoids - eugenol	: Anticonvulsant, antimitotic, antioxidant, hypothermic and spasmolytic activies. Also, it shows antiyeast and central nervous system depressant activities. It inhibits prostaglandin synthesis by human colonic mucosa, the metabolism of arachidonic acid by human polymorphonuclear leukocytes, smooth muscle activity *in vitro* (humans and animals), and carrageenan-induced foot inflammation in rats. Also, it inhibits induced platelet aggregation *in vitro.* It is used as an antiseptic and anesthetic in dentistry.
Terpenoids : Monoterpenoids - limonene	: Expectorant and sedative activities
: Iridoids - asperuloside	: Laxative activity. It shows seed germination and plant growth inhibiting activities.
: Carotenoids - carotene	: Vitamin A precursor

Table 2.4 *(continued)* Chemical constituents found in *M. citrifolia* fruit (Modified from Jeffrey, 1995 and Hirazumi, 1997)

Compounds	Documented effected of compounds
Minerals - calcium - potassium - sodium	: Essential dietary requirement in humans

In 2001, two novel glycosides, 6-*O*-(β-D-glucopyranosyl)-1-*O*-octanoyl-β-D-glucopyranose and asperulosidic acid (Figure 2.2), extracted from *M. citrifolia* fruit juice were investigated for their effects on 12-*O*-tedtradecanoylphorbol-13-acetate (TPA)- and epidermal growth factor (EGF)-induced AP-1 transactivation and cell transformation in mouse epidermal JB6 cells. The results showed that both compounds were effective on suppressing TPA- or EGF-induced cell transformation and the effects were associated to AP-1 activity (Lie *et al.*, 2001). Increase AP-1 activity is associated with malignant transformation and cancer promoting properties of several agents, such as UV radiation (Adler *et al.*, 1996), growth factors (Lamb *et al.*, 1997), phorbol esters (Dong *et al.*, 1994 and Huang *et al.*, 1997) and transforming oncogenes (Lamb *et al.*, 1997). On the other hand, inhibition of AP-1 activity has been shown to suppress cell transformation (Dong *et al.*, 1997). Some chemopreventive agents, including aspirin, sodium salicylate, tea polyphenols, perillyl alcohol and retinoic acid, have been reported to inhibit cell transformation and tumor promotion and were also found to suppress AP-1 transactivation (Liu *et al.*, 2001).

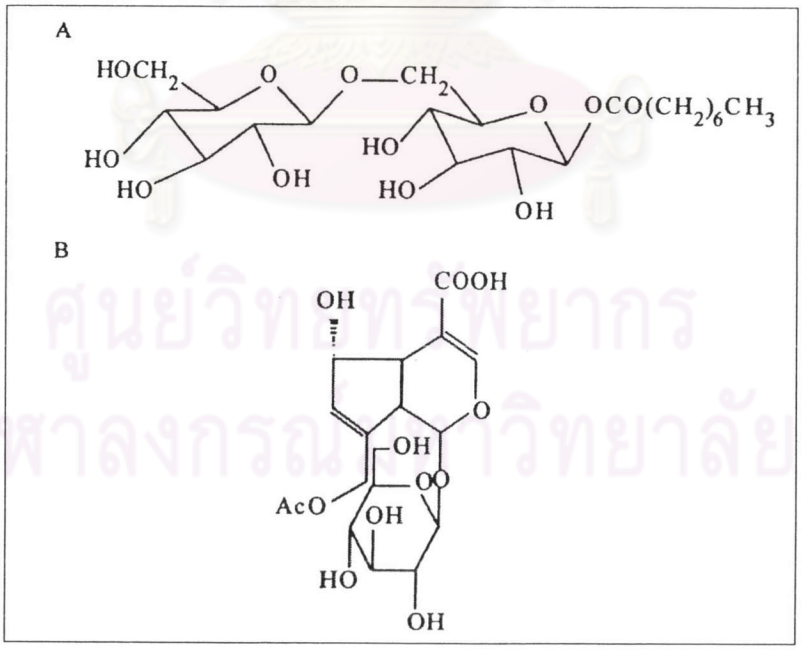

Figure 2.2 Structures of compounds identified in the fruits of *M. citrifolia*
(A) 6-*O*-(β-D-glucopyranosyl)-1-*O*-octanoyl-β-D-glucopyranose (B) asperulosidic acid (Wang *et al.*, 1999)

Physiological and pharmacological effects

1. Antiemetic and antidopaminergic activity

In animal study

Antiemetic and antidopaminergic activities of *M. citrifolia* fruits were evaluated in mice, rats and dogs treated with apomorphine. It was found that water extract of *M. citrifolia* fruits could not antagonize potent dopaminergic effects of apomorphine on the induction of emesis in dogs or on the delay of gastric emptying in mice. However, the extract at the dose of 40 g/kg BW could significantly reduce the duration of apomorphine-induced gnawing behavior in rats and at the dose of 10 and 20 g/kg BW could significantly increase gastric emptying in mice. The results suggested that *M. citrifolia* fruits might contain some water-soluble substances possessing weak antidopaminergic activity that might be responsible for its weak antiemetic action observed in humans (Chuthaputti *et al.*, 1996).

In clinical study

A clinical study was performed in malarial patients who experienced symptoms of nausea and vomiting. It was found that *M. citrifolia* juice drinking 30 ml every 2 hours could significantly reduce the number of times that patients vomited as compared to the control group which subjects received tea. The antiemetic effect of this juice was, however, much less effective than that of metoclopramide (วิชัย เอกพลากรและคณะ, 2530).

2. Anti-tumor/anticancer and immunomodulator activity

In animal study, *M. citrifolia* has an anti-tumor/anticancer and immunomodulator activities. Ethanol-precipitated fraction (EtOH-ppt) of *M. citrifolia* fruit exerted anti-tumor activity against Sarcoma 180 in mice following an intraperitoneally injection at a dosage of 500 mg/kg 24 hours after an inoculation of the tumor (Hirazumi *et al.*, 1996).

Likewise, anti-tumor activity against Lewis lung carcinoma (LLC) was also indicated when 15 mg of the EtOH-ppt was injected intraperitoneally to mice for 4-5 days. The EtOH-ppt, which contained a polysaccharide-rich substance, possessed anti-tumor activity while the ethanol soluble did not. Concomitant administration the

EtOH-ppt fraction with immunosuppressive agent, 2-chloroadenosine (Cl-Ade; a macrophage inhibitor) or cyclosporine A (Cys-A; a T-lymphocyte inhibitor) to mice resulted in diminishing the anti-tumor activity, thereby substantiating an immunomodulatory mechanism (Hirazumi *et al.*, 1994). Subsequently, Hirazumi and Furusawa (1999) found that the EtOH-ppt could induce several cytokines that were cytotoxic to tumor cells. Those cytokines included tumor necrosis factor-α (TNF-α), interleukin1β (IL-1β), IL-12p70, IL-10 and interferon-γ (IFN-γ) as well as nitric oxide (NO). Wang and Su (2001) found that *M. citrifolia* fruit juice was able to significantly reduce the 7,12-dimethylbenz(a)anthracene (DMBA)-DNA adduct formation in rats and mice *in vivo*. In an *in vitro* study, they found that *M. citrifolia* fruit juice possessed strong antioxidant activities, the characteristic that may contribute to the cancer preventive effect of this plant (Wang and Su 2001).

Effect of the M. citrifolia fruit juice against LLC peritoneal carcinomatosis

Graded doses of the crude *M. citrifolia* fruit juice were administered i.p. once daily (QD) or once every other day (QOD) for a total of 4-5 injections commencing from day 1 after i.p. LLC tumor inoculation in syngeneic inbred C57BL/6 mice. The results are shown in Table 2.5. The juice seemed to demonstrate curative effects from the doses between 3 to 20 mg/mouse. Statistically significant antitumor activity was shown at the doses between 6-15 mg/mouse *M. citrifolia* juice at between these doses range prolonged life span of mice by more than 75%. Ethanol fractionation of the juice demonstrated the antitumor activity in the ethanol-precipitated fraction (EtOH-ppt). EtOH-ppt prolonged the life span of tumor-bearing mice by more than 75%, whereas EtOH-sol was unable to elicit significant beneficial antitumor activity (Hirazumi and Furusawa 1999).

Table 2.5 Antitumor effect of *M. citrifolia* fractions on LLC peritoneal carcinomatosis (Hirazumi and Furusawa, 1999)

Agent	Dose(mg)/ mouse	MST±SEM (days)	No. mice survived/total	ILS(%)
Test 1				
Control		15.9 ± 0.8	0/55	
Crude juice	3	27.5 ± 5.0	1/10	73
	6	32.7 ± 3.2[a]	4/18[b]	106
	12	28.0 ± 3.6[b]	4/17[b]	76
	15	34.7 ± 3.3[a]	9/22[a]	119
	20	21.0 ± 4.5	2/11	32
Test 2				
Control		14.8 ± 0.9	0/58	
EtOH-ppt	0.8	32.2 ± 2.5[a]	15/39[a]	118
	1.6	29.0 ± 3.1[a]	5/22[b]	96
EtOH-sol	5.2	19.7 ± 2.2	0/12	33
	10.4	14.6 ± 1.1	0/19	0

Inbred C57BL/6 mice were inoculated i.p. with LLC (2-4 x 10^5 cells/mouse) on day 0. 0.1 ml of vehicle or *M. citrifolia* samples was administered i.p. at the indicated doses QD or QOD x 4-5 injections beginning on day 1. Survival of mice was recorded up to 50 days. Mice surviving 50 days were considered cured. [a] $p<0.001$, [b] $p< 0.01$ compared with control.

Effect of EtOH-ppt with concomitant treatment with immunosuppressive agents

Co-administration of immunosuppressive agents with the EtOH-ppt was conducted in order to determine if the antitumor acitivity involved host immune system. Cl-Ade (a macrophage inhibitior) or Cys-A (a T-lymphocyte inhibitor) and the EtOH-ppt were administered i.p. 1 day after tumor inoculation and continued every other day for a total of five injections or given as a single s.c. injection, in the case of Cys-A. The results shown in Table 2.6 indicated that Cl-Ade completely abolished the antitumor activity of EtOH-ppt, whereas Cys-A moderately attenuated the antitumor activity. The effect of a combination of Cys-A and EtOH-ppt was significantly higher than the control, but significantly less than EtOH-ppt treatment alone (Hirazumi and Furusawa 1999).

Table 2.6 Combination of immunosuppressive agents with EtOH-ppt against LLC peritoneal carcinomatosis (Hirazumi and Furusawa, 1999)

Agent	Dose/mouse Schedule(route)	MST±SEM (days)	No. mice survived/total	ILS (%)
Control		15.0±1.7	0/10	
EtOH-ppt	0.8mg,QODx 5(i.p.)	35.9±5.6[a]	5/9[b]	139
Cl-Ade	50μg,QODx5(i.p.)	12.2±0.9	0/9	-19
Cl-Ade+EtOH-ppt	same as above	12.8±0.8[c]	0/9	-15
Cys-A	2mg, day 1 (s.c.)	15.4±1.4	0/9	3
Cys-A + EtOH-ppt	same as above	19.2±1.6[b,d]	0/9	28

Inbred C57BL/6 mice were inoculated i.p. with LLC (2-4 x 10^5 cells/mouse) on day 0. 0.1 ml of vehicle or agents administered i.p. at the indicated doses beginning on day 1. Survival of mice was recorded up to 50 days. Mice surviving 50 days were considered cured. [a] $p<0.005$, [b] $p< 0.01$ compared with control. [c] $p < 0.01$, [d] $p < 0.05$ compared with EtOH-ppt. MST, mean survival time; ILS, increase in life span : 2-chloroadenosine (Cl-Ade), Cyclosporin (Cys-A)

Effect of chemoimmunotherapy of EtOH-ppt with standard chemotherapeutic agents

Effect of EtOH-ppt as a supplementary agent in combination with chemotherapy was assessed in view of its clinical application. Inbred C57BL/6 mice were inoculated i.p. with LLC (2-4x10^5 cells/mouse) on day 0. Chemotherapeutic agents (adriamycin; Adria, cisplatin; CDDP, 5-fluorouracil; 5-FU, methotrexate; MTX or vincristine; VCR) were given to mice as a single dose on day 1 accompanying with EtOH-ppt treatment beginning also on day 1 once daily for 5 days. (Table 2.7). Significant beneficial effects occurred with the combined regimen of EtOH-ppt with Adria, CDDP, 5-FU or VCR as compared to the chemotherapy alone. Lifespan of tumor bearing mice was dramatically increased to more than 150% in all cases and improved cure rats were also observed. The combined regimen with MTX was not effective. Therefore, it may be of benefit to cancer patients by enabling them to use lower doses of anticancer drugs to achieve the same or even better results (Hirazumi and Furusawa, 1999).

Table 2.7 Combination of suboptimal dose of chemotherapy with EtOH-ppt against LLC peritoneal carcinomatosis (Hirazumi and Furusawa, 1999)

Agent	Dose/mouse Schedule(route)	MST±SEM (days)	No. mice survived/total	ILS (%)
Exp.1				
Control		13.9±0.9	0/9	
EtOH-ppt	0.8mg,QODx 5(i.p.)	28.0±4.9[b]	2/9	101
Adria	2μg, day x1(i.p.)	24.2±3.9[b]	1/9	74
Adria+EtOH-ppt	same as above	42.3±4.0[c]	6/9[a]	204
Exp.2				
Control		14.9±1.4	0/10	
EtOH-ppt	0.8mg,QODx 5(i.p.)	28.4±5.3[b]	2/8	91
CDDP	10μg, day x1(i.p.)	25.2±5.0[b]	1/8	69
CDDP+EtOH-ppt	same as above	41.4±4.5[c]	5/8[a]	178
Exp.3				
Control		16.9±2.4	0/7	
EtOH-ppt	0.8mg,QODx 5(i.p.)	33.8±6.6[b]	4/8[b]	100
5-FU	300μg, day x1(i.p.)	33.4±6.3[b]	3/8	98
5-FU+EtOH-ppt	same as above	45.6±4.4[c]	7/8[a]	170
Exp.4				
Control		16.7±1.4	0/10	
EtOH-ppt	0.8mg,QODx 5(i.p.)	35.5±4.6[b]	3/8[b]	113
VCR	1μg, day x1(i.p.)	30.5±4.8[b]	2/8	83
VCR+EtOH-ppt	same as above	44.4±4.4[c]	6/8[a]	166

Inbred C57BL/6 mice were inoculated i.p. with LLC (2-4 x 10^5 cells/mouse) on day 0. 0.1 ml of vehicle or agents administered i.p. at the indicated doses beginning on day 1. Survival of mice was recorded up to 50 days. Mice surviving 50 days were considered cured. [a] $p<0.01$, [b] $p< 0.05$ compared with control. [c] $p < 0.05$ compared with chemotherapeutic agent or EtOH-ppt. MST, mean survival time; ILS, increase in life span : adriamycin (Adria), cisplatin (CDDP), 5-fluorouracil (5-FU) and vincristine (VCR)

3. Cancer preventive and antioxidant effects

Wang and Su (2001) demonstrated that *M. citrifolia* fruit juice was able to significantly reduced the DMBA-DNA adduct formation in rats and mice *in vivo*. In that study, rats were given 10% *M. citrifolia* fruit juice in drinking water for one week followed by intragastrically administration of 25 mg/kg DMBA on the 8th day. The results showed that levels of DMBA-DNA adduct were reduced by 30% in heart, 41% in lung, 42% in liver and 80% in kidney of female SD rats. Even more dramatic results were obtained in male C57BL/6 mice: 10% *M. citrifolia* fruit juice was able to reduced DMBA-DNA adduct formation by 60% in heart, 50% in lung, 70% in liver and 90% in kidney.

This group of researchers further performed an *in vitro* study. They found that *M. citrifolia* fruit juice possessed strong antioxidant activities. The antioxidant activities were examined *in vitro* by lipidhydroperoxide (LPO) and tetrazolium nitroblue (TNB) assays. *M. citrifolia* fruit juice showed a dose-dependent inhibition of both LPO and superoxide anion radicals (SAR). It was noted that in the TNB assay, SAR reduced TNB to formazan blue that was measured by spectrophotometric absorption at 602 nm. Moreover, antioxidant activity of *M. citrifolia* fruit juice was compared to the effects of vitamin C, grape seed powder (GSP) and pycnogenol (PYC) at the daily dose per serving level recommended by U.S. RDAs or manufacturers. The results showed that the SAR scavenging activity of *M. citrifolia* fruit juice was 2.8, 1.4, 1.1 times than that of vitamin C, PYC and GSP respectively.

From this study, the authors suggested that prevention of carcinogen-DNA adduct formation and the antioxidant activity of *M. citrifolia* fruit juice may contribute to the cancer preventive effect of this plant.

Toxicological effects

Acute toxicity

Following intraperitoneal administration of ethanol-soluble material (EtOH-sol) from the over ground parts of *M. citrifolia* to mice, it was shown that median lethal dose (LD_{50}) was varied between 0.75 to greater than 1 g/kg of body weight (Nakanishi *et al.*, 1965; Dhawan *et al.*, 1977). Likewise intraperitoneal administration of methanol-soluble material (MetOH-sol) from *M. citrifolia* leaves to mice, the LD_{50} was found to be greater than 1 g/kg of body weight (Nakanishi *et al.*, 1965).

Subchronic toxicity

A subchronic study was performed in swiss mice by giving orally 16 g/kg of water extract from root and intraperitoneal administration of this extract at various doses (1, 2, 4 and 8 g/kg). Toxic effect was not observed in the first 3 days after administration. However, body weights of mice were decreased within 14 days, after the oral administration of 16 g/kg of the extract and the intraperitoneal administration of this extract at doses of 4 and 8 g/kg (Yonos *et al.*, 1990).

Clinical reports

One case report of a man with chronic renal insufficiency who self-medicated with an alternative product known as *M. citrifolia* fruit juice. The patient presented to the clinic with hyperkalemia despite having low-potassium diet. Potassium concentration in *M. citrifolia* fruit juice was found to be approximately 56.3 mEq/L, the concentration that is equal to that found in orange juice and tomato juice. Therefore, herbal remedies and alternative medicinal products may be surreptitious sources of potassium in patients with renal disease (Mueller *et al.*, 2000).

Biotransformation of xenobiotics

Biotransformation appears to be an important process determining the consequences of xenobiotics or foreign chemicals as well as endogenous compounds in biological system. Enzymes involved in biotransformation have a particular subcellular localization. Many are found in smooth endoplasmic reticulum (SER). Some are located in cytosol and a few are found in other organelles such as mitochrondria. Xenobiotic-biotransformation enzymes convert chemicals of lipophilic property to metabolites of hydrophilic property that are more readily excreted in urine or feces (Timbrell, ed., 2000 and Parkinson, 2001). In general, the reaction catalyzed by these enzymes is divided into phase I and phase II reactions and sometimes, phase III reactions (Table 2.8).

Table 2.8 The major biotransformation reactions (Timbrell, ed., 2000)

Phase I	Phase II	Phase III
Oxidation	Sulphation	Further metabolism of
Reduction	Glucuronidation	glutathione conjugates
Hydrolysis	Glutathione conjugation	
Hydration	Acetylation	
Dehalogenation	Amino acid conjugation	
	Methylation	

Xenobiotics, after converted by specific enzymes to more reactive, more electrophilic intermediates, are capable of reacting covalently with biological macromolecules such as proteins, nucleic acids or lipids. Binding of xenobiotic metabolites to DNA may cause modification of genetic information, mutation and a consequent possibility of malignant growth.

Phase I reactions introduce a functional group (-OH, $-NH_2$, -SH or -COOH, etc.) to a molecule leading to a small increase in hydrophilicity as well as a suitable characteristic for further phase II biotransformations (Timbrell, ed., 2000 and Parkinson, 2001).

Phase I reactions

The most important reactions in phase I is oxidation, the reactions which mostly catalyzed by cytochrome P450(CYP) monooxygenase enzymes. These enzyme systems are predominantly localized in the membrane of SER of the liver cells, thus known as microsomal enzymes. These enzymes generally catalyze the oxidation reactions of a wide variety of both endogenous compounds and xenobiotics with overlapping substrate specificity (Potter and Coon, 1991 and Guengerich, 1991, 1992). Major CYP enzymes in human, their specific substrates and their percent participation in drug metabolism are shown in Table 2.9.

The basic reaction catalyzed by CYP is a monooxygenation reaction as following (Gibson and Skett, 2000):

$$\text{Substrate (RH)} + O_2 + \text{NADPH} + H^+ \xrightarrow{\text{CYP}} \text{Product (ROH)} + H_2O + NADP^+$$

Product from this reaction does not appear to be only a simple alcohol because rearrangement might be occurred. There are also documented that CYP may catalyze reduction reaction e.g. carbon tetrachloride, azo dyes and epoxides (Guengerich, 1991). However, biotransformation by CYP is not always a detoxification reaction. A variety of specific CYP isoforms, especially CYP in family 1, 2 and 3 are involved in the activation of certain chemical procarcinogens (Soucek and Gut, 1992; Parkinson, 2001).

CYPs in families 1, 2, and 3 play a major role in drug and xenobiotic metabolism. These three families account for about 70% of total CYPs in human livers while CYP4 is a family of enzymes involved in fatty acid and prostaglandins metabolism (Rendic and Di Carlo, 1997). CYP isoforms which play a role in the activation of xenobiotics to toxic metabolites include CYPs 1A1, 1A2, 2B1, 2B2, 2E1 in rats as well as CYPs 1A1, 1A2, 2B6, 2E1, 3A4 in humans. An example of rat and human CYPS that activate some potential carcinogens/metagens are demonstrated in table 2.10

CYP1A Subfamily

Enzymes in CYP1A subfamily are responsible for the metabolic activation of some known procarcinogenic environmental chemicals, toxins, and drugs. Important isoforms in this subfamily include CYP1A1 and CYP1A2. CYP1A1 is detected in lungs of smokers. *CYP1A1* gene expression is observed in several human cancer tissues including pulmonary carcinoma cells and malignant breast cancer. CYP1A1 is present at very low level but highly inducible (Gonzalez, 1994). In contrast to CYP 1A1, CYP1A2 is not expressed in extrahepatic tissues. CYP1A1 and CYP1A2 are found in both humans and rats. Function of CYP1A is fairly well conserved across species, although there are subtle differences (Parkinson, 2001). For instance, isolated and purified human CYP1A2 enzyme from the liver has been shown to display substrate specificity similar to the rat protein. These isozymes are undoubtedly the most significant in activation of carcinogens since they can activate more than 90% of known carcinogens (Rendic and Di Carlo, 1997), for example, cigarette smoke, charcoal-broiled meat (a source of polycyclic aromatic hydrocarbons), and cruciferous vegetables (a source of various indole) (Parkinson, 2001). The most potent inducing agent of the *CYP1A* genes is an environmental pollutant chemical TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin). Some drugs that are substrates (e.g. caffeine, bufuralol, propanolol and paracetamol), inducers (e.g. omeprazole and lansoprazole), or inhibitors (e.g. cimetidine), may also interact with enzymes belonging to other CYP families (Rendic and Di Carlo, 1997).

CYP2B Subfamily

CYP2B has been extensively studied in rats because it can be induced by phenobarbital. CYP2B1 and CYP2B2 are highly similar in nucleotide sequence and have similar substrate specificity. Rat CYP2B1 is analogous to human CYP2B6, which generally exists in small amount. CYP2B6 would be expected to be inducible by phenobarbital, however the level of isozyme is extremely low even in individuals treated with phenobarbital (Parkinson, 2001). It appears that the ability of phenobarbital to stimulate biotransformation of xenobiotics in human largely stems from its ability to induce other CYPs, CYP2C and CYP3A4 (Parkinson, 2001).

CYP2E Subfamily

CYP 2E1 is expressed constitutively in liver and possibly in extrahepatic tissues, such as kidney, lung, and lymphocytes. This enzyme is responsible for the formations of reactive metabolites from a number of laboratory and environmental chemicals such as benzene, aniline, polyhalogenated compounds, urethane, butadiene, chlorofluorohydrocarbons, fluorohydrocarbons (Guengerich ed., 1991). CYP2E1 substrates such as ethanol, isopropanol, acetone, toluene, and benzene, may also induce CYP2E1 itself. Isoniazid and imidazole compounds are also potent inducers. Diabetes may induce the activity of this enzyme (Rendic and Di Carlo, 1997). Human liver CYP2E1 is similar to rat CYP2E1 and rabbit CYP2E1 in structure, catalytic activity and regulatory characteristics (Wrighton ed., 1986). Thus, CYP2E1 are well conserved among mammalian species (Parkinson, 2001).

CYP3A Subfamily

CYP3A subfamily (CYP3A4, CYP3A5 and CYP3A7) is very frequently involved in the metabolism of drugs and other chemicals. Enzymes in this subfamily are the most abundantly expressed in both human liver and GI tract (Guengerich, 1995 and Kronbach, 1995). The level in liver is about 25-28% of total CYP enzymes but can be as high as 70%. CYP3A7 is the major CYP enzyme identified in human fetal liver and it is considered primarily as a fetal enzyme (Gibaldi, 1992).

More than 150 drugs belonging to about 38 classes are listed as substrates of CYP3A4. Examples of substrates are opioid analgesics, corticosteroids, immunosuppressants and antiarrhythmics. This enzyme also catalyzes the metabolism of endogenous steroids including androgens, anabolic hormones, cortisol, estradiol and progesterone (Table 2.9). Most CYP3A4 substrates are lipophilic compounds which undergo either N-dealkylation or aliphatic oxidation.

Many CYP3A4 inhibitors are usually also substrates for the enzymes. Other inhibitors require metabolic activation to achieve inhibition. After repeated doses, some inhibitors can induce the activity of CYP3A enzymes (e.g. macrolide antibiotics and corticosteroids).

Some dietary compounds can either stimulate or inhibit CYP3A4 *in vivo* and *in vitro*. For instance, flavonoids (queretin, kaempherol, tangeretin and naringenin) inhibit nifedipine and felodipine oxidation catalyzed by CYP3A4 in human liver microsomes. Grapefruit juice (containing the flavonoids quercetin, naringin, bergamottin and naringenin) was found to inhibit the oxidation of both nifedipine and felodipine as well as affect the disposition of cyclosporine, terfenadine, midazolam and 17 α-ethinylestradiol (Yang, 1994).

CYP3 enzymes activate some procarcinogens, drugs and dietary compounds by forming reactive metabolites. For instance, CYP3A catalyzes the N-dealkylation of cocaine, a pathway accounting for about 10% of its total metabolism. The formation of a pharmacologically active N-dealkylated metabolite and its further metabolism have been associated with cocaine hepatotoxicity (Rendic, 1997).

Table 2.9 Human CYP enzymes, their specific substrates and their percent participation in drug metabolism (modified from Rendic and Di Carlo, 1997)

CYP enzymes	Substrates	Participation in drug metabolism (%)
1A1	7-Methoxyresorufin R-Warfarin	2.5
1A2	Acetaminophen Caffeine 7-Ethoxyresorufin 7-Methoxyresorufin Phenacetin R-Warfarin	8.2
2B6	Cyclophosphamide 7-Benzyloxyresorufin S-Mephenytoin 7-Pentoxyresorufin Testosterone	3.4
2E1	Acetaminophen Aniline Chlorzoxazone Dapsone Halothane *p*-Nitrophenol	4.1
2C8, 9	Diclofenac Hexobarbital Phenytoin Tolbutamide S-Warfarin	15.8
2C18, 19	Diazepam S-Mephenytoin Omeprazole	8.3
2D6	Codeine	18.8

Table 2.9 *(continued)* Human CYP enzymes, their specific substrates and their percent participation in drug metabolism (modified from Rendic and Di Carlo, 1997)

CYP enzymes	Substrates	Participation in drug metabolism (%)
	Bufuralol	
	Debrisoquine	
	Dextromethophane	
	Sparteine	
3A4, 5	Carbamazepine	34.1
	Cortisol	
	Dapsone	
	Diazepam	
	Erythromycin	
	Midazolam	
	Nifedipine	
	Omeprazole	
	Testosterone	

Table 2.10 Role of rat and human CYPs in the activation of some potential carcinogens/ mutagens (Soucek and Gut, 1992; Guengerich, 1993; Gonzalez and Gelboin, 1994)

CYP	Potential mutagens/ carcinogens	
	Rat	Human
1A1	Aflatoxin B_1	Benzo(a)pyrene
	Benzo(a)pyrene	7,12-Dimethylbenz(a)anthracene
	7,12-Dimethylbenz(a)anthracene	6-Nitrochrysene
	2-Naphthylamine	
	4,4'-(bis) Methylene chloroaniline	
1A2		2-Acetylfluorene
		2-Aminoanthracene
		Aflatoxin B_1
		4-Aminobiphenyl
		2-Naphthylamine
		6-Nitrochrysene
2B1	2-Acetylfluorene	
	Aflatoxin B_1	
	Benzo(a)pyrene	
	3-Methylcholanthrene	
	4,4'-(bis) Methylene chloroaniline	
2B2	4,4'-(bis) Methylene chloroaniline	
2B6		6-Aminochrysene
2B7		Aflatoxin B_1
2E1	N-N'-Nitrosodimethylamine	Acrylonitrile
	N-Nitroso-N-diethylamine	Benzene
		Carbon tetrachloride
		Chloroform
		N-N'-Nitrosodimethylamine
		N-Nitroso-N-diethylamine
		Styrene
		Trichloroethylene
		Vinyl carbamate
		Vinyl bromide
		Vinyl chloride
3A4		Aflatoxin B_1
		Aflatoxin G_1
		Benzo(a)pyrene
		6-Nitrochrysene
		Sterigmatocystin

Phase II reactions

Most phase II reactions are recognized as detoxification pathway, which results in a large increase in hydrophilicity as well as an enhancement of foreign molecule excretion (Parkinson, 2001; Timbrell, ed., 2000). Most reactions in phase II are conjugation reactions such as glucuronide, glutathione and sulfate conjugations (Table 2.8) (Wattenberg, 1983). Enzymes of those reactions are mainly located in cytosol except for the UDPGTs, which are microsomal enzymes. Glucuronidation is a major pathway of xenobiotic biotransformations in most mammalian species (Parkinson, 2001). In addition, glutahione conjugation has been studied extensively as a major detoxification system and considered as an important protective mechanism against chemical induced carcinogenesis (Spranin, Venegas and Wattenberg, 1982; Wattenberg, 1983). GST catalyzes nucleophilic attack of glutahione thiolate anion (GS^-), derived from glutathione, to electrophilic xenobiotics (Parkinson, 2001). Moreover, products from phase II reaction may be further metabolized. This process is sometimes termed phase III reaction (Timbrell, ed., 2000).