ผลของสารสกัดจากพืชสมุนไพรสามชนิดในวงศ์ ASTERACEAE ต่อ *XANTHOMONAS CAMPESTRIS* PV. *GLYCINES* สาเหตุของโรคใบจุดนูนของถั่วเหลือง

นางสาวอัจฉราวรรณ์ คำแสน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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THE EFFECTS OF THREE MEDICINAL PLANT EXTRACTS IN ASTERACEAE FAMILY ON *XANTHOMONAS CAMPESTRIS* PV. *GLYCINES* CAUSING BACTERIAL PUSTULE OF SOYBEAN

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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จากการสกัดสารจากใบ ดอก และรากของพืชสามชนิดในวงศ์ Asteraceae คือ สาบเสือ (*Eupatorium odoratum* L.) สาบแร้งสาบกา (*Ageratum conyzoides* L.) และตีนตุ๊กแก (*Tridax procumbens* L.) ด้วยเครื่อง Soxhlet extractor แล้วทำให้แห้งด้วย rotary vacuum evaporator พบว่า ร้อยละของผลได้จากการสกัดจากน้ำหนักแห้ง 50 กรัมของใบ ดอก และราก ของ *E. odoratum*, *A. conyzoides* และ *T. procumbens* คือ 38.36, 24.71, 15.20, 29.24, 23.13, 13.34, 19.00, 18.70 และ 18.42 ตามลำดับ เมื่อนำสารสกัดมาทดสอบประสิทธิภาพในการยับยั้งการเจริญของเชื้อ *Xanthomonas campestris* pv. *glycines* (*Xcg*) สาเหตุของโรคใบจุดนูนของถั่วเหลืองจำนวน 5 ไอโซเลท ด้วยวิธี agar disc diffusion และในสภาพเรือนทดลอง พบว่า สารสกัดจากแอลกอฮอล์ของพืชทั้งสามชนิดแสดงประสิทธิภาพในการยับยั้งเชื้อ *Xcg* ได้ทั้ง 5 ไอโซเลท โดยสารสกัดจากใบและดอกของ *T. procumbens* ที่ความเข้มข้น 100,000 ppm แสดงค่าการยับยั้งได้ดีที่สุดคือ 22.00 มม. และผลการศึกษาในสภาพเรือนทดลอง พบว่า สารสกัดจากใบ ดอกของพืชทั้งสามชนิด และรากของ *T. procumbens* แสดงประสิทธิภาพในการลดการเกิดแผลใบจุดนูนของ *Xcg* 728 ในใบถั่วเหลืองที่ทดสอบอย่างมีนัยสำคัญ ($p<0.05$) เมื่อเปรียบเทียบกับกลุ่มควบคุม โดยที่ความเข้มข้น 100,000 ppm สารสกัดแสดงประสิทธิภาพในการป้องกันและการรักษาการเกิดโรคใบจุดนูนได้ไม่แตกต่างกัน แต่แสดงประสิทธิภาพในการป้องกันมากกว่าการรักษาโรคที่ความเข้มข้น 50,000 และ 30,000 ppm

สาขาวิชาชีววิทยา
ปีการศึกษา 2548

ลายมือชื่อนักศึกษา อัจฉราวรรณ์ คำแสน
ลายมือชื่ออาจารย์ที่ปรึกษา
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

ARDCHARAWAN KUMSAEN : THE EFFECTS OF THREE MEDICINAL PLANT EXTRACTS IN ASTERACEAE FAMILY ON *XANTHOMONAS CAMPESTRIS* PV. *GLYCINES* CAUSING BACTERIAL PUSTULE OF SOYBEAN. THESIS ADVISOR : ASST. PROF. GRIANGSAK EUMKEB, Ph.D. 118 PP. ISBN 974-533-494-4

ANTIBACTERIAL ACTIVITY/PLANT EXTRACT/ASTERACEAE/ *XANTHOMONAS CAMPESTRIS* PV. *GLYCINES*

Fifty grams of dried leaves, flowers and roots of *Eupatorium odoratum* L., *Ageratum conyzoides* L. and *Tridax procumbens* L. were extracted by using Soxhlet extractor apparatus and concentration to dryness by rotary vacuum evaporator. The result showed that the percentage of extracts yielded were 38.36%, 24.71%, 15.20%, 29.24%, 23.13%, 13.34%, 19.00%, 18.70% and 18.42%, respectively. Efficacy of the extracts were tested against five isolates of *Xanthomonas campestris* pv. *glycines* (*Xcg*) by the agar disc diffusion method and under greenhouse conditions. Results from the agar disc diffusion method showed that the 95% ethanolic extracts of the plant parts showed antibacterial activity against the five isolates of *Xcg*. The leaf and flower extracts of *T. procumbens* exhibited highest activity at 100,000 ppm, with an inhibition zone of 22.00 mm. Under greenhouse conditions, results showed significant ($p<0.05$) protective and curative effects for the leaf and flower extracts of the three plants, and root extracts of *T. procumbens*, in reducing the numbers of *Xcg* 728 bacterial lesions when compared to the negative control. In addition, these plant

extracts showed similar protective and curative effects at 100,000 ppm, but the protective effect was greater than the curative effect at 50,000 and 30,000 ppm.

School of Biology

Academic Year 2005

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Ardcharawan Kumsaen

CONTENTS

CONTENTS (Continued)

CONTENTS (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

ACF	*A. conyzoides* L. flowers
ACL	*A. conyzoides* L. leaves
ACR	*A. conyzoides* L. roots
CFU	Colony Forming Unit
CRD	Completely Randomized Design
CU	Copper oxychloride (Cupravit®)
DMSO	Dimethylsulfoxide
EOF	*E. odoratum* L. flowers
EOL	*E. odoratum* L. leaves
EOR	*E. odoratum* L. roots
mg/ml	milligram per milliliter
MIC	Minimal Inhibiory Concentration
ppm	part per million
TPF	*T. procumbens* L. flowers
TPL	*T. procumbens* L. leaves
TPR	*T. procumbens* L. roots
w/w	weight by weight
Xcg	*Xanthomonas campestris* pv. *glycines*

CHAPTER I

INTRODUCTION

Over the last decade, soybean [*Glycine max* (L.) Merrill] has become one of the most important crops in the world because of its multiple uses as a primary source of vegetable oil, food protein, animal feed and as a raw material for industry. In Thailand, demand for soybean has increased markedly due to expansion of the animal feed and vegetable oil industries, but the domestic production of soybean is still very low (Office of Agricultural Economics [OAE], Online, 2005). Soybean import to Thailand was 1,007,923 tons in 1999, increasing to 1,435,801 tons in 2004 (OAE, Online, 2004). There are several factors affecting soybean production in Thailand including quality of seed, lack of production technology, natural disasters, disease and pest problems (Chainuvati, Online, 1999). Soybean diseases are one of the factors contributing to low soybean production. More than 100 pathogens are known to affect soybeans (Sinclair, 1997). Bacterial pustule disease caused by *Xanthomonas campestris* pv. *glycines* is one of the important diseases of soybean in Thailand because of the warm weather and high rainfall during the crop growing season. The disease symptoms are due primarily to hypertrophy of host mesophyll cells, which can result in premature defoliation of infected plants. Yields are lowered because of reduced seed size and number, and the bacteria can proliferate greatly under suitable conditions (Hwang, Lim and Shaw, 1992). Methods, such as the improveed seed quality, crop rotation, use of resistant varieties and better

water management can help control the bacterial pustule disease. Chemical treatment has been one of choices in modern agriculture because of its obvious capacity for controlling diseases and because chemicals are used in small amounts per unit area. A data from Department of Agriculture (DOA), Office of Agricultural Regulation, Online, (2004), showed that value of fungicide imports was 673,209,591 baths in 2004 and it has been increasing. However, the impact of this practice on human health, and environment, and resistance of bacteria have become problems in many countries. Therefore, searching for new natural products from plant extracts to replace the use of chemical or antibiotic in plant disease control is important.

Medicinal plants have been used for centuries in medicine and pest control. Natural plant products such as wild herbs have been used against agricultural pests for many decades.

Eupatorium odoratum L., *Ageratum conyzoides* L. and *Tridax procumbens* L. in the Asteraceae family are medicinal plants with a long history of traditional uses in many countries, especially in tropical and subtropical regions. In Thailand, they are found in many areas including roadsides, agricultural fields, wastelands and forests. These plants have been used in traditional medicine for wound healing, as coagulants, and as antiseptic agents, and there have been various studies on their antibacterial properties. For example, leaves of *A. conyzoides* and *T. procumbens* were against *Bacillus cereus*, *Klebsiella aerogenas* and *Alkaligens viscolactis* (Perumal, Ignacimuthu and Raja, 1999). The ethanol extract of *E. odoratum* (*Chromolaena odorata*) showed antibacterial activity on *Escherichia coli*, *Pseudomonas* sp., *B. thuringensis*, *Klebsiella* sp. and *Streptococcus faecalis* (Irobi, 1997).

In this study, three plants in family Asteraceae were evaluated against *X. campestris* pv. *glycines*, causing bacterial pustules of soybean.

1.1 Research objectives

To study the effects of crude extracts of *E. odoratum*, *A. conyzoides* and *T. procumbens* on *X. campestris* pv. *glycines* both in vitro and in vivo.

1.2 Research hypothesis

The crude extracts of *E. odoratum*, *A. conyzoides* and *T. procumbens* could suppress the growth of *X. campestris* pv. *glycines* by disc diffusion method and may reduce the bacterial pustule number.

1.3 Scope of the study

In this study, the crude extracts of *E. odoratum*, *A. conyzoides* and *T. procumbens* were tested against *X. campestris* pv. *glycines*. The test was being done in vitro by disc diffusion method. The in vivo effect was being studied on SJ.5 soybean at greenhouse in Suranaree University of Technology (SUT), Nakhon Ratchasima province. The five isolates of bacteria used in this study were collected and isolated from Department of Agriculture (DOA), Ministry of Agriculture and Cooperatives, Thailand.

1.4 Expected results

1. Gaining the information on the effects of crude extracts of *E. odoratum, A. conyzoides* and *T. procumbens* on *X. campestris* pv. *glycines*.
2. If the extracts are effective in suppressing the in vitro growth of the bacteria and reduce the disease incidence in soybean, the plant extracts could be developed further as a bio-bactericide.

CHAPTER II

LITERATURE REVIEW

2.1 Medicinal plants in Asteraceae family

Family Asteraceae (Compositae) is the largest of flower plant families, containing 900 genera and more than 20,000 species (จันทร์เพ็ญ ประคองวงศ์, 2543). The family is divided into 13 groups. They have diversity of genera and species.

There are several groups of plant in this family, such as medicinal plants (*Chamomilla recutita*, *Chrysanthemum moriifolium*), food (lettuce, globe artichoke and sunflower), flowers (chrysanthemum) and noxious weeds (*Eupatorium odoratum*, *E. adenophorum* and *Ageratum conyzoides*).

Plants in Asteraceae are widely distributed in many regions of the world, especially tropical regions. Most of the members in this family are shrub or perennial shrub that have underground rhizome. Some of them are annual herb that have underground rhizome or climbing or succulent plants (Sriwatcharakul, 1998).

2.1.1 *Eupatorium odoratum* L.

Synonym: *Chromolaena odorata* (L.) R.M. King & H. Robins.

Vernacular name: Siam weed, bitter bush, eupatorium, paraffinbush, paraffinweed, Armetrong's weed, triffid weed, turpentine weed, Sap suea, Ya men Cha phak khrat, Yisun Thani, Phak khrat (Quattrocchi, 2000; เต็ม สมิตินันทน์, 2544)

Taxonomy classification:

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Asterales

Family: Asteraceae

Genus: Eupatorium, Chromolaena DC.

Species: *Eupatorium odoratum* L.

Chromolaena odorata (L.) King & H.E. Robins.

Figure 1 Leaves and flowers of *Eupatorium odoratum* L.

Description: *E. odoratum* is a perenial, densely branchin shrub. It forms tangled bushes 2-3 m tall in open land, but can reach up to 20 m as a scrambling climber on trees. The soft, hairy leaves are roughly trianglular in shape, and the stems are round with no prickles at all. It produces masses of white or lilac flowers that develop on all branches and side shoots to cover the whole plant. Small brown seeds

are released after flowering and can be blown long distances on their parachutes of white hairs (Hills and Ostermeyer, 2000).

Radanachaless and Maxwell (1994) described *E. odoratum* L. as follows:

"Erect, Branching scented, mostly annual herb to 1.5 m tall. Stems and branches terete, striate; younger part densely pubescent, older branches glabrescent; light green and turning brownish with age. Leaves opposite, decussate, simple. Blades thin, ovate, acute at the tip, base abruptly narrowed and becoming decurrent on the upper part of the petiole; margins broadly serrate except along the entire base; venation pinnate, tri-plinerved near the base, midnerve distinct; sparsely puberulous on both sides; dark green above, green underneath; 5.5-11.5 × 2-6.5 cm. Petioles moderately puberulous, 6-17 mm long. Inflorescence terminal, corymbose, often with its lower branches in the axils of reduced leaves, 3-4 × 6-8 cm. Axes similar to the branchlets, light green, "pedicels" 6-9 mm long. Heads numerous, ellipsoid, 8-9 × 4-5 mm. Involucral bracts in 3 whorls, with several in each, increasing in size towards the inside; scarious, sparsely puberulous, very pale light greenish with green nerves; outer ones ovate, tip obtuse, 2 × 1.5 mm; middle ones lanceolate, tip acute, 4-5 × 2 mm; inner ones linear, tip acute, 8 × 1 mm; chaff none. Flowers numerous in each head, all discoid (tubular) and bisexual, 10-11 mm long. Pappus consisting of a single whorl of free, rigid, erect, minutely barbed, white bristles 5 mm long. Corolla mostly glabrous, white and often with a lilac hue, 5 mm long; lobes 5, triangular, 0.75 mm long. Stamens 5 inserted on and included in the tube; anthers connate, base truncate, 2 mm long; filaments free, slender, 1 mm long. Stigmas 2, pigmented as the corolla, 5 mm long; style 1, 4 mm long. Ovary inferior, 1-locular and with 1 basal ovule. Achenes linear, flattened, sparsely puberulous, 3 mm long, crowned by the pappus."

Distribution: *E. odoratum* is native to the rain forest areas of Central and South America, from Mexico to Bolivia and possibly the West indies. It reached Malaysia and Thailand before World War I, but now exists as a major weed throughout much of South-East Asia and is still spreading, having recently reached Timor, Irian Jaya and Papua New Guinea (Hills and Ostermeyer, 2000).

Reproduction biology: Flowering is photoperiod controlled, even near the equator, and thus occurs synchronously in a region. There is no seed dormancy, and it can produce up to 87,000 seeds per plant each year, seeds germinate rapidly following rain and in suitable soil, large numbers of seedlings become established. It dose not reproduce vegetatively, but plants can survive fire and drought by resprouting from the root crown when favourable conditions return (Hills and Ostermeyer, 2000).

Traditional medicinal usage: Leaf decoctions were used to treat asthma and to lower body temperature in children infected with chicken-pox (Bouda, Tapondjou, Fontem and Gumedzoe, 2001). They were used in the treatment of skin infections (impetigo and ringworm), in the maintenance of homeostasis (Agu, 1980 quoted in Irobi, 1997). Fresh leaves or decoction has been used throughout Vietnam for many year as well as in other tropical countries for the treatment of leech bite, soft tissue wounds, burn wounds, skin infection and dento-alveolitis. In Ivory Coast, *E. odoratum* is used as traditional medicine as a wound healing and a local antiseptic agent (Bamba, Bessiere, Marion, Pelissier and Fouraste, 1993). In tropical Africa and Nigeria, *E. odoratum* L. has acquired a reputation as a medicinal herb for a variety of ailments including malaria (Akah, 1990 quoted in Thakong, 1999). In Thailand,

decoction of the root of this plant mixed with the root of lime and root of *Tiliacora triandra* is used for fever. The decoction of the leaf was used as cough remedy and stem decoction of the plant can be used in pulmonary hemorrhage. It is also used as hemostatic agent to arrest bleeding from fresh cuts and to stop nosebleeds (Thakong, 1999).

Pharmacological studies: Eupolin ointment, a formulation prepared from the aqueous extract of the leaves of *E. odoratum* has been licensed for clinical use in Vietnam. A clinical trial of eupolin ointment was performed in the National Institute of Burns in Hanoi, Vietnam between 1987 to 1991 on 136 patients with full thickness wounds and an average wound size of 79.9 cm^2. The stimulatory effects of eupolin ointment on the formation of granulation tissue and wound re-epithelialization were demonstrated clinically and histologically (Nghiem, 1992 quoted in Thang, Patrick, Teik and Yung, 2001).

Insecticidal and other biological activity: These plants have reported to have some insecticidal properties against insect pests. For example, essential oil extracts of *E. odoratum* had insecticide effect on the main grain weevil, *Sitophilus zeamais* (Bouda et al., 2001). *E. odoratum* showed strong repellent activity and thus deterred the insects from feeding. It reduced survival of *Tribolium castaneum* (Coleoptera: Tenebrionidae) *and Sitophilus oryzae* (Coleoptera: Curculionidae) less than 25% after 10 days of treatment at concentrations of 0.1 mg/ml (Owusu, 2001). Jacob and Shiela (1993) reported that powder prepared from the leaves of *E. odoratum* protected stored

rice from the attack of *Rhyzopertha dominica* (F.) when admixed at 5% w/w before storage.

Antibacterial activity studies: Ethanolic extract from natural dried leaves of *E. odoratum* showed ability to inhibit *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* sp., *Streptoccocus faecalis* and *Bacillus thuringensis* (Irobi, 1997). Essential oil of *E. odoratum* exhibited a notable activity towards the gram-negative species including *Klebsiella pneumoniae*, *E. coli* and specifically towards *Pseudomonas aeruginosa* and essential oil from Nigeria showed its greatest activity on gram-positive species, mostly in *S. aureus* (Bamba et al., 1993). The extract of *E. odoratum* showed strong inhibitory effects (zone of inhibition ≥ 15 mm) to *Propionibacterium acnes* and *Staphylococcus epidermidis* (Chomnawang, Surassmo, Nukoolkarn and Gritsanapan, in press). สรกนก วิมลมั่งคั่ง และสุรเชษฐ์ ตั้งธีระบัณฑิตกุล (2545) demonstrated that the crude extract of *E. odoratam* was active against *S. aureus* and *E. coli*. The activity against *S. aureus* of this plant extract was comparable to that of 2 μg/ml gentamicin.

Chemical constituents: Chemical analysis of this plant extracts reveal the presence of flavone and flavonoid, tannin, alkaloid, saponins, sesquiterpenes, and other phenolic compounds. The chemical composition of *E. odoratum* is summerized in Table 1.

Table 1 Chemical compounds of *E. odoratum* L.

Chemical compound	**Chemical group**	**Plant parts**	**References**
α-Elemene	sesquiterpene	whole plant	Bomba et al. (1993)
Acacetine	flavonoids	whole plant	Duke, Online (2005)
β-Amyrin	terpenoids	stem	Talapatra, Bhar and Talapatra (1974) quoted in Thakong (1999)
α-Cadinene	triterpenoids	whole plant	Bomba et al. (1993)
δ-Cadinene	sesquiterpene	whole plant	Bomba et al. (1993)
β-Caryophyllene	sesquiterpene	whole plant	Bomba et al. (1993)
α-Copaene	sesquiterpene	whole plant	Bomba et al. (1993)
ρ-Coumaric acid	coumarin	leaf	Phan,Wang, See, Grayer, Chan and Lee (2001)
Ceryl alcohol	terpenoids	leaf	Duke, Online (2005)
∂-decane	alkanes	whole plant	Bomba et al. (1993)
γ-Elemene	sesquiterpene	whole plant	Bomba et al. (1993)
β-Elemene	sesquiterpene	whole plant	Bomba et al. (1993)
α-Eremen	essential oil	whole plant	Bomba et al. (1993)
γ-Eudesmol	sesquiterpene	whole plant	Bomba et al. (1993)
β-Eudesmol	sesquiterpene	whole plant	Bomba et al. (1993)
α-Humurene	essential oil	whole plant	Bomba et al. (1993)
ρ-Hydroxybenzoic acid	hydroxybenzoic acid	leaf	Phan et al. (2001)
Σ-Muurolene	essential oil	whole plant	Bomba et al. (1993)

Table 1 (Continued)

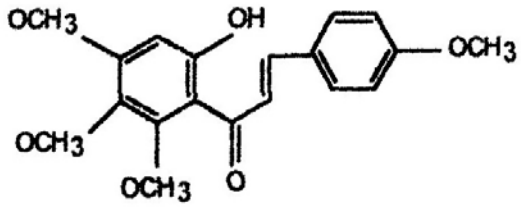
Chemical compound	Chemical group	Plant parts	References
β-Phelladrene	monoterpene	whole plant	Bomba et al. (1993)
α-Pinene	monoterpene	stem,	Bomba et al. (1993)
β-Pinene	essential oil	whole plant	Bomba et al. (1993)
α-Sitosterol	sterol	leaf	Duke, Online (2005)
β-Sitosterol	sterol	leaf	Talapatra et al. (1974) quoted in Thakong (1999)
γ- Sitosterol	sterol	leaf	Duke, Online (2005)
1-Hexenol	essential oil	whole plant	Bomba et al. (1993)
2-Hexenol (E)	essential oil	whole plant	Bomba et al. (1993)
3'-Acetylrinderine	pyrolizidine alkaloids	flower, root	Biller, Boppre, Witte and Hartmann (1994)
3-Hexenol (Z)	essential oil	whole plant	Bomba et al. (1993)
4-Terpineol	monoterpene	whole plant	Bomba et al. (1993)
d-Eupatene	sesquiterpene	whole plant	Areu, Pittit and Ode (1978) quoted in Thakong (1999)
Dodecane	alkanes	whole plant	Bomba et al. (1993)
Elemol	sesquiterpene	whole plant	Bomba et al. (1993)
Epoxylupeol	triterpenoids	whole plant	Talapatra et al. (1974) quoted in Thakong (1999)
Eupatol	sesquiterpene	stem	Talapatra et al. (1974) quoted in Thakong (1999)

Table 1 (Continued)

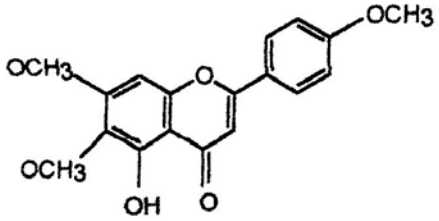
Chemical compound	Chemical group	Plant parts	References
Ferulic acid	hydroxycinnamic acid	leaf	Phan et al. (2001)
Geijierene	essential oil	whole plant	Bomba et al. (1993)
Germacrene	sesquiterpene	whole plant	Bomba et al. (1993)
Germacrene-D	sesquiterpene	whole plant	Bomba et al. (1993)
Hexadecane	alkanes	whole plant	Bomba et al. (1993)
Hexenal	essential oil	whole plant	Bomba et al. (1993)
Intermedine	pyrrolizidine alkaloids	flower, root	Biller et al. (1994)
Isosakuranetin	flavonoids	leaf, flower	Wollenwerer, Dorr and Muniappan (1995)
Kaempferide	flavonol	leaf	Wollenwerer et al. (1995)
Laciniatin	flavonoids	leaf	Wollenwerer et al. (1995)
l-Eupatene	sesquiterpene	stem	Areu et al. (1978) quoted in Thakong (1999)
Limonene	monoterpene	whole plant	Bomba et al. (1993)
Lupenol	sterol	stem, leaf	Talapatra et al. (1974) quoted in Thakong (1999); Duke, Online (2005)
Myrcene	monoterpene	whole plant	Bomba et al. (1993)
Ocimene	monoterpene	whole plant	Bomba et al. (1993)

Table 1 (Continued)

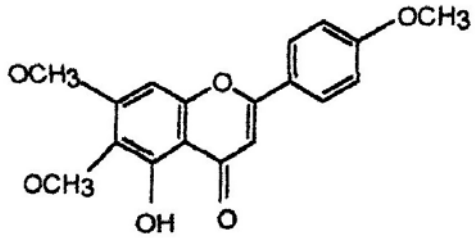
Chemical compound	Chemical group	Plant parts	References
Odoratin	flavonoid	leaf, plant, flower	Barua, Sharma, Thyagarajan and Hertz (1978)
Ombuin	flavonol	leaf	Wollenwerer et al. (1995)
Pentadecane	alkanes	whole plant	Bomba et al. (1993)
Pregaijerene	essential oil	whole plant	Bomba et al. (1993)
Rhamnetin	flavonol	leaf	Wollenwerer et al. (1995)
Rinderine	alkaloids	flower, root	Biller et al. (1994)
Sabinene	monoterpene	whole plant	Bomba et al. (1993)
Sakuranetin	flavonoids	whole plant	Iwu and chori (1984) quoted in Wollenwerer et al. (1995)
Salvigenin	flavone	stem, flower	Bose, Chakrabarti, Dutta Chakravarti and Barua (1973)
Sinensetin	flavonoids	leaf	Phan et al. (2001)
Tamarixetin	flavonoids	leaf, flower	Wollenwerer et al. (1995)
T-cadinol	essential oil	whole plant	Bomba et al. (1993)
Tetradecan	essential oil	whole plant	Bomba et al. (1993)
Tridecan	essential oil	whole plant	Bomba et al. (1993)
Undecane	alkanes	whole plant	Bomba et al. (1993)



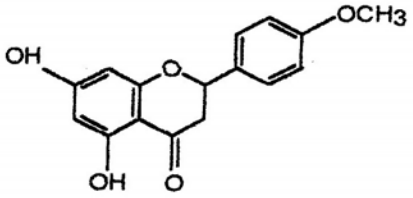
odoratin



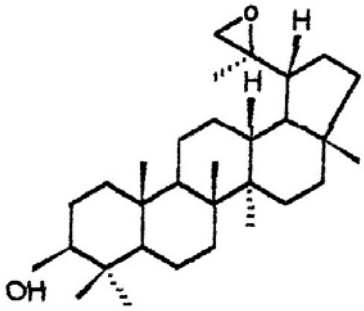
savigenin



pinene



isosakuranetin



epoxylupeol

Figure 2 Some chemical compounds of *E. odoratum* L.

(Lide and Milne, 1995; Thakong, 1999)

2.1.2 *Ageratum conyzoides* L.

Vernacular name: tropic ageratum, Billy-goat weed, blueweed, goatweed, white weed, appa grass, conyzoid floss-flower, bastard agrimony, mejorana, Sap raeng sap ga, Tap suea lek, Thiam mae hang, Ya sap haeng, Ya sap raeng (Quattrocchi, 2000; เต็ม สมิตินันทน์, 2544)

Taxonomy classification:

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Asterales

Family: Asteraceae

Genus: Ageratum L.

Species: *Ageratum conyzoides* L.

Figure 3 Leaves and flowers of *A. conyzoides* L.

Description: *A. conyzoides* L. is an annual branching herb, which grows to approximately 1 m in height. The stems and leaves are covered with fine white hairs, the leaves are ovate and up to 7.5 cm long. The flowers are purple to white, less than 6 mm across and arranged in close terminal inflorescences. Flower heads consist of 60 to 70 tubulous flowers arranged in clusters. The fruits are achene and are easily dispersed. The optimum germination temperature ranges from 20 to 25°C. The species has great morphological variation and appears highly adaptable to different ecological conditions.

Radanachaless and Maxwell (1994) described *A. conyzoides* L. as follows:

"Erect, glandular-sticky, scented, annual herb to 1 m tall. Stems terete, densely covered with soft. Gland-tipped, villous indumentum; light green and often with purplish in places. Leaves opposite decussate, simple. Blades thin, ovate, acute at the tip, base truncate and slightly decurrent on the upper part of the petiole; margins with shallow, rounded crenulations except at the nearly entire base; venation pinnate, tri-plinerved at the base; both surfaces sparsely convered with thin puberulence and more rigid hairs; green above, light green underneath; 4-7 × 3.5-4.5 cm. Petioles with indumentum as the stem 2-3 cm long. Inflorescence a terminal, multi-headed cyme 2.5-3.5 cm diameter; axes similar to the stem and petioles; individual heads ellipsoid, 5 × 4 mm. Peduncles with scattered, linear appendages 4-10 mm long. Involucral bracts in one series, free, lanceolate, sparsely puberulous, green with acute, purple tips, 2.5-3 mm long. Flowers numerous, all discoid (tubular), bisexual, 4-4.5 mm long. Pappus of 5 free, lanceolate, acuminate bristles with minutely barbed margins, 2.5 mm long. Corolla tubes with a few minute glandular hairs, light lilac to white, 2 mm long; margins minutely 5-lobed. Stamens 5, included in the corolla tube. Stigmas

2, divergent, 1 mm long, light lilac to white; style 2 mm long. Ovary inferior, cuneate and slightly curved, prominently 4-angled, 2 mm long. Achenes cuneate, angular, straight or slightly curved, the angles often with sparse, minute, antrorse bristles; black, 1.5 mm long; crowed by the 2 mm long, white, scarious, erect, pappus scales; the involucrum persistent."

Distribution: Originated in Central America and the Caribbean. It is now found in many places in tropical and sub-tropical regions. This species has great morphological variation and appears highly adaptable to different ecological conditions (Ming, 1999).

Reproduction biology: flowering and fruiting throughout the year. Seeds are positively photoblastic, and viability is often lost within 12 month (Ming, 1999).

Traditional medicinal usage: *A. conyzoides* has been used in various parts of Africa, Asia and South America for treatment of several diseases. Leaves of *A. conyzoides* are used in Cameroon to treat conjunctivitis, ophthalmia, headache and otitis (Bouda et al., 2001). In Central Africa, it is used to treat pneumonia, but the most common use is to cure wounds and burns (Durodola, 1977 quoted in Okunade, 2002). Traditional communities in India use this species as a bacteriocide, antidysenteric (Borthakur and Baruah, 1987 quoted in Ming, 1999), treatment of leprosy and as an oil lotion for purulent opthalmia (Kirtikar and Basu, 1991 quoted in Shirwaikar, Bhilegaonkar, Malini and Kumar, 2003), In Asia, South America, and Africa, aqueous extract of this plant is used as a bacteriocide (Almagboul, Farroq and

Tyagi, 1985 quoted in Ming, 1999). In Reunion, the whole plant is used as an antidysenteric (Vera, 1993 quoted in Ming, 1999). Aqueous extracts of leaves or whole plants have been used to treat colic, colds and fevers, diarrhea, rheumatism, spasms, or as a tonic (Ming, 1999). In Brazil folk medicine, medicinal teas of *A. conyzides* are used as anti-inflammatory, analgesic and anti-diarrhoeic (Okunade, 2002).

Pharmacological studies: Several pharmacological investigations have been conducted to determine efficacy. Oral administration of the ethanol extract at dose levels of 500 and 750 mg/kg significantly protected gastric lesions by 80.59% and 89.33%, respectively (Shirwaikar et al., 2003). The hydroalcoholic extract (HAE) of *A. conyzoides* leaves was studied for its antiinflammatory effect on sub-acute (cotton pellet-induced granuloma) and chronic (formaldehyde-induced arthritis) models of inflammation in rats. The results showed that the group of rats treated with HAE (250 mg/kg body wt.; PO) had a 38.7% reduction in cotton-pellet granuloma. The development of chronically induced paw edema was also reduced significantly ($p<0.05$) by the plant extract (Moura et al., 2005).

Insecticidal and other biological activity: *A. conyzides* has bioactivity that may have agricultural use, as shown by several research investigations in different countries. Vyas and Mulchandani (1986a) reported the action of cromenes (precocenes I and II), isolated from *Ageratum* plants, which accelerate larval metamorphosis, resulted in juvenile forms or weak and small adults. *A. conyzides* also induces morphogenetic abnormalities in the formation on mosquitoes larva (*Culex*

quinquefasciatus, Aedes aegypt and *Anopheles stephensi*). This has been verified by using petroleum ether extracts (5 and 10 mg/l) of the whole plants. The larvae showed intermediary stages between larvae-pupae, discoloured and longer pupae, as well as incompletely develop adults (Sujatha, Nisar and Jadhi, 1988 quoted in Ming, 1999). The crude extract of *A. conyzoides* was found to suppress to population of *Anopheles stephensi* (Diptera: Culicidae), a major vector for malaria in urban population at higher dosages (Saxena and Saxen, 1992).

The presence of *A. conyzoides* can also be used as seed inhibitor, decreasing development of several plants. Xuan, Shinkichi, Hong, Khanh and Min (2004) reported allelopathic effects of *A. conyzoides* that showed strong inhibition on germination and growth of *Raphanus sativus* L. (radish). The leaves of this plant applied at 2 t/ha reduced about 70% of the growth of *Echinochloa crus-galli* var. f*ormosensis* Ohwi. and completely inhibited emergence of *Monochoria vaginalis* and *Aeschynomene indica* L. in calcareous soil condition. In addition, application of *A. conyzides* leave at 2 t/ha in paddy field for 2 days after transplanting caused about 75% paddy weed reduction and increased yield by 14% compared with a herbicide treatment. As well as, Sriwatcharakul (1998) in Thailand, reported that crude extract (0.1 g) of this species inhibited growth of root and shoot of rice.

Antibacterial activity studies: Aqueous extract from leaves of *A. conyzides* showed a significant control of the growth of *Alkaligens viscolactis*, *Klebsiella aerogenes*, *Bacillus cereus* and *Streptococcus pyogenas* (Perumal et al., 1999). Methanolic extract of the whole plant also has antimicrobial activity (Almagboul et al., 1985 quoted in Ming, 1999). Essential oil inhibited 22 bacteria, including gram-

positive cocci, gram-positive rods and gram-negative rods (Pattnaik, Subramanyam and Kole, 1996 quoted in Okunade, 2002)

Chemical constituents: *A. conyzoides* are widely investigated, especially in chemical studies. Many compounds such as, flavonoids, coumarins, terpenoids, tannins and pyrolizidine alkaloids isolated from this plant, have been reported. The chemical compounds of *A. conyzoides* are shown in Table 2.

Table 2: Chemical compounds of *A. conyzoides* L.

Chemical compound	Chemical group	Plant part	References
α- Pinene	monoterpene	aerial part	Rana and Blazquez (2003)
β-Boubonene	sesquiterpene	aerial part	Rana and Blazquez (2003)
α-Bergamotene	sesquiterpene	shoot	Duke, Online (2005)
δ-Cadinene	sesquiterpene	aerial part	Rana and Blazquez (2003)
γ-Cadinene	sesquiterpene	not specified	Sharma, Gary, Girgyne and Jain (1980) quoted in Vajrodaya (1986)
β-Caryophyllene	sesquiterpene	leaf, stem, flower, shoot	Mensah, Sarpong, Baser and Ozek (1993); Rana and Blazquez (2003)
α-Copaene	sesquiterpene	aerial part	Rana and Blazquez (2003)
ρ-Coumaric acid	hydroxybenzoic acid	leaf	Xuan et al. (2004)
α-Cubenene	essential oil	aerial part	Rana and Blazquez (2003)
β-Cubenene	sesquiterpene	shoot	Duke, Online (2005)
ρ-Cymene	monoterpene	aerial parts	Rana and Blazquez (2003)
β-Farnesene	sesquiterpene	whole plant	Duke, Online (2005)
α-Humulene	sesquiterpene	aerial parts	Rana and Blazquez (2003)
ρ-Hydroxybenzoic acid	hydroxybenzoic acid	leaf, stem	Xuan et al. (2004)
α-Muurolene	sesquiterpene	shoot	Duke, Online (2005)

Table 2 (Continued)

Chemical compound	Chemical group	Plant part	References
β-Pinene	monoterpene	aerial parts	Rana and Blazquez (2003)
β-Sesquiphellandrene	sesquiterpene	aerial parts	Rana and Blazquez (2003)
β-Sinensal	sesquiterpene	aerial parts	Rana and Blazquez (2003)
β-Selinene	sesquiterpene	whole plant	Duke, Online (2005)
β-Sitosterol	sterols	whole plant	Hui and Lee (1971)
α-Spinasterol	sterols	whole plant	Horng, Lin and Chen (1976) quoted in Vajrodaya (1986)
α-Terpinene	monoterpene	whole plant	Duke, Online (2005)
1,8-Cineole	monoterpene	aerial parts	Rana and Blazquez (2003)
1-Oceten-3-ol	monoterpene	aerial parts	Rana and Blazquez (2003)
1,2-desifropirrolizidinic	alkaloids	not specifies	Ming (1999)
2-carene	essential oil	shoot	Duke, Online (2005)
2-Heptanone	monoterpene	aerial parts	Rana and Blazquez (2003)
2-Hexen-1-ol	monoterpene	aerial parts	Rana and Blazquez (2003)
2-Hexenal	monoterpene	aerial parts	Rana and Blazquez (2003)
3-Hexen-1-ol	monoterpene	aerial parts	Rana and Blazquez (2003)
3-Octanol	monoterpene	aerial parts	Rana and Blazquez (2003)
5,6,7,8,3',4',5'-heptamethoxyflavone	flavonoids	whole plant	Adesogan and Okunade (1979)
5,6,8,3',4',5'-hexamethoxyflavone	flavonoids	aerial part	Gonzales, Aguiar, Grillo, Luis, Rivera and Calle (1991b)
5'-methoxynobiletin	flavonoids	leaf, stem	Vajrodaya (1986)

Table 2 (Continued)

Chemical compound	Chemical group	Plant part	References
6,10,14-Trimethyl-2-pentadecanone	sesquiterpene	aerial parts	Rana and Blazquez (2003)
6-demthoxy-ageratochromene (Precocene I)	chromene	flower, leaf, stem	Riaz, Khalid and Chavdhary (1995)
6-Methoxyquinoline-1-oxide	terpenoids	aerial parts	Rana and Blazquez (2003)
6-Vinly-7-methoxy-2,2-dimethl-chromene	sesquiterpene	aerial parts	Rana and Blazquez (2003)
8-hydroxy-5,6,7,3',4',5'-hexamethoxyflavone	flavonoids	aerial part	Gonzales et al. (1991b)
Ageratochromene (prococene II)	chromene	leaf, stem, flower	Riaz et al. (1995); Rana and Blazquez (2003)
Benzaldehyde	monoterpene	aerial parts	Rana and Blazquez (2003)
Bergamotene	sesquiterpene	aerial parts	Rana and Blazquez (2003)
Borneol	monoterpene	aerial parts	Rana and Blazquez (2003)
Bornyl acetate	monoterpene	aerial parts	Rana and Blazquez (2003)
Bornyl-formate	monoterpene	shoot	Duke, Online (2005)
Caffeic acid	lignan	leaf	Ramachandran, Kotiyal and Subramania (1977) quoted in Vajrodaya (1986)

Table 2 (Continued)

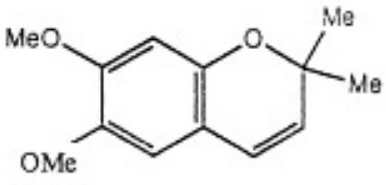
Chemical compound	Chemical group	Plant part	References
Cadinol	essential oil	shoot	Duke, Online (2005)
Camphene	monoterpene	aerial parts	Rana and Blazquez (2003)
Camphor	monoterpene	aerial parts	Rana and Blazquez (2003)
Caryophllene oxide	sesquiterpene	aerial parts	Rana and Blazquez (2003)
Catechin	terpenoids	stem	Xuan et al. (2004)
Conyzorigun	chromene	leaf	Andesogan and Okunade (1978)
Coumalic acid	hydroxybenzoic acid	leaf, stem, root	Xuan et al. (2004)
Coumarin	coumarin	leaf	Vajrodaya (1986)
Cubenol	sesquiterpene	aerial parts	Rana and Blazquez (2003)
D-neralidol	sesquiterpene	aerial parts	Rana and Blazquez (2003)
Demethoxy-encecalin	chromene	shoot	Duke, Online (2005)
Dihydrodemethoxy encecalin	chromene	shoot	Duke, Online (2005)
Dihydroencecalin	chromene	shoot	Duke, Online (2005)
Dotriacontene	terpenoids	whole plant	Duke, Online (2005)
Eicosane	alkaloids	shoot	Duke, Online (2005)
Eupalestin	flavone	whole plant, leaf	Vyas and Mulchandani (1986b); Vajrodaya (1986)
Farnesol	sesquiterpene	aerial parts	Rana and Blazquez (2003)
Friedlin	terpenoids	whole plant	Hui and Lee (1971)

Table 2 (Continued)

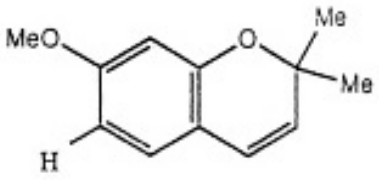
Chemical compound	Chemical group	Plant part	References
Fumaric acid	alkenes	leaf	Ramachadran et al. (1977) quoted in Vajrodaya (1999)
Gallic acid	phenolic acid	leaf, stem, root	Xuan et al. (2004)
Geraniol	monoterpene	aerial parts	Rana and Blazquez (2003)
Germacrene-D	sesquiterpene	aerial parts	Rana and Blazquez (2003)
Heptanal	monoterpene	aerial parts	Rana and Blazquez (2003)
Humulene oxide	sesquiterpene	aerial parts	Rana and Blazquez (2003)
Kaempferol	flavonoids	leaf	Ramachadran et al. (1977)
Kaempferol-3,7-di-o-β-D-glucoside	flavonoids	aerial parts	Gill, Mionskowski, Janczewska and Kapsa (1978) quoted Vajrodaya (1986)
Limonene	monoterpene	aerial parts	Rana and Blazquez (2003)
Linalool	monoterpene	aerial parts	Rana and Blazquez (2003)
Linderoflavone B	flavonoids	whole plant	Vyas and Mulchandani (1986b)
n-Hentriacontane	alkane	entire plant	Horng et al. (1976) quoted in Vajrodaya (1986)
n-Heptacosane	alkane	entire plant	Horng et al. (1976) quoted in Vajrodaya (1986)

Table 2 (Continued)

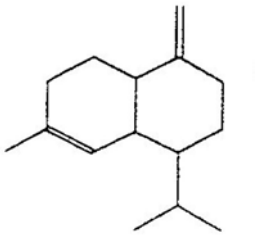
Chemical compound	Chemical group	Plant part	References
Nobiletin	terpenoids	whole plant	Vyas and Mulchandani (1986b)
Phenylethyl alcohol	monoterpene	aerial parts	Rana and Blazquez (2003)
Protocatechuic acid	-	leaf, stem, root	Xuan et al. (2004)
Quercitrin	flavonoids	aerial parts	Gill et al. (1978) quoted in Vajrodaya (1986)
Quercetin-3,7-diglucoside	flavonoids	whole plant	Duke, Online (2005)
Quercetin-3-o-rhamnosylglucoside	flavonoids	whole plant	Duke, Online (2005)
Sabinene	monoterpene	leaf	Xuan et al. (2004)
Sitosterol	sterol	leaf	Gonzales et al. (1991a)
Stigmast-7-en-3-β-ol	sterol	leaf	Ramachadran et al. (1977) quoted in Vajrodaya (1986)
Stigmasterol	sterol	whole plant	Horng et al. (1976)
Tetradecanal	essential oil	aerial parts	Rana and Blazquez (2003)
Tritriacontene	alkene	leaf	Andesogan and Okunade (1978)
Terpinolene	monoterpene	shoot	Duke, Online (2005)



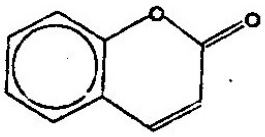
ageratochromene



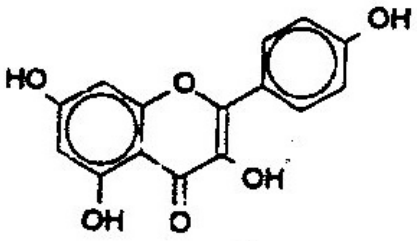
precocene I



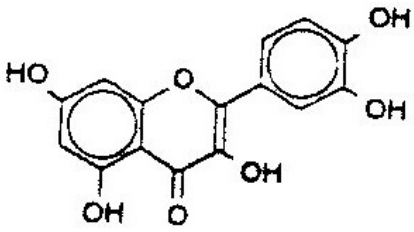
cadinene



coumarin



kaempferol



quercetin

Figure 4 Some chemical compounds of *A. conyzoides* L.

(Lide and Milne, 1995; Thakong, 1999)

2.1.3 *Tridax procumbens* L.

Vernacular name: coat buttons, tridax daisy, daisy, wild daisy, aster, Tin tukkae (Quattrocchi, 2000; เต็ม สมิตินันทน์, 2544)

Taxonomy classification:

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Asterales

Family: Asteraceae

Genus: *Tridax* L.

Species: *Tridax procumbens* Linn.

Figure 5 Aerial parts of *T. procumbens* L.

Description: *Tridax procumbens* L. is a semi-prostrate annual or short-lived perennial, with stems up to 50 cm long. It is a weed of pastures and a wide range of annual and perennial crop types. The persistent pappus enables the achenes to be carried by wind over a wide range. The large number of achenes produced per plant

(50-1500), as well as the plant's spreading stems, account for this species' weediness and widespread distribution (Scher, Online, 2005).

Radanachaless and Maxwell (1994) described *T. procumbens* L. as follows:

"Decumbent, annual herb to 50 cm tall. Stems terete, rooting at the lower nodes; with sparse, softly pilose hairs; green. Leaves opposite, decussate, simple. Blades subcoriaceous, elliptic, acute at the tip, base acute and decurrent on the upper part of the petiole; mostly deeply lobed above the lower ¼ which is entire and with gradually reduced serrations towards the tip, the opposite margins usually not matching; venation pinnate with gradually reduced serrations towards the tip, the opposite margins usually not matching; venation pinnate with a distinct midnerve and with a subbasal nerve on each side of it (3-plinerved); sparsely strigose and dark green above, sparsely puberulous and pale light green underneath; 3.5-6 × 1-4.5 cm. Inflorescence axillary, solitary; peduncle similar to and more slender than the stem, hollow, 4.5-21 cm long. Head 1 per peduncle, 9-11 mm long. Involucral bracts strigose, green and often becoming dull dark reddish, united at the base; in 2 whorls each with 4-5 subequal, thickened, ovate-oblong, acutely-tipped, erect lobes; outer ones 7 × 2.5 mm, inner ones similar but with scarious margins and gradually becoming thinner, stramineous and often reddish-tipped, apiculate chaff to 6 × 2 mm towards the inside of the head. Outer flowers 6-7, ligulate (ray), female, asymmetric; corolla tube puberulous, pale light yellowish, 3 mm long; limb spreading, orbicular, 3-lobed at the tip, glabrous, cream, 4 mm diameter. Stigmas 2, spreading, yellow, 1.5 mm long; style 1, pale yellowish, 5 mm long. Pappus of individual, soft, pilose, white bristles 2 mm long. All ovaries inferior, 1-locular, puberulous, 2 mm long, with 1 basal ovule. Disc (tubular) flowers much more numerous, regular, 5-merous, bisexual,

yellow, 9-10 mm long; tube narrow, finely puberulous to nearly glabrous, 5 mm long; lobes 5, spreading, tip acute, minutely papillose inside, 0.75 mm long. Anther 5, partly exserted from the corolla, orange, 2 mm long; filaments free, inserted near the base of the corolla tube, pale orangish, 4 mm long. Pappus similar to that of the ligulate flowers, 4-5 mm long. Achenes flattened, densely brown pilose, 3 × 1 mm, crowned by the pappus."

Distribution: native to tropical America, now naturalized throughout the rest of the tropics

Reproduction biology: flowering and fruiting throughout the year

Traditional medicinal usage: *T. procambens* is commonly used in Indian traditional medicine as anticoagulant, hair tonic, antifungal, insect repellent, in bronchial catarrh, diarrhea, and dysentery. More over it possesses wound healing activity and promotes hair growth (Saraf, Pathak and Dixit, 1991 quote in Ravikumar, Shivashangari and Devaki, in press).

Pharmacological studies: *T. procambens* has significantly reduced exudate volume leucocyte migration, edema fluid, granuloma tissue and glutamyl transpeptidase and indicate good anti-flammatory action (Diwan, Karwand, Margaret and Sattur, 1989). Ravikumar, Shivashangari and Devaki (in press) reported that *T. procumbens* extract afforded a significant protection against D-GalN/LPS-induced liver injury by maintaining the levels to near normal. Tiwari, Rastogi, Singh, Saraf

and Vyas (2004) also investigated the immunomodulatory properties of ethanol insoluble fraction of aqueous extract of *T. procumbens* L. (TPEIF). After intraperitoneal administration of TPEIF in dose of 0.25 and 0.5 g/kg body weight a significant increased in phagocytic index and leukocyte count.

Bioactivity studies: Chloroform extract of *T. procambens* has a certain herbicide effect on seedling growth of *Lactuca sativa* L. var Grand rapid (Krautmann and Riscala, Online, 2000).

Antibacterial activity studies: Aqueous extract from leaves of *T. procambens* were active against *B. cerues* and *Streptococcus pyogenas* (Perumal et al., 1999). Ethanol, water and dichloromethane extracts from aerial parts of *T. procambens* inhibited *P. aeruginosa*, *Salmonella typhi* and *S. aureus* (Caceres et al., 1998).

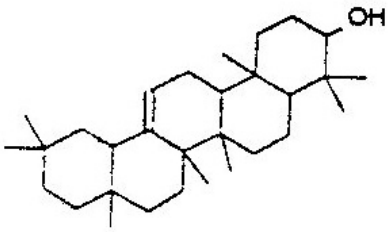
Chemical constituents: The chemical compounds of this plant are flavonoids, tannin and sesquiterpene hydrocarbon. The chemical composition of *T. procumbens* is shown in Table 3.

Table 3: Chemical compounds of *T. procumbens* L.

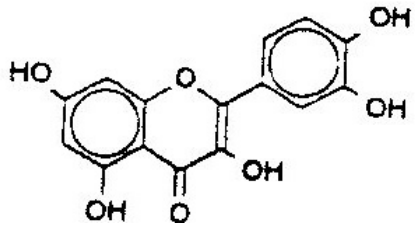
Chemical compound	Chemical grop	Plant parts	References
β-amyrin	triterpenoids	whole plant	Verna and Gupta (1988)
β-amyrone	triterpenoids	whole plant	Verna and Gupta (1988)
β-sitosterol	sterol	stem, leaf, flower	Kasture and Wadodkar (1971)quoted in ดนัย กิจชัยนุกูล และกมล สิกขมาน (2529)
β-sitosterol-3-o-β-D-xylopyranoside	saponin	flower	Saxena and Albert (2005)
β-sitosterylglucoside	-	flower	ดนัย กิจชัยนุกูล และกมล สิกขมาน (2529)
1-(2,2-dimethyl-3-hydroxypropyl)-2-isobutyl phthalate	lipids	whole plant	Verna and Gupta (1988)
10-oxoheptadecane	lipids	whole plant	Verna and Gupta (1988)
Δ_{12}-dehydrolupen-3-one	lipids	whole plant	Verna and Gupta (1988)
12-hydroxytetracosan-15-one	lipids	whole plant	Verna and Gupta (1988)
30–methyl-28-oxodotriacont-29-en-1-oic acid	lipids	whole plant	Verna and Gupta (1988)
32-methyl-30-oxotetratriacont-31-en-1-ol	lipids	whole plant	Verna and Gupta (1988)

Table 3 (Continued)

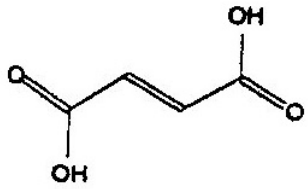
Chemical compound	Chemical grop	Plant parts	References
3-methylnonadecyl benzene	lipids	whole plant	Verna and Gupta (1988)
9-oxoheptadecane	alkanes	whole plant	Verna and Gupta (1988)
Fucosterol	sterol	whole plant	Verna and Gupta (1988)
Glucoluteolin	flavonoids	flower	Subramanian, Nair and Ramakrishman (1968)
Isoquercetin	terpenoids	flower	Duke, Online (2005)
Lupeol	sterols	whole plant	Verna and Gupta (1988)
Luteolin	flavonoids	flower	Subramanian et al. (1968)
Methlyl 14-oxononacosanoate	lipids	whole plant	Verna and Gupta (1988)
Methyl 14-oxooctadecanoate	lipids	whole plant	Verna and Gupta (1988)
Oleanolic acid	triterpene	whole plant	Ali, Jahangir, Hussan and Choudhary (2002)
Quercetin	flavonoids	flower	Subramanian et al. (1968)
procumbetin	flavonoids	aerial part	Ali, Ravinder and Ramchandram (2001)
Stigmasterol	sterols	flower	ดนัย กิจชัยนุกูล และกมล สิกขมาน (2529)



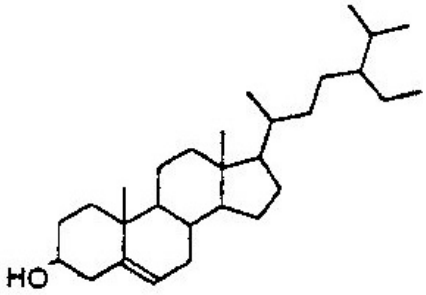
β-amyrin



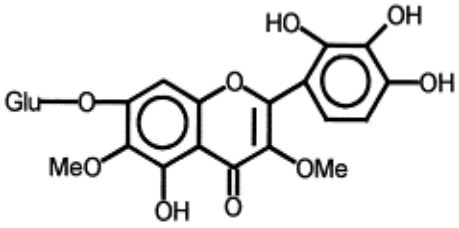
quercetin



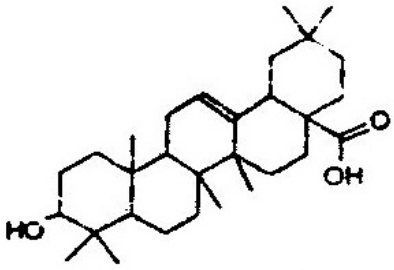
fumaric acid



oleanolicacid



procumbetin



β-sitosterol

Figure 6 Some chemical compounds of *T. procumbens* L.

(Ikan, 1991; Lide and Milne, 1995)

2.2 Soxhlet extraction

The soxhlet extractor (Figure 7) is one of the extraction systems. It is a type of laboratory glassware invented in 1879 by Franz von Soxhlet. It was originally designed for the extraction of lipid from a solid material. Typically, dry test material is placed inside a “thimble” made from filter paper, which is loaded, into the soxhlet extractor. The extractor is attached to a flask containing a solvent and condenser. The solvent is heated, causing it to evaporate. The hot solvent vapor travels up to the condenser. Where it cools and drips down onto the test material. The chamber containing the test material slowly fills with warm solvent until, when it is almost full, it is emptied by siphon action, back down to the flask. This cycle may be allowed to repeat many times (Wikipedia, Online, 2005).

Figure 7 Soxhlet extractor apparatus

2.3 Soybeans

Soybean (*Glycine max* (L.) Merr.) is one of the oldest crops of the Far East. For centuries the Chinese and other Oriental people, including Japanese, Korean, and Southeast Asian, have used the bean in various forms as one of the most important sources of dietary protein and oil. For this reason and because the amount of protein produced by soybeans per unit area of land is higher than that of any other crop.

Scientific Name: *Glycine max* (L.) Merr.

Taxonomy classification:

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Rosales

Family: Leguminosae

Genus: *Glycine* L.

Species: *Glycine max* (Merr.)

Description: Soybean is an annual plant 75 to 125 cm in height, sparsely branched, bush-type growth habit with pinnately trifoliolate leaves. The leaflets are broadly ovate, oval to elliptic-lanceolate. The purple or white flowers are borne on short axillary racemes on reduced peduncles. The pods are straight or slightly cured, usually hirsute. The one to three seeds per pod are usually ovoid to subspherical. The seed coats range in color from light yellow, olive green, and brown to reddish black. The seeds weigh from about 10 to 20 g per 100 seeds (Hymowitz and Singh, 1978).

Radanachaless and Maxwell (1994) described *Glycine max* (L.). Merr. as follows:

" Erect, cultivated, usually unbranched, annual crop herb to 75 cm tall. Older with globose nitrogen-fixing nodules 2.5-3.5 mm diameter. Stems terete, green, densely covered with brown, spreading hirsute indumentum, the hairs 2 mm long. Leaves spirally arranged, pinnately trifoliate, well-spaced on the stem, to 27 cm long. Leaflets thin, tip rounded to acute, with a terminal mucro, base rounded to slightly acute; entire; venation pinnate with distinct nerves; very finely and sparsely puberulous on both sides, dark green above, light green underneath; lateral leaflets opposite, asymmetrically ovate to suborbicular, 5-11 × 3.5-6.5 cm; terminal leaflet symmetrically elliptic, 4.5-10.5 × 3-6.5 cm. Leaf axes similar to the stem; petioles 8,5-15.5 cm long, petiolules of side leafltets 4-6 mm long, stalk of the terminal leaflet 14-30 mm long. Stipules ovate, tip acute, base rounded and basifixed, hirsute, green, 5-6 × 3 mm, persistant. Stipels subulate, 2.5-3 mm long; pulvini thickened. Inflorescences axillary, racemose, 12-25 mm long. Axes similar to the stems, pedicels 2 mm long. Bract 1 per flower, elliptic, tip acute, hirsute, 3 × 1.5 mm. Bracteoles 2 per flower, inserted at the base of the calyx, ovate-oblong, tip acute, sparsely hirsute, 3 × 1 mm. Flowers few to several per inflorescence; irregular, 5-merous, bisexual. Calyx campanulate, sparsely hispid, pale light green, 6 mm long; tube 2 mm long; lobes 5 (3+2); upper lobe 3 mm long, halfway bifid, tips acute, 1.5 mm long; lower 3 lobes subequal, ovate-lanceolate, tip acute, 3.5-4 × 1.5 mm. Petals thin, glabrous, rarely all white. Standard symmetrically orbicular, tip broadly rounded and shallowly emarginate, base clawed (2 mm), pink with violet veins and often with purple above the claw, 8 mm diameter. Wings asymmetrically oblanceolate, tip broadly rounded.

Base clawed (2 mm) and lobed on one side, white and with a very faint lilac hue, 8 × 3 mm. Kell petals asymmetrically obovate-oblong, ventrally connate in the upper part, tip rounded, base clawed (2 mm), lilac, 5 × 2 mm. Stamens 10, diadelphous (9+1), glabrous, 3.5 mm long. Anthers 2-locular, cream, 0.4 mm diameter; free tips of the filament tube of alternate lengths, white, 0.5 and 0.75 mm long; filament tube whitish, 3 mm long. Stigma capitate, cream, 0.4 mm diameter; style curved, basal part thickened, white, 2 mm long. Ovary superior. Hirsute, 2 mm long; 1-locular with several parietal ovules. Fruiting racemes slightly accrescent. Pods reflexed, flat, linear-lanceolate, tip rounded and with a mucro, slightly curved, both margins entire, light green with brownis, hispid indumentum otside, whitish and glabrous inside; seed areas slightly swollen; 4-6 cm × 9-12 mm; splitting into 2 pieces. Seeds 2-4 per pod, ellipsoid, biconvex, both ends rounded, smooth, pale light green with brownish, hispid indumentum outside, whitish and glabrous inside; seed areas slightly swollen; 4-6 cm × 9-12 mm; splitting into 2 pieces. Seeds 2-4 per pod. Ellipsoid, biconvex, both ends rounded, smooth, pale light yellowish-cream and later more tan, 9-10 × 7-8 mm; hillum distinct on one side."

Distributions: It is generally agreed that the species of soybean originated in China, Soybean expanded throughout China and its peninsular Korea by the first century A.D. Movement to Japan, Southeast Asia and South Central Asia occurred from the first century to the 16th century A.D. (Singh et al., Online, 1998).

Disease and pests: Soybeans are susceptible to attacks by various diseases and pests throughout their growing season. More than 100 pathogens are known to

affect soybeans; about 35 are important economically (Kennedy and Sinclair, 1989). All parts of the soybean plant are susceptible to a number of pathogens that reduce the quality and quantity of seed yields. The extent of losses depends upon the pathogen or condition involved, state of plant development and health when infection occurred, severity of the disease on individual plants and number of infected plants.

Brown leafspot, frogeye leafspot, brown stem rot, phytophthora root rot, stem canker, purple seed stain, pod and stem blight are major soybean fungal diseases. Bacterial blight, pustule, wildfire and wilt are major soybean diseases caused by bacteria. Major viral diseases include soybean mosaic, yellow mosaic, bud blight, and bean pod mottle. Many species of nematodes attack soybeans, with soybean cyst nematode and root knot nematode being the major problem.

Rotating crops, spraying chemicals and choosing resistant cultivars brought about by plant breeding have been the major tool used by farmers to control disease and pests (Liu, 1997).

Soybean growth stages: Vegetative stages are determined by counting the number of node on the main stem, beginning with the unifoliolate node, which have or have had a completely unrolled leaf. A leaf is considered completely unrolled when the leaf at the node immediately above it has unrolled sufficiently so the two edges of each leaflet are no longer touching. At the terminal node on the main stem, the leaf is considered completely unrolled when the leaflets are flat and similar in appearance to older leaves on the plant. Reproductive stages R1 and R2 are based on flowering, R3 and R4 on pod development, R5 and R6 on seed development and R7 and R8 on maturation (Fehr et al., 1971).

Table 4 Growth stage key for soybean disease evaluation (Fehr et al., 1971)

Stage	Description
V_1	Completely unrolled leaf at the unifoliolate node
V_2	Completely unrolled leaf at the first node above the unifoliolate node
V_3	Three nodes on the main stem, beginning with the unifoliolate node
V_N	*N* nodes on the main stem, beginning with the unifoliolate node
R_1	One flower at any node
R_2	Flower at node immediately below the upper mostnode with a completely unrolled leaf
R_3	Pod 0.5 cm (0.25 in.) long at one of the four uppermost nodes with a completely unrolled leaf
R_4	Pod 2 cm (0.75 in.) long at one of the four uppermost nodes with a completely unrolled leaf
R_5	Beans beginning to develop (can be felt when the pod is squeezed) at one of the four uppermost nodes with a completely unrolled leaf
R_6	Pod contains full-sized green beans at one of the four uppermost nodes with a completely unrolled leaf
R_7	Pods yellowing, 50% of leaves yellow (physiologic maturity)
R_8	95% of pods brown (harvest maturity)

2.4 Bacterial pustule disease

Xanthomonas campestris pv. *glycines* (*Xcg*) causes bacterial pustules. The disease symptoms are due primarily to hypertrophy of host mesophyll cells, which can result in premature defoliation of infected plants. Yields are lowered because of reduced seed size. Under certain environmental conditions, serious economic losses can occur.

Bacterial pustules have been reported in most soybean-growing areas of the world where warm weather and frequent showers prevail during the growing season. The disease has been observed in the USA since 1916 (Hedges, 1922 quoted in Hokawat and Rudolph, 1993) where it is now widespread. This disease is one of the limiting factors in improvement of soybean production in Thailand.

Taxonomy classification:

Kingdom: Proteobacteria

Class: Zymobacteria

Order: Xanthomonadales

Family: Xanthomonadaceae

Genus: *Xanthomonas*

Species: *Xanthomonas campestris* pv. *glycines* (Nakano) Dye

Morphology: *X. campestris* pv. *glycines*, the causal agent of the bacterial pustule disease, is a motile, gram-negative rod, 0.5-0.9 × 1.4-2.3 μm, with a single polar flagellum. Colonies on beef infusion agar are pale yellow (becoming deeper yellow with age), small, circular, and smooth, with an entire margin. The optimal

temperature for growth is 30-33°C. The bacteria quickly hydrolyses starch, produces acids from glucose and liquefies gelatin. This bacterium produces bacteriocin, exopolysaccharides (EPS) and auxin (Kennedy and Sinclair, 1989).

Symptoms: Symptoms are generally confined to leaves. Firstly, small yellowish-green areas with reddish-brown and elevated center appear on one or both leaf surfaces. Small, raised pustules are developed in the center of the lesions, especially on the lower leaf surface (Figure 8). Sometimes similar pustules also develop on pods. Spots may merge and result in large irregular dead area which sometimes fall off, giving the leaf a ragged appearance. Heavily infected leaves turn yellow and fall off. Heavy incidence on a susceptible plant may cause complete defoliation (Dunleavy, Chamberlain and Ross, 1966 quoted in CAB International, CD-ROM, 2000). The symptoms on seedlings in the greenhouse are initially white pustules later turning into reddish-brown spots that appear on hypocotyls, cotyledons and the primary leaves. Symptoms on resistant soybean cultivars have also been reported, such as occasional small chlorotic spots, but no well-defined pustules, or light green chlorosis and slight browning, or fewer and smaller pustules (Hartwig and Lehman, 1951 quoted in Hokawat and Rudolph, 1993).

Figure 8 Bacterial pustule disease symptoms show reddish-brown raised lesions surrounded by yellow halo on the upper leaf surface.

Transmission: *X. campestris* pv. *glycines* is able to be overwintering and transmitted through seeds since the pathogen was found alive in seeds after 30 months of harvesting. Under laboratory conditions, the pathogen is recovered from infected leaves after 9 month storage in non-sterile soil at 5°C and 17% or 52% water-holding capacity (Graham, 1953). Prathuangwong (1990) found that this bacterium survived in field soil for 35-42 days long at soil surface. This disease spreads rapidly in rainy conditions. Secondary transmission of the bacterium can occur via rain splash or water splash, agricultural implements, the movements of animals and humans, foliage contact and wind in wet conditions.

Insect transmission of the causative agent is not common, but in Russia, *Carpocoris fusispinus* was thought to play a key role in the spread of the disease (CAB International, CD-ROM, 2000). Also in Thailand, ชนิดา อัมระนันทน์ (2541) reported

that bean file (*Ophiomyia phaseoli* Tryon: Diptera, Agromyzidae) are potential vector to transmit *X. campestris* pv. *glycines*.

Disease cycle and epidemiology: The pathogen over seasons in soybean seeds, in surface crop debris, weed and in other hosts (Kennedy and Sinclair, 1989). Bacteria enter the host plant through natural openings, such as stomata and wounds and pass into the intercellular spaces where they multiply. After entering, bacteria require 5-7 days to form a pustule. Due to an extracellular enzyme produced by the bacteria, leaf cells at the infected site grow longer and multiply faster than normal, resulting in increased growth in a localized area and the mass of bacteria cause epidermal expansion of leaf surfaces. These raised areas rupture and become pustules. The degree of attack associated with the number of stomata on the under surface of leaves. Several factors which may contribute to the virulence of *X. campestris* pv. *glycines* have been described, such as production of extracellular polysaccharides, indoleacetic acid and cytokinin (Feaster, 1951 quoted in CAB International, CD-ROM, 2000).

Host range: Hosts other than *Glycine max* are *Brunnichia cirrhosa* (red vine), *Phaseolus vulgaris* (kidney bean), *Phaseolus lunatus* (lima bean), *Macrotyloma uniflorum* (horsegram) and a number of other *Phaseolus* and *Vigna* spp. including *Vigna unguiculata* (cowpea) (Kennedy and Sinclair, 1989).

Geographic distribution: Bacterial pustule disease probably occurs in all the important soybean growing regions of the world where warm and moist climatic conditions prevail during the crop growing season (Kennedy and Sinclair, 1989).

Economic impact: The premature defoliation occurs due to heavy infections, which in turn produces a reduction in seed size and number. Study of Hartwig and Johnson (1953) showed that soybean yield losses, due to this disease, were 8-11%. Pre-flowering appearance of disease causes economic losses in yield (Saxena, 1977 quoted in CAB International, CD-ROM, 2000). It can be estimated that soybean yield losses of 15, 21, 38 and 53% are encountered at the 10.1-25, 25.1-50, 50.1-75 and >75% infection rates, respectively (Shukla, 1990 quoted in CAB International, CD-ROM, 2000). In Thailand, Prathuangwong and Amnuaykit (1989) reported that yield losses due to natural dissemination in the susceptible soybean cultivar were 15-40%.

Diagnostic methods: Bacterial pustule of soybean is easily recognized on the basis of its characteristic symptoms. The ooze test and suitable staining tests can check the presence of the bacterium. Confirmation of disease identity is usually made by pathogenicity tests (CAB International, CD-ROM, 2000).

Control: Rotating crops, seed treatment, spraying chemicals or antibiotics and choosing resistant variety have been the major tools used to control the bacterial pustule disease. In chemical control, there are only a few bacteriocides because of the emphasis on control of fungal diseases compared to that of bacterial diseases and

rapid development of resistant strains of the bacterial pathogen (Mew and Natural, 1993).

Generally, growers in Thailand have controlled bacterial diseases with copper bactericides and streptomycin. The most widely used bactericides are copper-based, such as copper oxychloride, copper hydroxide, cuprous oxide and Bordeaux mixture. Antibiotics, such as streptomycin and oxytetracyclin are use in agricultural bactericide. ประพันธ์ โอสถาพันธุ์, นิรมิต กิจรุ่งเรือง และสมจิตต์ กิจรุ่งเรือง (2531) reported that copper oxychloride + zineb + maneb (four application) gave highest cost-effectiveness of spraying bacteriocides in SJ.1 and SJ.4 varieties. Antibiotics, such as streptomycin and oxytetracyclin are agricultural bactericides. สุดฤดี ประเทืองวงศ์, สฤษดิพร ชูประยูร และกิติศักดิ์ อำนวยกิจ (2526) demonstrated that Aureomycin (streptomycin), Cholromycetin (250-750 ppm) and Vitavax (500-1,000 ppm) inhibited the *X. campestris* pv. *glycines* growth effectively under laboratory conditions and Aureomycin at 500 ppm was the most promising antibiotic to control the soybean pustule by increasing yield up to 50.73% when compared with the control under field conditions. Also เชษฐพันธุ์ ชูเชื้อ (2529) reported that seed treatment of seven chemical compounds, namely, Tersan 75, Thane M-45, Cupravit, Terramycin, Aureomycin, Dumocycline and Agrimycin-100 were effective against *X. campestris* pv. *glycines* under laboratory conditions and increased germination and yield of soybean seeds. In another study, the combination between seed treatment and chemical spraying can reduce severity of bacterial pustule disease (Sangawong, 1991 quoted in สุพจน์ กาเซ็ม, 2545).

However, the agricultural use of chemical and antibiotics that are several disadvantages, such as expensive, chemical residues on plant and development of resistant bacteria. Several reports have shown that some phytopathogenic bacteria resistance to copper and antibiotics. สุดฤดี ประเทืองวงศ์, วิโรจน์ เติมวัฒนากร และศักดิ์ สุนทรสิงห์ (2535) reported that 31.50% and 3.94% of 127 strains of *X. campestris* pv. *glycines* were tolerant to $CuSO_4 \cdot 5H_2O$ and antibiotics (chloramphenical, streptomycin) at 0.4 mM and 25-100 ppm, respectively.

Because of these problems associated with the use of chemical (especially copper) and antibiotic treatments, study of biological control is alternative method to control bacterial diseases. For bacterial pustule disease control, several treatments with antagonistic bacteria have been studied such as, reduction of the disease incidence was obtained by using *Pseudomonas fluorescens* B29 and B39 (Suwanto, Friska and Sudirman, 1996 quoted in สุพจน์ กาเซ็ม, 2545). Also treatment with non-pathogenic mutant strain of *X. campestris* pv. *glycines* was found to be effective in disease suppression (Rukayadi, Suwanto, Tjahjono and Harling, 2000 quoted in สุพจน์ กาเซ็ม, 2545). Study by สุพจน์ กาเซ็ม (2545) demonstrated that four bacterial epiphytic isolates from healthy soybean leaves efficiently inhibited growth and reduced infection of *X. campestris* pv. *glycines* both in laboratory and field experiments.

2.5 Major groups of antimicrobial compounds from plant

Useful antimicrobial phytochemicals can be divided into several categories.

2.5.1 Phenols

The vast majority of the plant based aromatic natural products are phenols. Phenols are compounds in which one or more hydroxyl groups are directly attached to a carbon atom of an aromatic nucleus. They are widely distributed in plants, usually in combination with sugars as glycoside. Phenols are water soluble and mildly acidic in nature. Categories of these compounds include simple phenols, flavonoids, tannins and quinones.

Simple phenols and phenolic acids: Most of the simple phenols are monomeric components of the polymeric polyphenols and acids, which make up plant tissue. These individual components are obtained by acid hydrolysis of plant tissues. The components include ρ–hydroxybenzoic acid, protocatechuic acid, vanillic, syringic, sallicylic and gallic acids.

Catechol and pyrogallol are hydroxylated phenols, shown to be toxic to microorganisms. The site and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. The mechanisms thought to be responsible for phenolic toxicity to microorganism include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more non specific interactions with the proteins (Cowan, 1999).

Quinones: The quinones form a large group of natural pigments and are found mainly in plant. Quinones are aromatic rings with two ketones substitution. The naturallly occurring quinones are divided into the benzoquinone, 2,5-dihydroxybenzoquinones, naphthoquinones and anthraquinone. The potential of quinone antimicrobial effects is great. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides and membrane-bound enzymes.

Kazmi et al. (1994) described an anthraquinone from *Cassia italica*, which was bacteriostatic for *B. anthracis*, *Corynebacterium pseudodiphthericum* and *Pseudomonas aeruginosa* and bactericidal for *P. pseudomalliae* (Cowan, 1999).

Flavonoids: The flavonoid compounds can be regarded as C_6-C_3-C_6 compound, in which each C_6 moiety is a benzene ring, the variation in the state of oxidation of the connecting C_3 moiety determining the properties and class of each such compound. The different classes within the group are distinguished by additional oxygen containing heterocyclic rings and hydroxyl groups. These include the flavanones, flavanonols, flavones, flavonols, isoflavones, chalcones, anthocyanidins, catechins, leucoanthocyanidins and aurones. Flavonoids occur in all parts of plants.

Antimicrobial activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, as described for quinones. More lipophilic flavonoids may also disrupt microbial membranes. Catechins, the most reduced form of the C_3 unit in flavonoid compounds, These compounds inhibited in vitro *Vibrio cholerae* O1, *Streptococcus mutans*, *Shigella* and other bacteria. The catechins inhibited isolated bacterial glucosyltransferases in *S. mutant* (Cowan, 1999). In addition, Mori, Nishino, Enoki

and Tawata (1987) quoted in Cushnie and Lamb (2005) suggested that B ring of the flavonoids may play a role in intercalation or hydrogen bonding with the stacking of nucleic acid bases and that this may explain the inhibitory action on DNA and RNA synthesis.

Tannin: Tannins are chemically complex substances. They usually occur as a mixture of polyphenols. They comprise a large group of complex substances that are widely distributed in the plant kingdom. Their mode of antimicrobial action, may be related to their ability to inactivate microbial adhesins, enzymes, cell envelop transport proteins, etc. In addition, the toxicity of tannin on microorganism operates either by their direct action on the microbial membrane or by metal ion depletion (Nas, 2004).

Coumarins: Coumarins are phenolic substances made of fused benzene and α–pyrone ring. Several other coumarins have antimicrobial properties. Hydroxycinnamic acids, related to coumarins, seem to be inhibitory to gram- positive bacteria.

2.5.2 Alkaloids: Alkaloids are nitrogenous compounds occurring in plants. Alkaloids are generally insoluble in water and soluble in ether or chloroform and other nonpolar solvents. They very often form salts with plant acids such as quinic or meconic acid. Basic chemical structures generally found are phenylalkylamine, pyridine, piperidine, tropane, quinoline, isoquinoline, indole, carboline, imidazole, purine, phenanthrene and steroidal (De Amelio, 1999). Diterpenoid alkaloids are

commonly found to have antimicrobial properties. The mechanism of action of alkaloids such as berberine and harmane is attributed to their ability to intercalate with DNA.

2.5.3 Terpenoids and Essential oils: Terpenoids are widely distributed in nature, mostly in the plant kingdom. They may be regarded as derivatives of oligomers of isoprene. Terpene structure is $C_{10}H_{16}$ and they occur as sesquiterpenes ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$), triterpenes ($C_{30}H_{48}$), tetraterpenes ($C_{40}H_{64}$) and polyterpenes$(C_5H_8)_n$ (Ikan, 1991). Abundant sources of terpenoids are the essential oils. They consist of a complex mixture of terpenes or sesquiterpenes, alcohols, aldehydes, ketones, acids and esters. Terpenes or terpenoids are active against bacteria.

Investigations into the effects of terpenoids upon isolated bacterial membranes suggested that their activity is a function of the lipophilic properties of the constituent terpenes, the potency of their functional groups and their aqueous solubility. Their site of action appeared to be at the phospholipid bilayer, caused by biochemical mechanism catalysed by the phospholipid bilayers of the cell. These processes include the inhibition of electron transport, protein translocation, phosphorylation steps and other enzyme-dependent reaction. Their activity in whole cells appears more complex. Although a similar water solubility tendency is observed, specific statements on the action of single terpenoids in vivo have to be assessed singularly, taking in to account not only the structure of the terpenoid, but also the chemical composition of the cell wall (Dormand and Deans, 2000).

2.5.4 Polypeptides: Peptides that are inhibitory to microorganisms were first reported in 1942. They are often positively charged and contain disulfide bonds. Their mechanism of action may be the formation of ion channels in the microbial membrane or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors.

Thionins are peptides commonly found in barley and wheat. They are toxic to yeasts and gram-negative and gram-positive bacteria (De Caleya, Pascual, Olmedo and Carbonero, 1972 quoted in Cowan, 1999). Fabatin, a newly identified 47-rsidue peptide from fava beans, inhibits *E. coli*, *P. aeruginosa* and *Enterococcus hirae* (Zhang and Lewis, 1997 quoted in Cowan, 1999).

2.6 Antibacterial activity of plant extracts on *Xanthomonas* bacteria

Bacteria belonging to the genus *Xanthomonas* are important pathogens of many plants. Members of this genus have been shown to infect at least 124 monocotyledonous and 268 dicotyledonous plants (Chan and Goodwin, 1999).

Plant extracts are known to exhibit antimicrobial properties for a long time. There are several reports on using plant extracts to *Xanthomonas* diseases. Satish, Raveesha and Janardhana (1999) reported that eight plant species showed antibacterial activity to different pathovars of *X. campestris*. In addition, Akhtar, Rahber-Bhatti and Aslam (1997) demonstrated that plant diffusates of *Phyllanthus emblica*, *Acacia nilotica*, *Sapindus mukorossi* and *Terminalia chebula* inhibited *X. campestris* pv. *citri* at 50 g/l by hole-plate diffusion method and these diffusates at concentrations of 50, 20, and 10 g/l were significantly ($p<0.01$) more effective in reducing the number of canker lesions on detached leaves and fruits of Frost Marsh.

Assay conducted in Thailand by Vudhivanich (2003) showed efficacy of *Psidium guajava* leaf, *Terminalia bellerica* fruit, *Punica granatum* fruit peel, *Polyscias scutellaria* fruit and *Terminalia chebula* fruit inhibited the growth of *X. campestris* pv. *citri*, causing bacterial canker of citrus.

พรทิพย์ วงศ์แก้ว, ศุภลักษณ์ ฮอกะวัด และวรรณทนา สินสิริ (2540) reported that crude extract of *Cassia fistula*, *Cassia allota* and *Curcuma longa* could inhibit the growth of *X. campestris* pv. *vesicatoria*, *Erwinia carotovora* and *Pseudomonas solanacearum*.

กาญจนา ภิญโญภาพ (2540) demonstrated that *Alpinia galanga* have effective inhibition growth of *X. campestris* pv. *citri*. In other studies, the water extract of *Allium sativum*, *Spondias bipinnata*, *S. cytherea*, *Aster cordifolius*, *Tamarindus indica*,

Phyllanthus emblica, *Punica granatum*, *Eucalyptus globulus*, *Nymphaea stellata* and *Piper betle* could inhibit several pathovars of *X. campestris* (ชนิดา เล็กสมบรูณ์, นิพนธ์ ทวีชัย และวิชัย โฆสิตรัตน, 2543). Seed extracts of *Azadirachta indica* A. Juss. could inhibit *X. campestris* pv. *glycines* (นลินี ศิวากรณ์, สุเนตรา ภาวิจิตร, ชวรัตน์ ทับทิมไทย และวนิดา ฐิตะฐาน, อ้างถึงใน อุไรวรรณ ตวงสิน, 2544).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant materials

Three plant parts, comprising leaves, flowers and roots of *E. odoratum, A. conyzoides* and *T. procumbens* at flowering stage were collected from fields in Nakhon Ratchasima province. Excised plant parts were oven-dried at 37°C and kept in zip-lock bags at room temperature until used.

3.1.2 Bacterial isolation

Five isolates of *Xanthomonas campestris* pv. *glycines* (*Xcg*) were obtained from the Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. The isolates of *X. campestris* pv. *glycines* used in this study are listed in Table 5.

3.1.3 Culture media

- Nutrient agar Difco
- Nutrient broth Difco

Table 5 Five isolates of *Xanthomonas campestris* pv. *glycines* from the Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand

Bacterial Code	Cultivar/Line	Location	Year
239	-	Chachoengsoa	2526
241	-	Pisanulok	2525
285	-	Pisanulok	2519
553	OCB	Chaingrai	2525
728	-	Chaingmai	2530

3.1.4 Chemicals

95% Ethanol	Laboratory reagent
Dimethylsulfoxide (DMSO)	AR grade
Copper oxychloride 85% WP (Cupravit®)	Commercial grade
Sodium hypochlorite (Clorox®)	Commercial grade
Chemical Fertilizer (12-24-12, 15-15-15)	Commercial grade
Biochemical test agents	Laboratory reagent

3.1.5 Equipment

3.1.5.1 Apparatus

Soxhlet extractor apparatus	Buchi
Rotary evaporator	Buchi
Refrigerated incubator	VELP scientifica
Autoclave	Yamato

Spectrophotometer	Milton Roy
Laminar air flow	Woerden
Hot air oven	Shellab
Shaking incubator	Heto
Electric blender	National
Hot plate	VELP scientifica
Vortex mixer	Labinco
Paper disc	Whatman
Syringe filter	Whatman
Micropipettes (100-1000 microliters)	Witeg
Micropipettes (2-20 microliters)	Witeg

3.1.5.2 Glassware

Beakers (25, 50, 100, 250, 600, 1000 ml)	Pyrex
Measuring cylinders (5, 10, 25, 100, 250, 500 ml)	Simax
Petri dishes (9 cm diameter)	Petriq
Test tube	Pyrex

3.2 Methods

3.2.1 Preparation of plant extracts

Three plant parts, comprising leaves, flowers and roots of *E. odoratum, A. conyzoides* and *T. procumbens* were oven-dried at 37°C and then powdered by using electric blender. Each 50 g of the powdered plant parts were extracted with 500 ml of 95% ethanol using a Soxhlet extractor apparatus until the solution was colorless.

Subsequently, these extracts were evaporated by rotary vacuum evaporator to remove the solvent. The residues obtained were stored in refrigerator at 4°C prior to testing.

3.2.2 Incubation and subculturing of *X. campestris* pv. *glycines*

Five isolates of *X. campestris* pv. *glycines* were inoculated on nutrient agar slants and incubated at 30°C for 24 hours. These cultures were stored in a refrigerator at 4°C. Subculturing were done every 1-2 weeks. In addition, the bacterial isolates were stored in nutrient broth medium with 20% glycerol at -20°C.

3.2.3 Confirmation of isolates of *X. campestris* pv. *glycines*

3.2.3.1 Study of morphology and some biochemical characteristics of *X. campestris* pv. *glycines*

Criteria for confirmation of the bacterial isolates were gram stain, starch hydrolysis, gelatin hydrolysis, catalase test, MR-VP test, indole production, urea hydrolysis and acid production from carbohydrates (Forbes, Sahm and Weissfeld, 2002; วิชัย โฆสิตรัตน, 2531).

3.2.3.2 Pathogenicity confirmation of *X. campestris* pv. *glycine*

The bacterial suspension of each isolate was prepared by transferring the bacterial colony to nutrient broth and incubated in a shaking incubator at 30°C, 150 rpm for 24 hours (Basim, Basim and Ozcan, in press; บุษราคัม อุดมศักดิ์, 2543). The bacterial suspension was adjusted to 10^8-10^9 CFU/ml by using the absorption of bacterial suspension viable counts standard curve, and sprayed onto the foliage of the

SJ.5 soybean variety kept under greenhouse conditions. Bacterial pustule symptoms were evaluated after 3-14 days.

3.2.4 Bacterial suspension standard curve

Each isolate of *X. campestris* pv. *glycines* was inoculated into 20 ml of nutrient broth and incubated in a shaking incubator at 30°C, 150 rpm for 24 hours. The bacterial suspensions were diluted for 5 spectrophotometer readings with the absorbance range of approximately 0.05-0.25 at wavelength 600 nm. Viable counts for each absorbance reading were determined in triplicate using the spread plate technique (Eumkeb, 1999; Wistreich, 2003; วิชัย โฆสิตรัตน, 2531).

3.2.5 Antibacterial test by agar disc diffusion method

The plant extracts were dissolved in 100% dimethylsulfoxide (DMSO) to four different concentrations (10,000, 30,000, 50,000 and 100,000 ppm) and filter sterilized through a 0.45 μm filter. The density of bacterial suspension was adjusted to approximately 10^8 CFU/ml by using the absorption of bacterial suspension viable counts standard curve and 0.1 ml was spread on a nutrient agar medium. Paper discs (6 mm in diameter) were impregnated with 20 μl of the plant extracts at each concentration and placed on the inoculated agar. Negative control was DMSO and positive control was copper oxychloride at 4,000 ppm. The inoculated plate was incubated at 30°C for 24 hours. Antimicrobial activity was evaluated by measuring the zone of inhibition. The tests were conducted with three replications.

3.2.6 Antibacterial test in soybean leaves

Plant extracts at concentrations 30,000, 50,000 and 100,000 ppm dissolved in 50% ethanol were selected for further study based on the results of the in vitro test.

3.2.6.1 Protective effect of plant extract

To investigate the protective effect of the extract, trifoliate leaves of the soybean variety SJ.5 susceptible to the bacterial pustule disease were swabbed with the diluted extracts. After brief air drying, the trifoliate leaves (at V2 stage of soybean growth, approximately 3 weeks after planting) were inoculated by spraying 10^8 CFU/ml of *X. campestris* pv. *glycines* 728 suspension, after which the inoculated plants were placed in the greenhouse at a temperature range of 30-35°C for 14 days.

3.2.6.2 Curative effect of plant extract

To test the curative effect of the extracts, trifoliate leaves were inoculated with *X. campestris* pv. *glycines* 728 suspension. The inoculation was done in the same manner as for the protective effect. Subsequently, the inoculated leaves were incubated in the greenhouse for 72 hours under greenhouse conditions. After that, the trifoliate leaves were swabbed with each of the extract dilutions.

In both experiments, leaves swabbed with 50% ethanol were used as a negative control treatment, and leaves swabbed with copper oxychloride at 4,000 ppm were used as a positive control. The experiment was conducted in a completely randomized design (CRD) with three replications. Efficacy of the plant extracts was evaluated by comparing the number of lesions of *Xcg* 728 14 days after inoculation.

3.2.7 Experimental design and statistical analysis

The experimental design was a completely randomized design (CRD) and data were analyzed by analysis of variance (ANOVA) and Duncan's new multiple range test (DMRT).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 The extraction yield from each plant

The percentage of extraction yielded from each plant was calculated by using weight of dried residue extract per weight of dried plant. The results showed that leaves of *E. odoratum* presented the highest extraction yield (38.36%) and roots of *A. conyzoides* showed the lowest extraction yield (13.34%)(Table 6).

Table 6 Percentage of extraction yield obtained from each plant

Medicinal plants	Plant parts	95% Ethanolic extracts (% w/w)
Eupatorium odoratum L.	Leaves	38.36
	Flowers	24.71
	Roots	15.20
Ageratum conyzoides L.	Leaves	29.24
	Flowers	23.13
	Roots	13.34
Tridax procumbens L.	Leaves	19.00
	Flowers	18.70
	Roots	18.42

4.2 Confirmation of isolates of *Xanthomonas campestris* pv. *glycines*

4.2.1 Morphology and some biochemical characteristics of *X. campestris* pv. *glycines*

Five isolates of *X. campestris* pv. *glycines* were a gram-negative rod (Figure 9a). Colonies on nutrient agar were yellow, circular and smooth with and entire margin (Figure 9b). Catalase test was positive. Indole production and MR-VP test were negative. The bacteria could hydrolyse gelatin and starch. Acids were produced from many carbohydrates, namely glucose, lactose, arabinose, galactose, fructose, sucrose and mannose (Table 7). In this study, the characteristics of five isolates of *X. campestris* pv. *glycines* were similar to those of ภูษิต โชติธนะ (2535).

4.2.2 Pathogenicity of *X. campestris* pv. *glycines* on soybean leaves

In this study, symptoms of bacterial pustule disease appeared 6-7 days after inoculation with each isolate of *Xcg*. The lesions at first were small pale yellowish green spots with reddish brown and small pustules in the center of the lesions, especially on the lower leaf surface. Later, large irregular dead areas appeared, possibly through the merging of the lesions. Finally, the leaf became brownish and dropped off. The margin of lesions was often surrounded by a yellowish halo. The size of the lesions varied from 1-3 mm in diameter depending on soybean varieties and bacterial isolates (Figure 9c, 9d).

Table 7 Characteristic of isolates of *Xanthomonas campestris* pv. *glycines* obtained from the Department of Agriculture

Biochemical Tests	**isolates of *Xcg***				
	239	**241**	**285**	**553**	**728**
Gram reaction	-	-	-	-	-
Pigment	y	y	y	y	y
Catalase test	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+
Indole production	-	-	-	-	-
Gelatin liquefaction	+	+	+	+	+
MR test	-	-	-	-	-
VP test	-	-	-	-	-
glucose	+	+	+	+	+
lactose	+	+	+	+	+
arabinose	+	+	+	+	+
galactose	+	+	+	+	+
fructose	+	+	+	+	+
Sucrose	+	+	+	+	+
Mannose	+	+	+	+	+

Note: + Positive reaction

- Negative reaction

y Yellow

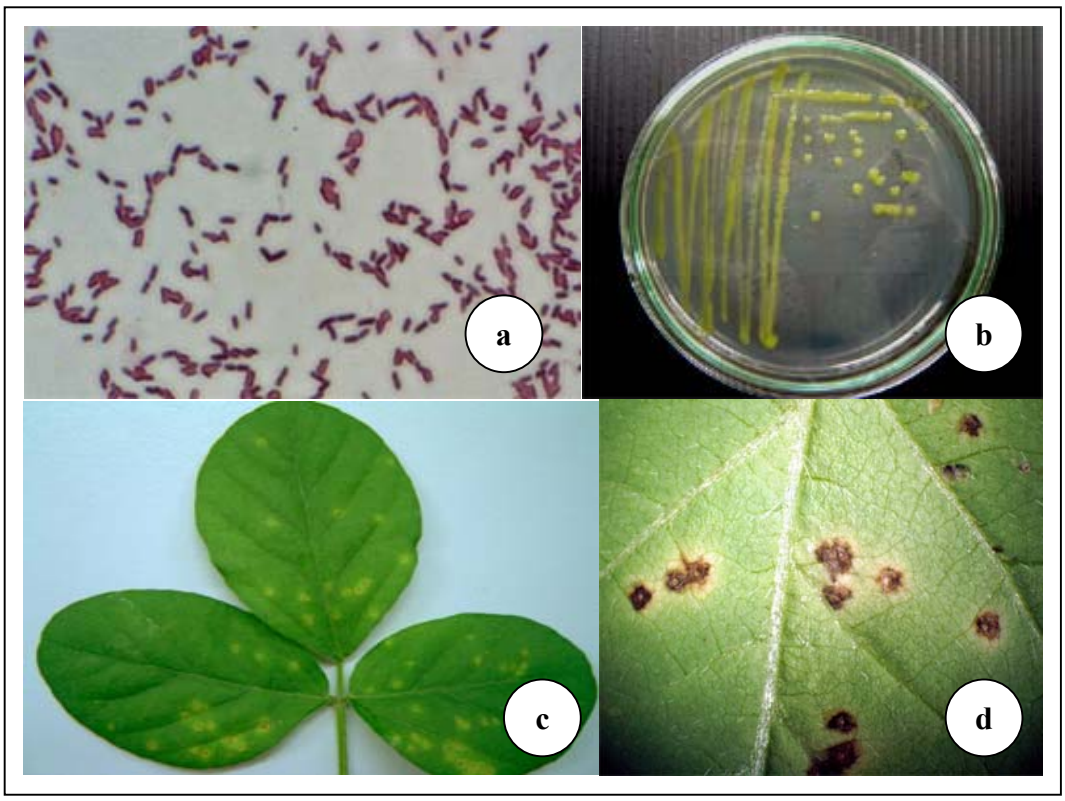


Figure 9 Bacterial pustule disease symptoms and characteristic of *Xanthomonas campestris* pv. *glycines* 728 from the Department of Agriculture

(a) Cell morphology of *X. campestris* pv. *glycines* by gram stain (1000×)

(b) Colonies of *X. campestris* pv. *glycines* on nutrient agar

(c) Small pale yellow green spots on the upper leaf surface of soybean, 7 days after inoculation with *Xcg* 728

(d) Reddish-brown pustule on the lower leaf surface of soybean, 14 days after inoculation with *Xcg* 728

4.3 Bacterial suspensions standard curve

The results of the bacterial suspensions standard curve for *Xcg* 239, 241, 285, 553 and 728 are presented in Figures 10 to 14: approximately 1×10^8 CFU/ml of isolates of *Xcg* 239, 241, 285, 553 and 728 have absorption of about 0.13, 0.2, 0.08, 0.16 and 0.13 respectively, at 600 nm.

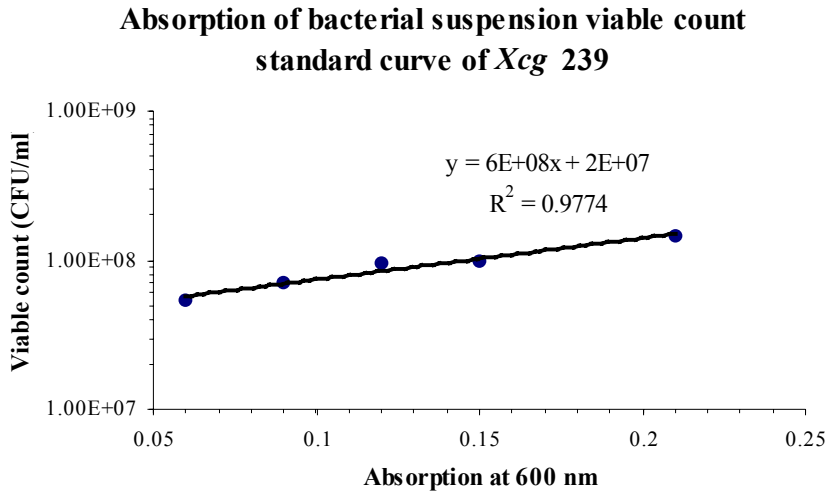


Figure 10 The standard curve for suspensions of *Xcg* 239

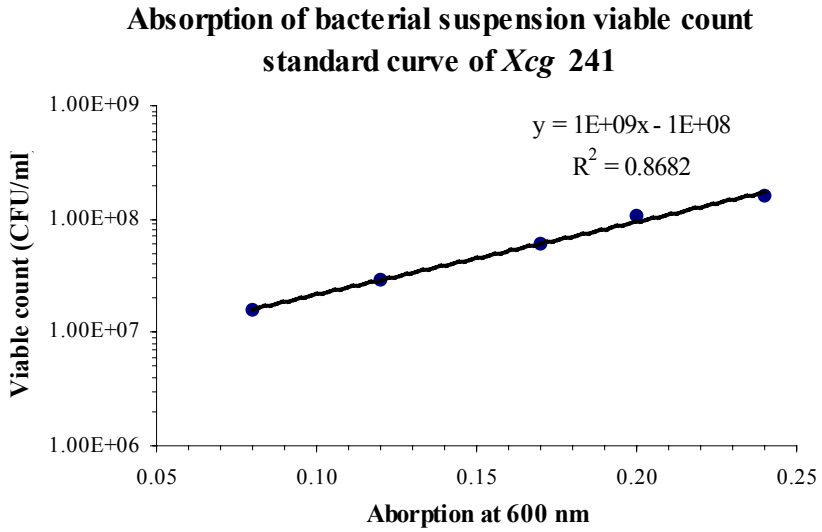


Figure 11 The standard curve for suspensions of *Xcg* 241

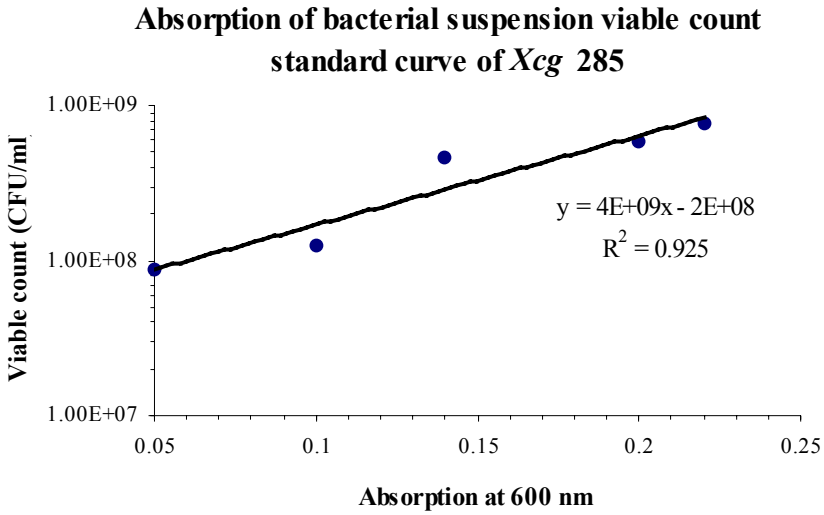


Figure 12 The standard curve for suspensions of *Xcg* 285

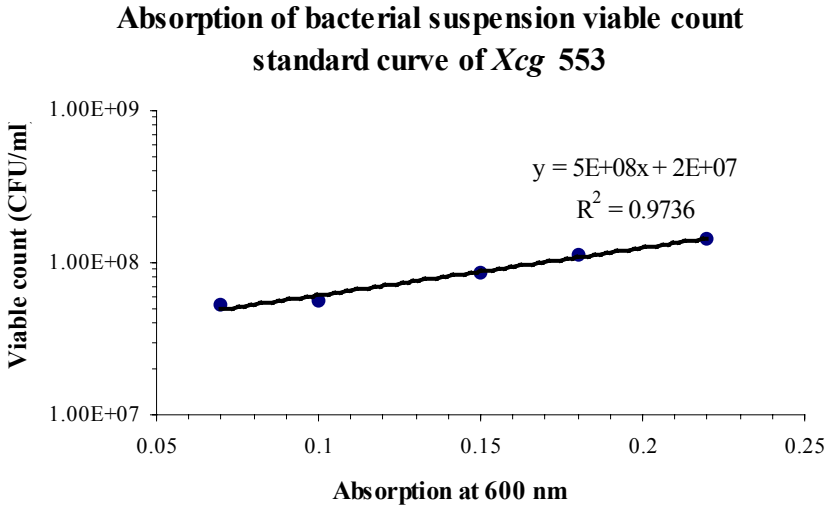


Figure 13 The standard curve for suspensions of *Xcg* 553

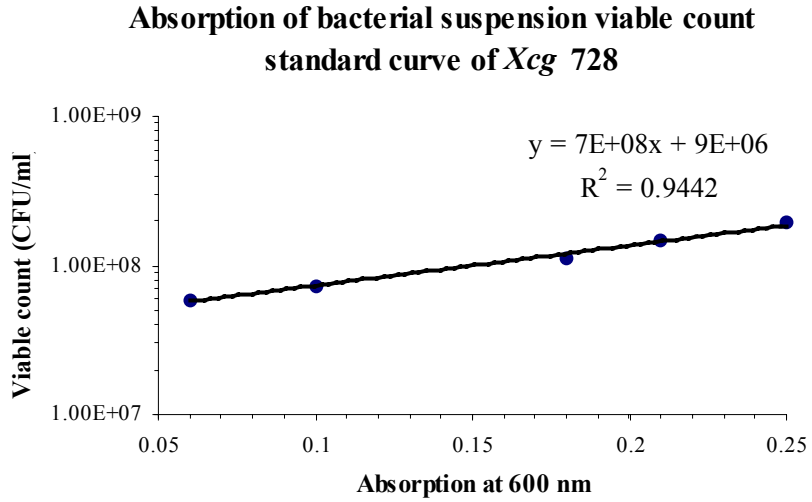


Figure 14 The standard curve for suspensions of *Xcg* 728

4.4 Antibacterial test by agar disc diffusion method

In the present study, all the extracts of *E. odoratum* leaves (EOL), *E. odoratum* flowers (EOF), *E. odoratum* roots (EOR), *A. conyzoides* leaves (ACL), *A. conyzoides* flowers (ACF), *A. conyzoides* roots (ACR), *T. procumbens* leaves (TPL), *T. procumbens* flowers (TPF) and *T. procumbens* roots (TPR) at concentrations 100,000, 50,000, 30,000 and 10,000 ppm were tested against five isolates of *X. campestris* pv. *glycines* (*Xcg* 239, 241, 285, 553 and 728) by the agar disc diffusion method.

Data on the effects of plant extracts on *Xcg* 239 are given in Figure 15 and Table 8. In *Xcg* 239, the results revealed that the leaf and flower extracts of three plants and root extracts of *T. procumbens* at all concentrations were most effective and could inhibit *Xcg* 239. The root extracts of *E. odoratum* and *A. conyzoides* also showed an inhibition effect against *Xcg* 239 at 100,000 and 50,000 ppm but did not

show inhibition zone at 30,000 and 10,000 ppm. The efficacy of crude extracts varied according to the concentration levels.

The efficacy of growth inhibition of the plant extracts at each concentration showed that at 100,000 ppm, the TPL and TPF extracts showed the largest inhibition zone (22 mm); At 50,000 ppm, the TPF extract showed the largest inhibition zone (20.33 mm); At 30,000 and 10,000 ppm, the TPL extract showed the largest inhibition zone of 16.67 and 11 mm, respectively.

From statistical analysis, these plant extracts significantly inhibited the growth of *Xcg* 239 ($p<0.05$). The extracts of TPL and TPF at 100,000 ppm showed higher inhibition against *Xcg* 239 than other plant extracts. As well as, the TPL extract indicated greater inhibition zone against this isolate than other treatments at 30,000 ppm but at 50,000 ppm, the extracts of TPL and TPF were not significantly different from the extracts of EOF, ACL and ACF. At 10,000 ppm, the plant extracts except the extracts of EOR and ACR were not different.

The growth inhibition of these plant extracts against *Xcg* 239 compared to copper oxychloride (positive control) at 4,000 ppm revealed that five plant extracts, (EOL at 100,000 ppm; ACF at 30,000 ppm; EOL at 50,000 ppm; TPF at 30,000 ppm and TPR at 50,000 ppm) were not significantly different from copper oxychloride. Twelve plant extracts, namely TPL, TPF, ACL, ACF and EOF at 100,000 and 50,000 ppm, TPR at 100,000 ppm and TPL at 30,000 ppm showed higher inhibition effect than copper oxychloride.

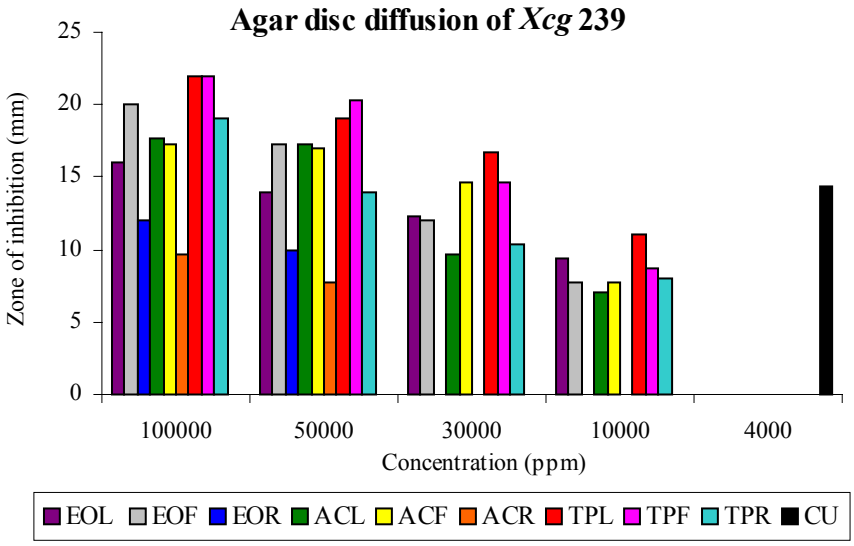


Figure 15 Antibacterial activity of *Xcg* 239 by agar disc diffusion assay

Notes: EOL = *E. odoratum* L. leaves

EOF = *E. odoratum* L. flowers

EOR = *E. odoratum* L. roots

ACL = *A. conyzoides* L. leaves

ACF = *A. conyzoides* L. flowers

ACR = *A. conyzoides* L. roots

TPL = *T. procumbens* L. leaves

TPF = *T. procumbens* L. flowers

TPR = *T. procumbens* L. roots

CU = Copper oxychloride

Table 8 Antibacterial activity of three plant parts extracts of *E. odoratum*, *A. conyzoides* and *T. procumbens* against *X. campestris* pv. *glycines* 239 by agar disc diffusion method

Plant Extracts	**Plant part**	**Mean inhibition zone diameter (mm)**[(1)]			
		Concentration (ppm)			
		100,000	**50,000**	**30,000**	**10,000**
E. odoratum	*leaves*	16.00 ^{DE}	14.00 ^{F}	12.33 ^{G}	9.33 ^{IJKL}
	flowers	20.00 ^{B}	17.33 ^{CD}	12.00 ^{GH}	7.67 ^{LM}
	roots	12.00 ^{GH}	10.00 ^{IJ}	0.00 ^{N}	0.00 ^{N}
A. conyzoides	*leaves*	17.67 ^{CD}	17.33 ^{CD}	9.67 ^{IJK}	7.00 ^{M}
	flowers	17.33 ^{CD}	17.00 ^{D}	14.67 ^{EF}	7.67 ^{LM}
	roots	9.67 ^{IJK}	7.67 ^{LM}	0.00 ^{N}	0.00 ^{N}
T. procumbens	*leaves*	22.00 ^{A}	19.00 ^{BC}	16.67 ^{D}	11.00 ^{GHI}
	flowers	22.00 ^{A}	20.33 ^{B}	14.67 ^{EF}	8.67 ^{JKLM}
	roots	19.00 ^{BC}	14.00 ^{F}	10.33 ^{HIJ}	8.00 ^{KLM}
Copper oxychloride[(2)]		14.33 ^{EF}			
DMSO		0.00 ^{N}			

Notes: Mean is the average of 3 replications, Coefficient of variation (C.V.) = 7.88 %

[(1)]Means with the same letter superscript is not significantly different at 5% level by DMRT

[(2)]Copper oxychloride at 4,000 ppm

Data on the effects of plant extracts on *Xcg* 241 are given in Figure 16 and Table 9. In *Xcg* 241, the results revealed that the extract of TPF at all concentrations inhibited *Xcg* 241 and EOL, EOF, ACL, ACF and TPL at 100,000, 50,000 and 30,000 ppm inhibited this isolate. The extracts of EOR, ACR and TPR at 100,000 and 50,000 ppm showed an inhibition effect against *Xcg* 241 and did not show inhibition zone at 30,000 and 10,000 ppm. The inhibition zones were varied related to different concentration levels of plant extracts.

The efficacy of growth inhibition of the plant extracts at each concentration showed that at 100,000 and 50,000 ppm, the extract of EOF showed largest inhibition zone of 18 and 15.33 mm respectively. At 30,000 ppm, the extract of TPL showed largest inhibition zone of 13.00 mm. At 10,000 ppm, the extract of TPF showed inhibition zone of 7.67 mm whereas the other extracts did not show inhibition zone against *Xcg* 241.

From statistical analysis, these plant extracts significantly inhibited the growth of *Xcg* 241 at 5% level ($p<0.05$). The EOF, ACF, TPL and TPF extracts at 100,000 ppm showed higher inhibition against *Xcg* 241 than other plant extracts. As well as, EOF, TPL and TPF at 50,000 ppm indicated greater inhibition zone against this isolate than other plant extracts. At 30,000 ppm, the EOR, ACR and TPR extracts were significantly different from the others. At 10,000 ppm, only TPF showed effects from other plant extracts. It showed that most plant extracts at 10,000 ppm were not effective to inhibit *Xcg* 241.

The growth inhibition of these plant extracts against *Xcg* 241 compared to copper oxychloride (positive control) at 4,000 ppm revealed that ten plant extracts, (ACL at 100,000 and 50,000 ppm; ACF at 50,000 and 30,000 ppm; ACR at 100,000

ppm; EOL at 50,000 ppm; EOF at 30,000 ppm; EOR at 100,000 ppm; TPL at 30,000 ppm and TPR at 100,000 ppm) were not statistically different from copper oxychloride. Eight plant extracts, comprising EOF, TPL, TPF at 100,000 and 50,000 ppm and EOL and ACF at 100,000 ppm showed a higher inhibition effect than copper oxychloride.

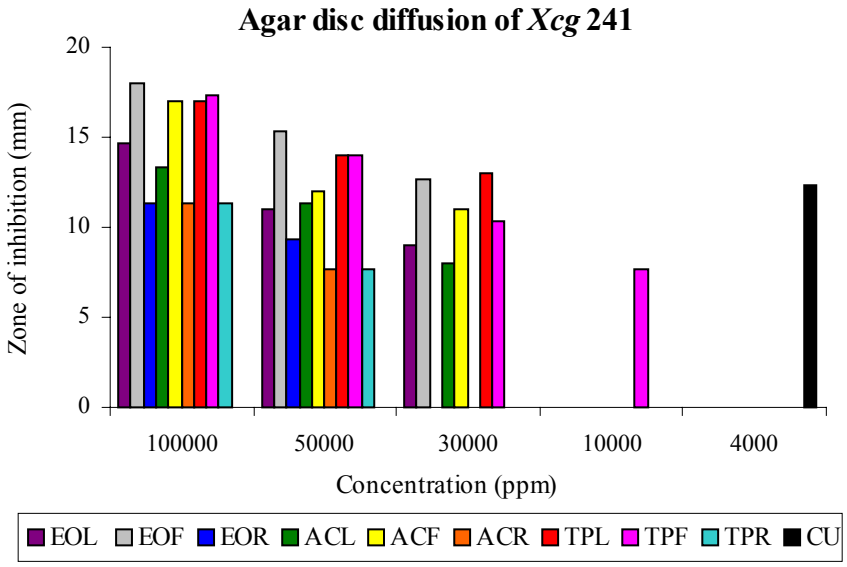


Figure 16 Antibacterial activity of *Xcg* 241 by agar disc diffusion assay

Table 9 Antibacterial activity of three plant parts extracts of *E. odoratum*, *A. conyzoides* and *T. procumbens* against *Xanthomonas campestris* pv. *glycines* 241 by agar disc diffusion method

Plant Extracts	Plant parts	Mean inhibition zone diameter (mm)[1]			
		Concentration (ppm)			
		100,000	**50,000**	**30,000**	**10,000**
E. odoratum	Leaves	14.67 ^{BC}	11.00 ^{GH}	9.00 ^{IJK}	0.00 ^{L}
	Flowers	18.00 ^{A}	15.33 ^{B}	12.67^{DEFG}	0.00 ^{L}
	Roots	11.33 ^{FGH}	9.33 ^{IJ}	0.00 ^{L}	0.00 ^{L}
A. conyzoides	Leaves	13.33 ^{CDE}	11.33 ^{FGH}	8.00 ^{JK}	0.00 ^{L}
	Flowers	17.00 ^{A}	12.00^{EFGH}	11.00 ^{GH}	0.00 ^{L}
	Roots	11.33 ^{FGH}	7.67 ^{K}	0.00 ^{L}	0.00 ^{L}
T. procumbens	Leaves	17.00 ^{A}	14.00 ^{BCD}	13.00 ^{DEF}	0.00 ^{L}
	Flowers	17.33 ^{A}	14.00 ^{BCD}	10.33 ^{HI}	7.67 ^{K}
	Roots	11.33 ^{FGH}	7.67 ^{K}	0.00 ^{L}	0.00 ^{L}
Copper oxychloride [2]		12.33 ^{EFG}			
DMSO		0.00 ^{L}			

Notes: Mean is the average of 3 replications, C.V. = 10.63 %

[1]Means with the same letter superscript is not significantly different at 5% level by DMRT

[2]Copper oxychloride at 4,000 ppm

Data on the effects of plant extracts on *Xcg* 285 are given in Figure 17 and Table 10. In *Xcg* 285, the results revealed that the extract of EOF, ACL and TPL at all concentrations inhibited *Xcg* 285. The extracts of EOL, EOR, ACF, TPF and TPR at 100,000, 50,000 and 30,000 ppm showed an inhibition effect against this isolate. The extract of ACR at 100,000 ppm showed inhibition effect against *Xcg* 285 and did not show inhibition zone at 50,000, 30,000 and 10,000 ppm. In addition, the inhibition zones were varied according to concentration levels of plant extracts.

The efficacy of growth inhibition of all plant extracts at each concentration showed that at 100,000 ppm, the extract of TPL showed the largest inhibition zone of 20.00 mm. At 50,000, 30,000 and 10,000 ppm, the extract of EOF showed the largest inhibition zone of 17.00, 14.33 and 11.33 mm, respectively. However, the extracts of EOL, EOR, ACF, ACR, TPF and TPR at 10,000 ppm did not inhibit growth of *Xcg* 285.

From statistical analysis, the plant extracts significantly inhibited the growth of *Xcg* 285 ($p<0.05$) at 100,000 ppm. As well as, the plant extracts except ACR were not significantly different at 50,000 ppm and 30,000 ppm. At 10,000 ppm, Only EOF, ACL and TPL extracts inhibited *Xcg* 285.

The growth inhibition of these plant extracts against *Xcg* 285 compared to copper oxychloride (positive control) at 4,000 ppm revealed that nine plant extracts, (ACF at 100,000, 50,000 and 30,000 ppm; ACL at 50,000 and 30,000 ppm; EOL and TPR at 50,000 ppm and EOF and TPL at 30,000 ppm) were not significantly different from copper oxychloride. Nine plant extracts, comprising EOF and TPL at 100,000 and 50,000 ppm, and EOL, EOF, ACL, TPF and TPR at 100,000 ppm showed higher inhibition effect than copper oxychloride.

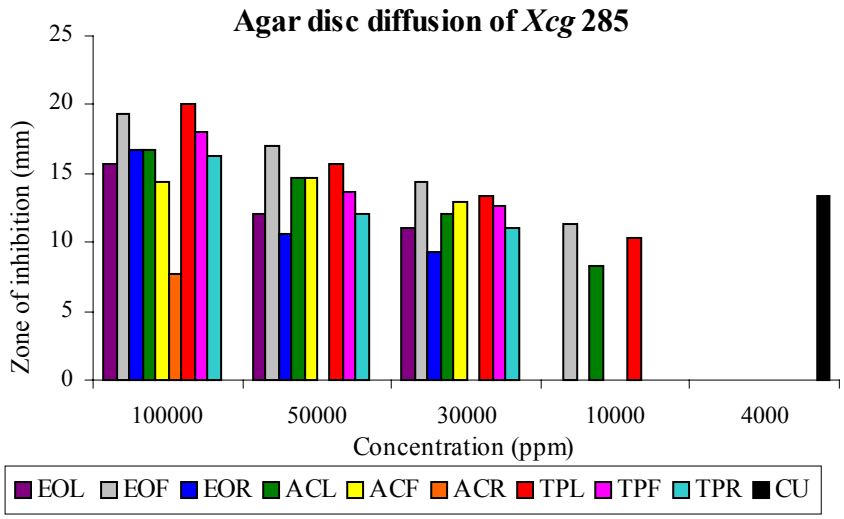


Figure 17 Antibacterial activity of *Xcg* 285 by agar disc diffusion assay

Table 10 Antibacterial activity of three plant parts extracts of *E. odoratum*, *A. conyzoides* and *T. procumbens* against *X. campestris* pv. *glycines* 285 by agar disc diffusion method

Plant Extracts	**Plant parts**	**Mean inhibition zone diameter (mm)**[(1)]			
		Concentration (ppm)			
		100,000	**50,000**	**30,000**	**10,000**
E. odoratum	Leaves	15.67 ^{DE}	12.00 ^{GHI}	11.00 ^{I}	0.00 ^{M}
	Flowers	19.33 ^{AB}	17.00 ^{CD}	14.33 ^{EF}	11.33 ^{HI}
	Roots	16.67 ^{CD}	10.67 ^{IJ}	9.33 ^{JK}	0.00 ^{M}
A. conyzoides	Leaves	16.67 ^{CD}	14.67 ^{EF}	12.00 ^{GHI}	8.33 ^{KL}
	Flowers	14.33 ^{EF}	14.67 ^{EF}	13.00 ^{FG}	0.00 ^{M}
	Roots	7.67 ^{L}	0.00 ^{M}	0.00 ^{M}	0.00 ^{M}
T. procumbens	Leaves	20.00 ^{A}	15.67 ^{DE}	13.33 ^{FG}	10.33 ^{IJ}
	Flowers	18.00 ^{BC}	13.67 ^{FG}	12.67 ^{GH}	0.00 ^{M}
	Roots	16.33 ^{D}	12.00 ^{GHI}	11.00 ^{I}	0.00 ^{M}
Copper oxychloride[(2)]		13.33 ^{FG}			
DMSO		0.00 ^{M}			

Notes: Mean is the average of 3 replications, C.V. = 8.50 %

[(1)]Means with the same letter superscript is not significantly different at 5% level by DMRT

[(2)]Copper oxychloride at 4,000 ppm

Data on the effects of plant extracts on *Xcg* 553 are given in Figure 18 and Table 11. In *Xcg* 553, the results revealed that the extracts of EOL, EOF, ACF, TPL and TPF at all concentrations and ACL and TPR at 100,000, 50,000 and 30,000 ppm inhibited this isolate. The extracts of EOR and ACR at 100,000 ppm inhibited *Xcg* 553 but did not show inhibition zone at 50,000, 30,000 and 10,000 ppm. The efficacy of crude extracts varied according to the concentration levels.

The efficacy of growth inhibition of the plant extracts at each concentration showed that at 100,000 ppm, the results revealed that the extract of ACF showed the largest inhibition zone (19.00 mm). The EOL and TPL extracts at 50,000 and 30,000 ppm showed the largest inhibition zone (13.67 and 12.00 mm, respectively). The ACF extract at 10,000 ppm showed the largest inhibition zone (8.67 mm).

From statistical analysis, the extract of ACF at 100,000 was not significantly different at 5% level ($p<0.05$) from the extracts of EOL, EOF, ACL, TPL and TPF but significantly different from the extract of EOR, ACR and TPR. Only EOR and ACR extracts at 50,000 ppm and 30,000 ppm showed significantly effects on *Xcg* 553. At 10,000 ppm, EOL, EOF, ACF, TPL and TPF extracts significantly different effects from EOR, ACL and TPR.

The growth inhibition of these extracts against *Xcg* 553 compared to copper oxychloride (positive control) at 4,000 ppm inferred that fifteen plant extracts, (TPL at 100,000, 50,000, 30,000; TPR at 100,000 and 50,000 ppm; EOL, ACL, ACF and TPF at 50,000 and 30,000 ppm; ACR at 100,000 ppm and EOF 50,000 ppm) were not significantly different from copper oxychloride. Five plant extracts (EOL, EOF, ACL, ACF and TPF at 100,000 ppm) showed higher inhibition effect than copper oxychloride.

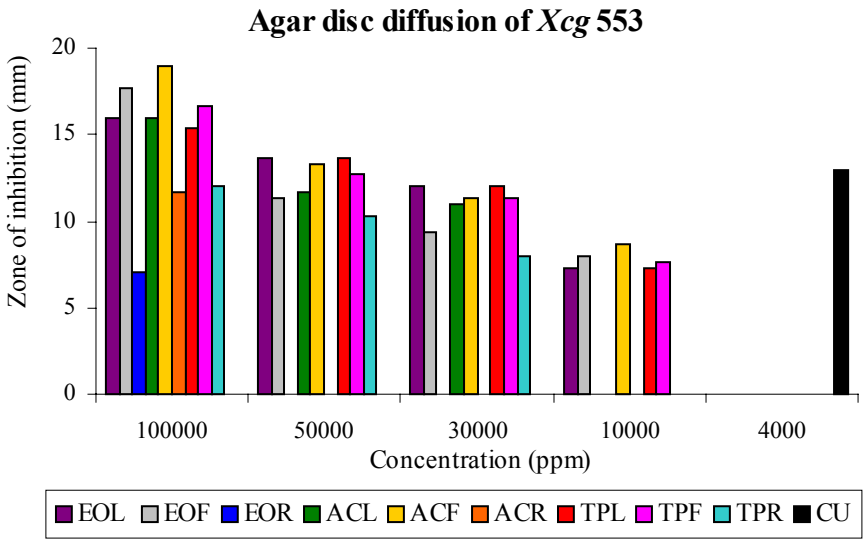


Figure 18 Antibacterial activity of *Xcg* 553 by agar disc diffusion assay

Table 11 Antibacterial activity of three plant parts extracts of *E. odoratum*, *A. conyzoides* and *T. procumbens* against *X. campestris* pv. *glycines* 553 by agar disc diffusion method

Plant Extracts	**Plant parts**	**Mean inhibition zone diameter (mm)**[(1)]			
		Concentration (ppm)			
		100,000	**50,000**	**30,000**	**10,000**
E. odoratum	Leaves	16.00 ^{BC}	13.67 ^{CDE}	12.00 ^{EFG}	7.33 ^{J}
	Flowers	17.67 ^{AB}	11.33 ^{EFG}	9.33 ^{GHIJ}	8.00 ^{IJ}
	Roots	7.00 ^{J}	0.00 ^{K}	0.00 ^{K}	0.00 ^{K}
A. conyzoides	Leaves	16.00 ^{BC}	11.67 ^{EFG}	11.00 ^{EFGH}	0.00 ^{K}
	Flowers	19.00 ^{A}	13.33 ^{DE}	11.33 ^{EFG}	8.67 ^{HIJ}
	Roots	11.67 ^{EFG}	0.00 ^{K}	0.00 ^{K}	0.00 ^{K}
T. procumbens	Leaves	15.33 ^{BCD}	13.67 ^{CDE}	12.00 ^{EFG}	7.33 ^{J}
	Flowers	16.67 ^{AB}	12.67 ^{EF}	11.33 ^{EFG}	7.67 ^{J}
	Roots	12.00 ^{EFG}	10.33 ^{FGHI}	8.00 ^{IJ}	0.00 ^{K}
Copper oxychloride[(2)]		13.00 ^{DEF}			
DMSO		0.00 ^{K}			

Notes: Mean is the average of 3 replications, C.V. = 15.63 %

[(1)]Means with the same letter superscript is not significantly different at 5% level by DMRT

[(2)]Copper oxychloride at 4,000 ppm

Data on the effects of plant extracts on *Xcg* 728 are given in Figure 19 and Table 12. In *Xcg* 728, the results revealed that the extracts of EOF, ACF and TPL at all concentrations inhibited *Xcg* 728. Extracts of EOL, ACL, TPF and TPR at 100,000, 50,000 and 30,000 ppm showed an inhibition effect against this isolate, as did the extract of ACR at 100,000 and 50,000 ppm. EOR extract inhibited *Xcg* 728 at 100,000 ppm but not at 50,000, 30,000 and 10,000 ppm. The efficacy of crude extracts varied according to the concentration levels.

The efficacy of growth inhibition of the plant extracts at each concentration showed that at 100,000 ppm, the EOF and ACF extracts showed the largest inhibition zone (19.33 mm); At 50,000 ppm, the ACF extract showed the largest inhibition zone (15.33 mm); At 30,000 ppm, the TPF extract showed the largest inhibition zone (13.67 mm) and At 10,000 ppm, the EOF extract showed an inhibition zone of 9.67 mm but the other extracts such as EOL, EOR, ACL, ACR, TPF and TPR did not inhibit the growth of *Xcg* 728.

From statistical analysis, the extracts of ACF, EOL, EOF and TPF at 100,000 ppm significantly inhibited *Xcg* 728 ($p<0.05$) compared with other plant extracts. At 50,000 ppm, the effects of EOL, ACF and TPF extracts were significantly different from the others, but at 30,000 ppm only EOR and ACR showed significant effects. At 10,000 ppm, EOF, ACF and TPL extracts showed significantly different effects from other plant extracts.

The growth inhibition of these plant extracts against *Xcg* 728 compared to copper oxychloride (positive control) at 4,000 ppm showed that eight plant extracts, (TPL at 50,000 and 30,000 ppm; ACL at 100,000 ppm; EOF and TPR at 50,000 ppm and EOL, ACF and TPF at 30,000 ppm), were not significantly different from copper

oxychloride. Nine plant extracts, comprising EOF, TPL and TPF at 100,000 ppm and EOL, ACF and TPF at 100,000 and 50,000 ppm showed a higher inhibition effect than copper oxychloride.

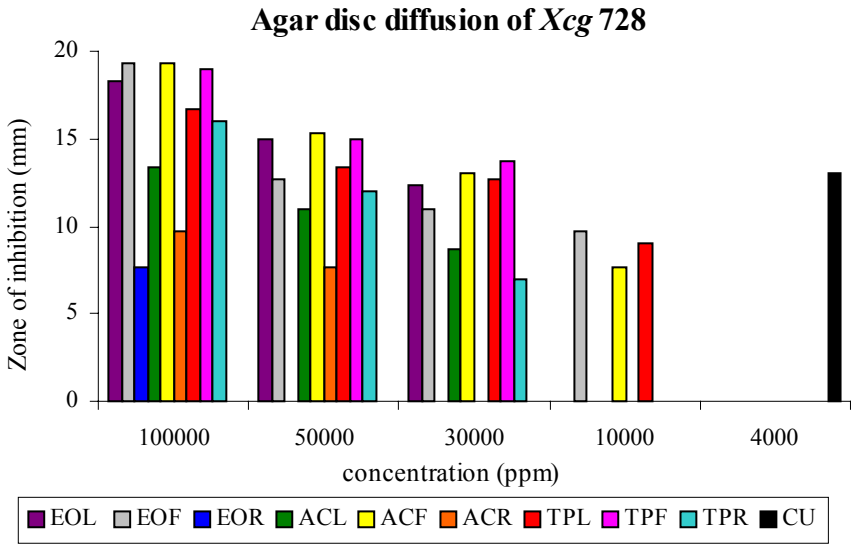


Figure 19 Antibacterial activity to *Xcg* 728 by agar disc diffusion assay

Table 12 Antibacterial activity of three plant parts extracts of *E. odoratum*, *A. conyzoides* and *T. procumbens* against *X. campestris* pv. *glycines* 728 by agar disc diffusion method

Plant Extracts	Plant parts	Mean inhibition zone diameter (mm)[1]			
		Concentration (ppm)			
		100,000	**50,000**	**30,000**	**10,000**
E. odoratum	Leaves	18.33 ^{A}	15.00 ^{BCD}	12.33 ^{EF}	0.00 ^{K}
	Flowers	19.33 ^{A}	12.67 ^{EF}	11.00 ^{FG}	9.67 ^{GH}
	Roots	7.67 ^{IJ}	0.00 ^{K}	0.00 ^{K}	0.00 ^{K}
A. conyzoides	Leaves	13.33 ^{DE}	11.00 ^{FG}	8.67 ^{HIJ}	0.00 ^{K}
	Flowers	19.33 ^{A}	15.33 ^{BC}	13.00 ^{E}	7.67 ^{IJ}
	Roots	9.67 ^{GH}	7.67 ^{IJ}	0.00 ^{K}	0.00 ^{K}
T. procumbens	Leaves	16.67 ^{B}	13.33 ^{DE}	12.67 ^{EF}	9.00 ^{HI}
	Flowers	19.00 ^{A}	15.00 ^{BCD}	13.67 ^{CDE}	0.00 ^{K}
	Roots	16.00 ^{B}	12.00 ^{EF}	7.00 ^{J}	0.00 ^{K}
Copper oxychloride [2]		13.00 ^{E}			
DMSO		0.00 ^{K}			

Notes: Mean is the average of 3 replications, C.V. = 10.25 %

[1]Means with the same letter superscript is not significantly different at 5% level by DMRT

[2]Copper oxychloride at 4,000 ppm

The results of this study are encouraging since the 95% ethanolic extracts of three plants in the Asteraceae family all showed antibacterial activity against five isolates of *X. campestris* pv. *glycines* by the agar disc diffusion method. The diameters of inhibition zones increased with the increase of concentration levels of plant extracts. Similar antibacterial properties of these plants have been reported in many studies, but most studied the antibacterial activity of the extracts against human pathogens. The various crude extracts of *E. odoratum*, *A. conyzoides* and *T. procumbens* showed significant activity against both positive and negative bacteria(Bamba et al., 1993; Irobi, 1997; Caceres et al., 1998; Perumal et al., 1999; Ming, 1999; Bouda et al., 2000; Okunade, 2002 and Chomnawang et al., 2005).

The antibacterial tests of this study also showed that the inhibition zone diameters were different according to plant parts used. The leaf and flower extracts of *T. procumbens* at 100,000 ppm exhibited the highest activity with an inhibition zone of 22.00 mm. The root extracts of *E. odoratum* and *A. conyzoides*, at all concentrations, showed minimal antibacterial activity or no inhibition zone in some bacterial isolates. However the root extract of *T. procumbens* showed an inhibition zone. Thus, it may be that the antimicrobial agent of these plants at the reproductive stage accumulates in the aerial part rather than the root. In addition, the use of leaf and flower for medicinal purposes may be more sustainable than that of other plant parts such as the root.

All isolates of *Xcg* were inhibited by all plant part extracts, but inhibition zone diameters were different according to severity of bacterial isolates. *Xcg* 239 was more susceptible to plant extracts than other isolates of *Xcg*. The growth of this isolate was more inhibited by EOF, ACL, TPL, TPF and TPR extracts at 100,000 ppm.

On the other hand *Xcg* 241 was more resistant to the extracts than other isolates of *Xcg*. Only extracts of EOR, ACR and TPR at 30,000 ppm, and no plant extracts at 10,000 ppm, could inhibit this isolate.

Antibacterial activity of these plant extracts may be due to the presence of secondary metabolites such as flavonoids, alkaloids, tannin, sesquiterpene hydrocarbon, phenolic compounds and essential oil (Subramanian et al., 1968; Kasture and Wadodkar, 1971; Talapatra et al., 1974; Horng et al., 1976; Adesogan and Okunade, 1979; Vyas and Mulchandani, 1986a, 1986b; Verna and Gupta, 1988; Gonzales et al., 1991a, 1991b; Bomba et al., 1993; Biller et al., 1994; Wollenwerer et al., 1995; Thakong, 1999; Ali, 2001; Phan et al., 2001; Ali, 2002; Rana and Blazquez, 2003; Xuan et al., 2004 and Doke, Online, 2005). Many reports supported that these compounds could inhibit many bacteria (Cowan, 1999; Cushnie and Lamb, 2005 and Dorman and Deans, 2000). For example, β-sitosterol and sitosterol-β-D-glucopyranoside inhibited *B. subtilis* at the concentration of 50 and 100 μg/ml, respectively (Beltrame et al., 2002). The study by Vijaya and Ananthan (1996), oral administration of either 142.9 mg/kg quercetin or 214.3 mg/kg quercetrin protected guinea pigs against an induced *Shigella* infection that killed untreated control animals (Cushnie and Lamb, 2005).

In general, most agar disc diffusion tests use the Mueller-Hinton medium as a standard medium. Usually, we do not use nutrient media in the agar disc diffusion tests because they tend to be complex substances that have yet to be completely characterized and chemically synthesized. In addition, this may affect the expression of the plant extract and bacterial growth.

However, the use of Mueller-Hinton medium in this study showed some disadvantages, such as the excessively slow growth of *Xcg*. Furthermore, the color of Mueller-Hinton medium was rather similar to the color of the *Xcg* colonies, and so it was difficult to measure the zone of inhibition. The fact that several previous researchers who published their work in international journals used a nutrient medium for *Xanthomonas* sp. test by agar disc diffusion method (Satish et al., 1999; Basim, Basim and Ozcan, in press and Karaman et al., 2003) indicates that the use of nutrient medium in this study should not affect *Xcg* determinations, and that the results of this agar disc diffusion test should be reliable.

4.5 Antibacterial test in soybean leaves

From previous results on the agar disc diffusion test, seven plant extracts, comprising *E. odoratum* leaves (EOL), *E. odoratum* flowers (EOF), *A. conyzoides* leaves (ACL), *A. conyzoides* flowers (ACF), *T. procumbens* leaves (TPL), *T. procumbens* flowers (TPF) and *T. procumbens* roots (TPR) at concentration of 100,000, 50,000 and 30,000 ppm were selected for an antibacterial test on soybean leaves inoculated by *X. campestris* pv. *glycines* 728 (*Xcg* 728).

4.5.1 Protective effect of plant extract

At 14 days after the inoculation of *Xcg* 728, the protective effects of the plant extracts at 100,000, 50,000 and 30,000 ppm appeared significant ($p<0.05$) in reducing the number of lesions on soybean leaves when compared to the negative control. The extract of ACF showed the highest efficacy in reducing the number of lesions (0.22 lesion/leaflet). However, there was no significant difference in the number of lesions on the soybean leaves among all concentrations of each plant extract.

When using these plant extracts to reduce incidence of bacterial pustule lesions compared to copper oxychloride at 4,000 ppm, the result revealed that thirteen of the plant extracts, comprising the extracts of EOF at 100,000 and 50,000 ppm, ACL at 30,000 ppm, ACF at 30,000 ppm, and TPL, TPF and TPR at 100,000, 50,000 and 30,000 ppm were not significantly more effective than copper oxychloride. However, seven plant extracts, comprising the extracts of EOL at 100,000, 50,000 and 30,000 ppm, ACL at 100,000 and 50,000 ppm and ACF at 100,000 and 50,000 ppm, were more significantly effective than copper oxychloride in reducing pustule lesions (Figure 21 and Table 13).

4.5.2 Curative effect of plant extract

14 days after inoculation with *Xcg* 728, all plant extracts at 100,000, 50,000 and 30,000 ppm, (with the two exceptions of EOL and TPL at 30,000 ppm) significantly reduced ($p<0.05$) the number of pustule lesions on soybean leaves in comparison to the negative control. The EOF extracts showed the highest efficacy in reducing the number of lesions (1.67 lesions/leaflet). The plant extracts at 100,000 ppm reduced the number of lesions more than the extracts at 50,000 and 30,000 ppm. The curative efficacy of these plant extracts steadily increased with the increase in concentration levels.

When using these plant extracts to reduce the incidence of bacterial pustule lesions compared to copper oxychloride (4,000 ppm). The results indicated that, in comparison to copper oxychloride, twelve plant extracts, those of ACL (100,000, 50,000 and 30,000 ppm), EOF and ACF (50,000 and 30,000 ppm), TPL (50,000 ppm) and TPF and TPR (50,000 and 30,000 ppm), had no significant effect in reducing bacterial pustule lesions. Six plant extracts at 100,000 ppm (all those except for the ACL extract) showed higher efficacy in reducing number of lesions than copper oxychloride treatment. The data are presented for the most effective plant extracts in Figure 21 and Table 14.

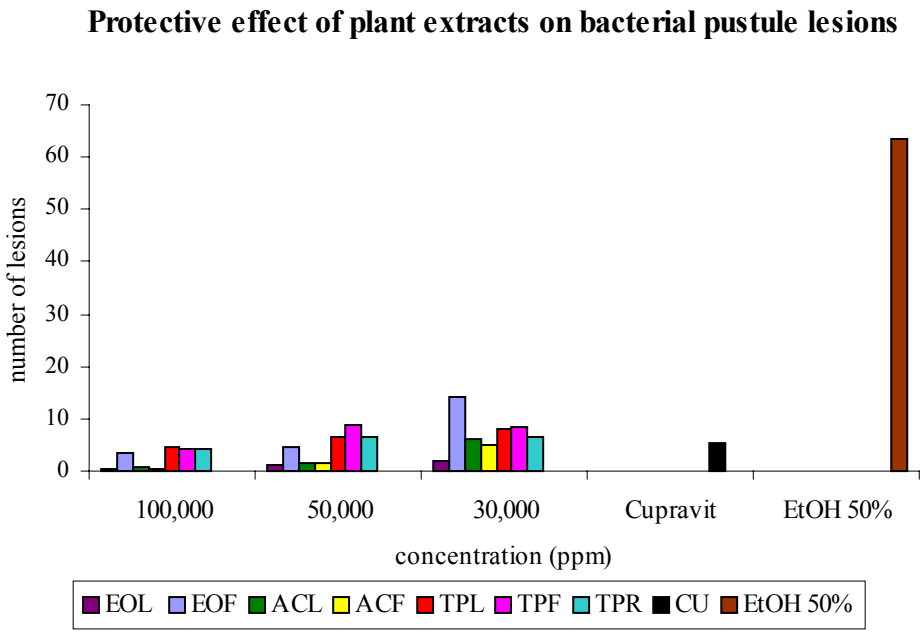


Figure 20 Protective effect of plant extracts on the bacterial pustule lesions

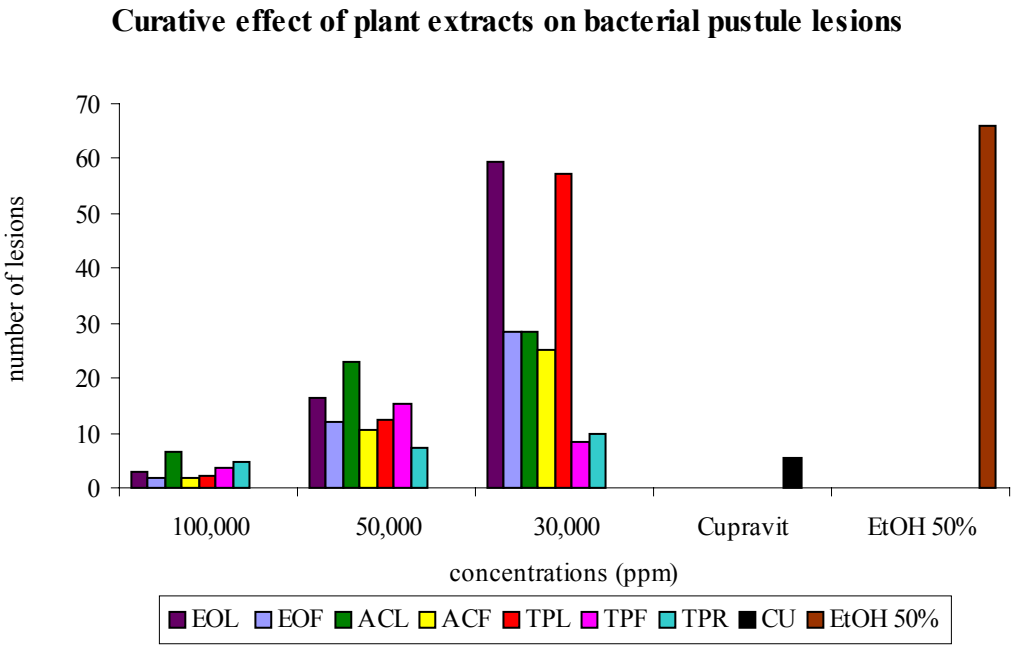


Figure 21 Curative effect of plant extracts on the bacterial pustule lesions

Table 13 Protective effect of plant extracts with different concentration on bacterial pustule disease lesions on soybean leaves

Plant Extracts	Plant parts	Number of lesion / soybean leaflet[1]		
		Concentration (ppm)		
		100,000	**50,000**	**30,000**
E. odoratum	Leaves	0.44 GH	1.22 FGH	1.89 EFG
	Flowers	3.33 DEF	4.78 CDE	14.11 B
A. conyzoides	Leaves	0.77 GH	1.44 FGH	6.11 BCD
	Flowers	0.22 GH	1.44 FGH	5.11 CD
T. procumbens	Leaves	4.78 CDE	6.33 BCD	7.89 BCD
	Flowers	4.22 CDE	8.78 BC	8.44 BC
	Roots	4.11 DEF	6.44 BCD	6.56 BCD
Copper oxychloride[2]			5.33 CD	
EtOH 50% (Negative control)			63.44 A	

Notes: Mean is the average of 3 replications, C.V. = 44.52 %

[1]Means with the same letter superscript is not significantly different at 5% level by DMRT

[2]Copper oxychloride at 4,000 ppm

Table 14 Curative effect of plant extracts with different concentration on bacterial pustule disease lesions on soybean leaves

Plant Extracts	Plant parts	Number of lesion / soybean leaflet[1]		
		Concentration (ppm)		
		100,000	**50,000**	**30,000**
E. odoratum	Leaves	2.89 ^{H}	16.44 ^{BCD}	59.56 ^{A}
	Flowers	1.67 ^{H}	11.89 ^{BCDE}	28.56 ^{B}
A. conyzoides	Leaves	6.44 ^{EFGH}	23.11 ^{BC}	28.33 ^{B}
	Flowers	1.89 ^{H}	10.67 ^{BCDE}	25.11 ^{BC}
T. procumbens	Leaves	2.22 ^{H}	12.44 ^{BCDE}	57.11 ^{A}
	Flowers	3.78 ^{GH}	15.44 ^{BCD}	8.44 ^{DEFG}
	Roots	4.56 ^{FGH}	7.11 ^{DEFG}	9.89 ^{DEF}
Copper oxychloride[2]			11.33 ^{BCDE}	
EtOH 50% (Negative control)			65.89 ^{A}	

Notes: Mean is the average of 9 replications, C.V. = 36.28 %

[1]Means with the same letter superscript is not significantly different at 5% level by DMRT

[2]Copper oxychloride at 4,000 ppm

Results suggest that the leaf and flower extracts of *E. odoratum*, *A. conyzoides* and *T. procumbens* and root extracts of *T. procumbens* at 100,000 ppm showed both protective and curative effects to control bacterial pustule disease. But, at lower concentration levels (50,000 and 30,000 ppm), the protective effect of these plant extracts was greater than the curative effect. This result may be because in the curative method bacteria could penetrate into plant tissues and increasing number prior to the application of the plant extracts. Thus, the plant extracts may have had less access to the pathogen, and less efficiency in reduction of the disease. On the other hand, the protective method involved treating plant extracts before inoculation of *Xcg* 728 on soybean leaves, so the active ingredients of the plant extracts may have had direct exposure to the pathogens in the bacterial suspension. Similarly, as reported by Curtis, Noll, Stormann and Slusarenko (2004) the application of garlic extracts at before inoculation was more effective than treatment after infection with plant pathogenic bacteria and fungi. Kagale, Marimuthu, Thayumanavan, Nandakumar and Samiyappan (2004) found that foliar application of leaf extract of *Datura metel* effectively reduced the incidence of sheath blight and bacterial blight diseases of rice under greenhouse condition. The pre-inoculation application of leaf extract was found better than post-inoculation application. As well as, the study of ธารทิพย ภาสบุตร (2540) reported that the used of Wann-Num extracts showed the best control before inoculation of *Colletotrichum gloeosporioides* on mango fruits.

From agar disc diffusion method and greenhouse results, it can be concluded that three plant extracts in Asteraceae family have potential for use in reducing the diseases caused by *Xanthomonas campestris* pv. *glycines*. Further studies should be

done to identify the biologically effective compound against *X. campestris* pv. *glycines* contained in these plant extracts, as well as the mechanism of action of these active ingredients against *Xcg*. Further investigation should also take place into how these extracts can be used in field conditions, and the suitable amounts to be used.

CHAPTER V

CONCLUSIONS

Bacterial pustule diseases caused by *X. campestris* pv. *glycines* are an important disease of soybean in Thailand. Disease management by using chemicals is expensive and affects non-target organisms; thus there is a need for alternative management of the disease. The leaf, flower and root extracts of *E. odoratum A. conyzoides* and *T. procumbens* were tested against the five isolates of *X. campestris* pv. *glycines* under in vitro and greenhouse conditions. Results from the agar disc diffusion method showed that the 95% ethanolic extracts of all plant parts in the Asteraceae family exhibited antibacterial activity. The leaf and flower extracts of *T. procumbens* at 100,000 ppm, exhibited the highest activity with an inhibition zone of 22.00 mm. The diameter of the zone was concentration dependent. However, the root extracts of *E. odoratum* and *A. conyzoides* at all concentrations, showed minimal antibacterial activity or no inhibition zone against some bacterial isolates. The negative controls did not show any inhibitory effect on any of the tested bacteria.

Under greenhouse conditions, seven plant extracts, comprising *E. odoratum* leaves (EOL), *E. odoratum* flowers (EOF), *A. conyzoides* leaves (ACL), *A. conyzoides* flowers (ACF), *T. procumbens* leaves (TPL), *T. procumbens* flowers (TPF) and *A. conyzoides* roots (TPR), at 100,000, 50,000 and 30,000 ppm, were selected to test for antibacterial activity on soybean leaves inoculated by *Xcg* 728. The results

indicated a significant protective and curative effect of the plant extracts ($p<0.05$) in reducing the number of lesions of *Xcg* 728 when compared to the negative control. Moreover, these plant extracts at 100,000 ppm showed both protective and curative effects against bacterial pustule disease. However, the protective effect of the plant extracts at 50,000 and 30,000 ppm was more marked than the curative effect. Although these plant extracts in the Asteraceae family may be used to inhibit the growth of *X. campestris* pv. *glycines* both in the agar disc diffusion method and greenhouse conditions, further investigation is necessary in actual field conditions.

From both agar disc diffusion and greenhouse results, it can be concluded that three plant extracts in the Asteraceae family have the potential to control *Xanthomonas campestris* pv. *glycines*. These results may lead to the development of botanical bactericides from these plant extracts.

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