Antioxidant Activity of Three Constituents Identified in Feces from *Zophobas atratus* (Order: Tenebrionidae)

WCU Biomodulation

Seoul National University

Hye Min Shin

ABSTRACT

Reactive oxygen species (ROS) are generated as by-products of cellular metabolism. An imbalance between ROS such as singlet oxygen, superoxide radical, hydrogen peroxide, and hydroxyl radical, causes oxidative stress leading to cellular and DNA damage as well as oxidation of low-density lipoproteins. Oxidative stress disorders caused by the actions of ROS are associated with many acute and chronic diseases such as inflammation and neurodegenerative conditions including Alzheimer's disease (AD).

Antioxidants stabilize or deactivate free radicals, often before they attack targets in biological cells. Thus, taking dietary antioxidants will also reduce the risks of numerous diseases.

In this study, an assessment was made of the antioxidant activity of constituents isolated from super mealworm feces using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

The antioxidant activities of these materials were compared with those of ascorbic acid, broad-spectrum antioxidant agent. The biologically active constituents were determined to be the simple 3-ethyl-5-methoxyanaphthalen-1(2*H*)-one, methyl (9*Z*,12*E*)-15-methoxypentadeca-9,12-dienoate and isopropyl(*Z*)-3-anilino-3-phenyl-2-propenoate by spectroscopic analysis, including EI-MS and NMR. 3-ethyl-5-methoxyanaphthalen-1(2*H*)-one, methyl (9*Z*,12*E*)-15-methoxypentadeca-9,12-dienoate and isopropyl(*Z*)-3-anilino-3-phenyl-2-propenoate were isolated from *Zophobas atratus* feces as new antioxidant activity principles. Based on fifty percent inhibition concentration (IC_{50}), isopropyl(*Z*)-3-anilino-3-phenyl-2-propenoate (96.21 μM) was the most antioxidant constituent. The IC_{50} of 3-ethyl-5-methoxyanaphthalen-1(2*H*)-one and methyl (9*Z*,12*E*)-15-methoxypentadeca-9,12-dienoate were 191.29 μM and 146.12 μM, respectively.

In conclusion, global efforts to reduce the level of antioxidant agents justify further studies on the feces of *Zophobas atratus* derived materials containing 3-ethyl-5-methoxyanaphthalen-1(2*H*)-one, methyl (9*Z*,12*E*)-15-methoxypentadeca-9,12-dienoate and isopropyl(*Z*)-3-anilino-3-phenyl-2-propenoate as potential antioxidant products of lead molecule for the eradication from oxidative stress.

Key words: Natural antioxidant, *Zophobas atratus*, Super mealworm feces, 3-ethyl-5-methoxyanaphthalen-1(2*H*)-one, Methyl (9*Z*,12*E*)-15-methoxypentadeca-9,12-dienoate, Isopropyl(*Z*)-3-Anilino-3-phenyl-2-propenoate

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INTRODUCTION

Reactive oxygen species (ROS) are generated as by-products of cellular metabolism. Reactive oxygen is essential to energy supply, detoxification, chemical signaling, and immune function (Pai, 2014). The detrimental effects of oxidative stress to human tissues and cells caused by reactive oxygen species (ROS) arising from aging and disease pathogenesis is well documented. Though the human body has inherent antioxidative mechanisms to counteract the damaging effects of free radicals, there is often a need to use dietary and medicinal antioxidant supplements, particularly during instances of disease attack. An imbalance between ROS such as singlet oxygen, superoxide anion radical, hydroxyl radical and hydrogen peroxide, and the natural detoxification capacity of the body in favour of the oxidant molecules causes oxidative stress leading to cellular and DNA damage as well as oxidation of low-density lipoproteins (Bouayed et al., 2007; Atmani et al., 2009). Oxidative stress disorders caused by the actions of ROS are associated with many acute and chronic diseases such as inflammation and neurodegenerative conditions including Alzheimer's disease (AD) (Hoozemans et al, 2006). Antioxidants can prevent oxidative damages and also reduce the risks of numerous diseases (Radimer et al., 2004). They are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Antioxidants inhibit oxidation of other molecules by limiting the initiation. Because oxidative stress has been highly correlated with AD, an age-related neurological disorder (Zhao et al., 2013). Natural antioxidants have been proposed as an

effective therapeutic approach for oxidative stress. Their protective effects have been attributed to their scavenging free radicals (Kameoka et al., 1999). There is a pressing need for the development of new improved antioxidants with novel target sites.

Natural compounds extracted from natural products such as animals, plants, and insects have been suggested as alternative sources for antioxidant products. This approach is appealing, in part, because the natural products constitute a potential source of bioactive secondary substances that have been perceived by the general public as relatively safe and often act at multiple and novel target sites (Raskin et al., 2002; Jassim and Naji, 2003). Insect are now being considered as a highly nutritious and healthy food source with high protein, fat, vitamin, fiber, and mineral contents (Van Huis et al., 2013). Certain insect preparations and their constituents are regarded as potential sources for developing commercial antioxidants. No work has been obtained concerning the potential use of *Z. atratus* feces for antioxidant activity.

In this study, an assessment was made of the antioxidant activity of 3-ethyl-5-methoxyanaphthalen-1(2*H*)-one, methyl (9*Z*,12*E*)-15-methoxypentadeca-9,12-dienoate and isopropyl(*Z*)-3-anilino-3-phenyl-2-propenoate identified in feces from *Zophobas atratus*. The antioxidant activity of these materials were compared with that of a commonly used broad-spectrum antioxidant ascorbic acid using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

Literature review

1. ROS and free radical

Reactive oxygen is essential to energy supply, detoxification, chemical signaling, and immune function. Although antioxidant enzymes such as super- oxide dismutase (SOD), catalase, and glutathione peroxidase play an important role in scavenging oxidants and preventing cell injury, these defense mechanisms are not adequate (Kuriakose, 2014). A free radical is an atom, molecule, or ion that contains one or more unpaired electrons and is more reactive than their parent species (Halliwell, 2001). In living systems, free radicals are generated as part of the body's normal metabolic process, and the free radical chain reactions are usually produced in the mitochondrial respiratory chain, liver mixed function oxidases, through xanthine oxidase activity, atmospheric pollutants and from transitional metal catalysts, drug (Tiwari, 2001). Free radicals attack the nearest stable molecules, stealing its electron. When the attacked molecule loses its electron, it becomes a free radical itself, beginning a chain reaction, finally resulting in the description of a living cell (Prior et al., 1998: Cao et al., 1996). Reactive oxygen species (ROS), such as superoxide radicals, hydroxyl radicals and peroxyl radicals, are natural byproducts of the normal metabolism of oxygen in living organisms with important roles in cell signaling (Aruoma et al., 1997). The human organism produces these ROS as a functional part of the harmonic balance between several physiological processes (Gupta and Verma, 2010). Excessive amounts of ROS may be harmful because they can initiate biomolecular oxidations which lead to cell injury and death, and create oxidative

stress which results to numerous diseases and disorders such as aging, cancer and stroke (Halliwell and Gutteridge, 2000).

2. Oxidative stress

Oxidative stress (OS) induced by reactive oxygen species (ROS) can be described as a dynamic imbalance between the amounts of free radicals generated in the body and levels of antioxidants to scavenge them and protect the body against their deleterious effects (Shirwaikar et al., 2006).

It is estimated that every day a human cell is targeted by the hydroxyl radical and other such species and average of 105 times inducing oxidative stress (Valko et al., 2004). The main targets of ROS, RNS and RSS are proteins, DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) molecules, sugars and lipids (Lü et al., 2010; Craft et al., 2012) (Fig. 2).

Regarding proteins, there are three distinct ways they can be oxidatively modified: 1) oxidative modification of a specific amino acid, 2) free radical-mediated peptide cleavage and 3) formation of protein cross-linkage due to reaction with lipid peroxidation products (Lobo et al., 2010). The damage induced by free radicals to DNA can be described both chemically and structurally having a characteristic pattern of modifications. An important reaction involved with DNA damage is the production of the hydroxyl radical through the Fenton reaction. The peroxyl and OH- radicals also intervene in DNA oxidation (Dizdaroglu et al., 2002; Valko et al., 2004).

Regarding sugars, the formation of oxygen free radicals during early glycation could

contribute to glycoxidative damage. The resulting chain reaction propagated by this radical can form α and β-dicarbonyls, which are well known mutagens (Benov and Beema, 2003).

Finally, another way to generate lipid peroxides is through the attack on their side chain by the singlet. This pathway doesn't probably qualify as initiation because the singlet oxygen reacts with the fatty acid instead of abstracting a hydrogen atom to start a chain reaction, making this a minor pathway when compared to the hydroxyl one (Halliwell and Chirico, 1993).

In a normal cell, there is appropriate oxidant: antioxidant balance. However, this balance can be shifted, when production of oxygen species is increased or when levels of antioxidants are diminished (Vinson et al., 1998; Cuvelier et al., 1992). Oxidative stress has been considered a mechanism involved in the pathogenesis of Alzheimer's disease. It has also played a major role in the aging process (Grassi et al., 2009; Stocker et al., 2004). Epidemiological research reveals that the consumption of antioxidant phytochemicals shows benefits for oxidative stress-induced damage (Dia et al., 2006).

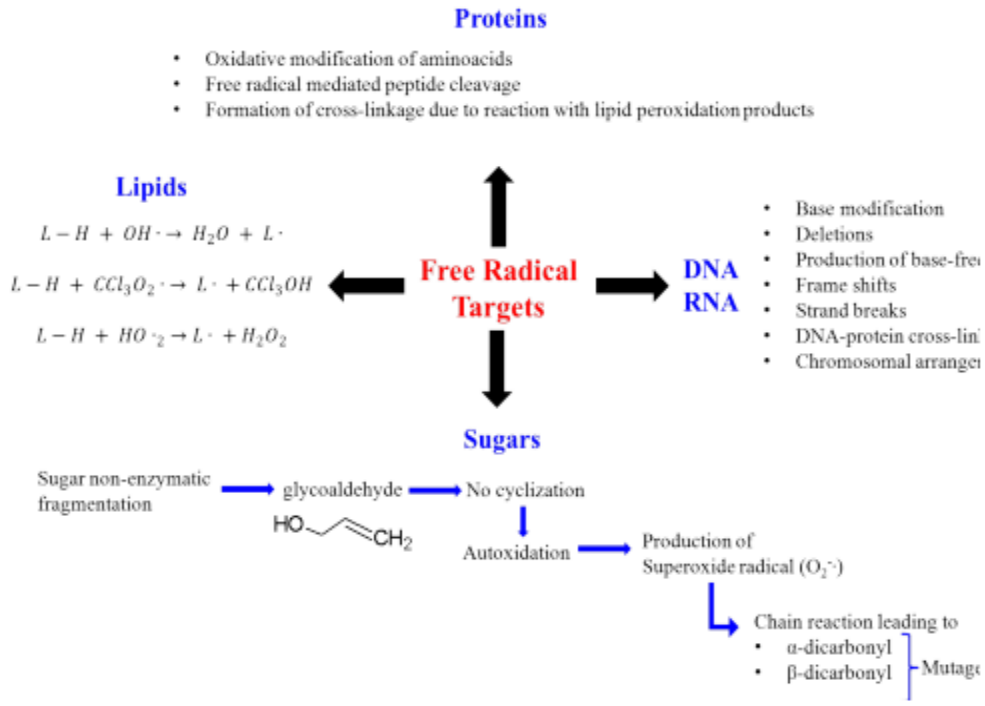

Fig. 1. Targets of free radicals

3. Antioxidants

Antioxidants stabilize or deactivate free radicals, often before they attack targets in biological cells. Although almost all organisms possess antioxidant defense and repair systems to protect against oxidative damage, they cannot prevent the damage entirely (Djeridane et al., 2006; Wannes et al., 2010).

Antioxidants are important inhibitors of lipid peroxidation not only for food protection but also as a defense mechanism of living cells against oxidative damage (Halliwell, 1991). Antioxidants have been shown to prevent or inhibit oxidation

processes n human body and food products (Diaz et al., 1997), and to prevent the destruction of β-cells (Slonim et al., 1983). Several studies have revealed that a major part of the antioxidant activity may be from compounds such as flavonoids, isoflavones, flavones, anthocyanins, and catechins and other phenolics with mechanisms involving both free radical-scavenging and metal chelation (Lien et al., 1999). Flavonoids are classified to wide group of chemical compounds – polyphenols. Amongst the most studied categories of antioxidants, dietary polyphenols and other natural antioxidants have rapidly gained attention as viable candidates for clinical testing in neurodegeneration and acute neuronal injury such as stroke (Mandel, 2004; Simonyi, 2005). It is produced in response to the immoderate UV radiation, ions of transition metals, thermal shock, or as the reaction to fungal infection (Wojcieszyńska, 2006). Recently, the alternative strategy of using natural plants or their extracts as antioxidants in animals has been confirmed to be effective and utilized extensively. The most effective constituents responsible for antioxidative properties of plants are phenolic compounds, including flavonoids, hydrolysable tannins, phenolic ads, and pholoc terpense (Gupta and Sharma 2006; Ogunlesi et al., 2009; Carlsen et al., 2010). The antioxidant activities of phenolic compounds are due to their structure and particularly ability to donate a hydrogen ion to the peroxy radical generated as a result of lipid peroxidation (Kashima 1999; Bisby et al., 2008).

3.1. Natural antioxidants

In nature, there are a wide variety of naturally occurring antioxidants which are

different in their composition, chemical properties and physical, mechanisms, and the site of action (Naik, 2003). Natural phenolic antioxidants, such as synthetics, can effectively scavenge free radicals, absorb light in the ultraviolet (UV) region (100 to 400 nm), and chelate transition metals, thus stopping progressive oxidative damage (Brewer, 2007). Food tissues, because they are living, are under constant oxidative stress from free radicals, ROS, and prooxidants generated both exogenously and endogenously (Brewer, 2007). For this reason, many of these tissues have developed antioxidant systems to control free radicals, lipid oxidation catalysts, oxidation intermediates, and secondary breakdown products (Agati et al., 2007; Brown and Kelly 2007; Chen 2008; Iacopini et al., 2008). Natural extracts with antioxidant activity generally quench free radical oxygen with phenolic compounds. Species and herbs, used in foods for their flavor and in medicinal mixtures for their physiological effects, often contain high concentrations of phenolic compounds that have strong H-donating activity (Lugasi et al., 1995; Muchuweti et al., 2007).

3.2. Synthetic antioxidants

Synthetic and natural food antioxidants are used routinely in foods and medicine especially those containing oils and fats to protect the food against oxidation (Carocho, 2013). Almost all processed foods have synthetic antioxidants incorporated, which are reported to be safe, although some studies indicate otherwise. There are a number of synthetic phenolic antioxidants, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) being prominent examples (Carocho, 2013). These compounds

haver been widely uses as antioxidants in food industry, cosmetics, and therapeutic industry. They effectively can inhibit oxidation, chelating agents, such as ethylene diamine tetra acetic acid, and bind metals reducing their contribution to the process (Brewer, 2011). Nordihydroguaiaretic acid, despite being a food antioxidant, is known to cause renal cystic disease in rodents (Evan and Gardner, 1979). Octyl gallate is considered safe to use as a food additive because after consumption it is hydrolyzed into gallic acid and octanol, which are found in many plants and do not pose a threat to human health (Joung et al., 2004).

3.3. Endogenous antioxidants

Endogenous antioxidants such as glutathione peroxidase, SOD and catalase are essentially enzymes that catalytically remove oxidants (Pouillot et al., 2011). These enzymes play a key role in decreasing the content of oxidants and preventing oxidative damage (Pouillot et al., 2011). The human antioxidant system is divided into two major groups, enzymatic antioxidants and nonenzymatic oxidants (Pouillot et al., 2011).

3.3.1. Enzymatic antioxidant

Regarding enzymatic antioxidants, they are divided into primary and secondary enzymatic defenses (Carocho, 2013). With regard to the primary defense, it is composed of three important enzymes that prevent the formation or neutralize free radicals: glutathione peroxidase, which donates two electrons to reduce peroxides by forming selenoles and also eliminates peroxides as potential substrate for the Fenton reaction

(Carocho, 2013). SOD converts superoxide anions into hydrogen peroxide as a substrate for catalase (Rahman, 2007). Catalase converts hydrogen peroxide into water and molecular oxygen and has one of the biggest turnover rates known to man, allowing just one molecule of catalase to convert 6 billion molecules of hydrogen peroxide. The secondary enzymatic defense includes glutathione reductase and glucose-6-phosphate dehydrogenase. Glutathione reductase reduces glutathione from its oxidized to its reduced form, thus recycling it to continue neutralizing more free radicals. Glucose-6-phosphate regenerates NADPH creating a reducing environment (Gamble and Burke, 1984; Ratnam et al., 2006).

3.3.2. Nonenzymatic antioxidant

Considering the nonenzymatic endogenous antioxidants, there are quite a number of them, such as enzyme cofactors (Q10), vitamins, nitrogen compounds (uric acid), and peptides (glutathione) (Carocho, 2013). β-Carotene is an excellent scavenger of singlet oxygen (Gupta et al, 2006). Vitamin A or retinol is carotenoid produced in the liver and results from the breakdown of β-carotene (Carocho, 2013). There are about a dozen forms of vitamin A. It is known to have beneficial impact on the skin, eyes, and internal organs (Palace et al., 1999; Jee et al., 2006). Coenzyme Q10 is present in all cells and membranes; it plays an important role in the respiratory chain and in other cellular metabolism. Coenzyme Q10 acts by preventing the formation of lipid peroxyl radicals, although it has been reported that this coenzyme can neutralize these radicals even after their formation. Another important function is the ability to regenerate vitamin E; some

authors describe this process to be more likely than regeneration of vitamin E through ascorbate (vitamin C) (Turunen et al., 2004). Glutathione is an endogenous tripeptide which protects the cells against free radicals either by donating a hydrogen atom or an electron. It is also very important in the regeneration of other antioxidants like ascorbate (Steenvoorden and Henegouwen, 1997).

3.4. Exogenous antioxidants

Exogenous antioxidants include antioxidants that cannot be synthesized by our body, such as vitamins, trace elements, and phytoantioxidants (Thigle and Nitave, 2014). Vitamin E (tocopherol) is the most powerful liposoluble antioxidant (Thigle and Nitave, 2014). It inhibits the peroxidation of membrane lipids. It reacts with free radicals to form the radical tocopheryl, a stable substance that stops the chain reaction of the membrane lipids. The chain reaction is propagation of free radicals (Thigle and Nitave, 2014).

4. Risk factors of synthetic antioxidant.

Synthetic antioxidants, such as BHT and BHA, have been used as antioxidants since beginning of this century (Hall and Cuppett, 1997). Synthetic antioxidants *tert*-butylhydroquinone (TBHQ) and Trolox are also widely used. TBHQ is a derivative of hydroquinone, substituted with a *tert*-butyl group (Nunes et al., 2012). It is a highly effective antioxidant used in foods as a preservative for unsaturated vegetable oils and many edible animal fats. In the food industry, synthetic antioxidants such as ascorbic

acid and BHT have been widely used as additives to preserve and stabilize foods and animal feed products for nutritive value, freshness, color (Nunes et al., 2012). Extensive research has shown high doses of this ingredient to cause significant damage to the lungs, liver and kidneys. Oral consumption of this ingredient has also been shown to have toxic effects on the body's blood coagulation system. For this reason, it has become very necessary to derive antioxidants from natural sources for use as supplements to human health.

5. Antioxidant activity of natural products

The growing relevance of medicinal products as possible sources for the discovery of novel antioxidant molecules is often based on their long historical utilization in natural products, especially in developing countries. Extensively studied sources of natural antioxidants are fruits and vegetables, seeds, cereals, berries, wine, tea, onion bulbs, olive oil, and aromatic plants (EI-Gharras, 2011). Food antioxidants such as α-tocopherol, ascorbic acid, carotenoids, amino acids, peptides, proteins, flavonoids and other phenolic compounds might also play a significant role as physiological and dietary antioxidants (Shahidi, 2000). Natural antioxidants are present in plants, and this is why the basic source of these compounds for humans is plant-derived products. Plant phenolics are multifunctional and can act as reducing agents, free radical terminators, metal chelators and singlet oxygen quenchers (Mathew and Abraham, 2006). Fat soluble vitamins and selenium also occur in food derived from animals (milk and fish lipids, eggs), but in smaller amounts, and depending on the kind of feed consumed.

Plant polyphenols with antioxidant capacity could scavenge reactive chemical species as well as minimize oxidative damage resulting from excessive light exposure (Oliveira et al., 2012). Several plants are reported to be produced various biological active compounds (Paudel et al., 2014). Some plant polyphenols are important components of both human and animal diets and are safe to be consumed (Gulçin et al., 2005). Lichens from the extreme environments such as high altitude, high UV, drought, and cold are believed to be synthesized unique types of secondary metabolites than the other one (Table 1).

The substrates for enzymatic hydrolysis are usually plants and aquatic animals (You et al., 2010). Some insects are also gaining attention because of their rich protein and bioactive peptides (Vercruysse et al., 2009). Antioxidative peptides are potential to be exploited as new natural antioxidant and functional food. There is a huge potential market in medical and cosmetic field as well.

Natural antioxidants are known to exhibit a wide range of biological effects including antibacterial, antiviral, anti-inflammatory, antiallergic, antithrombotic, and vasodilatory activities (Cook and Samman, 1996). Figure 2 revealed natural antioxidants separated in classes.

Table 1. IC_{50} values of natural plant's extract*

Natural product	IC_{50} (μg/mL)	Natural product	IC_{50} (μg/mL)
Rhododendron dauricum	0.45±0.02	*Ranunculus reptans*	7.3±0.23
Dryas grandis	0.52±0.03	*Thymus pavlovii*	7.5±0.45
Rhododendron redowskianum	0.61±0.02	*Sparganium hyperboreum*	8.2±0.43
Dryopteris fragrans	1.2±0.08	*Saxifraga punctata*	8.2±0.25
Saxifraga bronchialis	1.8±0.07	*Pinus pumila*	9.2±0.64
Aconogonon tripterocarpum	1.9±0.17	*Ribes fragrans*	9.5±0.38
Chamerion angustifolium	2.1±0.17	*Sedum sukaczevii*	9.8±0.49
Salix pulchra	2.1±0.15	*Thalictrum foetidum*	10.3±0.31
Chamerion angustifolium	2.2±0.06	*Sorbaria sorbifolia*	11±0.88
Betula divaricate	2.5±0.15	*Ptarmica salicifolia*	11.1±0.67
Artemisia vulgaris	2.8±0.14	Rheum compactum	11.2±0.34
Rhododendron lapponicum	3.1±0.31	*Artemisia lagocephala*	11.6±1.1
Andromeda polifolia	3.2±0.26	*Campanula rotundifolia* ssp. *langsdorffiana*	11.8±0.71
Vaccinium uliginosum	3.4±0.14	*Veratrum lobelianum*	12±0.72
Ribes triste	3.5±0.07	*Oxycoccus microcarpus*	12.1±0.48
Comarum palustre	3.6±0.18	*Beckmannia syzigachne*	13±0.39
Salix reptans	3.7±0.15	*Empetrum nigrum*	15.1±0.9
Ledum palustre	3.8±0.27	*Euprasia hyperborea*	15.1±0.45
Rosa acicularis	3.9±0.19	*Cassiope tetragona*	15.2±0.76
Pyrola rotundifolia	3.9±0.16	*Alopecurus roshevitzianus*	18±1.44
Sanguisorba officinalis	4.1±0.33	*Chosenia arbutifolia*	18.1±1.25
Carex aquatilis	4.1±0.08	*Dryas punctata*	18.2±0.91
Rubus matsumuranus	4.3±0.22	*Achillea millefolium*	19±1.33
Vaccinium vitis-idaea	4.7±0.19	*Thamnolia vermicularis*	5.2±0.16
Rubus chamaemorus	4.7±0.38	*Peltigera didactyla*	5.7±0.46

Veronica incana	4.8±0.14	*Peltigera malacea* 6.1±0.31
Pentaphylloides fruticosa	4.8±0.24	*Peltigera aphthosa* 14.7±1.03
Galium verum	4.9±0.34	*Sphagnum fuscum* 19.7±0.98
Cassiope ericoides	5.1±0.31	*Loeskypnum badium* 19.8±0.59
Parnassia palustris	5.4±0.11	*Hylocomium splendens* 19.8±1.19
Dracocephalum palmatum	6±0.48	*Polytrichastrum alpinum* 19.9±0.99
Orostachys spinosa	6.2±0.19	*Scorpidium scorpioides* >20
Salix tschuktschorum	6.3±0.25	*Paludella squarrosa* >20
Juniperus communis	6.8±0.14	

* From Paudel et al. (2014).

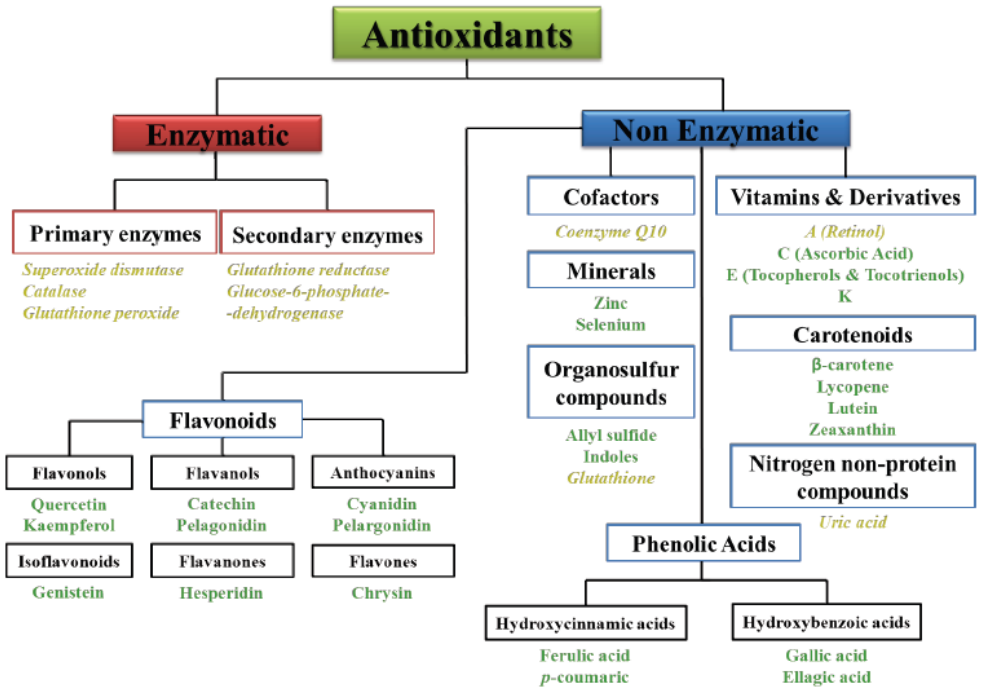

Fig. 2. Natural antioxidants separated in classes. Green words represent exogenous antioxidants, while yellow ones represent endogenous antioxidants.

5.1. Flavonoid polyphenols as neuroprotective agents

Phenolics are ubiquitous bioactive compounds and a diverse group of secondary metabolites universally present in higher plants (Robard et al., 1999). The action of phenolics as antioxidants is viewed as beneficial in both foods and the body where phenolics are oxidized in preference to other food constituents or cellular components and tissues (Robard et al., 1999). Quercetin is a flavonoid polyphenol found in many common foods such as apples and capers. *In vitro* studies show that quercetin increases cell survival in the presence of hydrogen peroxide (Dajas et al., 2003; Heo et al., 2004), *tert*-butyl hydroperoxide (Silva et al., 2008) and linoleic acid hydroperoxide(Sasaki et al., 2003).

5.2 Nonflavonoid polyphenols as neuroprotective agents

Resveratrol is a polyphenolic antioxidant found in many kinds of grapes and is known mostly for its cardiovascular benefits (Bertelli et al., 2009; Sadruddin et al., 2009). Research into the neuroprotective effects of the nonflavonoid polyphenol curcumin is less extensive than that for resveratrol. However, in Neuro2a mouse neuroblastoma cells infected with Japanese encephalitis virus, curcumin enhances cell viability by decreasing ROS and inhibiting proapoptotic signals (Dutta, 2009). Furthermore, curcumin displays an additive protective effect to that of catalase and SOD activities in the striatum and midbrain of MPTP-treated mice (Rajeswari, 2006).

6. Pharmacological significance of Insects

Insects make up about 80–90% of the largest and diverse group of organisms on the Earth. Approximately 950,000 species of insects have been studied out of estimating total species 4,000,000 (Berenbaum and Eisner, 2008). Insects secrete a wide variety of chemical substances to ward off attacks and these substances are likely to produce a wealth of useful information with applications in the fields of ecology, biochemistry, and biotechnology. For these reasons, insects and their constituents become a valuable source as new medicinal compounds (Dossey, 2010). Since early times, insects, their products and the substances have been used, directly and indirectly, in the medical systems of different human cultures throughout the world (Costa-Neto, 2002). Indeed, insects and the substances extracted from them have been used as therapeutic resources in the medical systems of many cultures (Costa-Neto, 2005). Also, insects have proven to be very important as sources of drugs for modern medicine since they have immunological, analgesic, antibacterial, diuretic, anesthetic, and antirheumatic properties (Yamakawa, 1998). The therapeutic use of insects and insect-derived products is known as entomotherapy. Commonly considered to be disgusting and filthy animals, many insect species have been used live, cooked, ground, in infusions, in plasters, in salves, and as ointments, both in curative and preventive medicines, as well as in magic-religious rituals (Costa-Neto, 2002). There are a number of articles describing the insect-derived substances with medically relevant properties. Insects and the substances are potentially important sources for natural product drug discovery.

6.1. Antioxidant activity of insect materials

Propolis and pollen have the highest antioxidant activities (Carpes et al., 2007). Bee venom is a potent antioxidant and possesses radio protecting actions. Honey is another insect-derived substance that varies widely throughout the world depending on the species of bee and plants the bees feed on, both of which influence the honey's antioxidant properties. Edible insects such as Blaptica dubia, Locusta migratoria and *Zophobas morio* are a valuable source of peptides with antioxidant activity as well as an ability to chelate metal ions and inhibit reducing power (Fig. 3) (Ewelina et al., 2016).

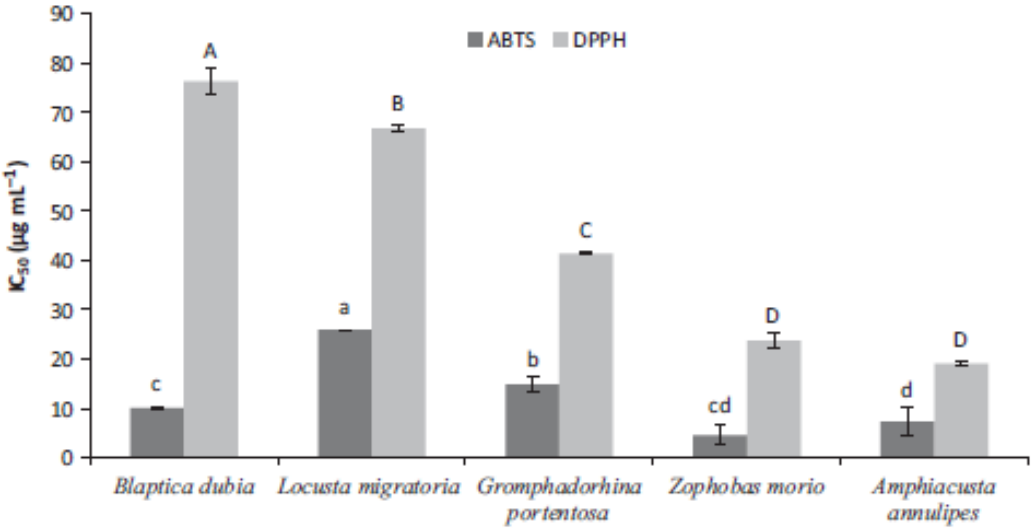

Fig. 3. Antioxidant activities of hydrolysates obtained by in vitro of selected insects.

6.2. Insect neurotoxins

Most numerous insect venoms contain neurotoxins which are used to paralyze prey or enemy. Acylpolyamines and peptides from a diverse spiders have potential use in treating pain and central nervous system (CNS) disease such as Huntington disease, Alzheimer's disease, and Parkinson's disease (Estrada et al., 2007). In fact, over 40 different polyamine neurotoxins have been isolated from the venoms of spider species (Nakanishi et al., 1994). Thus, even though spiders are an established source of toxins, they still represent a vast reservoir for discovery of useful substances such as potential pharmaceuticals. The venoms of many insects, particularly those of ants, bees, and wasps contain neurotoxins such as philanthotoxin which is originally discovered in the venom of the predatory wasp species *Philanthus triangulum* (Fab.) (Olsen et al., 2006). Philanthotoxin is a noncompetitive antagonist of both glutamate and nicotinic acetylcholine receptors (Nakanishi et al., 1994). These receptors in humans have a side diverse of functions, such as pre and postsynaptic neural transmission, memory formation, learning, and muscle contraction. Another example is a potent hymenopteran neurotoxin, the venom of the bullet ant (Paraponera clavata F.). This venom contains poneratoxin, a protein and this protein blocks ion channels of insects. They are, therefore, major drug targets for a lot of therapeutic applications, including treatment of neurodegenerative diseases (Dossey, 2010).

6.3. Other medicinally relevant properties of insect materials

Some insect-derived substances have been used to cure patients with diseases. The

substances from insect, particularly peptides, have effects against virus infection and replication. Melittin (derived from the sting venom of honeybees) and its analogs have anti-HIV activity and effects against other viruses such as herpes simplex virus and Junin virus (Matanic and Castilla, 2004). Another group of insect-derived antiviral peptides are the alloferons. Alloferons are discovered in the hemolymph of blowflies, *Calliphora vicina* Robineau-Desvoidy. These peptides have antiviral effects on influenza and herpes simplex virus (Chernysh et al., 2002). And, powdered silkworm larvae (silkworm powder) are often prescribed in Asian medicine and are commercially available. Silkworm powder has been tested and shown in modern bioassays to inhibit absorption of glucose in human intestinal epithelium cells (Han et al., 2007) and reduce vasopressin expression in the hypothalamus of diabetic mice (Kim et al., 2007). Also, bee venom therapy is commonly used to treat a variety of conditions such as arthritis, rheumatism, pain, and even cancer. It contains a variety of proteins and other substances with multiple pharmacologically relevant properties (Son et al., 2007). The use of bee venom and other natural products from bees is known as apitherapy. There is even an organization dedicated to promoting apitherapy, the American Apitherapy Society. With potential medicinal value, the insect materials are valuable to study.

7. *Zophobas atratus*

Zophobas is a genus of beetles in the family Tenebrionidae, the darkling beetles. In Cuba beetles of this genus are known as blind click-beetles. Perhaps the best known species is *Zophobas morio*, a beetle whose larvae are robust mealworms sold as food for

pets such as lizards. The larvae are known commonly as superworms. The superworm, as known the larva of *Zophobas morio*, has been officially imported from 2011 and bred commercially in Korea. But it is named as the corrected scientific name, *Zophobas atratus* by junior synonym throughout traditional taxonomy and newly designated Korean name as ‘a-me-ri-ca-wang-geo-jeo-ri’ in terms of resource management (Park et al., 2013). *Zophobas atratus* is also used as pet food, sold in pet stores under the name giant mealworms, but should not be confused with darkling beetle mealworms sprayed with juvenile hormone. These insect larvae resemble very large mealworms, about 1.7 to 2.25 inches long (50-60 mm) when full size. They have 6 small legs and two rudimentary hind prolegs. Once they reach adult size, the larvae pupate, and later emerge as large, black beetles. The larvae will not pupate if kept in a container with many other larvae and plentiful food, where they receive constant bodily contact. Keeping superworms this way is commonly used to hinder pupation. However, if you would like to mature the superworms into darkling beetles, you will have to separate each one individually for about 7-10 days. After the given amount of time, they will emerge from their pupae stage as darkling beetles (Clubfauna, 2014). Contrary to popular belief, the adult does not have fused elytra, since the beetle occasionally attempts to fly as an emergency measure against starvation. Superworms are accepted by lizards, turtles, frogs, salamanders, birds, koi and other insectivorous animals. Their hard chitin may make them less suitable for arachnids and some predatory insects. Their nutritional values are similar to those of mealworms, so it is possible that supplementation with calcium is necessary if they are used as a staple food item. The

larvae are odor-free (but the beetles possess a pungent chemical defense that may be released when overprovoked), and can be easily contained, making them ideal for raising at home to feed a collection of captive insectivores. However, there are a few reports of antioxidant agents from insects, which are the most diverse groups of organisms (Huang et al., 1997).

8. Perspectives

Natural product resource industry has many potential possibilities to grow up developing valuable sources of new medicinal compounds for human. Well-known examples of natural products that are part of today's armament against diseases include antibiotics, immune-suppressive agents, antitumor agents, and anticholesterolemic agents (Kai, 2001). Natural products and their derivatives have been and continue to be rich sources for drug discovery. An analysis of a number of chemotherapeutic agents and their sources indicates that over 60% of approved drugs are derived from natural compounds (Newman and Cragg, 2007). During the past decades, a lot of research has been carried out around antioxidants and their effects on health. It is true that antioxidants are beneficial and display a useful role in human homeostasis. The academic community should search deeper into the kinetics and *in vitro* mechanisms of antioxidants to uncover the optimal concentrations for desired functions in order to push forward against cancer, neurodegenerative diseases, and cardiovascular diseases. For many decades, humans were in search of effective drugs that will combat deadly diseases without any side effects. The world's biodiversity has been tested for biological

activity and have demonstrated a distinct difference in the structural properties of natural products relative to synthetic compounds. Compared with synthetic compounds, they generally have higher molecular weights and exhibit a different distribution of heteroatoms. To develop new drugs from natural products, it needs to be set vast libraries of compounds exactly. By contrast, the much more subtle use of natural-product templates combined with chemistry to produces elective analogs will have a much greater chance of success. In a sense, it will accept that nature has already carried out the combinatorial chemistry; all have to do is refined the structures (Mann, 2002). In addition, it needs to be broad portion natural products. Through these libraries database, researchers can take advantage of natural gift from natural products.

Although, insects make up about 80–90% of the largest and diverse group of organisms on the Earth, there are a few researches to develop new drugs utilizing insects and insects' metabolites. Insect resource industry has many potential possibilities to grow up developing valuable sources of new medicinal compounds for human. The experimental agents isolated from insects and insects' metabolites will give good opportunities to assess not only new chemical compounds families of anticancer agents, but also relevant mode of action potentially. And, it can identify the limitations of antioxidants and provide a perspective on the likely future trends in this field.

MATERIALS AND METHODS

1. Instrumental analyses

^{1}H and ^{13}C NMR spectra were recorded in MeOD on a AVANCE 600 spectrometer (Bruker, Rheinstetten, Baden-Württemberg, Germany) at 600 and 150 MHz, respectively, using tetramethylsilane as an internal standard, and chemical shifts are given in δ parts per million (ppm). Distortionless enhancement by polarization transfer (DEPT) spectra was acquired using the Bruker software. The ultraviolet (UV) spectra were obtained in methanol on a V-550 spectrophotometer (Jasco, Tokyo, Japan) and the mass spectra on a GSX 400 spectrometer (Jeol, Tokyo, Japan). Silica gel 60 (0.063–0.2 mm) (Merck, Darmstadt, Germany) was used for column chromatography. Merck precoated silica gel plates (Kieselgel 60 $F_{254,}$ 0.20 mm) were used for analytical thin layer chromatography (TLC). An Isolera one medium-pressure liquid chromatograph (Biotage, Uppsala, Sweden) and an Agilent 1200 high-performance liquid chromatograph with binary solvent pump (Santa Clara, CA, USA) were used for isolation of active principles.

2. Materials

A commercially-available antioxidant agent ascorbic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All of the other chemicals and reagents used in this study were of analytical grade quality and are available commercially.

3. Insect materials

Air-dried of adults, larvae, feces from *Z. atratus* were spplied by the Korea Beneficial Insects Labpratory (KBIL) (Soryong-gil, Okgwa-myeon, Gokseong-gun, Jeollanam-do, Republic of Korea (ROK)) in March 2015. Samples were stored at –4°C until use. Each voucher specimen (ZAA-01, ZAL-01, and ZAF-01) was deposited in the Research Institute of Agriculture and Life Sciences, College of Agriculture and Life Sciences, Seoul National University.

4. Sample preparation

The air-dried *Z. atratus* feces (7.6 kg) was pulverized, extracted with methanol (38 L) three times at room temperature for 3 days, and filtered through Whatman no.2 filter paper (Whatman, Maidstone, UK). The combined filtrate was concentrated to dryness by rotary evaporation at 40°C to yield approximately 380 g of a dark yellowish green tar. The extract was sequentially partitioned into with hexane- (121.98 g), chloroform- (24.68 g), ethyl acetate- (19.02 g), butanol- (24.73 g), and water-soluble (189.59 g) portions for subsequent bioassay (Fig. 4). The organic solvent-soluble portions were concentrated under vacuum at 40°C, and the water-soluble portion was concentrated at 50°C. To isolate the active principles, 500–125 mg/L of each *Z. atratus* feces-derived fraction was tested in a DPPH radical scavenging assay, as described previously by Oliveira et al. (2012).

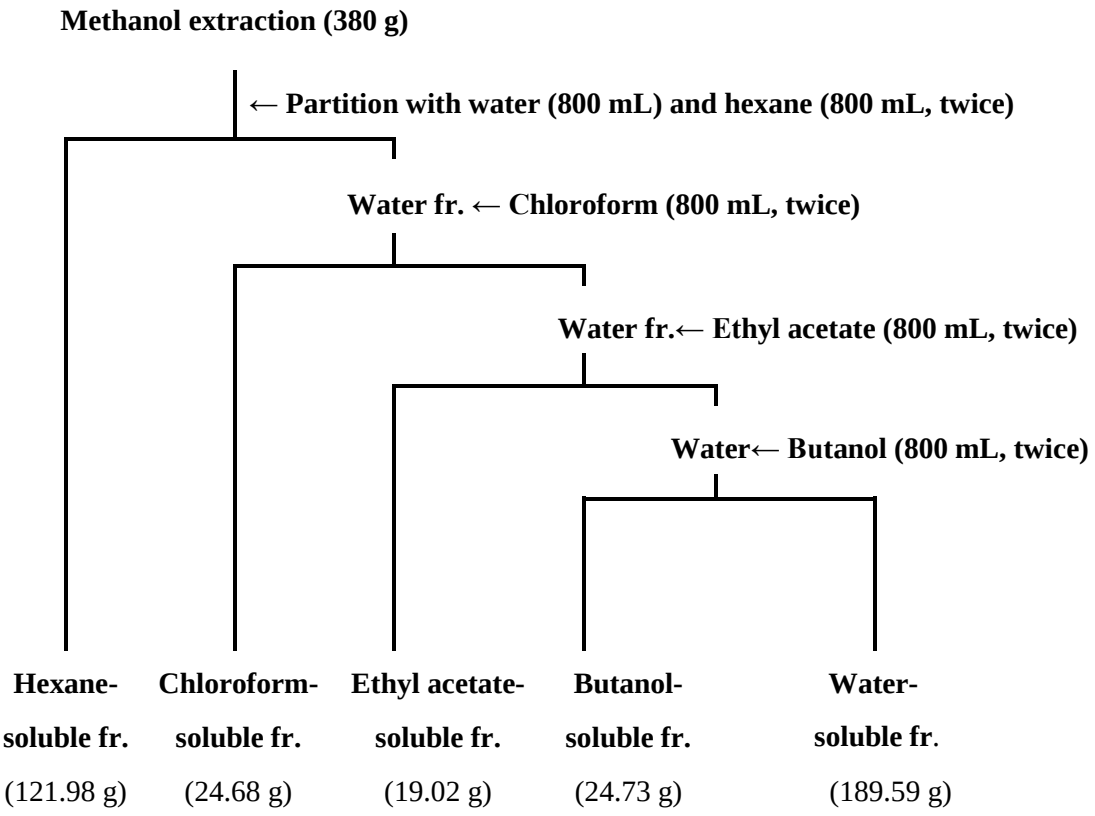

Fig. 4. Solvent fraction procedures of methanol extract from *Z. atratus* feces.

The *Z. atratus* feces methanol extract was sequentially partitioned into hexane-, chloroform-, ethyl acetate-, butanol-, and water-soluble portions.

5. Isolation of active constituents from *Z. atratus* feces

The ethyl acetate-soluble fraction (19.02 g) from the methanol extract of *Z. atratus* feces was the most biologically active fraction, and medium-pressure liquid chromatography (MPLC) was performed using an Isolera apparatus equipped with UV detector at 254 nm and 365 nm and a SNAP column cartridge (340 g silica gel) with a column volume of 510 mL (Fig. 5). Separation was achieved with a gradient of chloroform and methanol [100:0 (1785 mL), 98:2 (663 mL), 95:5 (1071 mL), 90:10 (2346 mL), 85:15 (969 mL), 80:20 (867 mL), and 0:100 (2 L) by volume] at a flow rate of 50 mL/min to provide 52 fractions (each approximately 200 mL). The column fractions were monitored by TLC on silica gel plates developed with a chloroform and methanol (90:10 by volume) mobile phase. Fractions with similar R_f values on the TLC plates were pooled. The spots were detected by spraying the plate with 2% sulfuric acid and the heating on a hot plate. Active fractions 13–38 (E4) was obtained. Fraction E4 was separated by MPLC with a UV detector and a column cartridge (100 g silica gel) with a column volume of 132 mL by elution with a gradient of chloroform and methanol [100:0 (383 mL), 98:2 (370 mL), 95:5 (528 mL), 90:10 (647 mL), 85;15 (396 mL), 80;20 (396 mL), 70;30 (277 mL), and 0:100 (800 mL) by volume] at a flow rate of 25 mL/min to provide 181 fractions (each approximately 20 mL). The column fractions were monitored by TLC on silica gel plates, as stated previously. Active fractions 30–43 (E42) was obtained. Fraction E42 was separated by MPLC with a UV detector and a column cartridge (25g silica gel) with a column volume of 33 mL by elution with a gradient of chloroform and methanol [100:0 (148 mL), 98:2 (280 mL), 95:5 (165 mL),

90:10 (112 mL), 80:20 (60 mL), and 0:100 (500 mL) by volume] at a flow rate of 25 mL/min to provide 82 fractions (each approximately 20mL). Active fractions 7–28 (E422) was obtained. Fraction E422 was separated by MPLC with a UV detector and a column cartridge (25 g silica gel) with a column volume of 33 mL by elution with a gradient of chloroform and methanol [100:0 (198 mL), 98:2 (429 mL), 95:5 (330 mL), 90:10 (148 mL), 80;20 (65 mL), and 0:100 (500 mL) by volume] at a flow rate of 25 mL/min to provide 89 fractions (each approximately 20 mL). The column fractions were monitored by TLC on silica gel plates, as stated previously. Active fractions E4222 and E4223 were obtained. Fractions E4222 and E4223 was separated by preparative TLC [chloroform:methanol (90:10) by volume]. Of the three fractions, active fraction E4222.2 (31 mg, R_f = 0.51) was obtained. Of the three fractions, the active fraction E4223.2 (26 mg, R_f = 0.63) were obtained. Fraction E4223.2 was separated by preparative TLC [hexane:ethyl acetate (30:70) by volume]. Of the three fractions, the active fraction E4223.23 (15.8 mg, R_f = 0.48) was obtained. A preparative high-performance liquid chromatograph was used to separate the constituents from active fraction E4222.2 from E4222 and active fraction E4223.2 from E4223. The column was a 7.8 mm i.d. × 300 mm Waters μBondapak C18 (Milford, MA, USA) with a mobile phase of methanol and water (40:60 by volume) at a flow rate 1 mL/min. Chromatographic separations were monitored using a UV detector at 222 nm and 216 nm, respectively. Finally, three active principles, **1** from E4222.22 and **2** and **3** from E4223.23, were isolated at retention times of 11.48, 11.46, and 11.38 min, respectively.

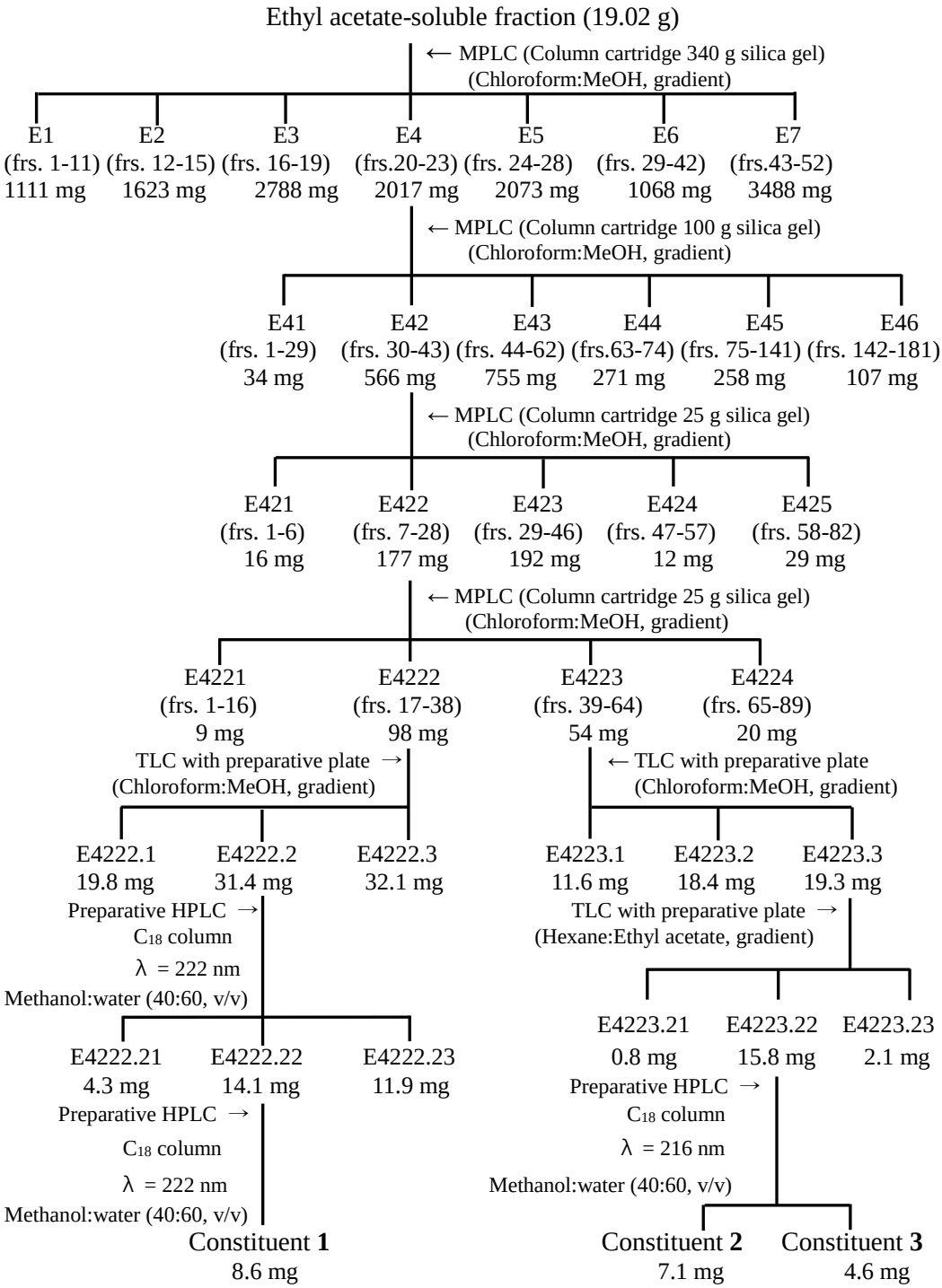
Ethyl acetate-soluble fraction (19.02 g)
← MPLC (Column cartridge 340 g silica gel)
(Chloroform:MeOH, gradient)
E1 (frs. 1-11) 1111 mg
E2 (frs. 12-15) 1623 mg
E3 (frs. 16-19) 2788 mg
E4 (frs.20-23) 2017 mg
E5 (frs. 24-28) 2073 mg
E6 (frs. 29-42) 1068 mg
E7 (frs.43-52) 3488 mg
← MPLC (Column cartridge 100 g silica gel)
(Chloroform:MeOH, gradient)
E41 (frs. 1-29) 34 mg
E42 (frs. 30-43) 566 mg
E43 (frs. 44-62) 755 mg
E44 (frs.63-74) 271 mg
E45 (frs. 75-141) 258 mg
E46 (frs. 142-181) 107 mg
← MPLC (Column cartridge 25 g silica gel)
(Chloroform:MeOH, gradient)
E421 (frs. 1-6) 16 mg
E422 (frs. 7-28) 177 mg
E423 (frs. 29-46) 192 mg
E424 (frs. 47-57) 12 mg
E425 (frs. 58-82) 29 mg
← MPLC (Column cartridge 25 g silica gel)
(Chloroform:MeOH, gradient)
E4221 (frs. 1-16) 9 mg
E4222 (frs. 17-38) 98 mg
E4223 (frs. 39-64) 54 mg
E4224 (frs. 65-89) 20 mg
TLC with preparative plate →
(Chloroform:MeOH, gradient)
← TLC with preparative plate
(Chloroform:MeOH, gradient)
E4222.1 19.8 mg
E4222.2 31.4 mg
E4222.3 32.1 mg
E4223.1 11.6 mg
E4223.2 18.4 mg
E4223.3 19.3 mg
Preparative HPLC →
C18 column
λ = 222 nm
Methanol:water (40:60, v/v)
TLC with preparative plate →
(Hexane:Ethyl acetate, gradient)
E4222.21 4.3 mg
E4222.22 14.1 mg
E4222.23 11.9 mg
E4223.21 0.8 mg
E4223.22 15.8 mg
E4223.23 2.1 mg
Preparative HPLC →
C18 column
λ = 222 nm
Methanol:water (40:60, v/v)
Preparative HPLC →
C18 column
λ = 216 nm
Methanol:water (40:60, v/v)
Constituent 1 8.6 mg
Constituent 2 7.1 mg
Constituent 3 4.6 mg

Fig. 5. Procedures to isolate the DPPH radical scavenging constituents of *Z. atratus* feces. The ethyl acetate-soluble fraction was the most biologically active fraction, and MPLC was performed. Each fraction (500–125 mg/L) was tested in a DPPH assay to isolate the active compounds from the fraction.

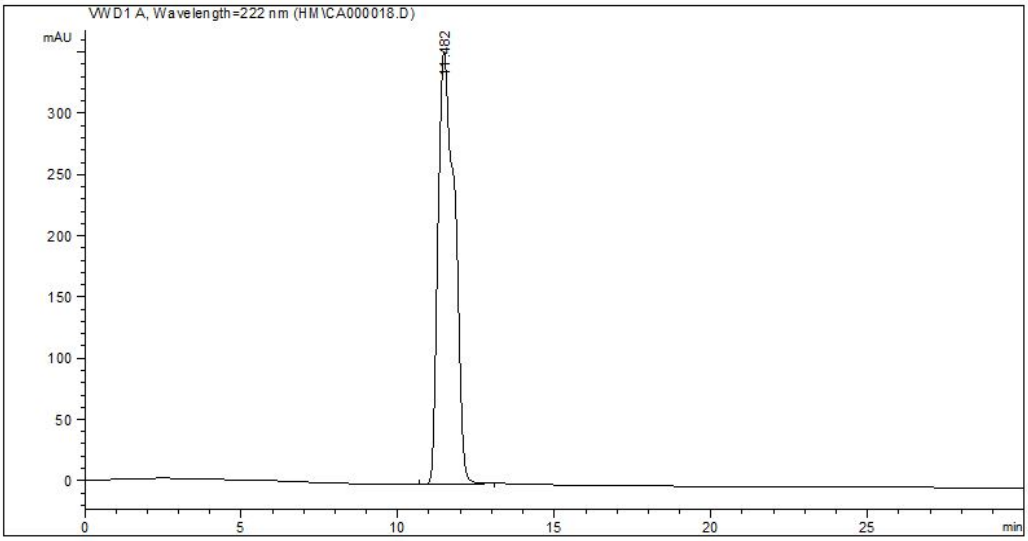

Fig. 6. HPLC chromatogram of constituent 1.

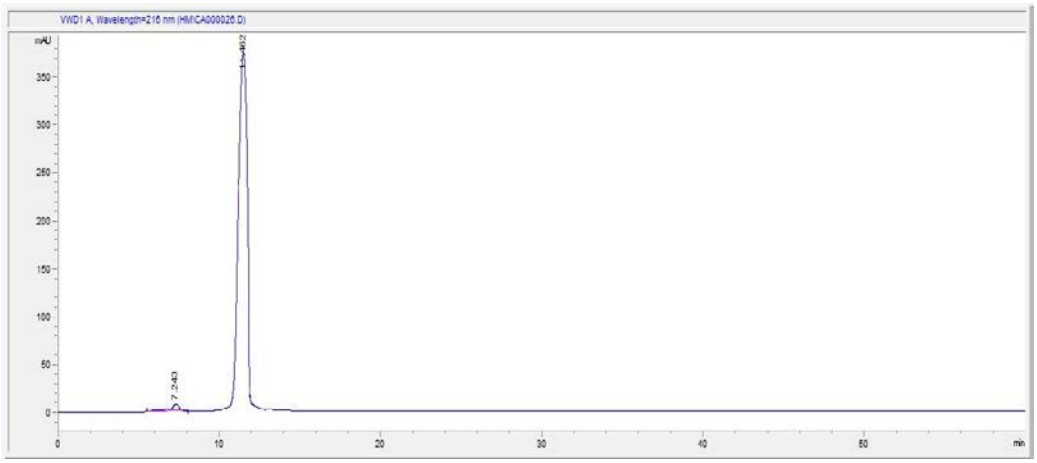

Fig. 6. HPLC chromatogram of constituent 2.

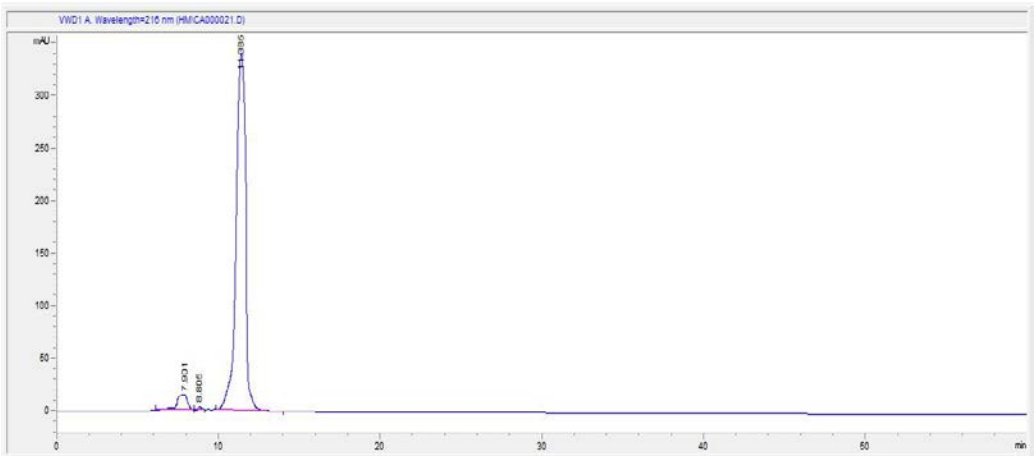

Fig. 7. HPLC chromatogram of constituent 3.

6. DPPH radical scavenging activity assay *in vitro*

DPPH radical scavenging activity was evaluated according to the method of Blois (1958). DPPH is a stable free radical that reacts with constituents that can donate a hydrogen atom. The electron donation ability of natural products can be measured by DPPH radical purple-colored solution bleaching. Each concentration of the test sample in 50 μL of methanol was added to 100 μL of 0.4 mM DPPH solution in 96-well microplates. After incubation at room temperature for 30 min, the mixture was determined by measuring the absorbance at 518 nm using a VersaMAX microplate reader (Molecular Devices, Sunnyvale, CA, USA). Ascorbic acid served as a positive control and was likewise formulated. Negative controls consisted of methanol solution only.

7. Data analysis

The DPPH radical scavenging activity was calculated according to the following formula: DPPH scavenging activity (%) = $\frac{(A0-A1)}{A0} \times 100$, where A0 is the absorbance of the control and A1 is the absorbance of the sample. Antioxidant activity was expressed as 50% inhibition concentration (IC_{50}) of the constituent that is required to cause a 50% DPPH inhibition. The IC_{50} values were determined using Prism 5 software program (GraphPad Prism Software, La Jolla, CA, USA). The IC_{50} values for the treatments were considered significantly different from one another when their 95% confidence limits (CLs) did not overlap. The inhibition was obtained by the following formula: inhibition rate (%) = $[1 - (S - S_0)/(C - C_0)] \times 100$, where C was the absorbance of the control after

30 min of incubation, C_0 was the absorbance of the control at zero time, S was the absorbance of each sample after 30 min of incubation, and S_0 was the absorbance of each samples at zero time. The results are expressed as the means ± standard errors (SEs) of triplicate samples from three independent experiments.

RESULTS

1. Antioxidant activity of materials derived from *Z. atratus*

The antioxidant activity of the methanol extracts from adults, larvae, and feces of *Z. atratus* was elucidated using a DPPH assay (Table 2). Significant differences in antioxidant activity in three materials of the extract were observed. The material from the methanol extract of *Z. atratus* feces was most biologically active.

Table 2. DPPH radical scavenging activity from the solvent hydrolysable of the methanol extract of adults, larvae and feces from *Z. atratus* using a DPPH assay

	Antioxidant activity, %		
Material.	2.0 mg of sample	1.0 mg of sample	0.5 mg of sample
Adults	65.74	36.18	14.25
Larvae	19.59	3.90	0.04
Feces	83.39	62.20	34.34

2. Isolation and identification of active principles from feces of *Z. atratus*

The fractions obtained from solvent partitioning of the methanol extract of *Z. atratus* feces were tested for antioxidant activity using a DPPH assay (Table 3). Significant differences in antioxidant activity were observed among the fractions and were used to identify peak activity fractions for the next step of purification of the extract. Based on the IC_{50} values, the ethyl acetate-soluble fraction (0.212 μg/mL) was the most potent

antioxidant and was a 4.3-fold less potent antioxidant. The IC_{50} of the chloroform-soluble fraction, *Z. atratus* feces extract, and butanol-soluble fraction was between 0.410 and 0.533 μg/mL. The water-soluble fraction exhibited moderate antioxidant activity, whereas no inhibition was obtained using the hexane-soluble fraction.

Table 3. DPPH radical scavenging activity of fractions obtained from solvent partitioning of the methanol extract of the feces from *Z. atratus* and antioxidant agent ascorbic acid using a DPPH assay

Material	IC_{50}, μg/mL (95% CL[a])	Slope ± SE	χ^{2b}	*P*-value
Feces methanol extract	0.506 (0.464–0.552)	1.5 ± 0.09	4.25	0.983
Hexane-soluble fr.[c]	>2000			
Chloroform-soluble fr.	0.410 (0.383–0.438)	1.3 ± 0.06	2.94	0.989
Ethyl acetate-soluble fr.	0.212 (0.193–0.232)	1.4 ± 0.09	3.77	0.976
Butanol-soluble fr.	0.533 (0.497–0.572)	1.1 ± 0.06	3.21	0.988
Water-soluble fr.	0.855 (0.785–0.930)	1.4 ± 0.08	3.80	0.982
Ascorbic acid	0.049 (0.036–0.066)	2.0 ± 0.47	4.14	0.984

[a] CL denotes confidence limit.

[b] Pearson's chi-square goodness-of-fit test.

[c] Fraction.

The antioxidant activities of each subfraction derived from the ethyl acetate-soluble fraction are given in Table 4.

Table 4. DPPH radical scavenging activity of each subfraction from ethyl acetate-soluble fraction derived from feces of *Z. atratus*

No.	Fraction	Antioxidant activity, %		
		Concentration, μg		
		500	250	125
1	E1	–	–	–
2	E2	24.9	14.7	–
3	E3	70.4	48.3	–
4	E4	88.6	82.1	–
5	E5	89.2	81.4	–
6	E6	89.8	67.9	–
7	E7	78.6	53.5	–
8	E41	–	36.3	–
9	E42	–	86.9	–
10	E43	–	58.6	–
11	E44	–	66.9	–
12	E45	–	26.7	–
13	E46	–	44.1	–
14	E421	–	48.6	–
15	E422	–	84.9	–
16	E423	–	58.6	–
17	E424	–	24.6	–
18	E425	–	24.5	–
19	E4221	–	71.2	–

Table 4. Continued

20	E4222	–	86.0	–
21	E4223	–	84.8	–
22	E4224	–	17.7	–
23	E4222.1	–	–	26.6
24	E4222.2	–	–	84.7
25	E4222.3	–	–	63.5
26	E4223.1	–	–	3.3
27	E4223.2	–	–	80.7
28	E4223.3	–	–	12.4
29	E4222.21	–	–	-
30	E4222.22	–	–	89.1
31	E4222.23	–	–	-
32	E4223.21	–	–	-
33	E4223.22	–	–	-
34	E4223.23	–	–	85.7
35	E4222.22-A	–	–	86.3
36	E4223.23-A	–	–	83.5
37	E4223.23-B	–	–	87.8

The DPPH assay-guided fractionation of the methanol extract from *Z. atratus* feces afforded three active principles (compound **1**, **2** and **3**) that were identified by spectroscopic analyses, including EI-MS, ^{1}H NMR, and ^{13}C NMR. Compound **1** was obtained as colorless powder. The mass spectrum of the isolate exhibited a molecular ion at *m/z* 202 $[M]^{+}$ (Fig. 9) and ^{1}H NMR spectra (Fig. 10) showed 14 protons. Its ^{13}C NMR spectra (Fig. 11) showed 13 carbons in the molecule comprising a naphthalene which has three chains containing one methoxy group, one ethyl group, one ketone group as indicated in DEPT (Fig. 12), suggesting the molecular formula $C_{13}H_{14}O_2$. This compound was characterized as 3-ethyl-5-methoxyanaphthalen-1(2*H*)-one (**1**) (Fig. 13). EI-MS (70 eV), *m/z* (% relative intensity): 202 $[M]^{+}$ (14), 198 (13), 168 (100), 153 (64), 125 (10), 97 (14). ^{1}H NMR (MeOD, 600 MHz) and ^{13}C NMR (MeOD, 150 MHz) : See Table 5 .

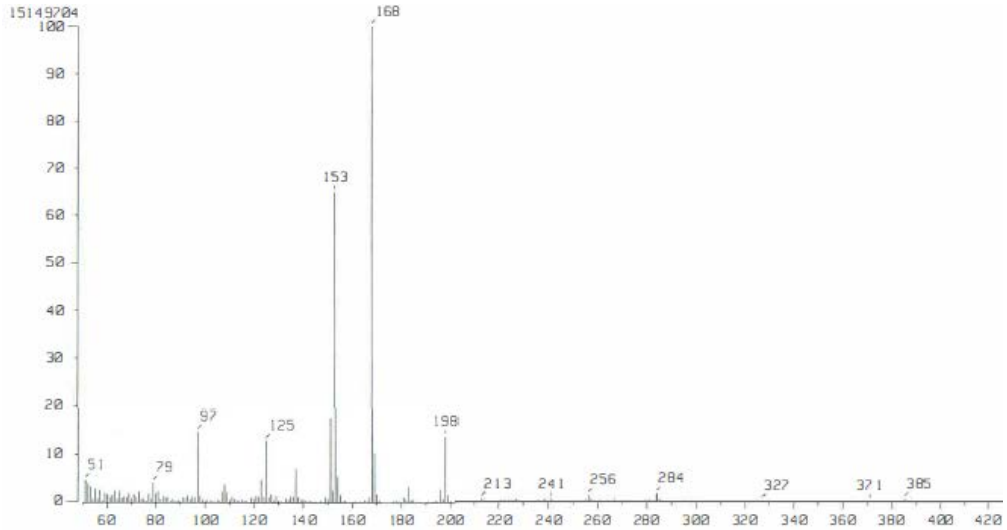

Fig. 9. EI-MS spectrum of compound 1.

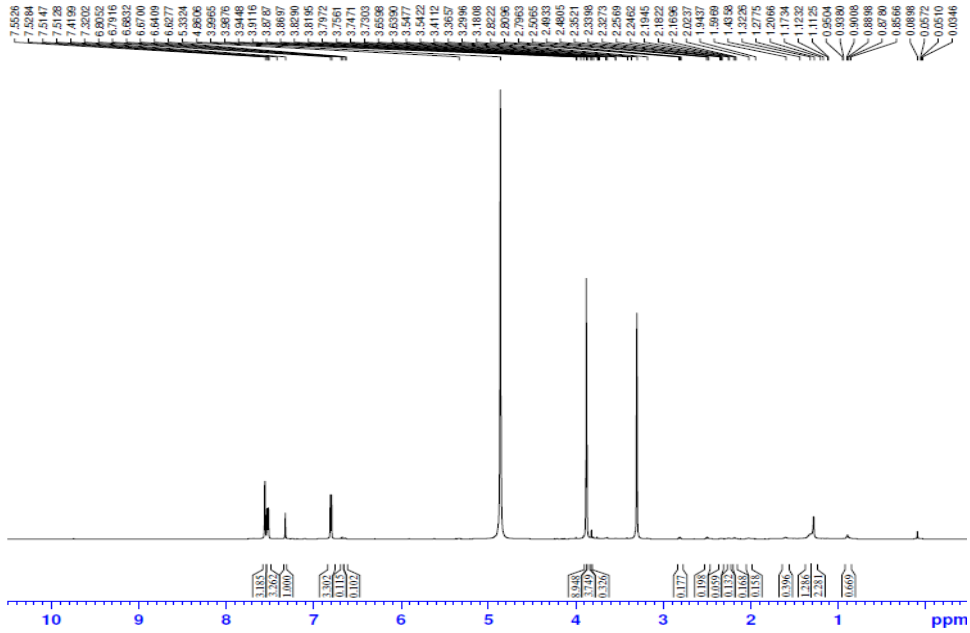

Fig. 10. ^{1}H NMR spectrum of compound 1.

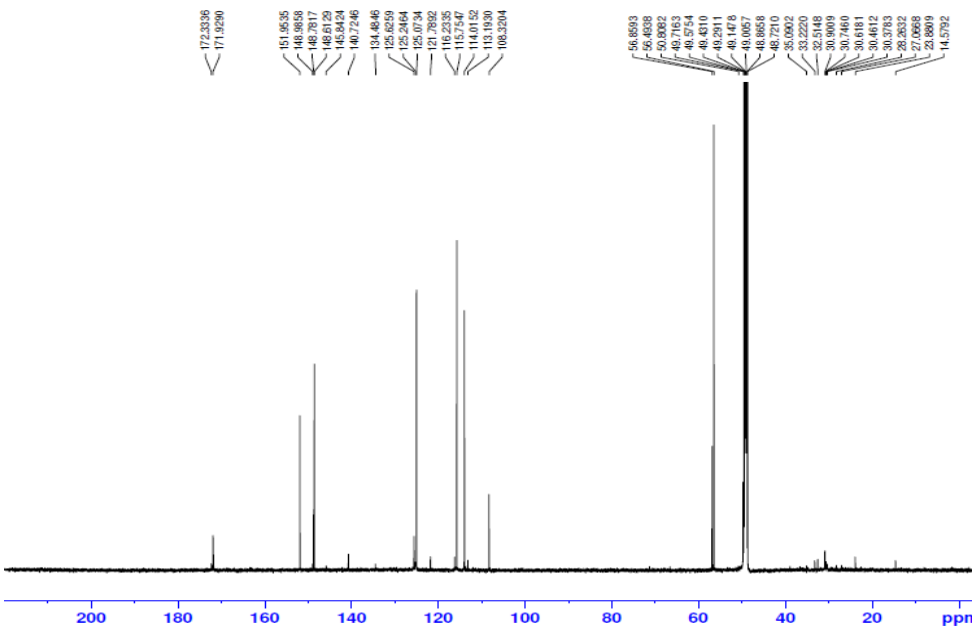

Fig. 11. ^{13}C NMR spectrum of compound 1.

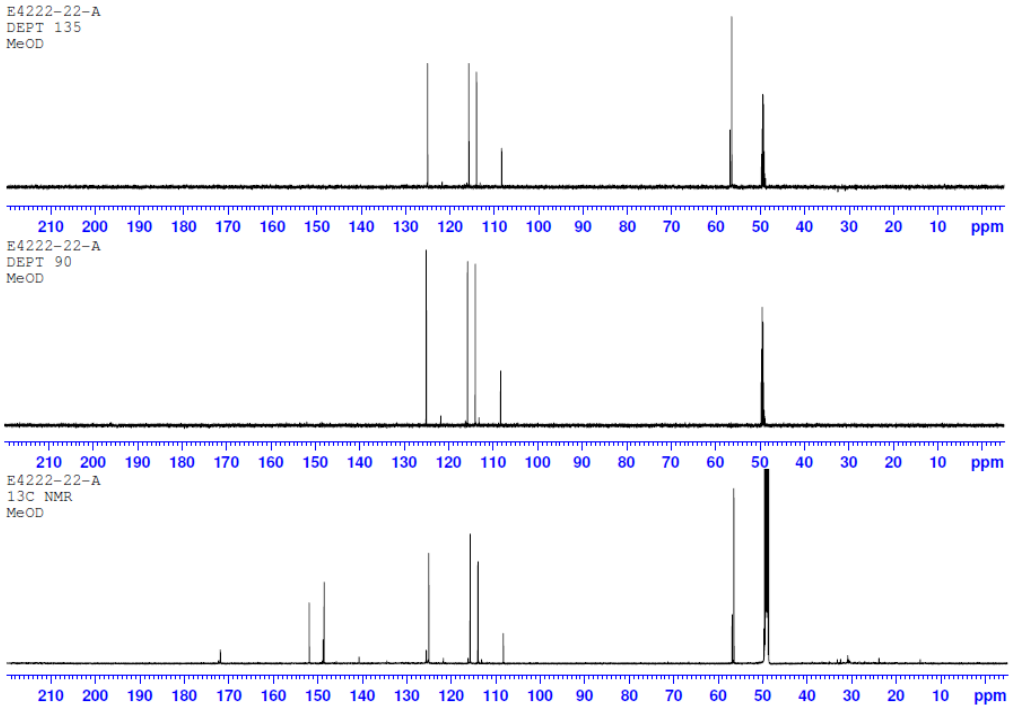

Fig. 12. DEPT spectrum of compound 1.

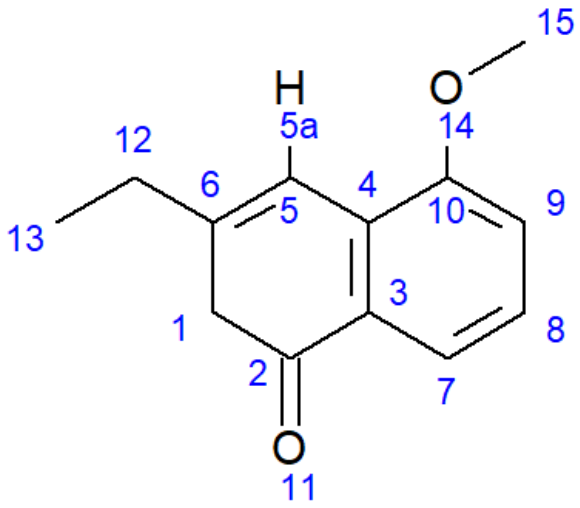

Fig. 13. Structure of 3-ethyl-5-methoxyanaphthalen-1(2*H*)-one.

Table 5. ^{1}H and ^{13}C NMR spectral data for compound 1 (MeOD, TMS, ppm)[a]

Position	Partial structure	δ_C (ppm)	δ_H (ppm)
1	CH_2	48.72	2.82, m
2	C	172.33	
3	C	134.48	
4	C	121.79	
5	CH	115.75	6.81, d (J = 8.16 Hz)
6	C	134.48	
7	CH	115.75	7.55, s
8	CH	125.63	7.53, d (J = 9.36 Hz)
9	CH	116.23	7.32, s
10	C	151.95	
11	O		
12	CH_2	30.90	2.02, s
13	CH_3	14.58	0.89, m
14	O		
15	CH_3	56.49	3.88, s

[a] Coupling constants (Hz) are in parenthesis.

Compound **2** was obtained as yellow powder. EI-MS revealed a molecular ion at m/z 282 $[M]^+$ (Fig. 14) and its ^1H NMR spectra (Fig. 15) showed 30 protons. Its ^{13}C NMR spectra (Fig. 16) showed 17 carbons in the molecule comprising a pentadeca-core containing one ester group, one methoxy group, two methyl groups as indicated in DEPT (Fig. 17), suggesting the molecular formula $C_{17}H_{30}O_3$. This compound was characterized as methyl (9*Z*,12*E*)-15-methoxypentadeca-9,12-dienoate (2). (Fig. 18). EI-MS (70 eV), *m/z* (% relative intensity): 282 M (5), 281 (25), 250 (
114 (11), 95 (11), 81 (20), 72 (68), 59 (100), 55 (32). ^1H NMR (MeOD, 600 MHz) and ^{13}C NMR (MeOD, 150 MHz): See Table 6.

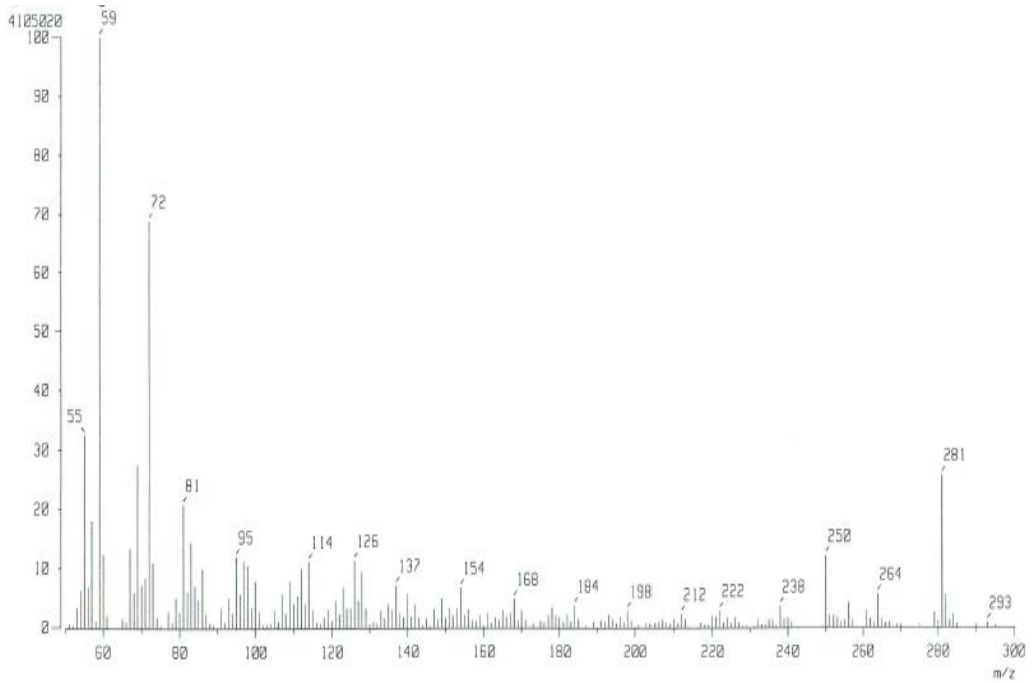

Fig. 14. EI-MS spectrum of compound 2.

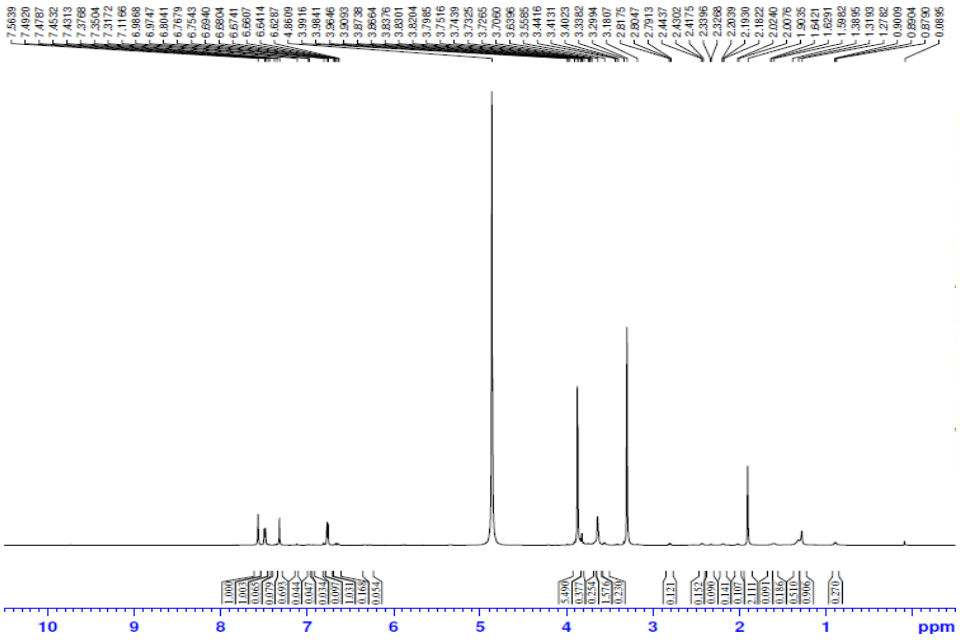

Fig. 15. ^{1}H NMR spectrum of compound 2.

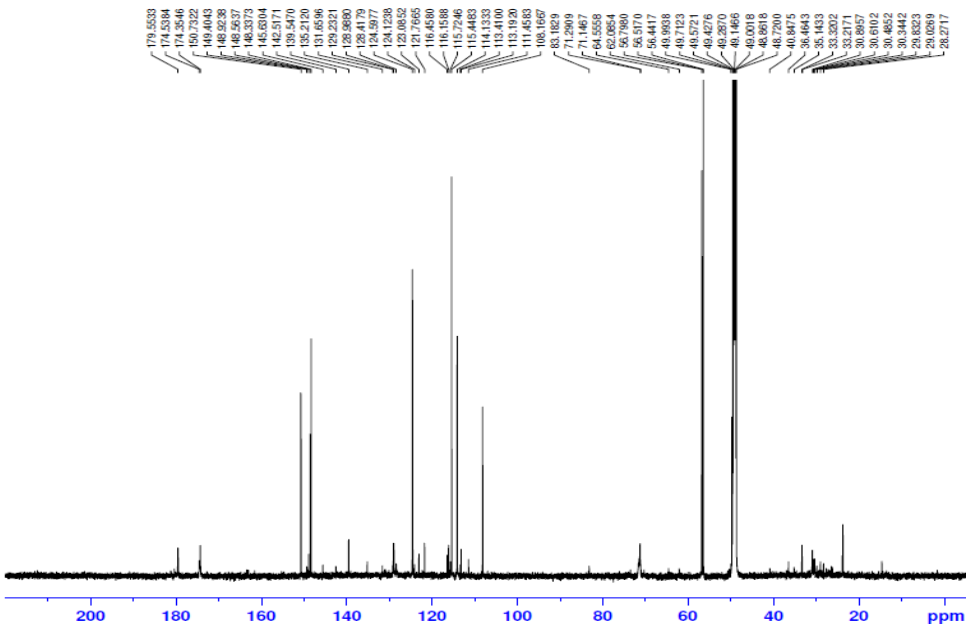

Fig. 16. ^{13}C NMR spectrum of compound 2.

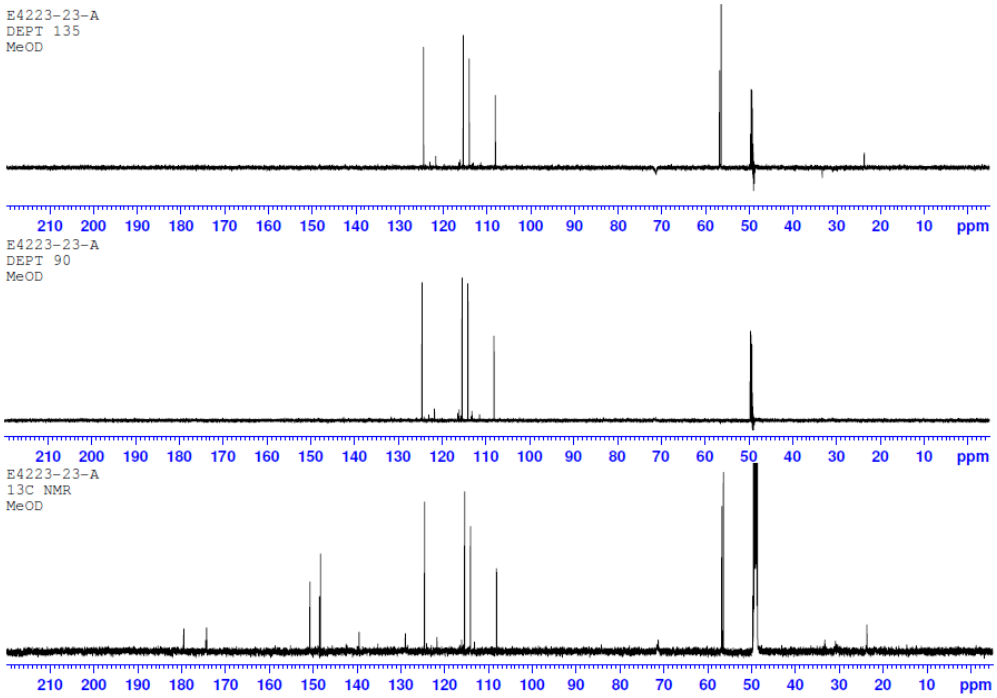

Fig. 17. DEPT spectrum of compound 2.

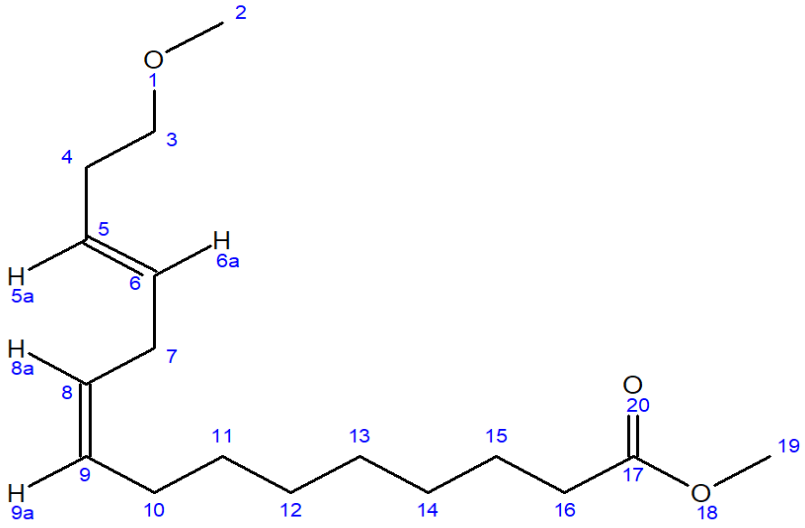

Fig. 18. Structure of methyl (9*Z*,12*E*)-15-methoxypentadeca-9,12-dienoate

Table 6. ^{1}H and ^{13}C NMR spectral data for compound 2 (MeOD, TMS, ppm)[a]

Position	Partial structure	δ_C (ppm)	δ_H (ppm)
1	O		
2	CH_3	56.80	3.56, s
3	CH_2	71.29	3.64, S
4	CH_2	33.32	2.02, d (J = 4.44 Hz)
5	CH	114.13	7.32, s
6	CH	128.99	7.56, s
7	CH_2	30.89	2.80, m
8	CH	115.45	7.49, d (J = 7.98 Hz)
9	CH	124.60	6.77, d (J = 8.16 Hz)
10	CH_2	27.61	2.18, m
11	CH_2	30.34	1.28, s
12	CH_2	29.83	1.32, s
13	CH_2	29.02	1.28, s

Table 6. Continued

14	CH_2	28.27	1.32, s
15	CH_2	23.72	1.90, s
16	CH_2	33.22	2.43, m
17	C	174.35	
18	O		
19	CH_3	56.44	3.87, d (J = 4.44 Hz)

[a] Coupling constants (Hz) are in parenthesis.

Compound **3** was obtained as yellow powder. EI-MS revealed a molecular ion at *m/z* 281 $[M]^+$ (Fig. 19) and its ^1H NMR spectra (Fig. 20) showed 19 protons. Its ^{13}C NMR spectra (Fig. 21) showed 18 carbons in the molecule two methyl groups, one aniline group, one ester group as indicated in DEPT (Fig. 22), suggesting the molecular formula $C_{18}H_{19}NO_2$. This compound was characterized as isopropyl(*Z*)-3-Anilino-3-phenyl-2-propenoate (3) (Fig. 23). The interpretations of proton and carbon signals were largely consistent with those of Fustero et al. (1998). Isopropyl(*Z*)-3-Anilino-3-phenyl-2-propenoate (**3**) was identified on the basis of the following evidence: yellow powder. EI-MS (70 eV), *m/z* (% relative intensity): 281 $[M]^+$ (24), 261 (12), 194 (56), 149 (12), 128 (10), 112 (16), 95 (11), 83 (16), 72 (68), 59 (100). ^1H NMR (MeOD, 600 MHz) and ^{13}C NMR (MeOD, 150 MHz): See Table 7.

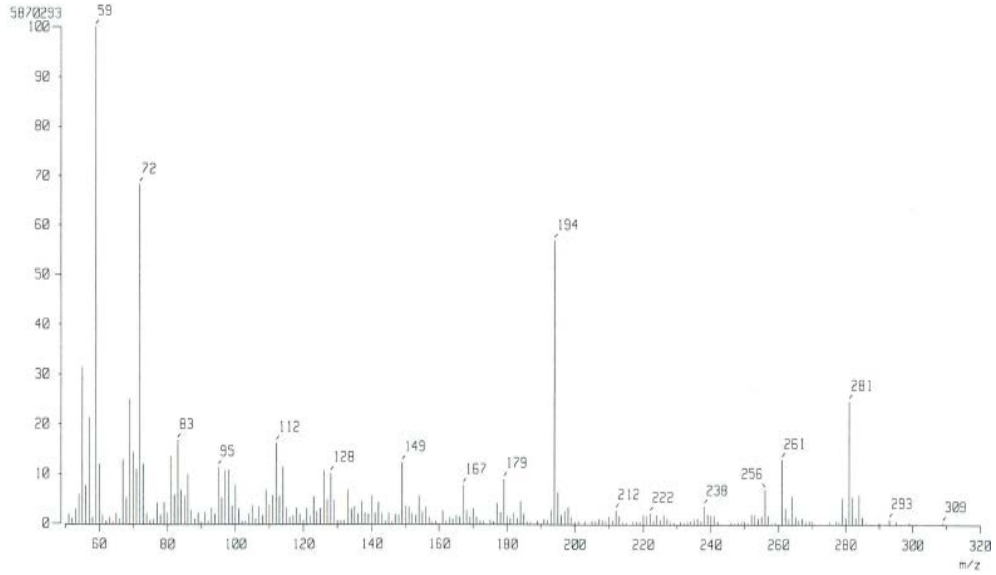

Fig. 19. EI-MS spectrum of compound 3.

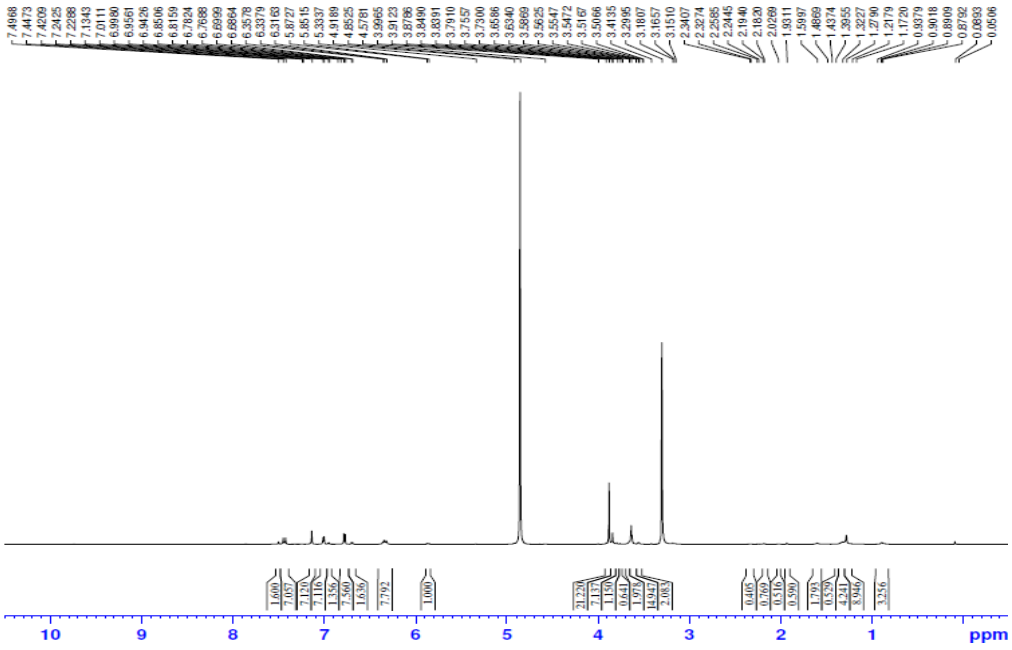

Fig. 20. ^{1}H NMR spectrum of compound 3.

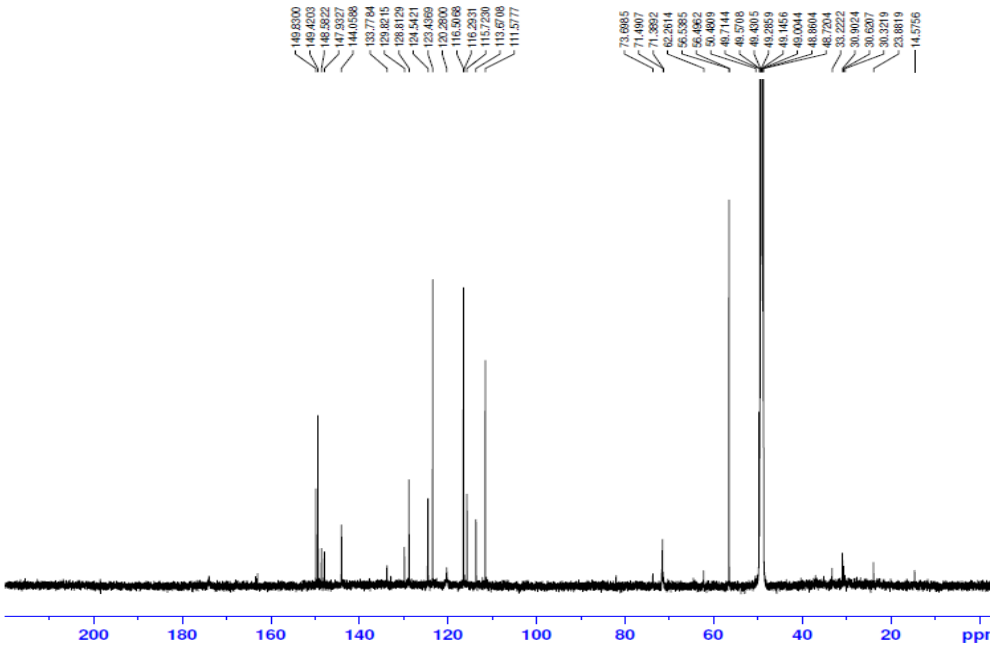

Fig. 21. ^{13}C NMR spectrum of compound 3.

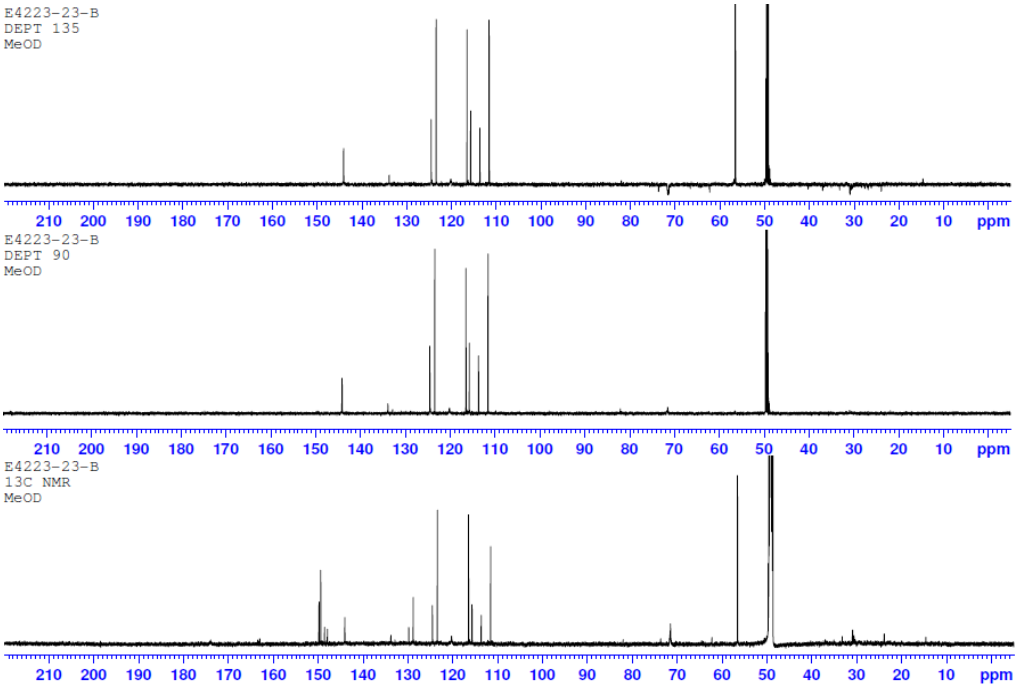

Fig. 22. DEPT spectrum of compound 3.

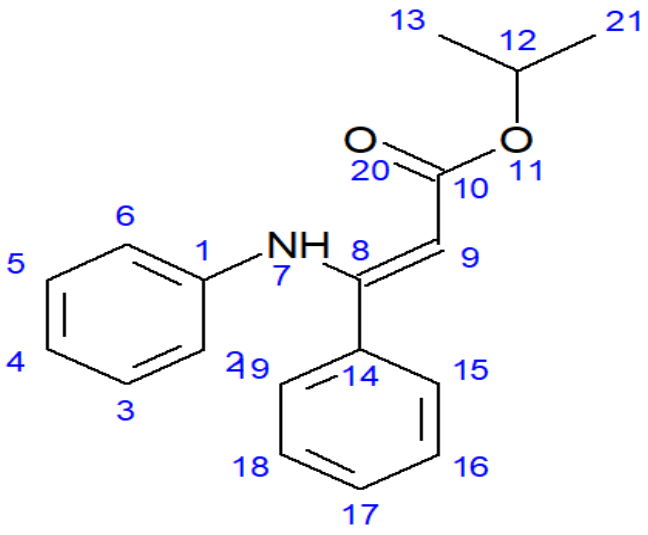

Fig. 23. Sturcture of isopropyl(*Z*)-3-Anilino-3-phenyl-2-propenoate.

Table 7. ^{1}H and ^{13}C NMR spectral data for compound 3 (MeOD, TMS, ppm)[a]

Position	Partial structure	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)
				Fustero et al. (1998)	
1	C	144.06		140.3	
2	CH	116.51	6.78, d	122.7	6.65-7.10 , m
			(*J* = 9.16 Hz)		
3	CH	129.82	7.00, d	129.2	6.65-7.10 , m
			(*J* = 7.86 Hz)		
4	CH	115.72	6.70, d	121.9	6.65-7.10 , m
			(*J* = 8.10 Hz)		
5	CH	129.82	7.00, d	129.2	6.65-7.10 , m
			(*J* = 7.86 Hz)		
6	CH	116.29	6.78, d	122.7	6.65-7.10 , m
			(*J* = 9.16 Hz)		
7	NH		(10.13)		10.38, br s
8	C	149.42		158.7	
9	CH	73.70	5.87, d	91.7	5.15, m
			(*J* = 12.72 Hz)		
10	C	149.83		169.5	
11	O				
12	CH	71.39	4.58, s	66.2	5.00, s

Table 7. Continued

13	CH_3	23.88	1.28, s	22.0	1.32 ,d (J = 7.2 Hz)
14	C	133.78		135.9	
15	CH	124.54	7.50, s	128.2	7.32, m
16	CH	128.81	7.42, d (J = 15.84 Hz)	128.4	7.32, m
17	CH	123.44	7.42, d (J = 15.84 Hz)	128.0	7.32, m
18	CH	128.81	7.42, d (J = 15.84 Hz)	128.4	7.32, m
19	CH	124.54	7.50, s	128.2	7.32, m
20	O				
21	CH_3	23.88	1.28, s	22.0	1.32 ,d (J = 7.2 Hz)

[a] Coupling constants (Hz) are in parenthesis.

3. *In vitro* DPPH radical scavenging activity of the isolated compounds

The antioxidant activity of the three isolated compounds and antioxidant agent ascorbic acid, which was used as a positive control, was evaluated using a DPPH assay (Table 8). As judged by the IC_{50} values, isopropyl(*Z*)-3-anilino-3-phenyl-2-propenoate (96.21 μM) was the most potent antioxidant constituent. The IC_{50} of 3-ethyl-5-methoxyanaphthalen-1(2*H*)-one and methyl (9*Z*,12*E*)-15-methoxypentadeca-9,12-dienoate were 191.29 μM and 146.12 μM, respectively. Overall, these compounds were significantly less potent antioxidants than ascorbic acid.

Table 8 DPPH radical scavenging activity of three isolated compounds and antioxidant agent ascorbic acid using a DPPH assay

Compound	IC_{50}, μM (95% CL[a])	Slope ± SE	χ^{2}[b]	*P*-value	RT[c]
EMNO[d]	191.29 (181.65–201.43)	1.2 ± 0.05	2.97	0.987	0.30
MMPD[e]	146.12 (141.24–151.19)	1.4 ± 0.03	1.56	0.997	0.40
IAPP[f]	96.21 (92.47–100.04)	1.4 ± 0.03	1.83	0.996	0.60
Ascorbic acid	58.19 (46.96–68.53)	2.0 ± 0.47	4.14	0.984	1.00

[a] CL denotes confidence limit.

[b] Pearson's chi-square goodness-of-fit test.

[c] Relative toxicity, IC_{50} of ascorbic acid/IC_{50} of test compound.

[d] 3-Ethyl-5-methoxyanaphthalen-1(2*H*)-one.

[e] Methyl (9*Z*,12*E*)-15-methoxypentadeca-9,12-dienoate.

[f] Isopropyl(Z)-3-anilino-3-phenyl-2-propenoate.

DISCUSSION

In most developed countries, human consumption of insects is infrequent, or even culturally inappropriate, although its nutritional value is comparable to conventional meat (Van Huis, 2013). In many regions and countries of the world, insects form part of the human diet and it is a misconception to believe that this is prompted by starvation (Van Huis, 2013).

With an increase in the world population, increased consumer demand for protein, and the amount of available agricultural land being constrained, the sustainable production of meat will represent a serious challenge for the future. Insects can be considered as an alternative protein source with less environmental impact (Van Huis, 2013). Insects can be consumed as a whole. However, they can also be processed in less recognizable forms, which may increase consumer acceptability.

ROS produced by from mitochondria and other cellular organelle have been traditionally regarded as toxic byproducts of metabolism leading to damage lipids, proteins, and DNA (Freeman, 1982; Thannickal and Fanburg, 2000). Free radical species mediate damage to proteins, lipids, mitochondria, and DNA and may activate the cell cycle; overwhelm endogenous antioxidant defenses in the brain; and contribute to neuronal damage(Lovell and Markesbery, 2007; Montine et al., 2002). Oxidative damage is associated with aging and is widespread in the brain in Alzheimer disease (Lovell and Markesbery, 2007).

Oxidative stress may be broadly defined as an imbalance between oxidant production

and the antioxidant capacity of the cell to prevent oxidative injury. Excessive free radicals result in the oxidation of cellular molecules, proteins, lipids, and DNA, ultimately culminating in cell death or tissue injury. Oxidative stress is recognized as a common factor in many neurodegenerative diseases and is a proposed mechanism for age-related degenerative processes as a whole (Lin and Beal, 2006). This phenomenon has been implicated in a variety of human degenerative phenomena, including aging, diabetes (Srivastava et al., 2005), and inflammation (Azad et al., 2008).

The antioxidants may be natural or synthetic ones. Polyhydroxy flavones, flavanones, flavanols, isoflavones, chalcones, and many members are of these groups of natural substances which proved to have a high degree of antioxidant activity and they are found to be widely spread in plant material (Rajani, 2004). Natural antioxidants include ascorbic acid, glutathione, uric acid, tocopherol, lipoic acid, and polyphenol metabolites. Synthetic antioxidant such as BHT and BHA have been used as antioxidants. However, the safety of synthetic additives has been questioned stimulating the evaluation of naturally occurring compounds with antioxidant properties. Oral consumption of this ingredient has also been shown to have toxic effects on the body's blood coagulation system. Even though there is no assurance of the safety of natural antioxidants, there is some comfort knowing that such antioxidants are purified from natural products that have been consumed. Natural antioxidant has been of great interest for their potential antioxidant effects for the preservation of the foods from the toxic effects of the oxidants. Antioxidant constituents derived from natural products include that scavenge the free radicals and detoxify the organism (Zygadlo et al., 1995; Maestri et al., 1996; Maestri et

al., 1998; Tepe et al., 2004).

Since early times, insects, their products, and their constituents have been used, directly and indirectly, in the medical systems of different human cultures throughout the world (Costa-Neto, 2002). Also, insects and the substances extracted from them have been used as therapeutic resources in the medicals systems of many cultures (Costa-Neto, 2005).

The superworm, as known the larva of *Zophobas morio*, has been officially imported from 2011 and bred commercially in Korea. But it is named as the corrected scientific name, *Zophobas atratus* by junior synonym throughout traditional taxonomy and newly designated Korean name as ‘a-me-ri-ca-wang-geo-jeo-ri’ in terms of resource management (Park et al., 2013). *Zophobas atratus* is also used as pet food, sold in pet stores under the name giant mealworms. Their nutritional values are similar to those of mealworms, so it is possible that supplementation with calcium is necessary if they are used as a staple food item. However, there are a few reports of antioxidant agents from insects, which are the most diverse groups of organisms (Huang et al., 1997).

In this study, the methanol extract of *Z .atratus* feces was proved to have antioxidant activity, although the extract was less antioxidant than the widely used antioxidant agent ascorbic acid. The antioxidant principle was determined to be the 3-ethyl-5-methoxyanaphthalen-1(2*H*)-one, methyl (9*Z*,12*E*)-15-methoxypentadeca-9,12-dienoate and isopropyl(Z)-3-anilino-3-phenyl-2-propenoate using DPPH assay. As judged by the IC_{50} values, 3-ethyl-5-methoxyanaphthalen-1(2*H*)-one, methyl (9*Z*,12*E*)-15-methoxypentadeca-9,12-dienoate and isopropyl(Z)-3-anilino-3-phenyl-2-propenoate

were between 96.21 and 191.29 μM, although these compounds were significantly less potent antioxidants than ascorbic acid. This original finding indicates that materials derived from *Z .atratus* feces can hold promise for the development of novel and effective naturally occurring antioxidant agent.

In conclusion, the *Z .atratus* feces-derived preparations containing 3-ethyl-5-methoxyanaphthalen-1(2*H*)-one, methyl (9*Z*,12*E*)-15-methoxypentadeca-9,12-dienoate and isopropyl(*Z*)-3-anilino-3-phenyl-2-propenoate could be useful as sources of potential antioxidants or lead molecules for treatment of several diseases in which there is an increase in free radical production. The antioxidant action of these constituents may be an indication of at least one of the pharmacological actions of *Z. atratus* feces. For practical use of the preparations as novel antioxidant products to proceed, further research is needed to establish their safety with respect to humans and whether this activity could be exerted *in vivo* after consumption of the *Z .atratus* fecees-derived product by humans. Lastly, detailed tests are needed to understand how to improve antioxidant potency and stability for eventual commercial development.

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아메리카왕거저리 분변토 유래 화합물의

항산화활성 효과

서울대학교 대학원

농업생명과학대학 WCU 바이오모듈레이션 전공

신 혜 민

초 록

자유라디칼을 가진 활성산소는 불안정하여 주위의 물질과 반응해 세포내 단백질이나 지질분자 그리고 DNA 등에 산화적 손상을 입히며 결과적으로 치명적인 피해를 입히는 것으로 알려져 있다. 이와 같이 산화 스트레스가 노화를 비롯하여 각종 질환을 일으키는 중요한 원인임이 입증됨으로써 활성산소 소거활성을 갖는 항산화성 생체 기능 물질의 노화억제 및 질환의 치료제로서 가능성이 크게 부각되고 있어, 새로운 항산화성 생체기능물질

개발이 요구되고 있다. 특히, 지금까지 알려진 항산화제는 독성 및 사용상의 한계로 인하여 문제점을 내포하고 있다. 따라서, 최근의 기술개발 동향은 안전하고 강한 활성을 지닌 천연 항산화제의 개발에 집중되고 있다.

본 연구에서는 아메리카왕거저리(*Zophobas atratus*) 배설물로부터 항산화 성분을 분리하였으며, 상용 항산화제인 아스코르브산(ascorbic acid)을 양성 대조군으로 하여 활성을 비교하였다. 아메리카왕거저리 배설물 7.6 kg에서 380 g의 메탄올 추출물을 얻었으며 각종 크로마토그라피 및 기기분석을 통하여 3개 물질, 3-ethyl-5-methoxyanaphthalen-1(2H)-one, methyl (9Z,12E)-15-methoxypentadeca-9,12-dienoate, isopropyl (Z)-3-anilino-3-phenyl-2-propenoate 를 분리 동정하였다. 이들 화합물의 IC_{50} 값은 각각 191.29 μM, 146.12 μM, 96.21 μM 으로서, 아스코르브산에 비해 낮은 항산화 활성을 보였지만 이들 성분들은 곤충 배설물 유래 화합물로서, 그 잠재적 가능성을 확인하였다.

본 논문은 아메리카왕거저리 배설물 및 그 성분들의 항산화 활성을 알아봄으로써, 항산화제로서의 가능성을 탐색하여 농업적, 산업적으로 그 활용 가능성이 높다고 판단되어 석사학위 논문으로써 충분한 가치가 있는 것으로 사료된다.

이상의 결과를 바탕으로 본 논문의 연구는 아메리카왕거저리 배설물에 함

유된 활성본체들을 분리동정 하였고, 활성 물질들의 작용기구를 연구했다는 데 그 의의가 있고, 구성 물질에 대한 생물검정을 통해 항산화제로써의 가능성을 탐색하고 아메리카왕거저리 배설물의 새로운 생리활성을 밝혀내어 농업적, 산업적으로 그 활용 가능성이 높다고 판단되며 이에 더하여 추가적 연구가 요구된다.

검색어: 천연항산화제, 아메리카왕거저리, 아메리카왕거저리 분변토, 3-ethyl-5-methoxyanaphthalen-1(2*H*)-one, Methyl (9*Z*,12*E*)-15-methoxypentadeca-9,12-dienoate, Isopropyl(*Z*)-3-anilino-3-phenyl-2-propenoate

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