Biological activities of mimosine isolated from Leucaena leucocephala and identification of bioactive compounds from subtropical plant Bidens pilosa

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BIOLOGICAL ACTIVITIES OF MIMOSINE ISOLATED FROM LEUCAENA LEUCOCEPHALA AND IDENTIFICATION OF BIOACTIVE COMPOUNDS FROM SUBTROPICAL PLANT BIDENS PILOSA

(Leucaena leucocephala から単離されたミモシンの生物活性及び亜熱帯性植物 Bidens pilosa からの生物活性物質の同定)

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ABSTRACT

Plants are rich source of bioactive compounds which have been utilized in the agrochemicals, foods, cosmetics and pharmaceuticals industries. *Leucaena leucocephala* and *Bidens pilosa* are the tropical and subtropical plants which grow abundantly in Okinawa, Japan. There is a peculiar phenomenon between these plants; *B. pilosa* grows frequently in *L. leucocephala* colony and in *B. pilosa* colony *L. leucocephala* abundance was often observed. To explain these interesting relationships, biological active compounds of *L. leucocephala* and *B. pilosa* were investigated in detail.

*L. leucocephala* shows strong phytotoxic activity on many plant species due to the presence of mimosine \( \beta-N-(3\text{-}\text{hydroxy}-4\text{-}\text{pyridone})-\alpha\text{-}\text{aminopropanoic acid}) \). Fifteen kinds of plants were examined by mimosine for its plant growth inhibitory activity. The chlorophyll and water content of those tested plants were also measured. The result revealed that the mimosine showed 90% plant growth inhibitory activity against the radicle of all plant seeds except *B. pilosa* of which hypocotyls growth was found the lowest by mimosine as compared to other fourteen tested plants. It was observed that the chlorophyll and water content of those tested plants were reduced by mimosine. By the screening process, it was demonstrated that *B. pilosa* itself had allelopathic activity. It was observed that fifteen kinds of phenolic compounds were identified in *B. pilosa*. 
Phenolic compounds are a class of secondary metabolites in plant that are involved in many functions including antioxidant, antimicrobial, allelopathic and enzyme inhibitory activities. Moreover, the extract of *B. pilosa* shows strong phytotoxic action against the growth of weeds and fungi. The extract of roots exhibited higher plant growth inhibitory activity than those of the leaves and stems. Before hydrolysis, a large amount of phenolic compounds were found in stem measured by Folin-Ciocalteu method. After hydrolysis, thirteen kinds of phenolic compounds were identified in roots by GC-MS. The content of caffeic acid was found highest among detected substances. These identified constituents may be responsible for the herbicide and fungicide activities of *B. pilosa*.

Tyrosinase is a key enzyme in the production of melanin in plants and animals. The inhibitory activities for mushroom tyrosinase using L-DOPA as substrate were investigated where the ethyl acetate fractions showed the highest activity among fractions and other extracts where mimosine and kojic acids were used as positive control. The ethylacetate fraction exhibited the strongest DPPH radical scavenging activity with an IC$_{50}$ value 25.2 μg/ml, when compared with methanol extract, other fractions and α-tocopherol as positive control. By the GC-MS, phenolic, flavanoid and hydroxycinnamic acid groups of compounds were identified from ethyl acetate fractions. The high phenolic content and anti-tyrosinase activity would make the ethyl acetate fraction to be the most competent candidate for food, pharmaceutical and cosmetic products.
Essential oil is a concentrated, hydrophobic liquid having volatile aroma compounds emitted from the plants called aromatic herbs or aromatic plants. The major volatile constituents of leaves and flowers from the essential oil of *B. pilosa* obtained by steam distillation were identified by GC-MS. Forty-six components were identified from *B. pilosa*, of which β-caryophyllene and τ-cadinene were the main compounds found in leaves and flowers, respectively. Antioxidant activity test of essential oils from leaves and flowers were carried out by DPPH (IC$_{50}$ 47.5 and 49.7 μg/ml, respectively) and β-carotene bleaching methods. The inhibitory activity of the flower essential oils against Gram-negative bacteria was significantly higher than Gram-positive. The essential oils and water extracts of *B. pilosa* possess antioxidant and antimicrobial activities that might be a natural potential source as a preservative in food and other related industries.
CHAPTER 1

GENERAL INTRODUCTION
INTRODUCTION

The therapeutic benefits of traditional medicines have been recognized for centuries. Although there is still lack of evidence for clarification of their typical mechanisms, unlike with Western medicine, it is still widely accepted by people from East Asia and beginning to be accepted by the rest of the world. Early cultures also recognized the value of using species and herbs in preserving foods and for their medicinal value. In recent years much interest has been focused on the biologically active compounds occurring in plants and herbs for their safety and effectiveness in prevention and treatment of human diseases. Great number of plant sources is being searched for their possible antimicrobial, antitumor, antioxidant etc activities. Most of the recent investigations have been carried out to isolate and identify the chemical nature of the substances responsible for these biochemical activities and many analytical techniques are improved for the isolation and characterization of numerous kinds of metabolites from various plants and their structure has been elucidated.

Primary plant metabolites can be considered as those metabolites essential to the life of the plant. The plant synthesizes sugars, amino acids and nucleotides. These simple molecules are used to produce polymers essential in the life of the plant. This aspect of the plants biochemistry can be considered as distinct from the production of more
complex molecules produced by more diverse pathways. Secondary metabolites are unique to plants, it was initially thought they were of use to a plant but morphine produced by poppies is of no use to the plant but is of use in medicine to heal people who are suffering. Secondary metabolites are also used as food, flavors, color dyes, poisons, perfumes, scented oils in aromatherapy, industrial products such as rubber and oils. It is estimated that 1/4 of prescription drugs contain at least one chemical originally identified and extracted from a plant. It can be seen that plant secondary metabolites have been used in a wide variety of uses; they have been used for centuries in cases and only more recently discovered in other instances. Herbal remedies have been used for centuries but more recently the compounds that are active have been identified and this has enabled them to be extracted and purified. Plants produce a high diversity of secondary metabolites for defense and survival in the ecosystem. Secondary plant metabolites, currently exceeding 100,000 identified substances, belong to three major chemical classes: terpenes (a group of lipids), phenolics (derived from carbohydrates) and alkaloids (derived from amino acids). Secondary metabolites are sought after because they are known to exhibit numerous biological activities that promote positive health effects. These activities include antibacterial, anticancer, antifungal and antioxidant. Additionally, plant secondary metabolites release a diversity of allelochemicals into the environment, which include phenolics, alkaloids, long chain fatty acids, terpenoids and flavonoids.
An explanation of the allelopathy mechanism has been attempted and the influence from a complex of phytochemicals synthesized in plants as secondary metabolites has been proposed. The allelochemicals can be broadly classified as plant phenolics and terpenoids, which show great chemical diversity and are involved in a number of metabolic and ecological processes. The most common effects of allelochemicals may occur through leaching, volatilization, root exudation, and the death and decay of the fallen plant parts are either via biotic or abiotic means (Anaya et al. 1990). Therefore, research in allelopathy focuses on isolation, identification and quantification of these active allelochemicals that may be used as natural herbicides or for developing new and environmentally-friendly herbicides (An et al. 1996). These compounds have been shown to have strong antioxidant and antimicrobial activities (Osbourn, 1999). These activities are utilized in the agricultural, food and pharmaceutical industries. As a consequence of these numerous applications, the world market for plant extracts and isolated secondary metabolites is increasing due to constant discoveries of their potential roles in health care and as lead chemicals for new drug development.

Phenolic compounds are widely found in the secondary products of medicinal plants, as well as in many edible plants (Hagerman et al. 1998). They are biochemically synthesized via the shikimate pathway (Singer et al. 2003). They can be divided into the following main groups: (1) benzoic acid derivatives (C₆-C₁) that are quite common in the
free form as well as combined as esters or glycosides; (2) cinnamic acid derivatives (C₆-
C₃) that are widely distributed and occur rarely in the free form and are very often
esterified, and (3) glycosidic phenylpropanoid esters (Škerget et al. 2005). Phenolic
compounds are important components of many fruits, vegetables and beverages and
contribute to their color and sensory properties such as bitterness and astringency
(Mozetič et al. 2002). They can act as antioxidants by donating hydrogen to highly
reactive radicals, thereby preventing further radical formation (Lapornik et al. 2005).
They are potentially responsible for allelopathic interferences in plants (Rebaz et al.
2001). Furthermore, phenolics are interesting antimicrobial agents (Holly and Patel,
2005) and they also play a significant physiological role in acclimation and tolerance
against oxidative damage caused by stress such as salinity (Wahid and Ghazanfar, 2006),
boron deficiency (Stavrianakou et al. 2006) and wounding (Choi et al. 2005).

As a result of the awareness of the consumers to the health impact of the synthetic
(artificial) additives added to the food and beverages, also to the side effect of many
drugs; demands for the natural products from herbs or plants lately increased, owing to
the fact that synthetic chemical compounds may be related to diseases such as
atherosclerosis and cancer.
It is also important to note that the medicinal herbs or plants consumed by many generations as a food and it shown to contain vitamins, essential oils and minerals in addition to the major food components. It could also improve the flavor, aroma and taste of the food or beverages when it included.
CHAPTER 2

LITERATURE REVIEWS
2.1. MEDICINAL PLANT

Historically, plants have played an important role in medicine. The practical plants are used as natural medicines since prehistoric times. Medicinal plants and herbs contain substances known to modern and ancient civilizations for their healing properties. Until the development of chemistry and, particularly, the synthesis of organic compounds in the 19th century, medicinal plants and herbs where the sole source of active principles capable of curing man ailments. In more recent history, the use of plants as medicines has involved the isolation of active compounds, beginning with the isolation of morphine from opium in the early 19th century (Kinghorn, 2001; Samuelsson, 2004). They continue to be important to people that do not have access to modern medicines and modern pharmaceuticals rely heavily on the same active principles; they will be natural or synthetic. The active compounds differ from plant to plant due to their biodiversity, i.e. to the plant’s genetic coding ability to produce them.

Plant medicines would be safer and better for human health than synthetic drugs. This is so because human beings have co-evolved with plants over the past few million years. We eat plants, drink their juices, ferment and distill libations from them, and consume them in a thousand forms. Ingredients in plants, carbohydrates, fats and protein to vitamins and minerals, are part of our body composition and chemistry. With thousands
of active compounds yet to be discovered or fully evaluated, it is no wonder that biodiversity is a fundamental topic on any nature preservation agenda. The genetic material of old and new herbs and plants are desired for their potential in discovering, combining, manipulating and synthesizing new medicine. Medicinal plants and herbs continue to be the source of proven medicaments and of new revolutionary drugs. If the active compounds of synthetic drugs are so important and can be found in many plants and herbs, cheaply and easily bought from market or herbalist, we can use them. If taken in the appropriate dose and form, they can be as effective as pharmaceutical drugs.

2.2. TROPICAL AND SUBTROPICAL PLANTS

Our daily dependence on plant products of tropical origin is astounding. Our lives are enriched by beautiful hardwoods, spices, essential oils and fruits. In addition, tropical countries export many fibers, gums, resins, dyes, and plant essences that we may never see directly, but which are widely used in medicine and industry. This section highlights tropical and subtropical important plants *L. leucocephala* and *B. pilosa*.

*L. leucocephala* (Family: Leguminaceae) is widely distributed in tropical and subtropical area (Yeung et al. 2004). It is nitrogen fixing multipurpose tree (Ramana et al. 2000), because it can serve many purposes, as foliage for livestock, as vitamin K and
Fig. 2.1. *Leucaena leucocephala* plant

Fig. 2.2. Mimosine from *L. leucocephala* plant
β-carotene supplements, as fuel wood, as green manure, or as drought resistance (Lalitha et al. 1993). However, the aqueous leaf extracts of *Leucanea* show strong phytotoxic activity on many plant species due to the presence of mimosine (Chou et al. 1986). Introduction of *L. leucocephala* outside its indigenous range has often led to acute and chronic toxicosis in animals. The agents of toxicity are the allelochemicals mimosine (Borthakur et al. 2003), a non protein amino acid tyrosine analog (Oppenheim et al. 2000), and its main degradative products, 3-hydroxy-4-pyridone (DHP) which cause goiter, loss of hair, reproductive disorders, epithelial damage and ultimately death when fed to grazing and experimental animals.

![Biodegradation of Mimosine](image)
Mimosine also inhibits growth and protein synthesis in microorganisms. Although mimosine was first isolated from *Mimosa pudica*, the mimosine concentrations in these plants are much lower than in *L. leucocephala* spp. (Lalitha *et al.* 2004). The concentrations of mimosine in air-dried *L. leucocephala* leaves were found to be in the range of 2.5 to 5.75%, and mimosine can be easily removed by soaking the leaves in water for 24 h. Soil extracts from around *L. leucocephala* trees are also toxic to other plants (Soedarjo *et al.* 1998).

On the other hand, *Bidens pilosa* (Family: Asteraceae alt. Compositae), commonly known as “Hairy beggar-ticks” or “Spanish needles”, is a pantropical weed which is well known for its medicinal properties (Sarker *et al.* 2000). It is a plant native to South America which at present is spread all over the world, particularly in tropical and subtropical regions. It grows up to 1.5 m and 5–15 mm in height and diameter respectively, and is widely used in African traditional medical practice. In Cameroon, the leaf aqueous extract is used in the treatment of jaundice, threatened abortion, conjunctivitis, toothache, cough, intestinal helminthiasis and leg ulcer (Tan *et al.* 2000). The Zulu of South Africa chew the leaves for their anti-inflammatory properties. Actually, all parts of the plant are medicinally used. The flower is used in the treatment of diarrhoea, dysentery and upset stomach in food poisoning.
Fig. 2.3. *Bidens pilosa* plant
In the Peruvian Amazon, *B. pilosa* is used for diabetes, dysentery, hepatitis, and water retention. *B. pilosa* also forms part of a compound mixture of herbs used for the treatment of hypertension (Dimo *et al.* 2001). Phytochemical studies of *B. pilosa* have revealed the presence of alkaloids, saponins, flavonoids, polyacetylenes and triterpens, acyl chalcones glucosides and 1-phenyl hepta 1,3,5-triyne (Abajo *et al.* 2004). *B. pilosa* is among the several plants used in Brazil to treat fevers and malaria. Experimental evidences have shown that crude extracts from roots, prepared with 80% ethanol by percolation, show in vitro activity against *Plasmodium falciparum*. Chemical study of the ethanol crude extract led to identification of two major groups of chemical constituents, the polyacetylenes and the flavonoids, the most likely active compounds (Oliveira *et al.* 2004).

The whole plant or its aerial parts are used in various folk medicines and as a popular ingredient in herbal tea for its antiseptic, liver-protective, blood-pressure lowering, and hypoglycemic effects. Topical application of this herbal extract is also used for skin disorders (Wu *et al.* 2004).

Previous studies on the genus *B. pilosa* reported the isolation of chalcones (Redl *et al.* 1993), phenylpropanoid glucosides (Sashida *et al.* 1991), polyacetylenes (Chang *et al.* 2000; Redl *et al.* 1994), a diterpene (Zulueta *et al.* 1995), flavonoids (Wang *et al.* 1997), and flavone glycosides (Brandão *et al.* 1998). Bioactivities of *B. pilosa* plant extracts,
including anti-hyperglycemic (Ubillas et al. 2000), antihypertensive (Dimo et al. 2002), immunosuppressive and anti-inflammatory (Jager et al. 1996; Pereira et al. 1999), anti-leukemic (Chang et al. 2001), anti-malarial (Brandão et al. 1997), anti-bacterial (Rabe et al. 1997), and anti-microbial (Khan et al. 2001) effects have been reported.

2.3. POLYPHENOL

Polyphenols embraces a wide range of substances which possess an aromatic ring bearing a hydroxyl constituent, including their functional derivatives. The subdivision of polyphenols into tannins, lignins, and flavonoids is derived from secondary plant metabolism of the shikimate pathway (Dewick, 1995). Phenolic compounds are widely found in the secondary products of medicinal plants, as well as in many edible plants (Hagerman et al., 1998). The ability of phenolic compounds to serve as antioxidants has been recognized, leading to speculation about the potential benefits of ingesting phenolic rich foods. Several studies have described the antioxidant properties of medicinal plants, foods, and beverages which are rich in phenolic compounds (Brown & Rice-Evans, 1998; Krings & Berger, 2001). Phenolic compounds are the most commonly investigated constituents among potential allelochemicals (Chon et al. 2005). They occur in plant tissues as free compounds (benzoic acid and cinnamic acid derivatives) and in bound forms (glycosidic phenylpropanoid esters).
2.3.1. BASIC POLYPHENOL UNIT

2.3.1.1. Basic Unit: Gallic Acid  
Class /polymer: Hydrolyzable Tannins

![Gallic Acid](image)

2.3.1.2. Basic Unit: Flavone  
Class /polymer: Flavonoid, condensed tannins

![Flavone](image)

2.3.1.3. Basic Unit: Cinnamic acid  
Class /polymer: Lignins
2.4. TERPENOIDS

Non-phenolic compounds (terpenes, alkaloids) were isolated from different plant sources and shown to possess many biochemical activities. Terpenes are hydrocarbons made up of 5-carbon units, often called isoprene units, atoms, the elementary composition $C_{10}H_{10}$ (corresponding to 2 isoprene units) are named monoterpenes. The isopentane units in most of these terpenes are easy to discern, and the compounds having the composition $C_{15}H_{24}$ are named sesquiterpene; and members of the $C_{20}H_{32}$ and $C_{30}H_{48}$ groups are named di- and tri-terpenes, respectively.

The mono- and sesqui-terpene occur as components of the essential oils obtained by steam distillation of the tissue of many plants; some of these terpenes are useful in the perfumes industry. Plant terpenoids are extensively used for their aromatic qualities. They play a role in traditional herbal remedies and are under investigation for antibacterial,
antineoplastic and other pharmaceutical effects. Limonene (present in citrus and other oils), menthol (from mint oil), have been used for medicinal purposes pain reliever and antipuretic (relieves itching) since before 2000 BC, and carvone from caraway are examples of monoterpene.

Diterpenes present in plants are found as constituents of the resins and balsams. The best known derivatives of this group of compounds are the resin acids abietic and sapietic acids obtained from pine oil. Vitamin A1, A2 and their aldehydes are derivatives of diterpenes. One of the most well-known medicinally valuable terpenes is the diterpene, taxol. Taxol was first isolated from the bark of the Pacific yew, Taxus brevifolia, in the early 1960’s, but it was not until the late 1980’s that its value as an anticancer drug was determined. It acts to stabilize the mitotic apparatus in cells, causing them to act as normal cells rather than undergo rapid proliferation as they do in cancer.

Triterpenes, although not widely distributed in nature, are of special interest because they are important in the biosynthesis of cholesterol. Triterpenes contain 30 carbones, derived essentially from coupling of two sesquiterpene precursors. Many of these compounds occur in plants as glycosides, often called sponins (molecules made up of sugars linked to steroids or triterpenes) due to their ability to make aqueous solutions appear foamy.
Fig. 2.4. Monoterpene hydrocarbons

Fig. 2.5. Oxygenated monoterpenes
Fig. 2.6. Sesquiterpene hydrocarbons

Fig. 2.7. Oxygenated sesquiterpenes and phytol
2.5. ANTIMICROBIAL ACTIVITY

Many of the plants used today were known to the people for their preservative and medicinal values. Scientific experiments on the antimicrobial properties of plants and their components have been documented in the late 19th century (Zaika, 1975). A vast majority of plant secondary metabolites have weak or narrow-spectrum antimicrobial activities, while bacteria, yeast, and fungi produce antibiotics that both are often effective and have broad spectra of activity. The nature of this disparity is puzzling. One possibility is that plant “antimicrobials” actually have other functions in the plant and their low level of antimicrobial activity is accidental and largely irrelevant. An even more extreme opinion, not uncommon and stemming from the same puzzle, is that plants
happen to make many secondary metabolites for no good reason, and some of them will inevitably have antimicrobial properties.

Wilkins and Board (1989), reported more than 1,340 plants are known to be potential sources of antimicrobial compounds that few have been studied scientifically. However, the use of plant secondary metabolites not used as systemic antibiotics at present. The main reason for this is their low level of activity, especially against gram negative bacteria. The reported MIC is often in the range of 100 to 1,000 µg/ml, orders of magnitude higher than those of common broad-spectrum antibiotics from bacteria or fungi.

2.6. ANTIOXIDANT ACTIVITY

Lipid oxidation is a chemical change of foods, which depends on the level of oxygen, degree of unsaturation of fatty acids, energy (heat/light) and metals. Lipid oxidation products are responsible for the development of rancidity by production of low molecular weight compounds that cause undesirable flavors, thus affecting the quality and limiting the shelf-life of food products (Frankel, 1985; Frankel et al. 1987). In addition, intake of fat oxidation products may be related to ageing, heart diseases and cancer (Marx, 1987; Addis and warner, 1991).
Synthetic antioxidants such as butylated hydroxyl tolune (BHT), butylated hydroxyl anisole (BHA), and propyl gallate (PG) are widely used in many foods to prevent food rancidity. These compounds are added at concentration ranging from 50-200 ppm to fats and pills to supreme the development of peroxides during food storage (Prasad et al. 1984). There has been some discussion recently of undesirable use of synthetic antioxidants. For example, dietary administration of BHT to rats caused fatal hemorrhages in the pleural and peritoneal cavities and in organs such as epididymis tests and pancreas (Farag et al. 1989). Also, BHT caused changes in rat thyroids, stimulation to DNA synthesis and induction of enzymes. BHA had toxic and carcinogenic effects (Farag et al. 1989). In addition, there is an increased preference for natural food, which are generally believed to be safer, healthier and less hazardous than food containing artificial food additives. Therefore, number of plant sources has been searched for their possible antioxidative activity; these include: ginger (Jitoe et al. 1992), oregano, thyme (Economou et al. 1991), rosemary, sage (Chang et al. 1977), fennel, rue (Amr, 1991) and clove (Kramer, 1985).

2.7. TYROSINASE INHIBITORY ACTIVITY

Tyrosinase (monophenol monooxygenase) (EC 1.14.18.1) is an enzyme that
catalyses the oxidation of phenols (such as tyrosine) and is widespread in plants and animals. Tyrosinase is a copper-containing enzyme present in plant and animal tissues that catalyzes the production of melanin and other pigments from tyrosine by oxidation, as in the blackening of a peeled or sliced potato exposed to air. In higher plants and fungi, tyrosinases occur in various isoforms such as immature, mature latent (Sa´nchez-Ferrer et al. 1990) and active forms; however, the biochemical description regarding the kinetic characterization and relationship between these isoforms is yet to be established. The biosynthetic pathway for melanin formation, operating in insects, animals, and plants, has largely been elucidated. The production of abnormal melanin pigmentation (melasma, freckles, ephelide, senile lentigines, etc.) is a serious esthetic problem in human beings. In fungi, the role of melanin is correlated with the differentiation of reproductive organs and spore formation, virulence of pathogenic fungi, and tissue protection after injury (Zimmerman et al. 1995). In addition, tyrosinase is responsible for the undesired enzymatic browning of fruits and vegetables (Martinez et al. 1995). A number of tyrosinase inhibitors from natural and synthetic sources that inhibited monophenolase, diphenolase, or both of these actives have been identified. Furthermore, tyrosinase inhibitors may be clinically used for the treatment of some skin disorders associated with melanin hyperpigmentation and are also important in cosmetics for skin whitening effects (Palumbo et al. 1991).
2.8. ALLELOPATHY ACTIVITY

The allelopathy phenomenon in nature has concerned farmers and scientists through ages, whereas allelopathy mechanisms of plant interaction are an important component of the aggressive nature of many species in both natural and agroecosystems. Allelopathic compounds that are released from decomposition and leaching of plant debris, volatilization of leaf, or exudation of root, can be beneficial or detrimental to the growth of receptor plants. In habitats with limiting environmental conditions, dominant species can suppress the growth of neighboring plants and compete for more resources by releasing allelochemicals into the environment. Allelopathy has been recognized as playing an appreciable role in plant dominance, succession, formation of plant communities and climax vegetation, and crop productivity (Tseng et al. 2003). It is an alternative weed control method whereby plants provide themselves with a competitive advantage by putting phytotoxins into the near environment, thereby reducing the dependence of synthetic herbicides (Wu et al. 2002; Wu et al. 2000). Herbicides continue to be a key component in most integrated weed management systems to control weeds. Nevertheless, extensive use of synthetic herbicides poses serious threats to the environment and public health. There is great incentive to discover biologically active natural products from higher plants that are better than synthetic agrochemicals and are much safer environmentally. The development of natural products such as herbicides and
fungicides and their role in biocontrol of plant disease promises to reduce environmental
and health hazards. Allelochemicals have been investigated as a possible alternative weed
management strategy. Biologically active natural products (allelochemicals) are now
being employed as herbicides and fungicides and in biocontrol of plant diseases (Oliva et
al. 1999). The common allelochemicals include phenolics, terpenoids, alkaloids,
coumarins, tannins, flavonoids, steroids, and quinines. Although most of the simple
phenolic acids and flavonoids are allelochemicals, they seem to be weakly phytotoxic in
soil and have little selectivity. Salicylic and \( p \)-hydroxybenzoic acids at high rates (56 to
112 kg/ha), are effective against weeds and relatively non-selective (Duke et al. 1993).
Phenolic derivatives such as dihydroquinone, sorgoleone, produced by sorghum
(\textit{Sorghum bicolor} L.), are extremely phytotoxic in hydrophonic cultures (Einhelling et al.
Fig. 2.9. Coexistence of *B. pilosa* with *L. leucocephala* plant colony
2.9. OBJECTIVE OF THE STUDY

The aims of this study are to evaluate the antibacterial, antioxidant, antifungal, tyrosinase inhibitory and allelopathic activities of different extracts from the Okinawan tropical plants *L. leucocephala* and *B. pilosa* in vitro, as well as to identify the phytochemical compounds responsible for such activities which are of great interest.
CHAPTER 3

PLANT GROWTH INHIBITORY ACTIVITY OF MIMOSINE
3.1. SUMMARY

*L. leucocephala* which is producing a non-protein aromatic amino acid mimosine that inhibited the growth of fifteen kinds of plant seeds and decreased content of chlorophyll. When treated with mimosine solutions at different concentrations, it was observed that the chlorophyll and water content of those tested plants were reduced gradually with the increase of mimosine concentration. Bioassays for the allelochemical activity of mimosine were conducted fifteen kinds of plant seeds for screening assays. Mimosine showed 100% plant growth inhibitory activity against the hypocotyl of *Brassica rapa* and the radicle of *Oryza sativa* at 100 ppm. *Raphanus sativus* was also inhibited above 97% at 100 ppm of mimosine. Mimosine also showed very little inhibitory activity against both shoot and root of *B. pilosa* as compared to other fourteen tested plants. It is concluded that the supply-orientation of chlorophyll was significantly inhibited by the mimosine, causing the shortage of chlorophyll. In antifungal assay, mimosine showed above 60% activity against *Fusarium solani*.

3.2. INTRODUCTION

A wide range of non-protein amino acids occur in the plant kingdom that exhibit a wider variety of chemical structures than do their amino acid counterpart. Mimosine is one of
such aromatic amino acid found in leaves, pods and seeds of tropical and subtropical
genus *L. leucocephala* and *Mimosa* sp. (D’Mello, 2000). The aqueous leaf extracts of
*Leucaena* show strong phytotoxic activity on many plant species due to the presence of
mimosine (Chou *et al.* 1986). Ingestion of mimosine and its dihydroxypyridinyl
metabolite (DHP) resulted in toxicity such as hair loss, goiter, reproductive disorders,
epithelial damage and ultimately death in grazing and experimental animals (Kamada *et al.* 1998). In studies with human lymphoblastoid and HeLa cell lines, mimosine was
reported to cause reversible inhibition of cell cycle at the late G1 phase (Hughes *et al.* 1996). This phytochemical inhibited some enzymes in vitro including tyrosinase (Hider *et al.* 1989), ribonucleotide reductase (Dai *et al.* 1994), serine hydroxymethyl transferase
(Lin *et al.* 1996) and prolyl–4–hydroxylase (Ju *et al.* 1998). Studies demonstrated
enzymatic biosynthesis of mimosine from O-acetyl serine and DHP in *L. leucocephala*
seedlings (Ikegami *et al.* 1990). Mimosine also inhibits growth and protein synthesis in
microorganisms (Chruscinska *et al.* 1999). Soil extracts including mimosine from around
*L. leucocephala* trees are also toxic to other plants. (Tawata *et al.* 1996). Mimosine is
reported to affect the growth of some bacteria and fungi when infused into growth
medium (Suda, 1960). Results on the incorporation of radioactivity from selective amino
acids into mimosine skeleton point to the physiological significance of this phytochemical
(Tiwari *et al.* 1967). Chemicals which are responsible for the allelopathic activity of a
plant may be exploited as herbicidal, pesticidal or fungicidal compounds. Since mimosine
is often degraded to DHP or HP by enzyme, which may play a role of phytoalexin
properties. Therefore, it is clear that mimosine has specific cytotoxic effects which
contribute to allelopathic activities in different species.

Even though a reduction of photosynthesis has been widely observed in the
allelochemical-targeted plants, the component of photosynthesis which is directly or
indirectly affected by the allelochemicals is still unknown. One possibility is that the
allelochemicals may partially block the biosynthetic pathway of chlorophyll (i.e. an
inhibition of supply-orientation), or stimulate the degradative pathway of chlorophyll (i.e.
a stimulation of consumption-orientation), or both, leading to a reduction of chlorophyll
accumulation, in turn causing a reduction of photosynthesis and finally diminished total
plant growth (Yang et al. 2002).

In the light of above facts, we are interested in investigating whether mimosine has
herbicidal effects on selected crops or weeds, primarily screening out the tested plant
species on allelopathic potential, and showing antimicrobial activity of mimosine as well.
To test the hypothesis, the present study is aimed at gauging the inhibitory effect of
mimosine on the chlorophyll biosynthetic pathway. A subsequent paper would be
reported the effect of the same compounds had allelopathic activity but it was first time
reported that mimosine inhibit the chlorophyll.

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3.3. MATERIALS AND METHODS

3.3.1. Herbicidal assay

Some plant seeds were collected from the campus of University of the Ryukyus and others were bought from local market in Okinawa. Mimosine was extracted from young *L. leucocephala* leaves which was purified in the Department of Bioscience and Biotechnology, Faculty of Agriculture, University of the Ryukyus, by following a simple method introduced by Tawata (1990). Fifteen kinds of seeds, *Brassica rapa*, *Brassica chinensis*, *Brassica campestris*, *Brassica juncea*, *B. pilosa*, *Chrysanthemum paludosum*, *Gypsophila elegans*, *Lepidium sativum*, *Melampodium paludosum*, *Lactuca sativa*, *Raphanus sativus*, *Raphanus spp.*, *Medicago sativa*, *Oryza sativa*, and *Echinochloa crus-galli*, were used to test the plant growth inhibition. The seeds of different plants were treated in advance with distilled water at 80°C for 2 min to stimulate germination. All seeds had a germination percentage > 90% in a germination test before the experiment was carried out. Before use, Petri dishes were incubated at 180°C for 1 hr. for sterilization. An aliquot of 8 ml of mimosine solution at different concentrations was poured into Petri dishes, and twenty seeds of the plant were sown in them, respectively. The treatment was kept for eight days at 27°C in the dark for 13 hours and in the light for 11 hours in the incubators (Sanyo MIR-152) during the experiment. After that the lengths of the roots and
shoots were measured. The plant growth inhibitory activity was expressed in comparison with that of the control. Assays were performed in triplicate, and data obtained from repetition of the experiments were combined to calculate the mean values.

3.3.2. Determination of chlorophyll concentration of plant

3.3.2.1. Extraction of chlorophyll from tissue

About 0.5-2.0g of 2 week old plant leaf was taken into a mortar and ca 0.1g of CaCO$_3$ or Na$_2$CO$_3$ was added into it. Then the tissue was macerated with pestle and ground short time. Small amount of 85% acetone was added to it and continued grinding until tissue is finely ground. The mixture was transferred to the funnel and filtered with suction. The residue was washed to mortar with 85% acetone, ground and filtered again as before until tissue is devoid of any green, and washings are colorless. When extraction was completed, the filtered extract was transferred to volumetric flask of appropriate size and filled the volumetric flask (100 ml) with 85% acetone up to the mark. Around 25 to 50 ml of extract was taken from volumetric flask and transferred it to the separating funnel containing 50 ml ether. Water was added carefully until it is apparent that all fat soluble pigments have entered ether layer. The separating funnel was shaken well, drained and discarded water layer. The procedure was repeated five times.
3.3.2.1.2. Spectrophotometric measurements

All absorbance measurements were carried out on Ubest-50 (Line VTG AC, 100V, 50/60 Hz) spectrophotometer, Japan Spectroscopic Co. Ltd., equipped with 1.0 cm quartz cells. A teaspoonful anhydrous Na₂SO₄ was added to 60 ml reagent bottle and filled it with ether (analytical reagent grade purchased from Wako Chemicals Co., Japan.) solution of pigment. When this solution is optically clear, it was transferred with pipette into another dry bottle and diluted with enough dry ether to give the value of 0.2 to 0.8 at the wavelength to be used. Most favorable value is near 0.6 at 660 nm, since such solution yields satisfactory value at 642.5 nm. Then the value of A at 660.0 and 642.5 nm were determined for each unknown solution and total chlorophyll (mg/g) was calculated by using the following formula as follows (Helrich 1990):

\[
\text{Total chlorophyll} = 7.12 A_{660.0} + 16.8 A_{642.5}
\]

3.3.3. Fungicidal assay

Fungi were collected from the Department of Plant Pathology, Faculty of Agriculture, University of the Ryukyus, Okinawa, Japan and were cultured in the laboratory. Five pathogenic fungi, *Fusarium solani*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Trichoderma viride*, and *Corticium rolfsii*, were used to test antifungal activity of mimosine. These phytopathogens were cultured on potato dextrose agar (PDA) (1.5%
agar) for 4 days at 27°C before the experiment was carried out. One ml volume of mimosine diluted solution was mixed with 8.0 ml of PDA and was then put in the Petri dish. Finally, the spores from a mycelia disk (5.7 mm in diameter) were put on the centre of the PDA plate. Controls contained distilled water only. All plates were transferred into an incubator (27°C) for 4 days. The antifungal activity was measured by agar dilution method (Taira et al. 1994) and expressed as percentage inhibition against the mycelia growth in diameter. All of the tests were carried out in triplicate and were taken the average of them.

3.4. RESULTS AND DISCUSSION

3.4.1. Herbicidal activity

More than 50% of tested plants were inhibited by 100 ppm mimosine found significantly different plant growth inhibitory activities against shoots (Fig. 3.1). Mimosine showed excellent plant growth inhibitory activity (above 80%) against the roots of most tested plant species. The result revealed that the mimosine showed 100% plant growth inhibitory activity against the shoot of Brassica rapa and the root of Oryza sativa at 100 ppm. It is also observed that mimosine showed very less inhibitory activity against the root growth of B. pilosa as compared to other fourteen tested plants at 100
ppm (Fig. 3.2). The same results were also found in the shoot of B. pilosa (Fig. 3.1). Mimosine inhibits the growth of several plants significantly but B. pilosa slightly, which is consistent with the previous report (Tawata et al. 1987). Fresh weight was measured against several plants treated with mimosine after fourteen days at 100 ppm (Table 3.1), and found that fresh weight of R. sativa (leaf) was drastically reduced compared with other tested plants, demonstrating that R. sativa is very sensitive against mimosine toxicity. The root and shoot growth of R. sativa was inhibited above 90% by mimosine at 50 and 75 ppm respectively, as shown in Table 3.2. Mimosine at 150 ppm applied to all plant species except B. pilosa showed almost similar value of root inhibitory activities. In case of B. pilosa, up to 50 ppm mimosine acts as a growth promoter and thereafter it acts as growth inhibitor at higher ppm. Chou and Kuo (1986) indicated that at 20 ppm, mimosine significantly suppressed growth of lettuce, rice and rye grass; however, Miscanthus floridulus was not inhibited by the mimosine at 200 ppm.
Fig. 3.1. Screening of shoots of different plant species by the application of allelochemical Mimosine at 100 ppm. Vertical bars represent standard deviations of the mean (n=3).
Fig. 3.2. Screening of roots of different plant species by the application of allelochemical mimosine at 100 ppm. Vertical bars represent standard deviations of the mean (n=3).
Table 3.1. Effects of mimosine on plant total fresh weight at 14 days after treatment at 100 ppm. Values are the means ± standard deviation of three replicates.

<table>
<thead>
<tr>
<th>Family</th>
<th>Group</th>
<th>Common name</th>
<th>Scientific name</th>
<th>Fresh weight (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asteraceae</td>
<td>Dicot</td>
<td>Hairy beggar’s tick/Spanish needle</td>
<td><em>B. pilosa</em></td>
<td>37.82 ± 4.78</td>
</tr>
<tr>
<td></td>
<td>Dicot</td>
<td>Million gold/Mini marguerite Pak choi</td>
<td><em>Chrysanthemum paludosum</em></td>
<td>69.78 ± 9.36</td>
</tr>
<tr>
<td>Brassicaceae</td>
<td>Dicot</td>
<td>Pak choi</td>
<td><em>Brassica chinensis</em></td>
<td>19.06 ± 5.44</td>
</tr>
<tr>
<td></td>
<td>Dicot</td>
<td>Mustard green</td>
<td><em>Brassica juncea</em></td>
<td>27.04 ± 8.07</td>
</tr>
<tr>
<td></td>
<td>Dicot</td>
<td>Garden cress/pepper weed Radish (leaf)</td>
<td><em>Lepidium sativum</em></td>
<td>47.52 ± 8.72</td>
</tr>
<tr>
<td></td>
<td>Dicot</td>
<td>Radish (stem)</td>
<td><em>Raphanus sativus</em></td>
<td>16.21 ± 0.23</td>
</tr>
<tr>
<td>Caryophyllaceae</td>
<td>Dicot</td>
<td>Showy baby’s breath</td>
<td><em>Gypsophila elegans</em></td>
<td>36.04 ± 8.59</td>
</tr>
<tr>
<td>Cruciferae</td>
<td>Dicot</td>
<td>Field mustard/broccoli raab</td>
<td><em>Brassica campestris</em></td>
<td>25.30 ± 6.25</td>
</tr>
<tr>
<td>Fabaceae</td>
<td>Dicot</td>
<td>Alfalfa</td>
<td><em>Medicago sativa</em></td>
<td>50.34 ± 0.55</td>
</tr>
<tr>
<td>Gramineae/Poaceae</td>
<td>Monocot</td>
<td>Rice/Paddy</td>
<td><em>Oryza sativa</em></td>
<td>51.87 ± 0.28</td>
</tr>
<tr>
<td>Poaceae</td>
<td>Monocot</td>
<td>Barnyard grass</td>
<td><em>Echinochloa crus-galli</em></td>
<td>57.04 ± 2.42</td>
</tr>
</tbody>
</table>
Table 3.2. Herbicidal activity of mimosine against selective plants species at different ppm

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Medicago sativa Shoot (mm)</th>
<th>Oryza sativa Shoot (mm)</th>
<th>Raphanus sativus Shoot (mm)</th>
<th>B. pilosa Shoot (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22.3 (0.0) a</td>
<td>42.6 (0.0) a</td>
<td>65.8 (0.0) a</td>
<td>26.4 (0.0) a</td>
</tr>
<tr>
<td>50</td>
<td>11.2 (49.8) b</td>
<td>35.2 (17.4) b</td>
<td>16.3 (75.2) b</td>
<td>27.8 (–5.3) a</td>
</tr>
<tr>
<td>75</td>
<td>9.0 (59.6) c</td>
<td>26.7 (37.3) c</td>
<td>3.63 (94.5) c</td>
<td>26.1 (1.14) a</td>
</tr>
<tr>
<td>100</td>
<td>6.0 (73.1) d</td>
<td>12.0 (71.8) d</td>
<td>2.13 (96.8) c</td>
<td>20.9 (20.8) b</td>
</tr>
<tr>
<td>150</td>
<td>5.33 (76.1) de</td>
<td>9.1 (78.6) de</td>
<td>1.13 (98.3) c</td>
<td>20.1 (23.9) b</td>
</tr>
<tr>
<td>200</td>
<td>3.47 (84.4) e</td>
<td>7.1 (83.3) e</td>
<td>0.00 (100) c</td>
<td>16.2 (38.6) c</td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td>2.17</td>
<td>4.06</td>
<td>5.47</td>
<td>2.29</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Medicago sativa Root (mm)</th>
<th>Oryza sativa Root (mm)</th>
<th>Raphanus sativus Root (mm)</th>
<th>B. pilosa Root (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>34.4 (0.0) a</td>
<td>90.0 (0.0) a</td>
<td>60.7 (0.0) a</td>
<td>31.3 (0.0) a</td>
</tr>
<tr>
<td>50</td>
<td>20.8 (39.5) b</td>
<td>28.3 (68.5) b</td>
<td>3.4 (94.4) b</td>
<td>26.4 (15.7) b</td>
</tr>
<tr>
<td>75</td>
<td>18.5 (46.2) b</td>
<td>13.4 (85.1) c</td>
<td>3.13 (94.8) b</td>
<td>20.2 (35.5) c</td>
</tr>
<tr>
<td>100</td>
<td>2.73 (92.1) c</td>
<td>0.63 (99.3) d</td>
<td>2.70 (95.6) b</td>
<td>19.4 (38.8) c</td>
</tr>
<tr>
<td>150</td>
<td>2.37 (93.1) c</td>
<td>0.00 (100) d</td>
<td>1.17 (98.1) b</td>
<td>17.8 (43.1) c</td>
</tr>
<tr>
<td>200</td>
<td>2.03 (94.1) c</td>
<td>0.00 (100) d</td>
<td>0.63 (99.0) b</td>
<td>8.6 (72.5) d</td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td>4.64</td>
<td>7.74</td>
<td>6.99</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Figures in the parenthesis with (–) indicate percentage of promotion over control. Means within the same column followed by the same letter are not significantly different at P<0.05.
3.4.2. Chlorophyll and water content

The growth inhibitions were monitored in the Petri dishes treated with mimosine solutions of different concentrations. In this case not only growth, even the chlorophyll content (Fig. 3.3) and water content (Table 3.3) were also reduced in the seedlings treated with mimosine, compared with untreated control. The reduction in chlorophyll content in response to allelochemicals has been reported in a number of plants. However, it is not clear whether the observed loss in chlorophyll was due to degradation of chlorophyll already present in the plant or to direct inhibition of chlorophyll biosynthesis. Nevertheless, the loss of chlorophyll is likely to reduce the photosynthetic ability and thereby the growth and development of the plant. Furthermore, the decrease of water content indicates that treatment with mimosine causes water loss due to root dysfunction. In this way, mimosine can cause considerable toxicity to tested plant species. Chlorophyll content was severely affected with the application of mimosine at 50 ppm and more against *Medicago sativa* as shown in Fig. 3.3. In case of *Raphanus sativus*, the effect was prominent when applied at 75 ppm or more. Chlorophyll reduction was observed in soybean plants treated with aqueous extract of velvetleaf (Colton *et al.* 1980). It has been suggested that some allelopathic compound may interfere with the synthesis of porphyrin, precursors of chlorophyll biosynthesis. (Rice, 1984). Furthermore, over 80% of the fresh weight of many plants is water. Plants uptake water continuously to survive; and plant
growth is inhibited when water level reaches to below 80% of its body weight. Plants give off water vapor continuously from all their above-ground parts but particularly their leaves, and through this process, known as transpiration; plants typically lose over 98% of all the water their roots absorbed. There are a number of pigments in the plant which absorb light energy for photosynthesis, but the most important are the chlorophylls, green pigments containing a magnesium atom in an otherwise organic molecule. The decrease of chlorophyll synthesis may be due, among other reasons, to a decrease of 5-amino laevulinic acid (ALA) accumulation. This acid is the precursor of all tetrapyrroles and is a precursor of protochlorophyllide (that is converted into chlorophyll when exposed to light). On the other hand, in chlorophyll degradation, the initial step is the removal of phytol by chlorophyllase that is considered to be the first enzyme acting in the pathway of chlorophyll degradation (Santos, 2004).
Fig. 3.3. Amount of total chlorophyll in two week seedlings of *Medicago sativa*, *Oryza sativa*, *Raphanus sativus*, and *Bidens pilosa* amended with mimosine. Vertical bars represent standard deviation of the mean (n = 3).
Table 3.3. The water content of two week seedlings of *Medicago sativa*, *Oryza sativa*, *Raphanus sativus*, and *Bidens pilosa* amended with mimosine.

<table>
<thead>
<tr>
<th>Different Concentration (ppm)</th>
<th><em>Medicago sativa</em> (mg/g)</th>
<th><em>Oryza sativa</em> (mg/g)</th>
<th><em>Raphanus sativus</em> (mg/g)</th>
<th><em>Bidens pilosa</em> (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>94.07 ± 0.16 a</td>
<td>66.96 ± 0.19 a</td>
<td>92.32 ± 0.24 a</td>
<td>94.16 ± 0.13 a</td>
</tr>
<tr>
<td>50</td>
<td>93.90 ± 0.08 a</td>
<td>65.47 ± 0.07 b</td>
<td>66.50 ± 1.12 b</td>
<td>92.41 ± 1.22 ab</td>
</tr>
<tr>
<td>75</td>
<td>92.96 ± 0.39 ab</td>
<td>57.21 ± 0.20 c</td>
<td>63.52 ± 0.90 c</td>
<td>91.96 ± 1.57 abc</td>
</tr>
<tr>
<td>100</td>
<td>92.39 ± 0.51 bc</td>
<td>45.51 ± 0.25 d</td>
<td>61.16 ± 0.31 d</td>
<td>90.24 ± 3.51bcd</td>
</tr>
<tr>
<td>150</td>
<td>91.42 ± 1.40 de</td>
<td>42.33 ± 0.14 e</td>
<td>59.22 ± 0.17 e</td>
<td>88.68 ± 2.35 cd</td>
</tr>
<tr>
<td>200</td>
<td>90.96 ± 1.10 d</td>
<td>38.96 ± 0.28 f</td>
<td>55.41 ± 1.12 f</td>
<td>86.89 ± 1.75 d</td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td>1.38</td>
<td>0.30</td>
<td>1.36</td>
<td>3.62</td>
</tr>
</tbody>
</table>

Data are mean ± SD of three replications. Within columns, means within the same experiment followed by the same letter are not significantly different according to Fisher’s protected least significant difference test at the 5% level of probability.
3.4.3. Fungicidal activity

The antifungal activities of mimosine showed significantly different results in case of \textit{F. solani, R. solani, T. viride,} and \textit{C. rolfsii} at different concentrations as shown in Fig.3.4. Most of the tested fungi did not respond markedly by mimosine. It was found that at 100 ppm of mimosine showed above 60% antifungal activity against \textit{F. solani} among five fungi. The lowest activity of mimosine was found in case of \textit{F. oxysporum} at the same concentration. Murugesan and Radha (1994) demonstrated that mimosine inhibited mycelial growth of different fungi. A similar inhibitory activity was observed in our studies against \textit{F. solani, T. viride, and C. rolfsii}. The inhibitory action of mimosine observed in our studies may be due to the arrest of DNA replication (Gilbert \textit{et al.} 1995).

3.5. CONCLUSION

We concluded that allelochemical mimosine inhibit the chlorophyll biosynthesis, the stimulation of chlorophyll degradation or both. In chlorophyll biosynthesis, Mg-chelatase may be the major target of mimosine interference, resulting in the shortage of the supply orientation of chlorophyll. We may further conclude that both the supply and consumption orientation of chlorophyll may be affected. In conclusion, the present investigation that was aimed at determining primary screen for mimosine indicating that mimosine has a promising phytotoxin for the development of natural product herbicide.
From the present study, it could be concluded that mimosine possesses weed suppressing ability that can be utilized for future weed management strategies.
Fig. 3.4. Antifungal activity of mimosine at 100 ppm. Vertical bars represent standard deviation of the mean (n = 3).
CHAPTER 4

HERBICIDAL AND FUNGICIDAL ACTIVITIES AND IDENTIFICATION OF POTENTIAL PHYTOTOXINS FROM *B. PILOSA* LINN. var. RADIATA
4.1. SUMMARY

This study was carried out to examine possibility of utilizing B. pilosa, a weed widely distributed in the subtropics and the tropics, for weed and plant fungus controls. The extract of B. pilosa shows strong phytotoxic action against growth of Raphanus sativus and Echinochloa crus-galli, as well as antifungal activity against phytopathogens in bioassays. The extract of roots exhibited plant growth inhibitory activity than those of the leaves and stems. Fifteen phenolic compounds including pyrocatechin, salicylic acid, p-vinylguaiacol, dimethoxyphenol, eugenol, 4-ethyl-1,2-benzenediol, iso-vanillin, 2-hydroxy-6-methylbenzaldehyde, vanillin, vanillic acid, p-hydroxybenzoic acid, protocatechuic acid, p-coumaric acid, ferulic acid, and caffeic acid were identified by GC-MS. Content of caffeic acid was the highest among detected substances. Dimethoxyphenol and vanillic acid were found only in the roots of B. pilosa. Ferulic acid and p-hydroxybenzoic acid were higher in roots than in leaves and stems. These identified constituents may be responsible for the phytotoxic and fungitoxic activities of B. pilosa.

4.2. INTRODUCTION

B. pilosa Linn. var. Radiata is an annual weed distributed widely in tropical and subtropical regions of the world. It is well known as hairy beggar ticks, sticks tights, or Spanish needles and is reported to be a weed of 31 crops in more than 40 countries (Holm
Rich plant ecosystems in the tropics may provide many plants with strong allelopathic potential, and several among them may be exploited as a source for paddy weed suppression (Hong et al. 2004). Major researches so far conducted on *B. pilosa* have focused on the pharmaceutical properties of this shrub. The plant is used in various folk medicines which have a popular ingredient in herbal tea for anti-inflammatory, antiseptic, liver-protective, blood-pressure lowering, and hypoglycemic effects (Dimo et al. 2002). *B. pilosa* herbal extract is also used for skin disorders on the topical application (Levin et al. 1974). In Africa, this plant was taken as food and they believed to contribute to the etiology of human esophageal cancer (Mirvish et al. 1985). Phenylpropanoid glucosides (Sashida et al. 1991), polyacetylenes (Wu et al. 2004), a diterpenes (Zulueta et al. 1995), flavonoids (Wang et al. 1997), and flavone glycosides (Brandão et al. 1998) have been identified from the extract of *B. pilosa*. The extracts showed anti-hyperglycemic (Ubillas et al. 2000), antihypertensive (Dimo et al. 2002), immunosuppressive and anti-inflammatory (Pereira et al. 1999), anti-leukemic (Chang et al. 2001), anti-malarial (Brandão et al. 1997), anti-bacterial (Rabe et al. 1997), and antimicrobial activities (Khan et al. 2001). Despite numerous chemicals involved in the pharmaceutical properties of this plant has been identified, compounds of phytotoxins in *B. pilosa*, which may be responsible for its strong invasiveness, has remained unknown.
In Japan, *B. pilosa* grows abundantly in southern Kyushu and regions of Ryukyu, and Ogasawara islands. Concerns on this weed have been increased since it was noted as an invasive species (Shimizu *et al.* 2001). With strong invasive strength and effects from climate changing, this noxious weed may be able to extend its dominance to other regions in Japan. Since a successful establishment of an effective strategy against this invasive species has not been approached yet, research on the utilization of *B. pilosa* for eco-friendly agricultural production is indispensable. This study was therefore conducted to examine possibility of exploiting this tropical shrub for control of weeds and phytopathogens in agricultural fields. Identification of phytotoxic and antifungal compounds in *B. pilosa* was also carried out.

### 4.3. MATERIALS AND METHODS

#### 4.3.1. Plant seeds

Two indicator plants including radish (*Raphanus sativus* cv. KSM016) and barnyardgrass (*Echinochloa crus-galli* Beauv.) were used. Commercial radish seeds were purchased from Wakaba Company, Naha City, Japan. Seeds of barnyardgrass were collected from paddy fields in Miyazaki Prefecture, Japan, November 2003. The germination of the seeds was randomly checked and was always more than 80%. 
4.3.2. Sample preparation

*B. pilosa* at vegetable stage was collected in nature around the campus of University of the Ryukyus, Okinawa, Japan, in July 2005. The fresh leaves, stems, and roots were well cleaned and separated, and each (400g) was taken in a conical flask. An amount of 800 ml of sterilized water was added into the flasks. After boiling for one hour and cooling at room temperature, the filtrates were dried under vacuum by rotary evaporator at 40°C. Each of the dried crude extract of leaves 13.0g, stems 19.1g and roots 5.2 g were hydrolyzed with 50 ml 4 N NaOH and shaken in a water bath at 40°C for 4 h. The suspension was adjusted to pH 2 by 6 N HCl and centrifuged at 10,000 rpm for 10 min, and then filtrated. The supernatants were extracted 3 times with 50 ml ethyl acetate and then treated with anhydrous sodium sulfate. The acidic ethyl acetate fraction was filtrated and evaporated to dryness on a rotary evaporator at 40°C. It was dissolved in water to examine the effects against growth of plant and phytopathogens. Identification and quantification for its chemical component were achieved by using GC-MS and HPLC.

4.3.3. Measurement of total phenolic compounds

The total phenolics in each plant parts including leaves, stems and roots of *B. pilosa* were determined by Folin–Ciocalteu method (Kähkönen *et al.*1999). This method was selected for the determination of water-soluble phenolic compounds (polyphenols and
simple phenolics), because it can rapidly evaluate phenolic compounds in the plant extracts (Luximon-Rahman et al. 2003). The acidic fraction of *B. pilosa* as mentioned above was diluted with ethanol at 500 ppm. Diluted solution (200 µl) was put into a test tube added with 1 ml of Folin-Ciocalteu’s reagent and 0.8 ml of sodium carbonate (7.5%). The mixture was allowed to stand for 30 min at room temperature. Absorbance was measured at 765 nm by using an UV spectrophotometer (UV-160A Shimadzu, Kyoto, Japan). The total phenolic compound was expressed as gallic acid equivalent (GAE) in mg per g extracts.

### 4.3.4. Herbicidal bioassay

Aqueous solution from the acidic fraction of *B. pilosa* was used and the diluted solution with water (100, 200 and 500 ppm) were prepared. *R. sativus* and *E. crus-galli* were used as indicator plants to test the plant growth inhibition activity of *B. pilosa*. Ten seeds of each plant were sown on Petri dishes (90 mm in diameter) lined with filtered paper, and 8 ml of each diluted solution of *B. pilosa* was added. Treatment with distilled water was as controls. The plates were kept for 7 days at room temperature (25-28°C). Germination (%) and lengths of the shoots and roots of the indicator plants were determined. The plant growth inhibitory activity was expressed as percent of inhibition against the controls. The bioassays were performed with 3 replicates.
4.3.5. Fungicidal bioassay

The antifungal activities of all extracts were tested against Corticum rolfsii f.sp. (Curzi, Roma N.S.), Fusarium solani (MAFF 237472) and F. oxysporum (mormodiace, S.K. Sun & J.W. Huang). The antifungal activity was measured by agar dilution method (Taira et al.1994) as described in Chapter 2.

4.3.6. Identification by GC-MS

One μl aliquot of each sample of B. pilosa and reference chemicals dissolved in acetone was injected into GC-MS (QP-2010, Shimadzu Co., Kyoto, Japan). The DB-5MS column was 30 m in length, 0.25 mm i.d., and 0.25 μm in thickness (Agilent Technologies, J & W Scientific Products, Folsom, CA). The carrier gas was helium. The operating condition of GC oven temperature was maintained as: the initial temperature 50°C for 6 min, the programmed rate 5°C min⁻¹ up to final temperature 280°C with isotherm for 5 min. The injector and detector temperatures were set at 250°C and 280°C, respectively. The mass range was scanned from 20 to 900 amu. The control of the GC-MS system and the data peak processing were carried out by means of Shimadzu’s GC-MS solution Software, version 2.1.
4.3.7. Quantification by HPLC

All reference compounds were purchased from Tokyo chemical Co. Ltd., Nacalai tesque, Kyoto and Wako Pure Chemical Industries Ltd. Osaka, Japan. The phenolic compounds were detected at 280 nm using a HPLC (SCL-10 A vp, Shimadzu Co., Kyoto, Japan). The HPLC column was RP-18 ZORBAX ODS (25 x 0.46 cm i.d.; 5 µm particle size; Agilent Technologies, USA). The mobile phase consisting of a two solvent system, of which solvent A was water and acetic acid (99:1) (v/v); solvent B was methanol, acetonitrile, and acetic acid (95:4:1, v/v). The flow rate was 0.8 ml min\(^{-1}\), and injection volume was 5 µl. A gradient elution was as follows: 5% B at 0-2 min., 5-25% B at 2-10 min., 25-40% B at 10-20 min., 40-50% B at 20-30 min., 50-100% B at 30-40 min., 100% B at 40-45 min., 100-5% B at 45-55 min. The identification of the phenolic compounds was carried out by comparing their retention times to those of standards and mass spectra from GC-MS results. The standard curves were obtained from each standards reference chemicals (r > 0.99). Peak areas between the standards and samples were compared to quantify detected compounds. The content was expressed in µg/g fresh weight. All analyses were performed in triplicate.
4.3.8. Statistical analysis

Means and standard errors (SE) of the samples were calculated. Mean differences were determined by using Fisher’s Protected LSD test at 5% level of significance. All statistical analyses were performed using SAS version 8.2 (SAS Institute, 1999-2001).

4.4. RESULTS

4.4.1. Total phenolic compounds

The total phenolic compounds were significantly different among plant parts of *B. pilosa*, of which the stem had the maximum amount of phenolic compounds (76.1 mg GAE g\(^{-1}\) extract), followed by the leaves and roots (47.6 and 30.7 mg GAE g\(^{-1}\) extract,) respectively (Fig. 4.1). These variations are expected due to the presence of different types of phenolic compound in different parts of the plant.
Fig. 4.1. Total phenolics in different parts of *B. pilosa*. GAE represents gallic acid equivalent. Each value represents means ± S.E (n=3)
4.4.2. Herbicidal activity

The extracts of *B. pilosa* showed strong reduction against growth of the indicator plants (Table 4.1). However, inhibition against germination was not strong as compared with the controls, as it did not exceed 20% in all treatments. Inhibitions caused by the stem and root extracts against *E. crus-galli* and *R. sativus* were about 70-90% in all tested concentrations. The suppressive magnitude against indicator plants was almost same to applied doses, as growths of hypocotyl and radicle of *E. crus-galli* and *R. sativus* were almost inhibited at 500 ppm. In general, *B. pilosa* was a little bit more suppressive against *E. crus-galli* than *R. sativus*. 
Table 4.1. Herbicidal activity of the acidic fraction from *B. pilosa* against *Echinochola crus-galli* and *Raphanus sativus*.

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Leaves Germination</th>
<th>Hypocotyl</th>
<th>Radicle</th>
<th>Stems Germination</th>
<th>Hypocotyl</th>
<th>Radicle</th>
<th>Roots Germination</th>
<th>Hypocotyl</th>
<th>Radicle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>83.3 b</td>
<td>67.1 b</td>
<td>71.5 c</td>
<td>96.7 a</td>
<td>89.2 b</td>
<td>80.8 c</td>
<td>83.3 a</td>
<td>81.8 b</td>
<td>89.4 c</td>
</tr>
<tr>
<td>200</td>
<td>96.7 a</td>
<td>72.7 b</td>
<td>78.1 b</td>
<td>100.0 a</td>
<td>92.2 b</td>
<td>85.6 b</td>
<td>83.3 a</td>
<td>88.5 b</td>
<td>91.9 b</td>
</tr>
<tr>
<td>500</td>
<td>93.3 ab</td>
<td>86.6 a</td>
<td>90.2 a</td>
<td>93.6 a</td>
<td>97.5 a</td>
<td>93.8 a</td>
<td>83.3 a</td>
<td>98.5 a</td>
<td>99.3 a</td>
</tr>
<tr>
<td>LSD(0.05)</td>
<td>11.5</td>
<td>5.9</td>
<td>4.7</td>
<td>9.4</td>
<td>4.4</td>
<td>3.3</td>
<td>11.5</td>
<td>8.4</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*Echinochola crus-galli*

*Raphanus sativus*

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Leaves Germination</th>
<th>Hypocotyl</th>
<th>Radicle</th>
<th>Stems Germination</th>
<th>Hypocotyl</th>
<th>Radicle</th>
<th>Roots Germination</th>
<th>Hypocotyl</th>
<th>Radicle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>86.7 a</td>
<td>71.3 c</td>
<td>75.1 c</td>
<td>83.3 a</td>
<td>61.1 c</td>
<td>73.9 b</td>
<td>86.7 a</td>
<td>69.6 c</td>
<td>81.1 c</td>
</tr>
<tr>
<td>200</td>
<td>86.7 a</td>
<td>77.1 b</td>
<td>79.9 b</td>
<td>83.3 a</td>
<td>70.4 b</td>
<td>78.2 b</td>
<td>83.3 a</td>
<td>72.7 b</td>
<td>85.7 b</td>
</tr>
<tr>
<td>500</td>
<td>93.3 a</td>
<td>85.0 a</td>
<td>92.6 a</td>
<td>93.3 a</td>
<td>90.6 a</td>
<td>95.8 a</td>
<td>86.7 a</td>
<td>91.4 a</td>
<td>96.5 a</td>
</tr>
<tr>
<td>LSD(0.05)</td>
<td>11.5</td>
<td>3.4</td>
<td>3.0</td>
<td>11.5</td>
<td>3.0</td>
<td>5.2</td>
<td>11.5</td>
<td>5.1</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Means within the same column followed by the same letter are not significantly different.
4.4.3. Fungicidal activity

The extracts from all plant parts of *B. pilosa* were fungi toxic to the growth of the indicator fungi (Table 4.2). *C. rolfsii* was most suppressed as its growth was almost reduced by all tested doses, followed by *F. oxysporum* and *F. solani*. However, the fungicidal activities of the stems and roots were greater than the leaves. At 200-500 ppm, the extracts of *B. pilosa* appeared to be effective against growth of these phytopathogens; despite *F. solani* being less affected than *C. rolfsii* and *F. oxysporum*.

4.4.4. Determination of chemical components

By GC-MS, 15 compounds were detected from *B. pilosa* extracts, including pyrocatechin, salicylic acid, *p*-vinylguaiacol, dimethoxyphenol, eugenol, 4-ethyl-1,2-benzenediol, *iso*-vanillin, 2-hydroxy-6-methylbenzaldehyde, vanillin, vanillic acid, *p*-hydroxybenzoic acid, protocatechuic acid, *p*-coumaric acid, ferulic acid, and caffeic acid (Table 4.3). However, composition of these chemicals varied among different parts of *B. pilosa*. All of these compounds were found in the root extracts, except for vanillin and *iso*-vanillin. However, demethoxy phenol, eugenol, *iso*-vanillin and vanillic acid were not found in the stems of *B. pilosa*. In case of the leaves, salicylic acid, dimethoxy phenol and vanillic acid were not observed.
Table 4.2. Antifungal activities of the leaves, stems and roots extract of *B. pilosa* against *C. rolfsii, F. solani,* and *F. oxysporum.*

Means within the same column followed by the same letter are not significantly different (*P*=0.05).

<table>
<thead>
<tr>
<th>Plant parts</th>
<th>Concentrations (ppm)</th>
<th>Corticium rolfsii</th>
<th>Fusarium solani</th>
<th>Fusarium oxysporum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>100</td>
<td>63.1 f</td>
<td>30.2. f</td>
<td>36.3 f</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>82.1 d</td>
<td>44.9 f</td>
<td>68.5 d</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>87.0 c</td>
<td>62.0 cd</td>
<td>81.2 bc</td>
</tr>
<tr>
<td>Stems</td>
<td>100</td>
<td>68.2e</td>
<td>47.7 e</td>
<td>57.4 e</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>85.1 c</td>
<td>67.3 c</td>
<td>90.3 ab</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>93.1 b</td>
<td>86.7 b</td>
<td>98.5 a</td>
</tr>
<tr>
<td>Roots</td>
<td>100</td>
<td>90.9 b</td>
<td>57.7 d</td>
<td>72.3 cd</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>97.8 a</td>
<td>82.5 b</td>
<td>92.2 a</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>98.9 a</td>
<td>95.5 a</td>
<td>97.2 a</td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td>2.8</td>
<td>7.1</td>
<td>9.9</td>
<td></td>
</tr>
</tbody>
</table>
There were 9 phenolics including pyrocatechin, \( p \)-vinylguaiacol, 4-ethyl-1,2-benzenediol, 2-hydroxy-6-methylbenzaldehyde, \( p \)-hydroxybenzoic acid, protocatechuic acid, \( p \)-coumaric acid, ferulic acid, and caffeic acid were present in all parts of \( B. \) pilosa.

Among these detected substances, only six compounds including pyrocatechin, vanillin, \( p \)-hydroxybenzoic acid, \( p \)-coumaric acid, ferulic acid and caffeic acid were quantified. As compared with the leaves and stems, the root of \( B. \) pilosa contained maximum amounts of phenolic compounds, except for pyrocatechin and \( p \)-coumaric acid (Table 4.4). In all plant parts, caffeic acid was present in much greater quantity than other phenolics (117.4, 298.7, and 350.3 \( \mu \)g g\(^{-1}\) in leaves, stems, and roots,) respectively. In the roots ferulic acid was greater than pyrocatechin, in contrast, it was much lower in the leaves and stems than pyrocatechin. Total amount of these phenolic acids in roots was higher than the leaves and stem.
Table 4.3. Chemical components in the extract of *B. pilosa* detected by GC-MS

<table>
<thead>
<tr>
<th>Phenolic compounds</th>
<th>Retention time</th>
<th>Plant parts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Leaves</td>
</tr>
<tr>
<td>Pyrocatechin</td>
<td>20.2</td>
<td>+</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>23.4</td>
<td>-</td>
</tr>
<tr>
<td><em>p</em>-Vinylguaiacol</td>
<td>23.9</td>
<td>+</td>
</tr>
<tr>
<td>Dimethoxy phenol</td>
<td>24.9</td>
<td>-</td>
</tr>
<tr>
<td>Eugenol</td>
<td>25.1</td>
<td>+</td>
</tr>
<tr>
<td>4-Ethyl-1,2-benzenediol</td>
<td>25.8</td>
<td>+</td>
</tr>
<tr>
<td><em>iso</em>-Vanillin</td>
<td>26.4</td>
<td>+</td>
</tr>
<tr>
<td>2-Hydroxy-6-methyl benzaldehyde</td>
<td>27.5</td>
<td>+</td>
</tr>
<tr>
<td>Vanillin</td>
<td>28.0</td>
<td>+</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>30.7</td>
<td>-</td>
</tr>
<tr>
<td><em>p</em>-Hydroxybenzoic acid</td>
<td>34.6</td>
<td>+</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>35.3</td>
<td>+</td>
</tr>
<tr>
<td><em>p</em>-Coumaric acid</td>
<td>36.3</td>
<td>+</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>37.7</td>
<td>+</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>38.1</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) detected;
(-) not detected;
Table 4.4. Phenolic compound contents in leaves, stems and roots of *B. pilosa*

<table>
<thead>
<tr>
<th>Compounds (μg /g fresh weight)</th>
<th>Leaves</th>
<th>Stems</th>
<th>Roots</th>
<th>L.S.D (0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrocatechin</td>
<td>18.5 b</td>
<td>32.9 a</td>
<td>29.6 a</td>
<td>10.7</td>
</tr>
<tr>
<td><em>p</em>-Hydroxybenzoic acid</td>
<td>8.3 b</td>
<td>6.1 b</td>
<td>17.6 a</td>
<td>6.1</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>117.4 b</td>
<td>298.7 a</td>
<td>350.3 a</td>
<td>60.1</td>
</tr>
<tr>
<td>Vanillin</td>
<td>2.4 a</td>
<td>2.5 a</td>
<td>-</td>
<td>0.2</td>
</tr>
<tr>
<td><em>p</em>-Coumaric acid</td>
<td>9.5 a</td>
<td>4.7 c</td>
<td>5.6 b</td>
<td>0.7</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>6.1 b</td>
<td>6.2 b</td>
<td>37.1 a</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Means within row by same letter are not significantly different (*P*=0.05).

(-) Not detected;
4.5. DISCUSSION

Phenolic compounds are the most commonly investigated constituents among potential allelochemicals (Chon et al. 2005). They occur in plant tissues as free form of compounds (benzoic acid and cinnamic acid derivatives) and as bound forms (glycosidic phenylpropanoid esters). All types of phenolic compounds act as toxins and deterrents to pathogens and herbivores (Kainulainen et al. 2002). These phytotoxins are putative allelochemicals (Turk et al. 2003). Appropriate utilization of plant allelopathy may help to reduce the dependence on synthetic herbicides (Wu et al. 2002; Xuan et al. 2004). Phenolic acids such as p-hydroxybenzoic acid, vanillic acid, p-coumaric acid, syringic acid, and ferulic acid are among main category of allelochemicals in nature (Blum et al. 1991). Ferulic acid, p-hydroxybenzoic acid and p-coumaric acid inhibit hydraulic conductivity and nutrient uptake of plant roots, resulting in growth inhibition (Blum et al. 1991). Catechol, a potent weed inhibitor, belongs to phenolic compounds found in plants (Topal et al. 2006). Phenolic compounds also inhibit seed germination, plant growth, and influence other physiological processes (Djurđević et al. 2004). On the other hand, membrane-associated disturbances are the common phenomenon attributed to phenolic acids (Barkosky et al. 2000). Caffeic acid is one of many phenolic compounds considered to be an important part of the general defending mechanisms of plants against infection and predation (Faulds et al. 1999). This phenolic compound has been
reported as a potential inhibitor to a variety of crops and weeds (Matsuo et al. 2002). In bioassay, it suppressed growth of plants (Miller et al. 1991), fungi (Ravn et al. 1989), bacteria (Bowls et al. 1994), and insects (Summers et al. 1994). Furthermore, caffeic acid can hinder root activity by inhibiting phosphate and potassium uptake and depolarization of root membranes (Barkosky et al. 2000). Phenolic compounds found in all plant parts of *B. pilosa* may be responsible for the phytotoxic action against Barnyardgrass and Radish. The herbicidal and fungicidal activities of each plant parts of *B. pilosa* were found to be proportional to quantity and amount of phenolic compounds. All of phenolic compounds found were first time identified in *B. pilosa*, and they are well known plant growth inhibitors. Salicylic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, vanillic acid, ferulic acid and caffeic acid were isolated from different plant and identified as potential allelochemicals (Chung et al. 2001). Caffeic acid is one of the major allelochemicals in *Leonurus*, having dose dependant inhibitory activity (Mandal, 2001). It may play a major role, but the phytotoxic and fungitoxic action may be attributed to all compounds present in *B. pilosa*.

*B. pilosa* is an invasive plant not only in Japan but also in many countries. It grows abundantly in Southeast Asia and was known as a noxious weed (Holm et al. 1991). In our previous work (Hong et al. 2003), a preliminary screening on evaluating weed suppressing potential of plants from plant ecosystem in Southeast Asia was conducted. Several plants were
suggested as paddy weed suppressing sources including *B. pilosa* (Hong *et al.* 2004). In this study, the herbicidal potential of *B. pilosa* was reconfirmed and the antifungal activity of this tropical weed was simultaneously observed. *R. sativus* is more sensitive against phytotoxins at low concentration than *E. crus-galli*, as this noxious weed has stronger resistance against plant phytotoxins than other common paddy weeds (Xuan *et al.* 2004). The roots of *B. pilosa* may contain either particular phytotoxins against growth of *E. crus-galli* which differ from the leaves and stems, or similar compounds but in higher contents. Chemical analysis of this tropical weed could help to elucidate this question. The roots were also very toxic to the tested fungi, suggesting that the roots of *B. pilosa* might be a useful source for treatment of noxious fungi. On the basis of our results and earlier findings, the fungicidal activity of different plant parts of *B. pilosa* having phenolic compounds, may be valuable for possible safer way of disease control.

Despite many phytotoxins were detected from *B. pilosa*, but it is unclear that what allelochemicals and their amounts were released into environment. Since the actual allelopathic activity of *B. pilosa* in nature could be clarified, it might provide helpful aid for the exploitation of this weed for eco-friendly agricultural production.
CHAPTER 5

CHEMICAL COMPOSITION AND ANTIOXIDANT, ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES OF THE ESSENTIAL OILS FROM B. PILOSA
5.1. SUMMARY

The present study describes the chemical composition, antibacterial and antifungal activities of essential oils in *B. pilosa*, an invasive plant widely distributed in the subtropics and tropics. The essential oils from the fresh leaves and flowers of *B. pilosa* were analyzed by GC-MS. Forty-six components were identified, of which β-caryophyllene (10.9 and 5.1%) and τ-cadinene (7.82 and 6.13%) were the main compounds in leaves and flowers, respectively. By DPPH and β-carotene bleaching methods for the antioxidant testing, the essential oils from leaves and flowers reduced the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) with the IC₅₀ 47.5 and 49.7 μg/ml, respectively. The oils from *B. pilosa* exerted significant antibacterial and antifungal activities against six bacteria and three fungal strains. The inhibitory activity of the flower essential oils against Gram-negative bacteria was significantly higher than in Gram-positive. The findings demonstrate that the essential oils and water extracts of *B. pilosa* possess antioxidant and antimicrobial activities that might be a natural potential source as preservative in food and other allied industries.

5.2. INTRODUCTION

The food industry at present is facing a tremendous pressure from consumers for using chemical preservatives to prevent the growth of food borne and spoiling microbes. To reduce
or eliminate chemically synthesized additives in their foods is a current demand worldwide. A new approach to prevent the proliferation of microorganism or protect food from oxidation is the use of essential oils as preservatives. Essential oils of plants are growing interest both in the industry and scientific research because of their antibacterial, antifungal, and antioxidant properties and make them useful as natural additives in foods (Pattnaik et al. 1997). Free radical oxidation of the lipid components in food due to the chain reaction of lipid peroxidation is a major strategic problem for food manufacturers. Due to undesirable influences of oxidized lipids on the human organisms, it is essential to decrease lipid peroxidation product in food (Karpinska et al. 2001). Reactive oxygen species are reported to be a causative agent of various diseases such as arthritis, asthma, dementia, mongolism, carcinoma and Parkinson’s disease (Perry et al. 2000). Plant essential oils and their extracts have had a great usage in folk medicine, food flavoring, fragrance, and pharmaceutical industries (Kusmenoglu et al. 1995).

*B. pilosa* Linn. var. *Radiata* (family Asteraceae) is widely distributed in the subtropical and tropical regions of the world. It is 30-100 cm in height with yellow flowers and is well known as hairy beggar ticks, sticks tights, and Spanish needles. The plant is used in various folk medicines such as anti-inflammatory, antiseptic, liver-protective, blood-pressure lowering, and hypoglycemic effects (Dimo et al. 2002). Phenylpropanoid glucosides, polyacetylenes, a
diterpenes, flavonoids, and flavone glycosides have been identified as bioactive components of this plant (Chiang et al. 2004). These compounds were suggested to involve in the pharmacological property of *B. pilosa* (Chiang et al. 2004), anti-bacterial, anti-microbial activities (Rabe et al. 1997). The plant has been widely used in Taiwan as a traditional medicine and as a major ingredient of herbal tea, which is believed to prevent from the inflammation and cancer (Yang et al. 2006).

To the best of our knowledge, there are no available reports on chemical composition and biological activities of the essential oils from *B. pilosa*. Therefore, the aim of the present work was carried out to study *in vitro* antioxidant, antibacterial, and antifungal activities of the essential oils as well as the water extract of *B. pilosa* in addition to evaluate the component of essential oils by GC-MS. Essential oils obtained by steam-distillation were analyzed for their possible antioxidant activities by two complementary methods, namely DPPH free radical-scavenging and β-carotene bleaching methods due to identify all possible mechanisms characterizing an antioxidant.
5.3. MATERIALS AND METHODS

5.3.1. Plant material, extraction of essential oils, and preparation of the water extract

The fresh leaves and flowers of *B. pilosa* at vegetable stage were collected at the campus of University of the Ryukyus, Okinawa, Japan in April 2006. Fresh leaves or flowers (each 400 g) of *B. pilosa* were steam-distilled for 4 hr. Each distillate was extracted with 200 ml diethyl ether twice. The solvent was carefully removed under vacuum at 30°C. The obtained essential oils were stored at 4°C until experiments were carried out. After completion of steam-distillation, the aqueous mixtures of leaves or flowers was filtered to collect water extract which was evaporated to dryness on a rotary evaporator at 40°C.

5.3.2. Identification by GC-MS

An aliquot of 1 µl oils dissolved in diethyl ether and adjusted to 1000 ppm was injected into GC-MS (QP-2010, Shimadzu Co., Kyoto, Japan). The DB-5MS column was 30 m in length, 0.25 mm i.d., and 0.25 µm in thickness (Agilent Technologies, J & W Scientific Products, Folsom, CA). The carrier gas was helium. The operating condition of GC oven temperature was maintained as: initial temperature 50°C for 5 min, programmed rate 5°C/ min up to final temperature 280°C with isotherm for 5 min. The injector and detector temperatures were set at 250°C and 280°C, respectively. The essential oil components were identified by comparing their retention times and mass fragmentation pattern with those of standards and
MS library (Shimadzu’s GCMS solution software, version 2.4). The quantity of oil components was compared using peak area measurements.

5.3.3. Antioxidant activity

5.3.3.1. DPPH assay

In this assay, antioxidant activity of essential oils was evaluated by measuring the bleaching of the purple-colored ethanol solution of DPPH (Burits et al. 2000). The radical scavenging ability was determined according to a method described by Abe, Murata, and Hirota (1998). One ml from 0.5 mM ethanol solution of the DPPH radical was mixed to 2.0 ml from the samples of different concentrations of essential oils from leaves and flowers and was added 2 ml of 0.1 M sodium acetate buffer (pH 5.5). The mixtures were well shaken and stand at room temperature in the dark for 30 min. The absorbance was measured at 517 nm using a Shimadzu UV-Vis spectrophotometer mini 1240, Kyoto (Japan). The authentic α-tocopherol and butyl hydroxytoluene (BHT) were used as a positive control while ethanol was as negative one. Inhibition (I) of DPPH radical was calculated using the equation:

\[ I(\%) = 100 \times \frac{(A_o - A_s)}{A_o} \]

\( A_o \) is the absorbance of the control (containing all reagents except the test compound), and \( A_s \) is the absorbance of the tested sample. The IC\(_{50}\) value represented the concentration of the
essential oils that caused 50% inhibition.

5.3.3.2. β-Carotene bleaching assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius et al. 1998). Antioxidant activity was carried out according to the β-carotene bleaching method (Siddhuraju et al. 2003) with minor modifications. β-Carotene (2.0 mg) was dissolved in 10 ml chloroform. Linoleic acid (20 μl) and Tween-40 (200 mg) were mixed with 1 ml of the chloroform solution. The chloroform was evaporated under vacuum at 45 ºC, then 50 ml oxygenated water was added, and the mixture was vigorously shaken. The emulsion obtained was freshly prepared before each experiment. The β-carotene-linoleic acid emulsion (250 μl) was transferred to a 96-well microplate. Ethanolic solutions (30 μl) of the sample extracts, BHT and α-tocopherol at 1000 ppm were added onto plate. An equal amount of ethanol was used as control. Absorbance was taken at 492 nm after incubation for every 15 min until 180 min at 45 ºC using a micro plate reader (Benchmark plus micro plate 170-6930j1, BIO-RAD Company).
5.3.4. Antibacterial activities

All bacterial strains have been kindly provided by Laboratory of Microbiology, University of the Ryukyus, Okinawa, Japan. Antibacterial activities of all extracts using ampicillin as the reference standard were tested against *Bacillus subtilis*, *Bacillus cereus*, *Bacillus pumilus*, *Escherichia coli* and *Pseudomonus ovalis*. Essential oils, water extracts and ampicillin were dissolved in 10% Tween 80 which was used as the control. Antibacterial activity was determined by using the disc diffusion method (Karaman *et al.* 2003). One hundred microliters of test organisms [10^6 colony forming units CFU/ml] grown in nutrient broth media for 24 h were spread over the surface of solid nutrient agar medium in 9 cm diameter Petri dishes. Filter paper discs (6 mm in diameter) loaded with each of the samples and ampicillin were placed on the surface of the nutrient agar which were incubated at 37°C for 24 hr, and then the diameters of inhibition zones were measured in millimeters.

5.3.5. Fungicidal bioassay

The antifungal activity was measured by agar dilution method (Taira *et al.* 1994) as described in Chapter 2. Before 5 strains are used but 3 strains in this chapter.
5.3.6. Statistical analysis

Means and standard errors (SE) of the samples were calculated. Each treatment was carried out with 3 replicates. Mean differences were determined by using Fisher’s Protected LSD test at 5% level of significance. All statistical analyses were performed using SAS version 8.2 (SAS Institute, 1999-2001).

5.4. RESULT AND DISCUSSION

5.4.1. Chemical composition of the essential oils

Fresh leaves and flowers part of *B. pilosa* were subjected to steam-distillation and the colorless and yellowish essential oils were obtained of 0.08 and 0.06%, w/w, respectively. The results obtained by GC-MS analysis of the essential oils from *B. pilosa* are presented in Table 5.1. Forty-six components were identified and the major essential oils belonging to terpenes were β-caryophyllene (10.9 and 5.1%) and τ-cadinene (7.82 and 6.13%), in the leaves and flowers, respectively. The other chemical components were α-pinene, limonene, β-trans-ocimene, β-cis-ocimene, τ-muurolene, β-bourbonene, β-elemene, β-cubebene, α-caryophyllene, caryophyllene oxide, and megastigmatrienone.

To the best of our knowledge, the essential oils of *B. pilosa* have never been reported. This work is therefore the first report on the essential oils from these tropical and subtropical
species. Among identified compound, $\beta$-caryophyllene is well-known for its anti-inflammatory and local anaesthetic activities (Ghelardini et al. 2001). This compound is also used in spice blends, soaps, detergents, creams and lotions, and is widely used in food products and beverages (Skold et al. 2006).
Table 5.1. Essential oil components of fresh leaves and flowers of *B. pilosa*

<table>
<thead>
<tr>
<th>Components</th>
<th>RI ¹</th>
<th>Peak area %</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Leaves</td>
<td>Flowers</td>
</tr>
<tr>
<td>Acetal</td>
<td>815</td>
<td>0.03</td>
<td>0.09</td>
</tr>
<tr>
<td>cis-3-Hexen-1-ol</td>
<td>854</td>
<td>0.10</td>
<td>-</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>930</td>
<td>0.99</td>
<td>5.97</td>
</tr>
<tr>
<td>Camphene</td>
<td>947</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>trans-Verbenol</td>
<td>951</td>
<td>- b</td>
<td>0.05</td>
</tr>
<tr>
<td>β-Phellandrene</td>
<td>971</td>
<td>0.01</td>
<td>0.15</td>
</tr>
<tr>
<td>β-Pinene</td>
<td>975</td>
<td>0.07</td>
<td>0.39</td>
</tr>
<tr>
<td>β-Myrcene</td>
<td>988</td>
<td>0.29</td>
<td>1.54</td>
</tr>
<tr>
<td>cis-3-Hexenyl acetate</td>
<td>1005</td>
<td>0.77</td>
<td>-</td>
</tr>
<tr>
<td>3-Carene</td>
<td>1006</td>
<td>-</td>
<td>0.65</td>
</tr>
<tr>
<td>m-Cymol</td>
<td>1024</td>
<td>0.08</td>
<td>0.11</td>
</tr>
<tr>
<td>Limonene</td>
<td>1029</td>
<td>0.34</td>
<td>2.12</td>
</tr>
<tr>
<td>β-trans-Ocimene</td>
<td>1036</td>
<td>0.55</td>
<td>1.64</td>
</tr>
<tr>
<td>β-cis-Ocimene</td>
<td>1047</td>
<td>1.45</td>
<td>1.46</td>
</tr>
<tr>
<td>γ-Terpinene</td>
<td>1059</td>
<td>NF</td>
<td>0.05</td>
</tr>
<tr>
<td>β-Linalool</td>
<td>1100</td>
<td>0.43</td>
<td>0.09</td>
</tr>
<tr>
<td>(4E,6Z)-2,6-Dimethyl-2,4,6-octatriene</td>
<td>1129</td>
<td>0.24</td>
<td>0.70</td>
</tr>
<tr>
<td>trans-Verbenol</td>
<td>1144</td>
<td>-</td>
<td>0.11</td>
</tr>
<tr>
<td>cis-Verbenol</td>
<td>1148</td>
<td>0.11</td>
<td>0.18</td>
</tr>
<tr>
<td>4-Terpineol</td>
<td>1183</td>
<td>0.15</td>
<td>0.41</td>
</tr>
<tr>
<td>p-Cymen-8-ol</td>
<td>1198</td>
<td>0.26</td>
<td>0.35</td>
</tr>
<tr>
<td>Bornyl acetate</td>
<td>1287</td>
<td>0.15</td>
<td>0.18</td>
</tr>
<tr>
<td>Elixene</td>
<td>1313</td>
<td>0.26</td>
<td>0.32</td>
</tr>
<tr>
<td>α-Cubebene</td>
<td>1318</td>
<td>0.17</td>
<td>0.21</td>
</tr>
<tr>
<td>Ylangene</td>
<td>1326</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td>τ-Muurolene</td>
<td>1329</td>
<td>1.04</td>
<td>0.78</td>
</tr>
<tr>
<td>β-Bourbonene</td>
<td>1332</td>
<td>1.10</td>
<td>0.99</td>
</tr>
<tr>
<td>β-Elemene</td>
<td>1333</td>
<td>1.00</td>
<td>0.67</td>
</tr>
<tr>
<td>α-Bergamotene</td>
<td>1341</td>
<td>0.12</td>
<td>0.07</td>
</tr>
<tr>
<td>β-Caryophyllene</td>
<td>1345</td>
<td>10.9</td>
<td>5.1</td>
</tr>
<tr>
<td>β-Cubebene</td>
<td>1348</td>
<td>2.23</td>
<td>1.77</td>
</tr>
<tr>
<td>β-Farnesene</td>
<td>1353</td>
<td>0.72</td>
<td>0.29</td>
</tr>
<tr>
<td>α-Caryophyllene</td>
<td>1357</td>
<td>1.55</td>
<td>1.00</td>
</tr>
<tr>
<td>(+)-Epi-bicyclesquiphellandrene</td>
<td>1358</td>
<td>0.27</td>
<td>0.25</td>
</tr>
</tbody>
</table>

¹: Retention index
Continuation of Table 5.1

<table>
<thead>
<tr>
<th>Substance</th>
<th>Retention Index</th>
<th>Area %</th>
<th>Retention Index</th>
<th>Area %</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoledene</td>
<td>1362</td>
<td>0.67</td>
<td>0.47</td>
<td></td>
<td>Insect attractant</td>
</tr>
<tr>
<td>α-Cadinene</td>
<td>1365</td>
<td>7.82</td>
<td>6.13</td>
<td></td>
<td>Antifungal</td>
</tr>
<tr>
<td>β-Bisabolene</td>
<td>1372</td>
<td>0.31</td>
<td>0.03</td>
<td></td>
<td>Common cold</td>
</tr>
<tr>
<td>β-Gurjunene</td>
<td>1375</td>
<td>0.44</td>
<td>0.24</td>
<td></td>
<td>Insecticidal</td>
</tr>
<tr>
<td>(-)-β-Cadiene</td>
<td>1376</td>
<td>0.82</td>
<td>0.23</td>
<td></td>
<td>Antimalaria</td>
</tr>
<tr>
<td>2,5,9-Trimethylcycloundeca-4,8-dienone</td>
<td>1387</td>
<td>0.82</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>trans-Nerolidol</td>
<td>1388</td>
<td>0.39</td>
<td>0.13</td>
<td></td>
<td>Carrying plant pathogenic fungi</td>
</tr>
<tr>
<td>Ent-spathulenol</td>
<td>1396</td>
<td>0.33</td>
<td>0.18</td>
<td></td>
<td>Artefact</td>
</tr>
<tr>
<td>Caryophyllene oxide</td>
<td>1398</td>
<td>1.47</td>
<td>1.03</td>
<td></td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>Megastigmatrienone</td>
<td>1444</td>
<td>5.35</td>
<td>2.04</td>
<td></td>
<td>Fragrance</td>
</tr>
<tr>
<td>Diphenylenemethane</td>
<td>1450</td>
<td>1.94</td>
<td>1.77</td>
<td></td>
<td>Photo stabilizing agent</td>
</tr>
</tbody>
</table>

\^ Retention index relative to \( n \)-alkanes on the DB-5 column;
\^ Not detected;
5.4.2. Antioxidant activity

5.4.2.1. DPPH free radical scavenging activity

Free radical scavenging capacities of the extracts, measured by DPPH assay are shown in Fig. 5.1. There is no significant difference between leaves and flower essential oils. The activity of the extracts is proportional to the concentrations and lower IC\textsubscript{50} value reflects better protective action. The leaves and flowers essential oils were able to reduce the stable free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) to the yellow–colored diphenylpicrylhydrazine with IC\textsubscript{50} of 47 and 50 \(\mu\)g/ml, respectively, whereas those of the synthetic and natural antioxidant activity were 21 and 36 \(\mu\)g/ml. *B. pilosa* essential oils are monoterpenes and oxygenated terpenes such as \(\alpha\)-pinene, ocimene, verbenol, \(\beta\)-myrcene, which have simple phenolic structures. Based on the structure-activity relationship related to antioxidant effects, it could be deduced that polymeric phenol are stronger antioxidant than monomeric phenolic compounds (Moure *et al.* 2001). Moreover, trying to correlate the observed activity with the chemical composition of the oils of plant origin, it is noteworthy to cite the work of Ruberto and Baratta (2000), who studied the antioxidant activity of 98 pure essential oils chemical components and showed that monoterpe hydrocarbons had a significant protective effect, with several variants due to the different functional groups.
Fig. 5.1. Free radical scavenging activities of the essential oil and water extracts from *B. pilosa*. BHT and α-tocopherol as a positive controls. Values are means of three replications±SE.
5.4.2.2. Antioxidant activity measured by $\beta$-carotene bleaching method

In the $\beta$-carotene bleaching method, the degree of linoleic acid oxidation is determined by measuring oxidation products (lipid hydroperoxides, conjugated dienes, and volatile by-products) of linoleic acid which simultaneously attack $\beta$-carotene, resulting in bleaching of its characteristic yellow color in ethanolic solution. All extracts of $B. \ pilosa$ inhibit the oxidation of linoleic acid, which is an important issue in food processing and preservation (Fig. 5.2). Inhibition of the breakdown of lipid hydroperoxides to unwanted volatile products allows us to determine secondary antioxidants in related mechanisms. It has been well reported that phenolic compounds are able to donate a hydrogen atom to the free radicals thus stopping the propagation chain reaction during lipid oxidation process (Sanchez-Mareno et al. 1998). Leaves essential oils and aqueous extracts of leaves and flowers exhibited the higher antioxidant activities than those of the flower oils. The lower activity of the flower essential oils of $B. \ pilosa$ may also be due to its volatility at higher temperature. The antioxidant effect of essential oils depends not only on the temperature but also on many factors such as their structural features, the character of the lipid system, and on the binding of the fatty acids.
Fig. 5.2. Antioxidant activity of essential oil and water extracts from *B. pilosa*, BHT and α-tocopherol measured by β-carotene bleaching method at 1000 ppm.

L, leaves; F, flower;
5.4.3. Antibacterial activities

As can be seen in Table 5.2, essential oils and the water extracts obtained from *B. pilosa* showed to have moderate antimicrobial activity against all microorganisms tested. In general, leaves essential oils exhibited stronger activity than the others. Among all essential oils and extracts, the flower essential oils were the most active against *E. coli* inhibition. It has been demonstrated that monoterpene hydrocarbons and oxygenated monoterpenes in the flower essential oils are able to destroy cellular integrity, and thereby, inhibit respiration and ion transport processes. This is strongly supported by the study on the effects of different essential oils components on outer membrane permeability in Gram-negative bacteria (Helander *et al.* 1998). The sesquiterpene β-caryophyllene is known to possess a critical part in plant defense (Ulubelen *et al.* 1994). The maximum activity of leaves essential oils was observed against Gram-positive bacteria *B. cereus* and *B. subtilis* but this oil had poor activity on the growth of Gram-negative bacteria *E. coli* and *P. ovalis*. Most studies investigating the action of essential oils against food spoilage organisms and food borne pathogens agree that essential oils are slightly more active against Gram-positive than Gram-negative bacteria (Lambert *et al.*, 2001). The antibacterial activity of the essential oils of *B. pilosa* against bacteria studied may be due to the presence of a high concentration of β-caryophyllene, since the antimicrobial properties of caryophyllene and caryophyllene oxide were found in *Salvia sclarea* also observed by
Ulubelen et al. (1994). The antimicrobial nature of the essential oils of *B. pilosa* is apparently related to its phenolic components, such as pine, myrcene, limonene and ocimene (Table 5.2), since there is a relationship between the chemical structures of the most abundant compounds in oils and the antimicrobial activity. The essential oils containing phenolic compounds are widely reported to possess high levels of antimicrobial activity (Lambert et al. 2001), which are consistent with our present studies.
Table 5.2. Antimicrobial activity of the essential oil and extracts of *B. pilosa*.

<table>
<thead>
<tr>
<th>Test bacteria</th>
<th>Essential oil and Extracts a</th>
<th>Zone of inhibition (mm)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lc. essential oil</td>
<td>Fm. essential oil</td>
</tr>
<tr>
<td><em>Micrococcus flavus</em> f</td>
<td>12.7 ± 0.3 b</td>
<td>8.7 ± 0.3 d</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> f</td>
<td>17.3 ± 1.9 b</td>
<td>11.7 ± 0.2 c</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> f</td>
<td>19.0 ± 1.4 a</td>
<td>11.2 ± 0.3 b</td>
</tr>
<tr>
<td><em>Bacillus pumilus</em> f</td>
<td>12.3 ± 0.7 b</td>
<td>10.8 ± 0.2 c</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>13.7 ± 0.4 c</td>
<td>20.3 ± 0.7 b</td>
</tr>
<tr>
<td><em>Pseudomonas ovalis</em> f</td>
<td>12.5 ± 0.8 ab</td>
<td>13.7 ± 1.5 a</td>
</tr>
</tbody>
</table>

Values are means of three replications ± SE. Means with the same letter are not significantly different at *P* ≤ 0.05.

a 400 μg/disc.
b Diameter of disc 6 mm.
c Leaves.
d Flowers.
e 30 μg/disc.
f gram positive bacteria
g gram negative bacteria
5.4.4. Antifungal activities

The results of antifungal activity assays showed that the essential oils and aqueous extract of *B. pilosa* had the inhibitory effects on the growth of fungi (*Table 5.3*). *F. solani* was most suppressed as its growth was almost reduced by all tested doses, followed by *F. oxysporum* and *C. rolfsii*. On the other hand, the toxic activities of the flower essential oils were higher than those of the leaves. At 100 ppm, the essential oils of *B. pilosa* appeared to be effective against growth of these phytopathogens above 80%. The volatile oils consist of complex mixtures of numerous components. The major or trace compound(s) might give rise to the antifungal activity. Possible synergistic and antagonistic effects of compounds also play an important role in fungi inhibition. Previous papers on the antifungal activities of essential oils of some species of various genera have shown that they have varying degrees of growth inhibition effects against some agricultural pathogenic fungal species (Alvarez-Castellanos *et al.* 2001). Although the different compounds exhibited varying degrees of antifungal activity, β-caryophyllene and caryophyllene oxide was very toxic against the studied *Fusarium* species (Cakir *et al.* 2004).

The present study indicates that *B. pilosa* essential oils contain β-caryophyllene and caryophyllene oxide (*Table 5.1*). Therefore, β-caryophyllene and caryophyllene oxide detected in *B. pilosa* essential oils, may affect their antifungal activities. α-Pinene, which was found to be an appreciable amounts in the oils of *B. pilosa*, has been reported to be the cause of the antifungal activity of oils from *Pistacia lentiscus* (Magiatis *et al.* 1999). Another minor monoterpene alcohol, linalool, is reported to have a wide range of antibacterial and antifungal activities (Pattnaik *et al.* 1997).
Table 5.3. Antifungal activities of essential oil and water extracts of *B. pilosa*. L, leaves; F, flower.

<table>
<thead>
<tr>
<th>Plant parts</th>
<th>Concentrations (ppm)</th>
<th><em>Corticium rolfsii</em> (%)</th>
<th><em>Fusarium solani</em> (%)</th>
<th><em>Fusarium oxysporum</em> (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. essential oil</td>
<td>100</td>
<td>85.7 ± 0.9 c</td>
<td>68.2 ± 0.0 d</td>
<td>74.9 ± 1.7 d</td>
</tr>
<tr>
<td>L. essential oil</td>
<td>250</td>
<td>96.0 ± 0.8 a</td>
<td>77.9 ± 1.8 c</td>
<td>87.9 ± 0.4 b</td>
</tr>
<tr>
<td>F. essential oil</td>
<td>100</td>
<td>60.4 ± 0.9 e</td>
<td>89.2 ± 0.4 b</td>
<td>86.9 ± 0.5 b</td>
</tr>
<tr>
<td>F. essential oil</td>
<td>250</td>
<td>89.4 ± 1.2 b</td>
<td>98.0 ± 0.3 a</td>
<td>94.9 ± 0.6 a</td>
</tr>
<tr>
<td>L. water extract</td>
<td>100</td>
<td>44.6 ± 1.7 f</td>
<td>60.5 ± 2.1 e</td>
<td>71.6 ± 0.7 d</td>
</tr>
<tr>
<td>L. water extract</td>
<td>250</td>
<td>94.2 ± 0.3 a</td>
<td>68.9 ± 0.7 d</td>
<td>82.4 ± 1.9 c</td>
</tr>
<tr>
<td>F. water extract</td>
<td>100</td>
<td>33.1 ± 1.1 g</td>
<td>71.4 ± 0.7 d</td>
<td>57.3 ± 2.2 e</td>
</tr>
<tr>
<td>F. water extract</td>
<td>250</td>
<td>66.1 ± 1.4 d</td>
<td>91.2 ± 0.0 b</td>
<td>90.0 ± 0.7 b</td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td>3.3</td>
<td>5.1</td>
<td>3.8</td>
<td></td>
</tr>
</tbody>
</table>
The essential oils from this plant exhibited antibacterial, antifungal and antioxidant activities. The antioxidant activity may be ascribed to the presence of the same chemical components. Monoterpenes found in these essential oils may act as radical scavenging agents. It seems to be a general trend that the essential oils which contain monoterpane hydrocarbons, oxygenated monoterpenes and/or sesquiterpenes have greater antioxidative properties (Tepe et al. 2004). These activities may be attributed to the presence of \( p \)-cymene-8-ol, \( \alpha \)-pinene, \( \beta \)-pinene, ocimene, limonene, terpinene, and camphene found in \( B. pilosa \) essential oils. Enantiomers of \( \alpha \)-pinene, 2-\( \beta \)-pinene and limonene have a strong antibacterial activity (Sökmen et al. 2003). These chemical components exert their toxic effects against studied microorganisms through the disruption of bacteria or fungal membrane integrity (Knobloch et al. 1989). The possible mechanisms of other essential oils components, such as \( \text{trans} \)-caryophyllene, limonene, camphene, and \( \alpha \)-humulene have not yet been elucidated. The mechanism of antifungal activity of these essential oils is still unknown.

Antioxidants, antibacterial and antifungal properties of the essential oils and various extracts from many plants have recently been of great interest in both academia and the food industry, because their possible use as natural additives emerged from a growing tendency to replace synthetic antioxidants with natural ones. Owing to strong antifungal and protective features exhibited in antioxidant activity tests, the essential oils and aqueous extracts of \( B. pilosa \) could be considered a natural herbal source that can be freely used in the food and pharmaceutical industries. This is the first report on the essential oils composition, antioxidants, antibacterial and antifungal activity of \( B. pilosa \). Further
studies are needed to obtain more information regarding the practical effectiveness of these oils in animal models.
CHAPTER 6

FREE RADICAL SCAVENGING AND TYROSINASE-INHIBITION ACTIVITIES OF B. PILOSA ROOT
6.1. SUMMARY

Methanol extract and three different fraction of B. pilosa root were evaluated for their free-radical-scavenging, \( \beta \)-carotene-linoleic acid bleaching assay and tyrosinase inhibitory activities. The ethyl acetate fraction showed highest activity against free-radical-scavenging and tyrosinase inhibitory. By the GC-MS fourteen phenolic compounds are identified in these fractions. Therefore, the antioxidative and tyrosinase inhibition activities exhibited by ethyl acetate fractions of root samples may be attributable to the contents of phenolic compounds.

6.2. INTRODUCTION

It is well-known that antioxidants have a range of applications in human health care. There is evidence that free radicals are involved in chemical liver injury and implicated in the etiology of several diseases such as stroke, rheumatoid arthritis, diabetes, cancer and aging process (Harish et al. 2006). In recent years, the prevention of cancer and cardiovascular diseases has been associated with the ingestion of fresh fruits, vegetables or teas rich in natural antioxidants (Wang et al. 2000). Proteins are modified by glucose through the glycation reaction, resulting in the formation of advanced glycation end-products (AGEs). The contribution of AGEs to diabetes, aging and Alzheimer’s disease has received considerable attention in recent years and free radicals have been shown to participate in AGEs formation (Halliwell, 2001). It has been reported that antioxidants and radical scavengers inhibit these processes (Nakagawa et al. 2002). Free radicals also upregulate the m-RNA level for tyrosinase, the rate-limiting enzyme in melanin biosynthesis. Increased productions of melanin possess a serious aesthetic problem.
Tyrosinase inhibitors may be clinically useful for the treatment of some dermatological disorders associated with melanin hyperpigmentation and important in cosmetics for depigmentation (Shiino et al. 2001).

*B. pilosa* belongs to the Asteraceae family, the largest flowering plant family in the world. It is widely distributed in tropical areas which have been used to treat infections in South Africa and Taiwan, stomach illness in Mexico, malaria and liver disorders in Brazil and diabetes in Taiwan and South America (Brandao et al. 1997; Pereira et al. 1999; Ubillas et al. 2000). Moreover, some phytochemicals purified from *B. pilosa* have been claimed to possess anti-inflammatory, immunosuppressive, anti-bacterial and antimalarial functions and an inhibition for the prostaglandin synthesis pathway (Geissberger et al. 1991; Jager et al. 1996; Rabe et al. 1997; Chiang et al. 2005). However, these plant components are insufficient to explain the habitual use of *B. pilosa* as an immunomodulator or anti-infectious agents in various regions of the world.

The purpose of the present work was to study the antioxidant and tyrosinase inhibition properties with an objective to propose the various bioactive roles of a new class of compound from *B. pilosa* root.

### 6.3. MATERIALS AND METHODS

#### 6.3.1. Plant material, extraction of different fraction and preparation of the methanol extract

The roots of *B. pilosa* were collected in nature around the campus of University of the Ryukyus, Okinawa, Japan in February 2006. Dry powders of the roots (300 g) were subjected to extraction three times with 1.5 L methanol under the shaking for 24 h.
methanol extracts were concentrated and evaporated to dryness on a rotary evaporator at 40°C. The crude methanol extracts was dissolved in water and the solution was subjected to fraction with hexane and ethyl acetate. The aqueous, hexane and ethyl acetate fraction was evaporated under reduced pressure.

6.3.2. Identification by GC-MS

An aliquot of 1 µl of hexane or ethyl acetate fraction dissolved in acetone and adjusted to 1000 ppm was injected into GC-MS (QP-2010, Shimadzu Co., Kyoto, Japan). The DB-5MS column was 30 m in length, 0.25 mm i.d., and 0.25 µm in thickness (Agilent Technologies, J & W Scientific Products, Folsom, CA). The carrier gas was helium. Same as describe in Chapter 4.

6.3.3. Antioxidant activity

6.3.3.1. DPPH assay

The radical scavenging activity was evaluated as described previously in Chapter 5. Inhibition (I) of DPPH radical was calculated using the equation:

\[ I(\%) = 100 \times \frac{(Ao - As)}{Ao} \]

Ao is the absorbance of the control (containing all reagents except the test compound), and As is the absorbance of the tested sample. The IC₅₀ value represented the concentration of the extract and fraction that caused 50% inhibition.
6.3.3.2. β-Carotene-linoleic acid bleaching assay

Antioxidant activity was carried out according to the β-carotene bleaching method (Siddhuraju et al. 2003) as described in chapter 5.

6.3.4. Mushroom tyrosinase inhibitory assay

Tyrosinase inhibitory activity of plant extracts and fractions was determined by spectrophotometric method. Tyrosinase inhibition assays were performed according to a modification of the method developed by Vanni, Gastaldi, and Giunata (1990). All fractions or extracts were first dissolved in DMSO at 1.0 mg/mL and then diluted to different concentrations using DMSO. Each sample (300 μl) was diluted with 970 μl 0.05 mM sodium phosphate buffer (pH 6.8) in a test tube. This was followed by addition of 1 mL (2.5mM) of L-DOPA solution and finally 1.0 mL mushroom tyrosinase (200 units/mL) was added. The test mixture (3.0 mL) was mixed by Vortex and incubated for 20 min at 37°C. The enzyme reaction was terminated by immersing the mixture in an ice bath and take the absorbance at 490 nm was measured. The absorbance of the same mixture without the test sample was used as the control. The same experiment was repeated at least twice with three replication and they gave similar results. The percent inhibition of tyrosinase activity was calculated as follows:

\[
\% \text{ of inhibition} = \frac{(A-B)}{A} \times 100
\]

Where, \( A = \) absorbance at 490 nm without test sample, and \( B = \) absorbance at 490 nm with test sample.
6.4. RESULT AND DISCUSSION

6.4.1. Identified the compound of the B. pilosa root by GC-MS

The yield of the crude methanol extracts, hexane and ethyl acetate fraction was 35.4, 1.6 and 2.4g, respectively. The results obtained by GC MS analysis of hexane and ethyl acetate fraction are presented in Fig. 6.1 and 6.2. The identified compounds are benzoic acid, 1,2-benzenediol, coumaran, p-coumaric acid, ferulic acid, isovanillin, 2-hydroxy-6-methylbenzaldehyde, vanillic acid, ethamvian, eugenol, syringaldehyde, 4-hydroxy-2-methoxycinnamaldehyde, 2,5-dimethoxy-4-hydroxycinnamaldehyde, 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol, 4-hydroxy-3-methylacetophenone, p-hydroxyl benzalacetone.
Figure 6.1: GC-MS Chromatogram of hexane fraction of \textit{B. pilosa} roots.

Peaks:
1. Coumaran
2. 4-Hydroxy-3-methylacetophenone
3. Eugenol
4. Isovanillin
5. 2-Hydroxy-6-methylbenzaldehyde
6. $p$-Hydroxybenzalacetone
7. 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol
Figure 6.2: GC-MS chromatogram of ethyl acetate fraction of *B. pilosa* roots.

Peaks:
1. Benzoic acid
2. 1,2-Benzenediol
3. Coumaran
4. *p*-Coumaric acid
5. Ferulic acid
6. Isovanillin
7. 2-Hydroxy-6-methylbenzaldehyde
8. Vanillic acid
9. Ethamvian
10. Eugenol
11. Syringaldehyde
12. 4-Hydroxy-2-methoxycinnamaldehyde
13. 3,5-Dimethoxy-4-hydroxycinnamaldehyde
14. 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol
6.4.2. DPPH free radical scavenging activity

DPPH is a stable radical that is used in popular method for screening free radical-scavenging (RSA) ability of compounds or antioxidant activity of plant extracts. DPPH radical scavenging activities of crude methanol extract and its three fractions hexane, ethyl acetate and aqueous from *B. pilosa* roots were determined to search which fractions had high antioxidant activities. The 50% inhibitory concentration (IC$_{50}$) of each extracts was calculated from the results. As shown in Fig. 6.3, the crude methanol extract from *B. pilosa* root exhibited significant DPPH RSA with an IC$_{50}$ value of 82 μg/ml. Among the three fractions, the ethyl acetate fraction exhibited the strongest DPPH RSA (IC$_{50}$ = 25.2 μg/ml), followed by the hexane fraction (IC$_{50}$ = 105.8 μg/ml) and aqueous fraction (IC$_{50}$ = 215.4 μg/ml), in descending order. The results imply that these active extracts may contain constituents with strong proton-donating abilities (Sawai *et al.* 2000).
Figure 6.3. IC$_{50}$ (μg/ml) of different fractions and crude methanol extract from *B. pilosa* by DPPH method. BHT and α-tocopherol are used as positive control. Values are means of three replications ± SE.
6.4.3. β-Carotene-linoleic acid bleaching

In this assay, oxidation of linoleic acid produces hydroperoxide-derived free radicals which attack the chromophore of β-carotene, resulting in a bleaching of the reaction emulsion. An extract capable of retarding/ inhibiting the oxidation of β-carotene may be described as a free radical scavenger and primary antioxidant (Liyana-Pathirana et al. 2006). As can be seen in Fig. 6.4, all the extracts and positive control BHA and α-tocopherol at 1000 ppm were capable of inhibiting the bleaching of β-carotene by scavenging linoleate-derived free radicals. The order of decreasing efficacy was ethyl acetate, aqueous, hexane and crude methanol. None of the extracts was as effective as the positive control BHA and α-tocopherol. It has been suggested that the polarity of an extract is important in water: oil emulsions, non-polar extracts are more effective antioxidants than polar extracts due to a concentrating effect within the lipid phase (Frankel et al. 1994; Koleva et al. 2002). Thus, it would be expected that the more nonpolar extracts would be more potent. This phenomenon was not observed in the case of all these extracts, a finding which has been reported previously (Koleva et al. 2003). According to the β-carotene-linoleic acid bleaching data, the extracts are capable of scavenging free radicals in a complex heterogeneous medium. This suggests that the extracts may have potential use as antioxidative preservatives in emulsion-type systems.
Figure 6.4. Antioxidant activity of the fraction and extracts of *B. pilosa* roots measured by β-carotene bleaching method at 1000 ppm.
6.4.4. Inhibition of tyrosinase activity

Inhibitory effects of three fraction and crude methanol extract on mushroom tyrosinase activity using L-DOPA as the substrates were determined, and their inhibitory activity values are shown in Fig 6.5. Ethyl acetate fraction showed highest activity than other fraction and extract. By the GC-MS identification in Fig. 6.2, ethyl acetate fraction contains highest amount of phenolic and hydroxycinnamic acid derivatives. Many scientific papers have also reported the tyrosinase inhibitory activity of phenolic and hydroxycinnamic acid derivatives. The ethyl acetate fraction showed that lower activity than mimosine and kojic acid. Mimosine had serious side effects on animal and human. Kojic acid is an excellent skin whitening agent but has been accused of serious side effects, such as cytotoxicity, skin cancer, dermatitis and has been banned in cosmetics in many countries (Tiedtke et al. 2004). They demonstrated that methoxylation and hydroxylation of cinnamic acid derivatives may have played an important role in exhibiting the tyrosinase inhibition activity (Billaud et al. 1996; Lee et al. 2002). However, there is so far no report on the effect of different fraction and extract from B. pilosa root on tyrosinase activity. We also examined the inhibitory effects of phenol and hydroxylcinnamic derivatives on mushroom tyrosinase as presented in Fig. 6.6.

The order of activity against tyrosenease activity eugenol > p-coumaric acid > ferulic acid > catechol.
Figure 6.5: Tyrosinase inhibitory activity of *B. pilosa* root. Values are means of three replications ± SE.

Figure 6.6: Tyrosinase inhibitory activity of identified phenolic compound found in *B. pilosa* root. Values are means of three replications ± SE.
In current epidemiological studies, phenolics have been found to be one of the most plentiful classes of constituents in the plant kingdom, and they have been reported to have multiple biological effects (Rao, 2003). Some literatures have reported that many phenolic compounds are in plants showing tyrosinase inhibitory activity (Boissy et al. 2004). The results showed that, in general, the stronger the antioxidant and tyrosinase inhibitory activities of ethyl acetate fraction, the higher the phenolic content. Thus, phenolics present in the fraction may play a major role in producing the results we obtained with the present studies. There are also reports that phenolic compounds may be used as depigmenting agents because they have a similar chemical structure to tyrosine, the substrate of tyrosinase (Boissy et al. 2004).

In conclusion, we found that the ethyl acetate fraction exhibited the highest degree of free radical scavenging and tyrosinase inhibition activities. In addition, the highest amount of phenolic compounds was identified in ethyl acetate fraction. These results suggest that ethyl acetate fraction possess antioxidative and tyrosinase-inhibition, which may useful as raw materials for pharmaceutical and cosmetic industries. However, their in vivo antioxidant activity and the mechanism of action need to be further studied.
CHAPTER 7

GENERAL CONCLUSION
Plants are potential major natural sources that have strong herbicidal, antimicrobial and antioxidant activities through either extracts or isolated compounds from the higher plants. Antibacterial, antifungal, antioxidant and tyrosinase inhibitory activities on bioactive compounds from tropical and subtropical plants *L. leucocephala* and *B. pilosa* were investigated.

The leguminous plant *L. leucocephala* grows abundantly in Okinawa Japan having great potential as animal feed for high protein content, but its use was limited owing to the presence of mimosine, which causes diseases in animals. To observe the inhibitory effect of mimosine in plant parts, chlorophyll and water content of tested plants were measured. As compared to other fourteen tested plants, mimosine showed less inhibitory activity against both hypocotyl and radicle of *B. pilosa*, indicating that *B. pilosa* itself may have more allelopathic activity than mimosine and the resistance mechanism of action may be new or not like as mimosine. Chlorophyll and water content of *B. pilosa*, *R. sativus*, *M. sativa*, and *O. sativa* were also measured. During growth inhibition monitoring in the Petri dishes treated with mimosine solutions of different concentrations, it was observed that the growth, the chlorophyll, and water content were also reduced in the seedlings growing in treated plants in Petri dish compared with control. It was also observed that the chlorophyll and water content of those tested plants were reduced gradually with the increase of mimosine concentration. From the present study, it could be concluded that mimosine possesses weed suppressing ability that can be utilized for future weed management strategies.

On the other hand, *B. pilosa* grows abundantly in southern Kyushu, regions of Ryukyu, and the Ogasawara islands in Japan. The extract of *B. pilosa* shows strong
phytotoxic action against the growth of R. sativus and E. crus-gali and antifungal activity against C. rolfsii, F. solani, and F. oxysporum in bioassay. Fifteen kind of phenolic compounds were identified in different plant parts of B. pilosa by GC-MS analyses. Caffeic acid was found in the highest amount among the detected chemicals components. Dimethoxyphenol and vanillic acid were found only in the roots. The extract of the roots exhibited plant growth inhibitory activity than those of the leaves and stems. Since many phytotoxins were detected in B. pilosa, there is a possibility of exploiting this tropical shrub for the control of weeds and phytophathogens in agricultural fields.

To reduce or eliminate chemically synthesized additives from foods is a current demand worldwide. A new approach to prevent the proliferation of microorganisms or protect food from oxidation is the use of essential oils as preservatives. Essential oils of plants are of growing interest both in the industry and scientific research because of their antibacterial, antifungal, and antioxidant properties and make useful as natural additives in foods. The essential oils from the fresh leaves and flowers of B. pilosa were analyzed by GC-MS. Forty-four components were identified, of which β-caryophyllene and τ-cadinene were the main compounds in leaves and flowers, respectively. The essential oil from leaves and flowers were found to be superior to all aqueous extract measured by DPPH radical scavenging activities, whereas all extracts and essential oils seemed to inhibit the oxidation of linoleic acid. The oils from B. pilosa exerted significant antibacterial and antifungal activities against six bacteria and three fungal strains. The findings demonstrated that the essential oils and aqueous extract of B. pilosa possess antioxidant and antimicrobial activities that might be a natural potential source of preservative used in food and other related industries.
The ethyl acetate fractions prepared from the roots of *B. pilosa* possessed marked antioxidant activity and tyrosinase inhibitory effect against mushroom tyrosinase. The highest activity of the extract could be attributed to the identified phenolic compounds.

In conclusion, mimosine of *L. leucocephala* could be used as natural potent bio herbicide on the basis of their allelopathic effects and chlorophyll inhibitions. On the other hand, *B. pilosa* can be suggested to be used as natural supplements on the basis of their antioxidant and antimicrobial effects. On the basis of these data and some earlier findings, it can be concluded that the ethyl acetate fraction of *B. pilosa* roots evidently have potential to be an effective anti-browning agent for foodstuffs or a skin-whitening agent in cosmetics.
LITERATURE CITED
References


methanol extract of B. pilosa pilosa prevents and attenuates the hypertension induced by high-fructose diet in Wistar rats. *J. Ethnopharma.* 83: 183–191.


Jones, E. and Halsall, T. (1955). Fortschritte der Chemie organischer naturstoffe, 12, p.44.


Soedarjo, M. and Borthakur, D. (1998) Mimosine, a toxin produce by the tree legume *L.*


JAPANESE ABSTRACT
要旨

ギンネムとセンダングサは日本国内、特に沖縄で広く繁殖している熱帯・亜熱帯性の帰化植物である。これらの植物をよく観察するとセンダングサはギンネムの群落内でも生育しており、また、センダングサ群落内にもギンネムが生育しているという特異な共生現象が見られる。これらの興味深い関係を明らかにするため、ギンネムとセンダングサに含まれる生物活性物質について詳細な研究を行った。

ギンネムにはミモシン[β-N-(3-hydroxy-4-pyridone)-α-aminopropanoic acid]が含まれているため、多くの植物種に対する強い成長阻害活性を示す。ミモシンによる植物生長阻害活性を15種類の植物について試験を行ったところ、ミモシンはセンダングサを除いたすべての植物の幼根に対して90%の生長阻害を示したが、センダングサに対しては他の14種類の試験に用いた植物よりも非常に低かった。それらの植物に含まれるクロロフィルと水分含量を測定した結果、クロロフィルと水分の含有量はミモシンの濃度に依存して減少が見られた。また、スクリーニングの過程で、センダングサ自体がアレロパシー活性を持っていることが明らかになった。

そこで、センダングサの成分として15種類のフェノール化合物を同定し、さらにセンダングサの抽出物は雑草と植物病原菌に対して強い生育阻害活性を示すことを明らかにした。フェノール化合物の含有量は茎が最大であったが、根の抽出物は葉と茎の抽出物よりも高い阻害活性を示した。加水分解処理後、GC-MSにより13種類のフェノール化合物を根の抽出物から同定し、また、検出された物質の中ではカフェー酸の含有量が最も高いことを明らかにした。

チロシナーゼは動植物中のメラニン生成において重要な酵素である。対照としてミモシンとコウジ酸、基質としてL-DOPAを用いてチロシナーゼ阻害活性を調査したところ、センダングサの酢酸エチル画分が他の画分よりも高い活性を示した。また、α-2トコフェノールを対照として、メタノール抽出物と他の画分の抗酸化能を比較したところ、酢酸エチル画分はIC50値で25.2μg/mlという強いラジカル除去能を示した。この画分に含まれるフェノール性化合物、フラボノイド、ヒドロキシケイ皮酸などの化合物をGC-MSで同定した。酢酸エチル画分にはこれらの生物活性物質が多く含まれることが示唆され、食品、製薬、化粧品の天然素材として有用であると思われた。

水蒸気蒸留によって得られるセンダングサの葉と花の精油成分をGC-MSで分析し、46種類の化合物を同定した。その中ではβ-caryophylleneとτ-cadineneが葉と花におけるそれぞれの主要物質であった。それら精油の抗酸化試験をDPPHとβ-カロテン脱色法で行ったところ、葉と花についてのIC50値は47.5と49.7μg/mlであった。グラム陽性菌に対して花の精油の生育阻害活性はグラム陰性菌よりも著しく高い値を示した。センダングサの精油および水抽出物は抗酸化と抗
菌活性を有しており、食品や他の関連産業で保存料として利用できる可能性がある。