Purification and Characterization of Tyrosinase Inhibitor from an Edible Brown Alga, *Hizikia fusiformis*

식용 톳으로부터 Tyrosinase 저해제의 정제 특성

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by

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A dissertation

by

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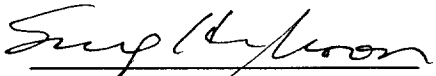

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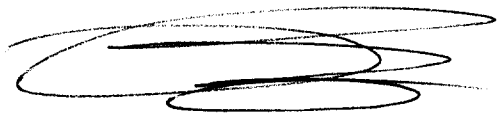

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Abstract

This study was carried out to purify a potent tyrosinase inhibitor from an edible brown alga, *Hizikia fusiformis* as a part of our efforts to isolate non-hazardous natural tyrosinase inhibitors considering their potential utilization in the cosmetic, medicinal, and agricultural industries. Components in methanol extract of *Hizikia fusiformis* were purified using polarity fractionation and different chromatographic techniques. Structural characterization with ^{1}H, NMR, ^{13}C NMR, FAB-MS, IR and UV-Vis spectroscopic techniques revealed the purified inhibitor as a flavanoid glycoside (Compound-1). Compound-1 inhibited monophenolase activity of tyrosinase dose dependently and IC_{50} value was 0.52 mM. Further, enzyme kinetics assay using a Lineweaver-Burk plot proved that compound-1 inhibited tyrosinase in a non-competitive manner. These results suggest that compound-1 derived from brown alga, *Hizikia fusiformis* is one of potent natural tyrosinase inhibitors.

Key words: *Hizikia fusiformis*, tyrosinase inhibitor, purification, flavonoid glycoside

Table of Contents

List of Tables

List of Figures

List of Schemes

List of Abbreviations

1D NMR	One Dimensional Nuclear Magnetic Resonance
2D NMR	Two Dimensional Nuclear Magnetic Resonance
^{13}C NMR	^{13}Carbon Nuclear Magnetic Resonance
COSY	Correlation Spectroscopy
DEPT	Distortionless Enhancement by Polarisation Transfer
DMSO-d_6	Dimethylsulfoxide
DOPA	3,4-dihydroxyphenylalanine
Dopachrome	2-carboxy-2,3-dihydroxyindole-5,6-quinone
^{1}H NMR	Proton Nuclear Magnetic Resonance
HMBC	Heteronuclear Multiple-Bond Correlation
HMQC	Heteronuclear Multiple-Quantum Correlation
HPLC	High-Performance Liquid Chromatography
HREIMS	High Resolution Electron Impact Mass Spectroscopy
Hz	Hertz (sec^{-1})
IC_{50}	50% inhibitory concentration
LREIMS	Low Resolution Electron Impact Mass Spectroscopy
NOESY	Nuclear Overhauser Effect Spectroscopy
HRFAB MS	High Resolution Fast Atom Bombardment Mass Spectroscopy
IR	Infra Red
K_i	Inhibitory constant
K_m	Michaelis constant
UV	Ultraviolet
V_{max}	Maximal velocity

List of Symbols

J	Coupling constant (Hz)
M^+	Molecular ion
m/z	Mass to charge ratio
Rf	Retention factor
δ	Chemical shift

Introduction

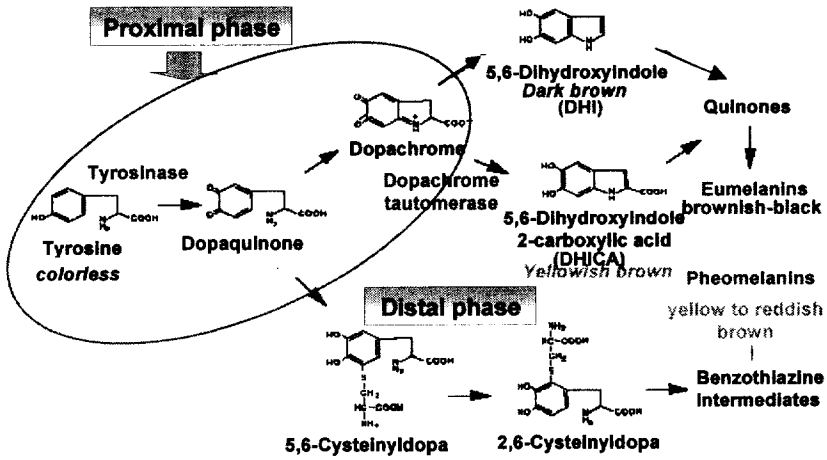
Pigments are the substances that impart colors to materials and pigmentation is one of the most obvious phenotypical characteristics in the natural world. Of the pigments, melanin is one of the most widely distributed among bacteria, fungi, plants, and animals (Sanchez-Ferrer et al, 1995). It is a nitrogen containing, heterogeneous, amorphous, irregular polyphenole like biopolymer and its color can be varied from yellow to black (Prota, 1988). Covalently linked indoles represent the basic structural unit of melanin. Although it must be emphasized that the overall structure is not known, most melanin molecules appear to be mixed polymers based on indoles, but containing variable amounts of other pre-indolic products of the synthetic pathway (Rilley, 1997). Melanin has many roles in biological systems owing to its structure.

Due to biological significance of melanin in biological systems, their synthesis, melanogenesis, is being studied over a long period. Melanin is synthesized in specialized pigment-producing cells known as melanocytes in the basal layer of the mammalian skin. Within the melanocytes, these compounds are biosynthesized in the membranous organelles named melanosomes, which are created by relatively amorphous and spherical vesicles that blebbed from endoplasmic reticulum. The mature melanasomes located in the dendrites of melanocytes are then phagocytosed by the surrounding keratinocytes. Thus, melanogenesis process is responsible for the variety of colors in human skin, hair, and eyes (Sanchez-Ferrer et al, 1995).

Melanogenesis pathway (Scheme 1) was firstly described by Raper in 1928 and confirmed and extended by Mason in 1965. The Raper-Mason pathway of melanogenesis explains the synthesis of two types of melanin in mammals, the eumelanins, brown to black melanin, and the pheomelanins, red to yellow melanin,

as end products. During the process of melanogenesis, amino acid, tyrosine undergoes series of enzymatic and chemical reactions in order to produce melanins. According to Raper and Mason, melanogenesis pathway can be divided into two phases as proximal and distal. The proximal phase consist of enzymatic oxidation of tyrosine (monophenol) and/or *o*-diphenol (L-DOPA) to its corresponding o-quinones. These conversions are catalyzed by a copper-containing enzyme called, tyrosinase. The end products of proximal phase, *o*-quinone, DOPAchrome, undergo a series of chemical and enzymatic reactions, which lead to synthesize eumelanin during distal phase. The availability of sulphihydryl compounds, glutathione or cysteine help to synthesize pheomelanin, initially reacting with *o*-DOPAchrome followed by subsequent enzymatic and chemical reactions. Out of all reactions, enzymatic conversion, tyrosinase activity in the proximal phase is considered to be the key step as it has noted that absence of functional tyrosinase prevent melanogenesis (Hearing, 2005). Therefore, enzyme tyrosinase has been studied thoroughly over a long period.

Tyrosinase (EC 1.14.18.1) a multicopper oxygenase is widely distributed in the phylogenetic scale, from bacteria to mammals and also known as polyphenol oxidase, monophenol monooxygenase, monophenol dihydroxyphenylalanine and dioxygen oxidoreductase. So far, tyrosinase is the most well studied multicopper oxigenase due to its pivotal role in synthesis of multifunctional melanin. It appears to reside in the plastids and chloroplasts of plants, cytoplasm of senescing or ripening fruits, as well as in the cuticle and heamolymph of insects (Marshall, Kim and Wei, 2000). In mammals tyrosinase is concentrated in melanosomes where melanins are produced. It is known that the characteristics of tyrosinase from various sources are different; including tyrosinase find in different organs of the same organism even they possess similar function (Sanchez-Ferrer et al, 1995). By now, properties of some tyrosinases are

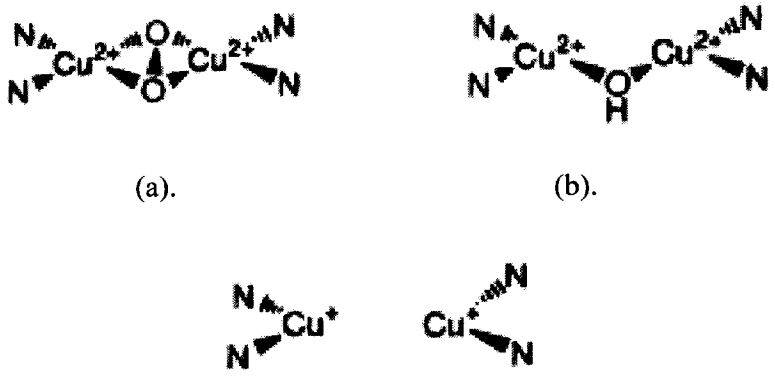


Scheme 1. Schematic melanogenesis pathway including the proximal and distal phase.

well characterized. Even though, the complete crystal structure for tyrosinase is unknown up-to-date, crystal structures of different active sites and their interactions with substrates or potential inhibitors have been well studied (Soloman, Sundaram and Machonkin, 1996). Further, these findings would help to understand the role of tyrosinase in melanogenesis pathway.

Chemical and spectroscopic studies of tyrosinase have shown that presence of a coupled binuclear copper active site and it is very similar to that found in heamocyanins (Soloman, Sundaram and Machonkin, 1996). This binuclear copper site can be appeared in variety of forms, *oxy*tyrosinase (E_{oxy}), *met*tyrosinase (E_{met}), and *deoxy*tyrosinase (E_{deoxy}) (Fig. 1). *Oxy*tyrosinase is oxygenated form of tyrosinase and it consist of two tetragonal Cu^{2+} ions, each coordinated by to strong equatorial and one weaker axial N_{His} ligand. The exogenous oxygen molecule is bound as peroxide and bridges the two Cu centers. *Met*tyrosinase like the *oxy* form contains two tetragonal Cu^{2+} ions antiferromagnetically coupled through an endogenous bridge, although hydroxide exogenous ligands other than peroxide are bound to the copper site. This derivative can be converted by addition of peroxide to oxytyrosinase, which in turn decay back to *met*tyrosinase, when the peroxide is lost. The resting form of tyrosinase, i.e. the enzyme as obtained after purification, is found to be mixture of $\geq$85% *met* and $\leq$15% *oxy* forms. *Deoxy*tyrosinase in contrast to other two forms has a bicuprous structure (Cu^{+}-Cu^{+}) (Sanchez-Ferrer et al, 1995).

Tyrosinase catalyzes two important reactions in proximal phase of melanization in which monophenolase activity is considered as the first step and it involves the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA). During the second step, diphenolase activity, tyrosinase catalyses the oxidation of DOPA to DOPAquinone (Fig. 2). Three copper states in the active site of tyrosinase have led to propose a structural model for the reaction mechanism



(a). (b).

(c).

Figure 1. Three states of binuclear copper active site of tyrosinase
(a). *Oxy*tyrosinase (b) *Met*tyrosinase and (c) *Deoxy*tyrosinase

involved in the monophenolase and diphenolase activity. During the monophenolase activity, monophenolic substrate, tyrosine initially coordinates to an axial position of one of the coppers of E_{oxy}. Rearrangement through a trigonal bipyramidal intermediate leads to *o*-hydroxylation of monophenol by the bound peroxide, loss of H_2O and formation of the $E_{met}D$ complex. The observed characteristic lag phase present in the monophenolase activity derives from the fact that the resting form of tyrosinase contains ≤15% *oxy* sites and monophilic substrate can only react with this *oxy* component. $E_{met}D$ complex formed during monophenolase activity has two fates, either render free diphenol to fulfill the equilibrium $E_{met} + D \rightleftharpoons E_{met}D + 2H^+$ as a first step in the diphenolase cycle, or undergo oxidation of the diphenolate intermediate bound to the active center,

giving a free quinone and a reduced binuclear cuprous enzyme site (E_{deoxy}). *Oxy*tyrosinase is then, regenerated after the binding of molecular oxygen to E_{deoxy}. During the diphenolase activity, diphenols can be bound to either E_{met} or E_{oxy}. If it binds to E_{oxy} form to render $E_{oxy}D$ complex, which oxidizes the diphenol to *o*-quinone and yields the antiferromagnetically coupled tetragonal Cu^{+2} form of the enzyme (E_{met}). The latter form transforms another *o*-diphenol molecule to *o*-quinone and is reduced to the bicuprous $E_{deoxy}D$ form. Characteristic lag phase is absent in diphenolase activity as diphenol can bind to both forms, *oxy* and *met* in the resting tyrosinase (Fig. 3) (Sanchez-Ferrer et al, 1995).

Understanding the mechanism in which how melanins are formed in various biological systems would help to control them, when their formation is beyond desirable level. Hyperpigmentation is the condition where darkening of skin resulting from increased level of melanin in the skin. This is a common and distressing problem caused by chronic sun exposure and various inflammatory skin disorders, such as eczema, and irritant contact dermatitis. Acne s also an inflammatory disease of the skin considered as a frequent cause. Papulosquamous (relating to an eruption composed of papules and scales) disorders in general commonly predispose a patient to post inflammatory hyperpigmentation (Halder and Richards, 2004). Melasma, a localized facial hyperpigmentation, caused by either pregnancy or the use of hormones (e.g., oral contraceptive pills) is a common form of noninflammatory hyperpigmentation (Stulberg, Clark and Tovey, 2003). Melanin pigments are also found in mammalian brain. Tyrosinase may have a role in neuromelanin formation in the human brain and could be central to dopamine neurotoxicity as well as contribute to the neurodegradation associate with Parkinson's disease (Xu et al, 1997).

Enzymatic browning is one of the most devastating reactions for many exotic fruits, vegetables, in particular tropical and subtropical varieties. It is

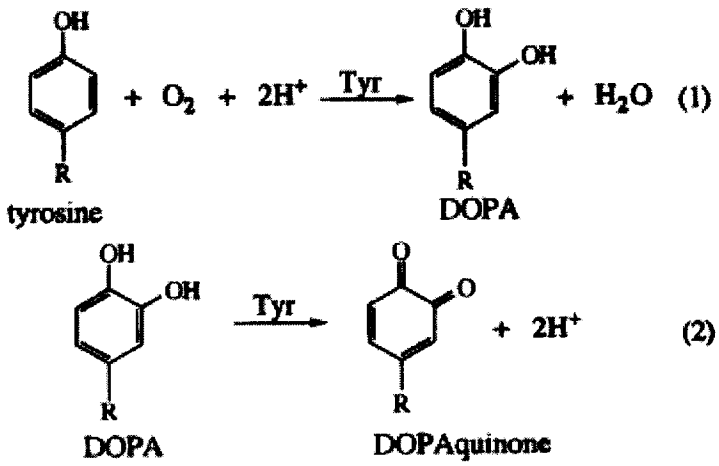


Figure 2. Two important reactions catalyzed by tyrosinase during the proximal phase of melanogenesis pathway. (1). Monophenolase activity; hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA). (2). Diphenolase activity; oxidation of DOPA to DOPAquinone.

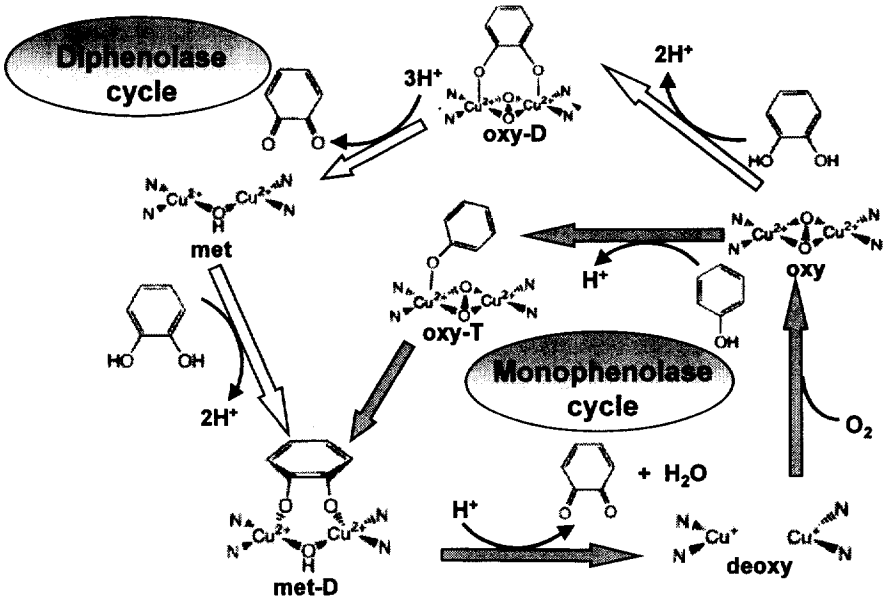


Figure 3. Catalytic cycle for the monooxygenation of monophenols and the oxidation of *o*-diphenols to *o*-quinones by tyrosinase. T=tyrosisine and D=DOPA bound form (adapted from ref. Wilcox et al, 1985).

estimated that over 50 percent losses in fruit occur as a result of enzymatic browning. Also enzymatic browning in aquatic foods post harvest occurs primarily in crustaceans. These highly prized and economically valuable products are extremely vulnerable to enzymatic browning. Such losses have prompted considerable interest in understanding and controlling phenoloxidase enzymes in foods. Lettuce, other green leafy vegetables, potatoes and other starchy staples, such as sweet potato, breadfruit, yam, mushrooms, apples, avocados, bananas, grapes, peaches, and a variety of other tropical and subtropical fruits and vegetables, are susceptible to browning and therefore cause economic losses for the agriculturist. These losses are greater if browning occurs closer to the consumer in the processing scheme, due to storage and handling costs prior to this point. The control of browning from harvest to consumer is therefore very critical for minimizing losses and maintaining economic value to the agriculturist and food processor. Browning can also adversely affect flavor and nutritional value (Marshall, Kim and Wei, 2000).

Tyrosinase is considered as an economically important enzyme due to its negative impacts on various biological systems. Therefore, over the years attempts are being made to find out methods that can be employed to inhibit tyrosinase. Especially in food industry blanching, refrigeration, freezing, dehydration, irradiation, high-pressure treatments are abundantly used to control tyrosinase, thereby to prevent undesirable browning. But none of these methods proved to be sufficient enough to control enzymatic browning. Therefore, searching for enzyme inhibitors from natural sources is considered to be a good remedy and so far hydorquinone, kojic acid, azelaic acid, resinoids, electron-rich phenols, arbutin, Vitamin C, etc. are abundantly used in cosmetics and pharmaceutical industries (Miyazawa et al, 2003). Currently available tyrosinase inhibitors like hydroquinone and kojic acid may result in serious side effects from long-term

exposure (Nakagawa and Kawai, 1995, Wei, et al, 1991). A widely used method in the food and beverage industries to control browning is the addition of reducing agents (sulfites, ascorbic acid, etc.), which chemically reduce the *o*-quinones to the less reactive, colorless diphenols. However, these compounds can also have adverse health effects (specially sulfites) and are also relatively reactive compounds, which may react with other components in the food system, resulting in unwanted effects (McEvily, Iyengar and Otwell, 1992). Since the losses due to pest attacks in agriculture crops and products play a significant role in deterioration in quality and market value, controlling measures of insects are more important. Use of tyrosinase inhibitors can be used as an alternate method for controlling them in order to produce quality agricultural products.

Therefore, there is a concerted effort to search for naturally occurring potent tyrosinase inhibitors from plants, because plants constitute a rich source of bioactive chemicals and many of them are largely free from harmful adverse effects (Kubo, Yokikawa, and Kinst-Hori, 1995). The isolated compounds would have been used in the betterment of pharmaceutical, cosmetic and food industries. So far, there are many evidences on various tyrosinase inhibitors isolated from various plant sources are found in the literature. But, most of them are seemed to be non-practical and there applications on industries are restricted. In continuing investigating of tyrosinase inhibitors from natural sources, it is noted that isolation of tyrosinase inhibitors from marine algae sources are limited. It is well known fact that marine algae are good sources of biologically active substances like antioxidants (Jang et al, 2005, Chidambara Murthy et al, 2005), lectins (Nagano et al, 2005, Leite, 2005), anticoagulants (Matsubara, 2004), antimicrobials (Gupta et al, 1991) etc. Therefore, in this study an attempt was made to assay the ability of selected marine algae to inhibit tyrosinase and to isolate potent tyrosinase inhibitor.

Experimental Procedures

1. Materials

Fourteen marine algal species and one marine flowering plant species were collected from two coastal areas in Busan, South Korea during the period of January to March 2005 (detailed information is listed in Table 1). Mushroom tyrosinase (2034 units/mg) and L-tyrosine were purchased from Fluka, Switzerland where, kojic acid was purchased from Aldrich Chem. Co, WI. Silica gel 60 (70-230 mesh, Merck, Germany) was used in open column chromatography and for thin layer chromatography pre-coated Kieselgel 60 F_{254} (Merck, Germany) Aluminium sheets were used. All other chemicals and reagents used were of analytical grade commercially available.

2. Methods

The following experimental procedures were used to purify and characterize tyrosinse inhibitor derived from marine algae.

2.1. Tyrosinase enzyme inhibitory assay

Inhibition of mushroom tyrosinase was determined by spectrophotometrically measuring the rate of dopachrome formation at 475 nm. L-tyrosine was used as a substrate in the tyrosinase inhibitory assay and tyrosinase inhibitory activity was measured according to the method described by Kobayashi et al. (1995) with modifications. Briefly, 0.5 ml of sodium phosphate buffer solution (pH 6.8), 0.5 ml of L-tyrosine, dissolved in sodium phosphate buffer (0.3 mg/ml) and 0.45 ml of sample solution (dissolved in 10% DMSO/DW) were

mixed thoroughly and preincubated at 30°C for 10 min. Then, 0.05 ml of 1000 units/ml of mushroom tyrosinase was added and incubated for 10 min. Finally, 0.5 ml of 1 M sodium azide was added to stop the reaction, and the absorbance was measured at 475 nm by using a Varian UV-Visible recording spectrophotometer (Cary 1C, Australia). A control test was run with 10% DMSO solution and kojic acid was used as positive control in the test. The inhibitory activity was calculated according to the following formula:

$$\text{Tyrosinase inhibitory activity (\%)} = \frac{\{C-(S-CC)\}}{C} \times 100$$

where, C is the absorbance of the control at 475 nm, CC is the absorbance of color control at 475 nm and S is the absorbance of the sample at same wavelength.

2.2. Preparation of plant extracts and preliminary screening for tyrosinase inhibitory activity.

Following the collection, algal samples were rinsed carefully with fresh water to remove sand, epiphytes and associated debris and then frozen at -70°C. The frozen samples were lyophilized and ground into powder by using a Waring blender. Powdered samples (20 g) were extracted with methanol (250 ml) by stirring overnight. Extraction was repeated thrice and the combined extracts were evaporated under reduced pressure to yield semi solid methanol extracts. Residue remained after methanol extracts were extracted with distilled water (500 ml) by stirring over night. The resulted extracts were centrifuged at 4500 × g for 15 min and supernatant were collected. The water extraction was repeated twice. Combined supernatants were evaporated under the vacuum in order to get dried

Table 1. List of marine plant species used in the screening assay for tyrosinase inhibition.

Phylum	Species	Family	Sampling site	Sampling date
Chlorophyta	**A**. *Ulva pertusa*	Ulvophyceae	Jangsan, Busan	Jan 2005
Phaeophyta	**B**. *Colpomenia sinuosa*	Phaeophyceae	Song Jung, Busan	Mar 2005
	C. *Costaria costata*	Laminaraceae	Song Jung, Busan	Mar 2005
	D. *Hizikia fusiformis*	Sargassaceae	Song Jung, Busan	Mar 2005
	E. *Laminaria japonica*	Laminariaceae	Jangsan, Busan	Feb 2005
	F. *Myelophycus simplex*	Phaeophyceae	Song Jung, Busan	Mar 2005
	G. *Sargassum fulvellum*	Phaeophyceae	Jangsan, Busan	Jan 2005
	H. *Sargassum horneri*	Sargassaceae	Song Jung, Busan	Mar 2005
	I. *Undaria pinnatifida*	Alariaceae	Jangsan, Busan	Feb 2005
Rhodopyta	**J**. *Carpopeltis crispate*	Florideophyceae	Jangsan, Busan	Jan 2005
	K. *Lomentaria catenata*	Lomentariaceae	Jangsan, Busan	Jan 2005
	L. *Pachymeniopsis elliptica*	Halymeniaceae	Jangsan, Busan	Jan 2005
	M. *Symphyocladia latiuscula*	Rhodophyceae	Jangsan, Busan	Jan 2005
Anthophyta	**N**. *Zostera marina*	Zosteraceae	Song Jung, Busan	Mar 2005
	O. Unidentified species		Jangsan, Busan	Jan 2005

water extracts and stored at –20°C until used (Fig. 4). Collectively, 15 methanol and 15 water extracts of marine algae were prepared for preliminary screening of tyrosinase inhibitory activity.

2.3. Isolation and purification of tyrosinase inhibitor

The most potent plant species was selected based on the tyrosinase inhibitory activity and dark colored, thick mass of methanol extract (15 g) was prepared using the same procedure described above. During each step of purification, potent fractions were selected based on tyrosinase inhibitory activity. The methanol extract was chromatographed on a silica gel column (Merck 70-230 mesh, 400 g, Ø 4.5 × 65 cm) and successively eluted with a stepwise gradient of n-Hexane, ethyl acetate and methanol. Eluted fractions were analyzed by thin layer chromatography (TLC) and fractions with identical R_f values were combined. The potent fraction selected was subjected to silica gel column chromatography (Merck 70-230 mesh, 75 g, Ø 2.5 × 40 cm) and successively eluted with a stepwise gradient of ethyl acetate/methanol. Eluted fractions with similar R_f values were pooled after TLC analysis. The pooled fraction with highest tyrosinase inhibitory activity was further purified using reverse-phase high performance liquid chromatography (RP-HPLC). The sample was injected to a preparative Capcell Pak C_{18} UG 120 (Ø 2 × 25 cm) column (Shiseido Co. Ltd, Japan) and elution was performed with a linear gradient of acetonitrile (0-100% v/v in 30 min) in methanol. Flow rate was maintained at 4 ml/min and fraction peaks were pooled at 254 nm. Finally, peak with the highest tyrosinase inhibitory properties was rechromatographed on Nucleosil 100-5 C_{18} (Ø 0.46 × 25 cm) column (Macherey Nagel GmbH & Co., Germany) using a linear gradient of

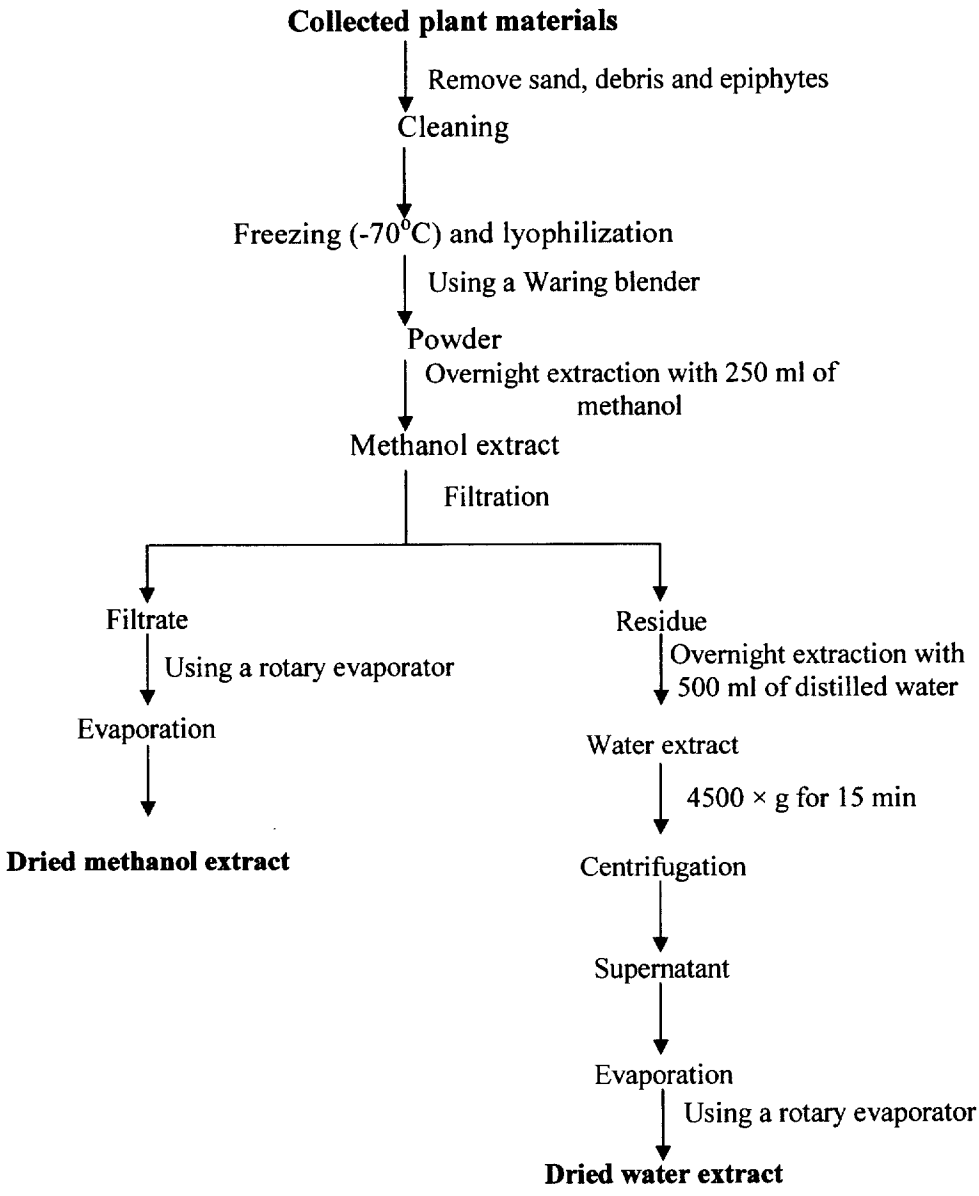


Figure 4. Experimental protocol of preparing plant extracts for preliminary screening.

methanol (0-100% v/v in 30 min) in distilled water at a flow rate of 0.4 ml/min. Eluted single peak was recognized as a purified compound and purity of isolated compound was confirmed by TLC analysis.

2.4. Thin-Layer Chromatography (TLC)

TLC was performed on a silica gel plate (5 × 20 cm, Kieselgel 60 F, 0.25 mm, Merck). An aliquot of each fraction was spotted on the silica gel plate with a solvent system of dichloromethane/methanol (10:1 v/v). The spots were visualized by spraying the plates with spraying solution of 0.5 g of vanillin in 100 ml of sulfuric acid/methanol (40:10).

2.5. Infrared Spectrum (IR)

The infrared spectrum (400-4000 cm^{-1}) of purified compound was recorded in potassium bromide (KBr) disks with a Fourier transform IR spectrophotometer (Brucker FT-IR model IFS-88 spectrometer). One milligram of dry sample was mixed with 100 mg of dry KBr, and the mixture was pressed into a disk.

2.6. Ultraviolet and Visible (UV-Vis) Spectrophotometry

UV-Vis absorption of purified compound dissolved in ethanol was recorded in a spectrophotometer (Varian UV-Visible spectrophotometer, Cary 1C, Australia).

2.7. Mass spectrometry

Electron Impact Mass Spectrometric (EIMS) data and HR-FABMS data were obtained from a JEOL JMS-700 spectrometer and a JEOL JMS HX 110A/HX110A Tandem mass spectrometer respectively.

2.8. Specific rotation-$[\alpha]_D$

Specific rotation of the purified compound was measured using a Pekerin Elmer model 341 polarimeter.

2.9. Nuclear Magnetic Resonance

The 1D NMR (^1H, ^{13}C, and DEPT) and 2D NMR (HMQC, HMBC, and COSY) spectra were taken by using a JEOL JNM-ECP 400 NMR spectrometer, and DMSO-d6 peak was used as reference standards.

2.10. Determination of the Inhibition pattern of the isolated compound

Inhibitory mechanism of the isolated compound on tyrosinase was assayed according to tyrosinase inhibitory assay described above. Two different concentrations of the inhibitor (0.25 and 0.5 mg/ml) and tyrosine at varying concentrations (0.75, 1.5, and 3.0 mM) were used during the kinetic analysis. Michaelis constant (K_m) and maximal velocity (V_{max}) of tyrosinase were determined by Lineweaver-Burk plots.

3. Results and Discussion

3.1. Tyrosinase enzyme inhibitory assay

Mushroom tyrosinase was utilized during the purification procedure due to its ready availability, though it differs from animal tyrosinase and other available tyrosinases. Because of this, over the years, mushroom tyrosinase has been widely used as the target enzyme for isolating and characterizing potential tyrosinase inhibitors (Huang et al 2005). Tyrosine was used as the substrate in this study; therefore, the activity studied in this paper was concerned with *o*-monophenolase inhibitory activity of mushroom tyrosinase. In the activity test, colors of the most extracts and dopachrome produced by tyrosine both contributed to give spectrophotometric values at 475 nm and those led to exceed spectrophotometric value of control at 475 nm. Therefore, color control was used during the activity test in order to prevent the hindrance of the activity of methanol and water extracts due to their color.

3.2. Screening of marine algal extracts for tyrosinase inhibition

During the screening for tyrosinase inhibitory activity, fifteen methanol extracts and fifteen water extracts of respective marine algae were tested. (Fig. 5). It was observed that only four methanol extracts and one water extract could inhibit tyrosinase activity considerably (more than 30% inhibition) at the sample concentration of 1 mg/ml. Comparison made between tested methanol extracts and water extracts revealed that, tyrosinase inhibitory activities of methanol extracts were more potent than that of water extracts. It is well known fact that most bioactivities in the plant extracts are due to phenolic compounds (Kahkonen

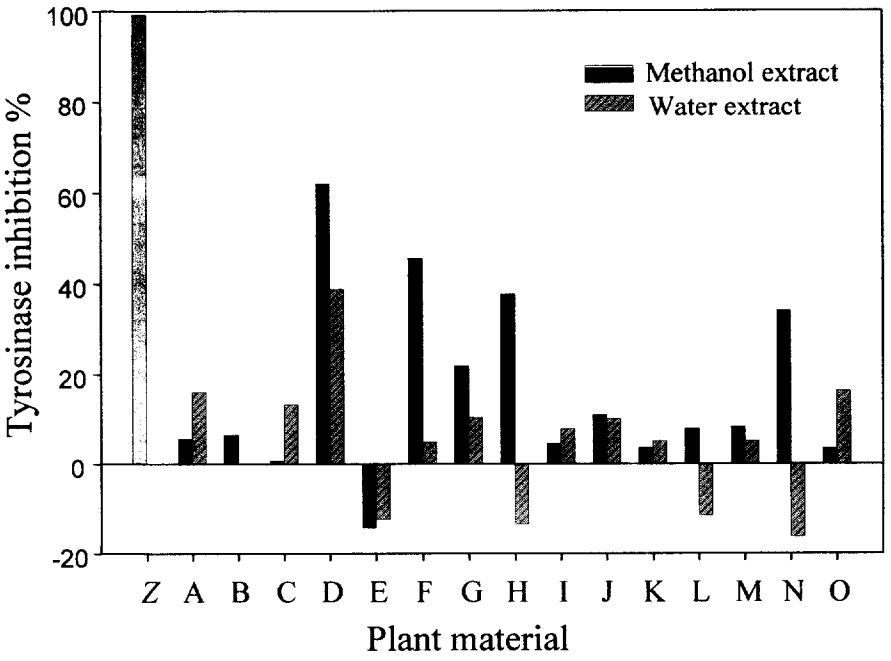


Figure 5. Tyrosinase inhibitory activity of methanol and water extracts derived from marine plants. Each derived extract was tested in a tyrosinase inhibitory assay as described in the text at the concentration of 1 mg/ml. Kojic acid was used as a positive control and it is denoted *Z* in the *x*-axis and the letters from A to O affixed to *x*-axis correspond to those marine plants listed in Table 1. *Y*-axis represents tyrosinase activity related to the control in that 10% DMSO tested, but not plant extract was added.

et al, 1999) and their solubility is high mostly in organic solvents, therefore, organic plant extracts could have expressed higher tyrosinase activities than that of water extracts. The similar observations have been reported in the literature (Hellio, 2001). Methanol extracts of *Hizikia fusiformis, Myelophycus simplex, Sargassum horneri,* and *Zostera marina* inhibited tyrosinase at 62%, 46%, 38% and 34% respectively, where as 39% inhibition could observe in water extract of *Hizikia fusiformis.* Most of other methanol extracts and water extracts had little effect or did not affect the enzyme activity, but some extracts (e.g., methanol and water extracts of *Laminaria japonica*) elevated the enzyme activity. This could be due to the fact that some plant species contain phenolic compounds other than tyrosine and L-DOPA, which can act as substrate to enzyme, tyrosinase (Marshall et al, 2000) and enhance the activity of tyrosinase to yield *o*-quinone, dopachrome.

Based on tyrosinase inhibitory activity, *Hizikia fusiformis* was selected for further purification. Though this is being used as a food source (an edible brown algae) over a long period of time, mainly in East Asian countries like China, Japan, and Korea, utilization of *Hizikia fusiformis* in isolating biologically active compounds is difficult to trace in the literature. But crude extracts of that have been shown its ability to posess bilogical activites such as immunomodulatory (Shan et al, 1999 and Liu et al 1997), and anticancer (Kim et al 2002). These indicated that its capcity to act as a potential source of biologically active material. Additionally, Kang et al, 2004 also have reported tyrosinase inhibitory activity of *Hizikia fusiformis* in methanol extract and inihibitory activiy is more or less similiar to IC_{50} value of 0.8 mg/ml (Fig. 6). The comparison made with potive control, a potent tyrosinase inhibitor, kojic acid, reveald that the activity of crude extract of *Hizikia fusiformis* is 60-folds less than that of kojic acid.

3.3. Isolation and purification of tyrosinase inhibitor from *Hizikia fusiformis*

The crude methanol extract was purified using polarity fractionation and different chromatographic techniques (Fig. 7). During the first purification, silica gel open column chromatography was utilized. Methanol extract was successively eluted with stepwise gradient of three solvents at 10 different ratios (n/Hexane:ethyl acetate: 100, 80, 60, 50, 0% and ethyl acetate:methanol: 80, 60, 50, 40, 0%). 1000 ml of each solvent ratio was applied and 100 ml of eluting fractions were collected. Out of 97 collected fractions, 9 major fractions were formed based on thin layer chromatography. Among them, fractions from A to E showed low inhibitory activities compared to fractions from F to I. It could observe that, assessing of tyrosinase inhibitory activity of non-polar fractions was difficult due to their low solubility in water based enzymatic media. This could have resulted in expressing lower enzyme inhibition, as there is less chance for enzyme inhibitory compounds present in non-polar fractions to interact with enzyme, which is activated in water-based medium. Analyzing the activities of all fractions, fraction G was selected for the further purification on second silica gel column chromatography as it exhibited the highest tyrosinase inhibitory activity of 82% at the concentration of 1 mg/ml.

The potent G fraction was loaded into second silica gel column and successively eluted with a stepwise gradient of ethyl acetate:methanol (50, 45, 40, 35, 30, 25, 20, 15, 10, 5, 0%). Eluted fractions were collected to form 4 major fractions according to thin layer chromatographic patterns. Out of four major fractions, fraction G3 expressed the highest tyrosinase inhibition of 93% at the concentration of 1 mg/ml.

Reversed-phase C_{18} HPLC column was used to separate the potent fraction G3, obtained from second silica gel column chromatography. Six fractions (Fig.

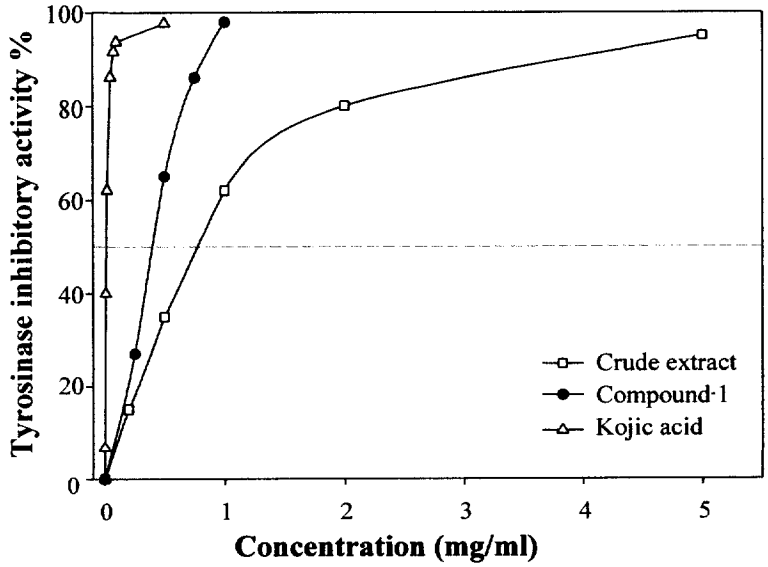


Figure 6. Dose dependent inhibition of mushroom tyrosinase by crude extract of *Hizikia fusiformis*, compound 1 and kojic acid. Tyrosinase activity was measured using L-tyrosine as the substrate.

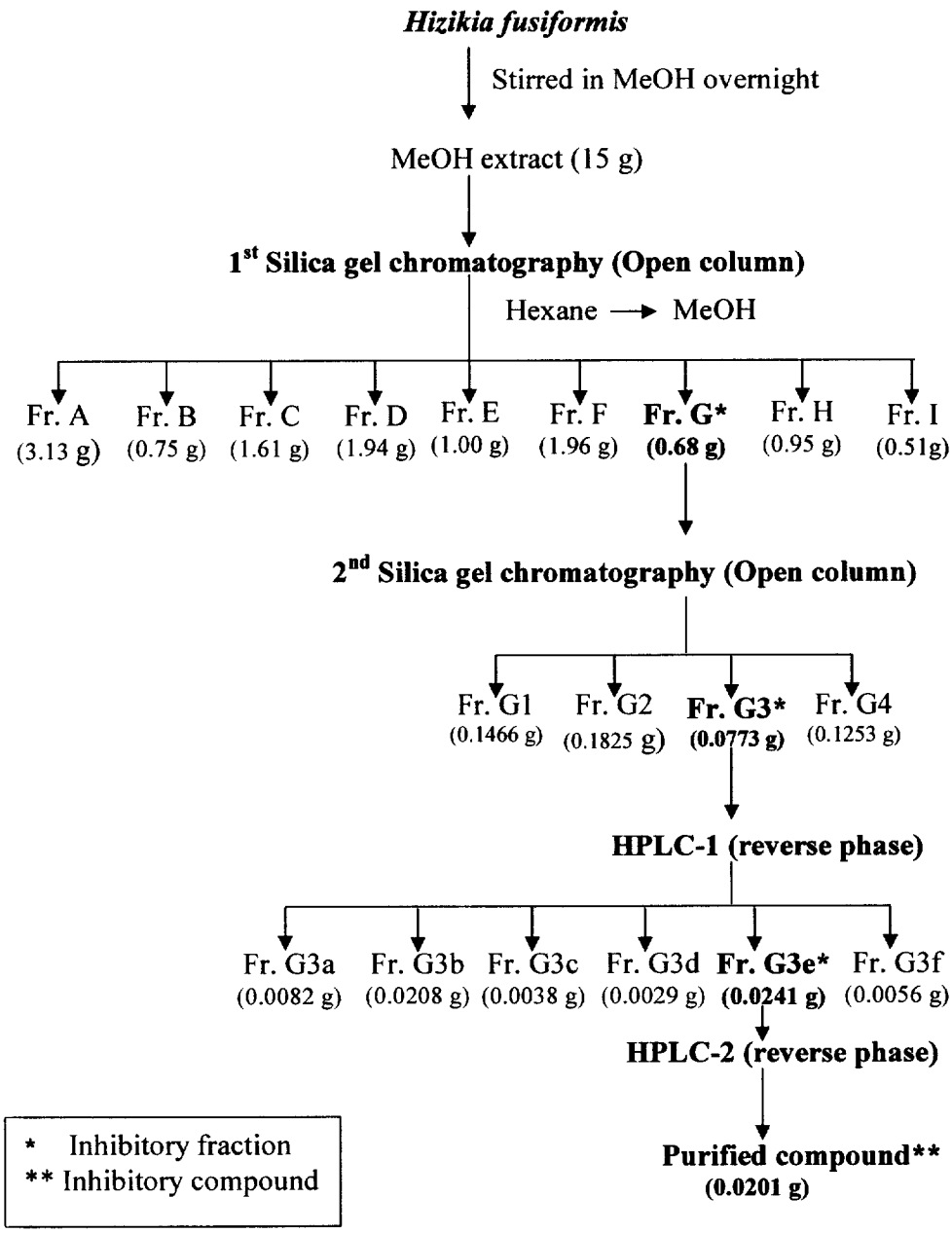


Figure 7. Purification procedure of tyrosinase inhibitor from *Hizikia fusiformis*

8) derived from HPLC were tested for tyrosinase inhibitory activity at the concentration of 0.5 mg/ml and it was observed that fraction G3e expressed the highest inhibition of 47%. The most effective fraction, G3d was finally chromatographed on an analytical C_{18} HPLC column and purity of resultant fraction was confirmed by using thin layer chromatography. This was designated compound-1. Final purification was carried out using a linear gradient of methanol (0-100% v/v in 30 min) in distilled water and it was observe that resultant fraction eluted at around 75 % concentration of methanol.

3.4. Elucidation of chemical structure of compound-1

Compound-1 was obtained as a yellowish powder and it exhibited optical rotation of –0.058°. A molecular formula of $C_{34}H_{44}O_{18}$ was obtained from the HRFAB MS (Fig. 9) $[M+Na]^+$ at m/z 762.4 and from the ^{13}C-NMR analysis (Fig. 10). The IR spectrum of compound-1 (Fig. 11) revealed that absorptions at 3510-3690 cm^{-1} and 1667 cm^{-1} were attributable to free hydroxyl and conjugated carbonyl groups respectively. An examination of its 1D NMR of ^{1}H NMR (Fig. 12), ^{13}C NMR and DEPT [at 45° (Fig. 13), 90° (Fig. 14) and 135° (Fig. 15)] and 2D NMR of HMQC (Fig. 16), HMBC (Fig. 17) and COSY (Fig. 18) data and a comparison with the literature (Singh, et al, 1999, Kang et al, 2000, and Hammami et al 2004) suggested that compound-1 (Fig. 19) was a flavanoid glycoside. Further, two peaks of 350 and 260 nm observed in the UV spectrum (Fig. 20) revealed that the presence of A and B rings in the structure of flavonoid, compound-1. The presence in the ^{1}H-NMR spectrum of signals at δ 5.59 (1H, d, J= 2.72), δ 2.76 (1H, d, J= 2.72) in conjunction with the ^{13}C-NMR signals at δ 79.1, and 130.4 ppm, inferred from HMQC and HMBC spectra (Table 2), pointed to the presence of an -O-CH-CH2-CO- system in the C ring and Hammani et al 2004 also have observed similar pattern of molecular arrangement in a reported

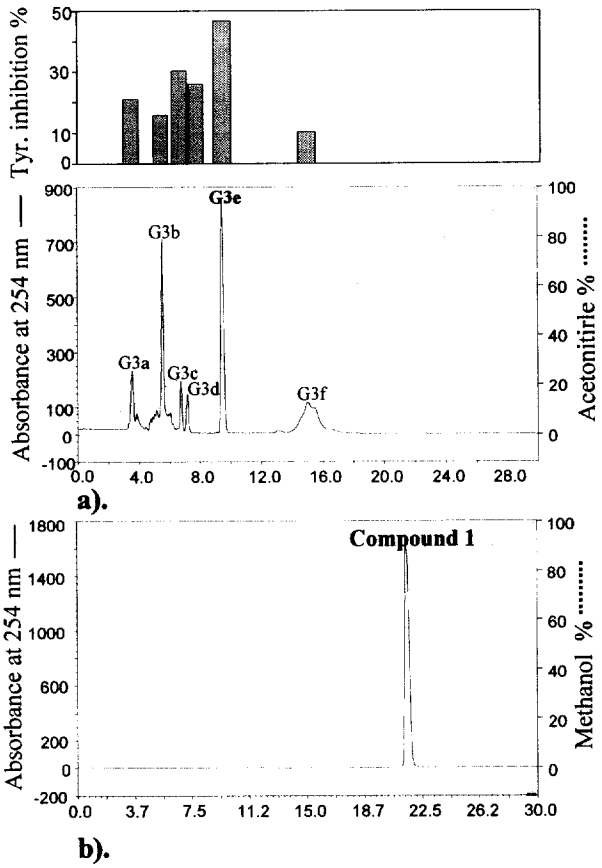


Figure 8. Purification of tyrosinase inhibitor, compound-1, using HPLC chromatography. (a). Reverse-phase HPLC on Capcell Pak C_{18} UG 120 column. The potent fraction, G3 obtained from 2nd silica gel column chromatography was separated into six fractions and based on their tyrosinase inhibitory activity, potent fraction G3e was selected. Upper panel of (a). represents the tyrosinase inhibitory activity of respective fractions, measured by using tyrosinase inhibitory assay at the sample concentration of 0.5 mg/ml.

(b). Reverse-phase HPLC on Nucleosil 100-5 C_{18} column. Finally, G3e was chromatographed and purified fraction of compound-1 was eluted under a linear gradient of methanol (0-100% v/v in 30 min) in distilled water.

flavonone compound. The EI mass spectrum (Fig. 21) showed a molecular ion peak at m/z 286 and had characteristic ion peak at m/z 119 as Singh et al 1999 observed in the flavonone, naringenin 5-methyl ether. It is believed that those characteristics peaks are due to retro-Diel's-Alder type fragmentation and that indicates presence of a methoxyl group in the ring A. A typical methoxyl signal at δ 3.76 ppm (3H, s) was observed in the ^{1}H spectrum and it was correlated with C-7 at δ 162.9 according to a HMBC relationship. Therefore, position of methoxyl group in the A ring was confirmed. Three anomeric carbons at δ 100. 5, 99.3 and 99.2 indicated that the presence of three sugar moieties attached to the compound-1. Further, HMQC relationships revealed that the anomeric protons were of δ 4.51 (1H, s), 4.98 (1H, s), and 4.96 (1H, s) attached to the three anomeric carbons respectively (Table. 2). Presence of rhamnose moiety was confirmed using the ^{13}C NMR at δ 17.8 and ^{1}H NMR at δ1.08 (3H,s) and it was concluded to be attached to C-6' of B ring as there were two HMBC relationships with C-2 of ring C and C''-5 of deoxyribose sugar moiety (Fig. 22). Methylene group at δ 41.8 helped to identify deoxyribose moiety and its attachment to ring A was confirmed using HMBC relationship with anomeric carbon (C-1'') with proton 6.13 (1H, s). HMBC and H-H COSY relationships were used to elucidate the structures of three sugar moieties and position of glucose moiety. The FAB mass spectrum of compound-1 showed fragments having m/z 762 $(M+Na)^{+}$ for $C_{34}H_{43}O_{18}Na$ and mass spectrum peaks of m/z 617, 441 indicates loss of rhamnose and loss of both rhamnose and glucose moieties respectively.

Table 2. ^{1}H- and ^{13}C NMR spectral data for compound-1[a]

Position	^{13}C NMR (ppm)	Multiplicity	^{1}H NMR δ (ppm), *J* (Hz)	HMBC
2	79.1	d	5.59 (1H, d, *J* 2.72)	H-2', H-1''
3	42.2	q	2.80 (1H, d, *J* 3.08), 2.76 (1H, d, J 2.72)	
4	197.0	s		H-6
5	165.1	s		
6	96.4	d	6.13 (1H, s)	H-8, H-OCH_3
7	162.9	s		
8	95.4	d	6.13 (1H, s)	
9	159.4	s		
10	103.4	s		H-8
-OCH_3	55.1	t	3.76 (3H, s)	H-2', H-5'
1'	130.4	s		
2'	128.4	d	7.47 (1H, d, *J* 8.56)	H-2
3'	159.4	s		H-2',H-5'
4'	128.2	d	7.46 (1H, d, *J* 8.52)	
5'	113.9	d	6.99 (1H, s)	
6'	162.9	s		
Deoxyribose				
1''	99.3	d	4.98 (1H, s)	H-6
2''	41.8	q	2.80 (2H, s)	
3''	68.2	d	3.39 (1H, s)	
4''	76.2	d	3.26 (1H, s)	
5''	65.9	q	3.76 (1H, s)	H-1''''
6''	48.5	t	3.16 (3H, s)	
Glc				
1'''	99.2	d	4.96 (1H, s)	
2'''	70.6	d	3.42 (1H, s)	
3'''	76.2	d	3.33 (1H, s)	
4'''	72.0	d	3.33 (1H, s)	
5'''	72.0	d	3.14 (1H, s)	
6'''	66.0	q	3.33 (2H, s)	
Ram				
1''''	100.5	d	4.51 (1H, s)	
2''''	75.5	d	3.54 (1H, m)	
3''''	69.5	d	3.11 (1H, s)	H-1'''', H-6'''' (-CH_3)
4''''	72.9	d	3.21 (1H, m)	H-1'''', H-6'''' (-CH_3)
5''''	68.2	d	3.39 (1H, s)	
6''''	17.8	t	1.08 (3H,s)	

[a] (DMSO-*d*6)

[b] d = doublet; s = singlet; q = quartet; t = triplet

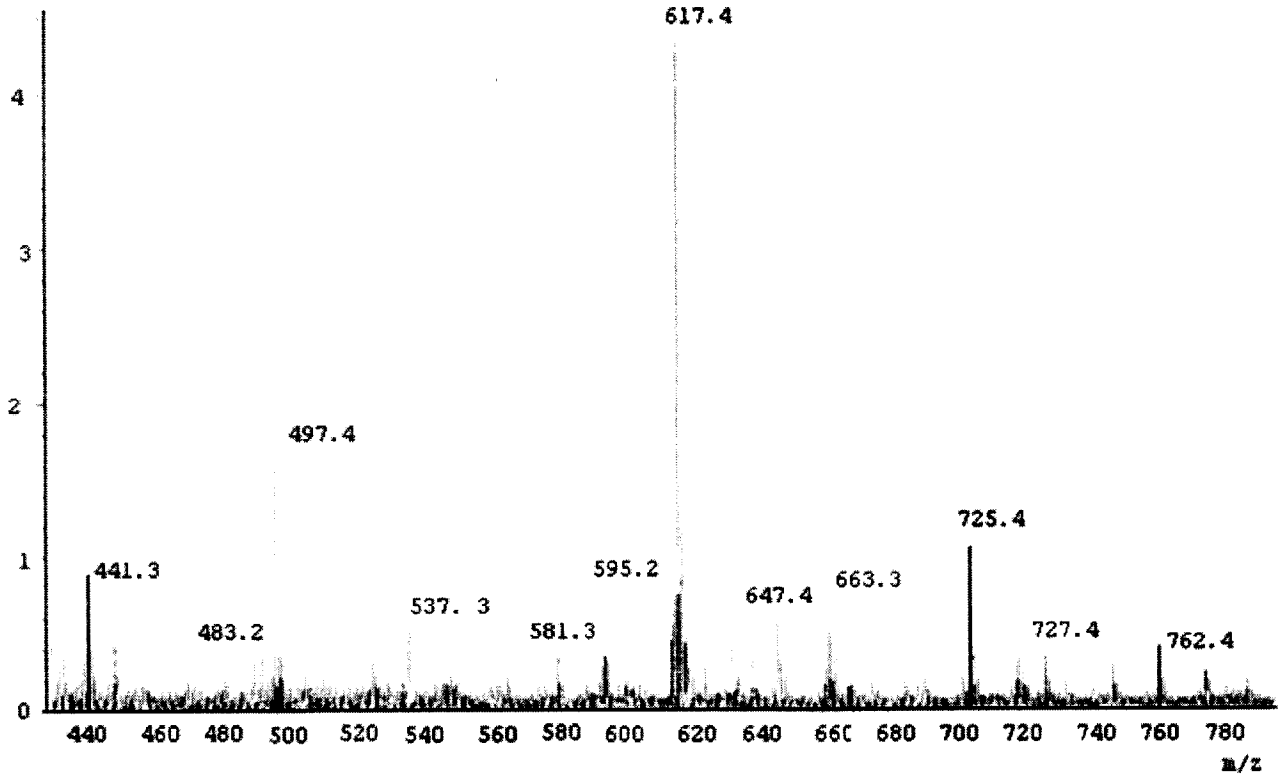


Figure 9. Positive FAB mass spectrum of compound-1

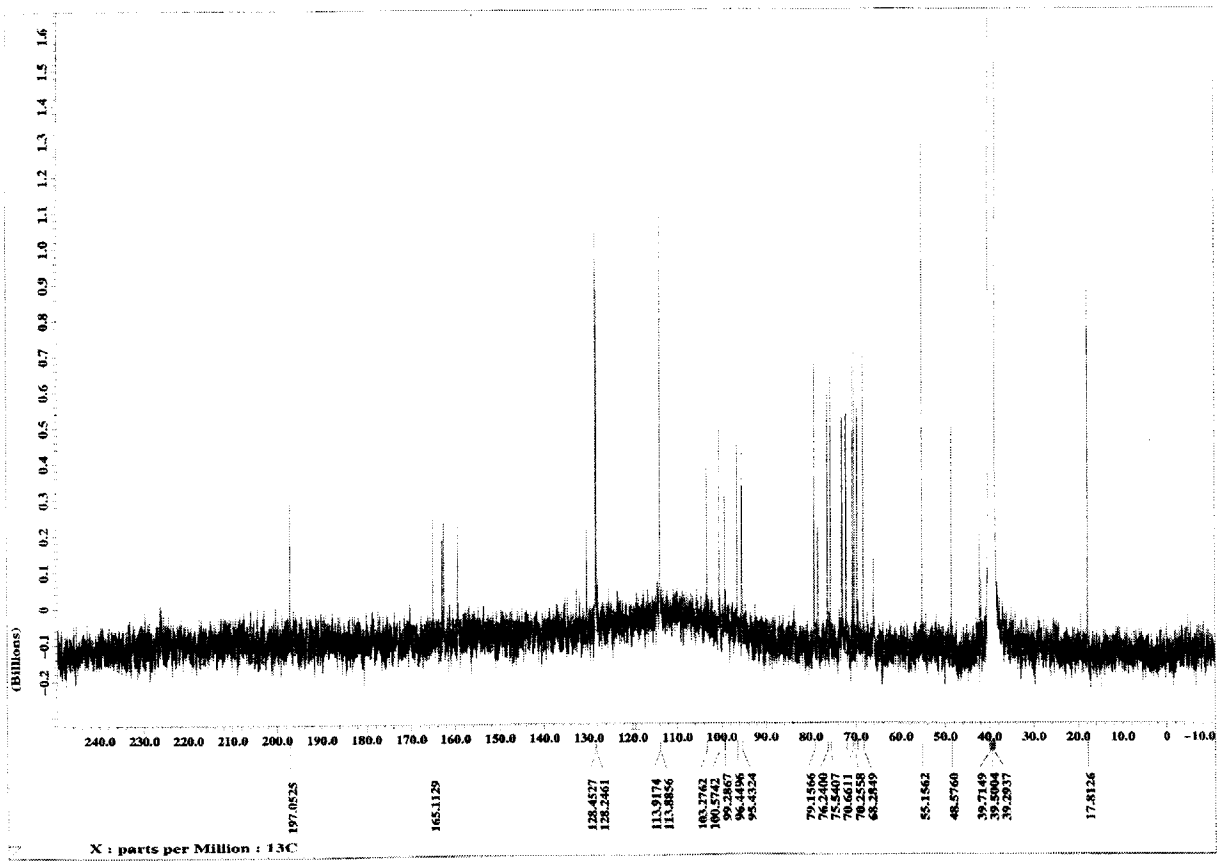


Figure10. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound-1

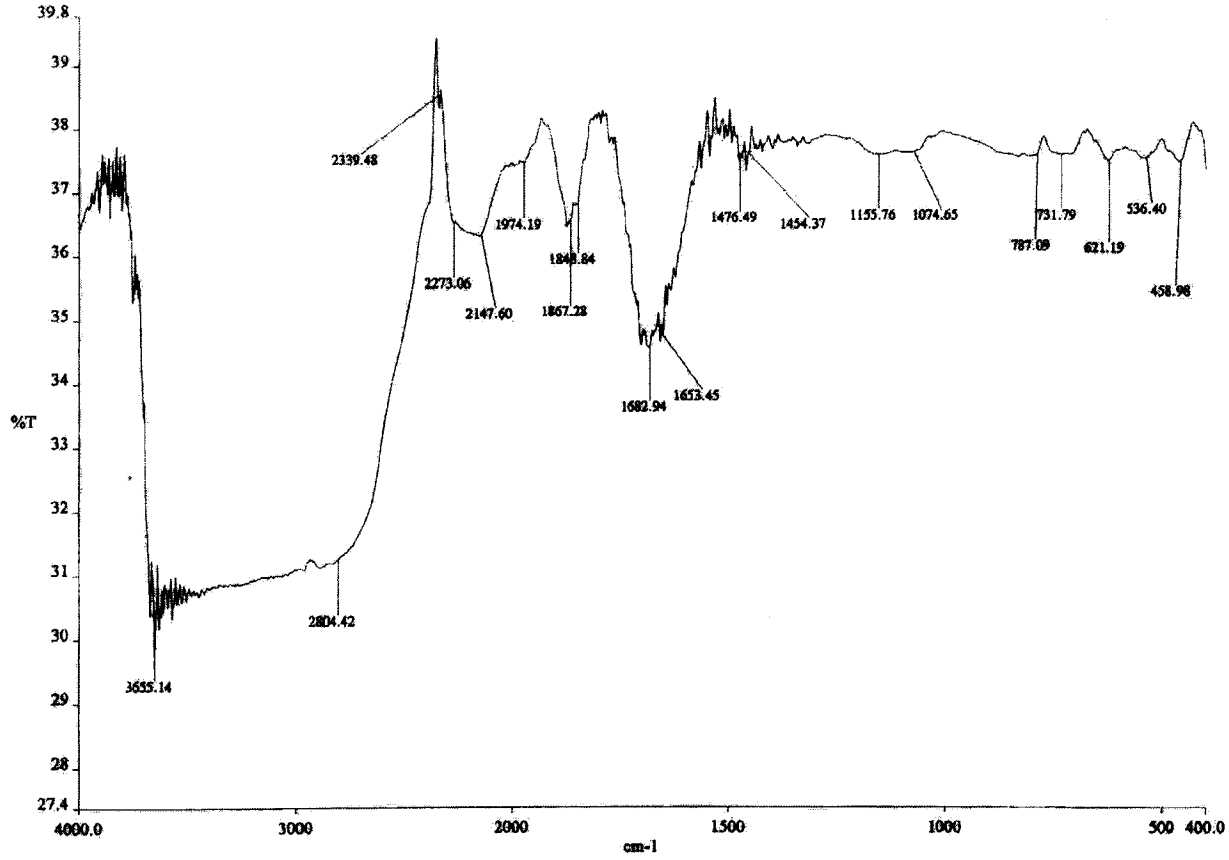


Figure 11. IR spectrum of compound-1

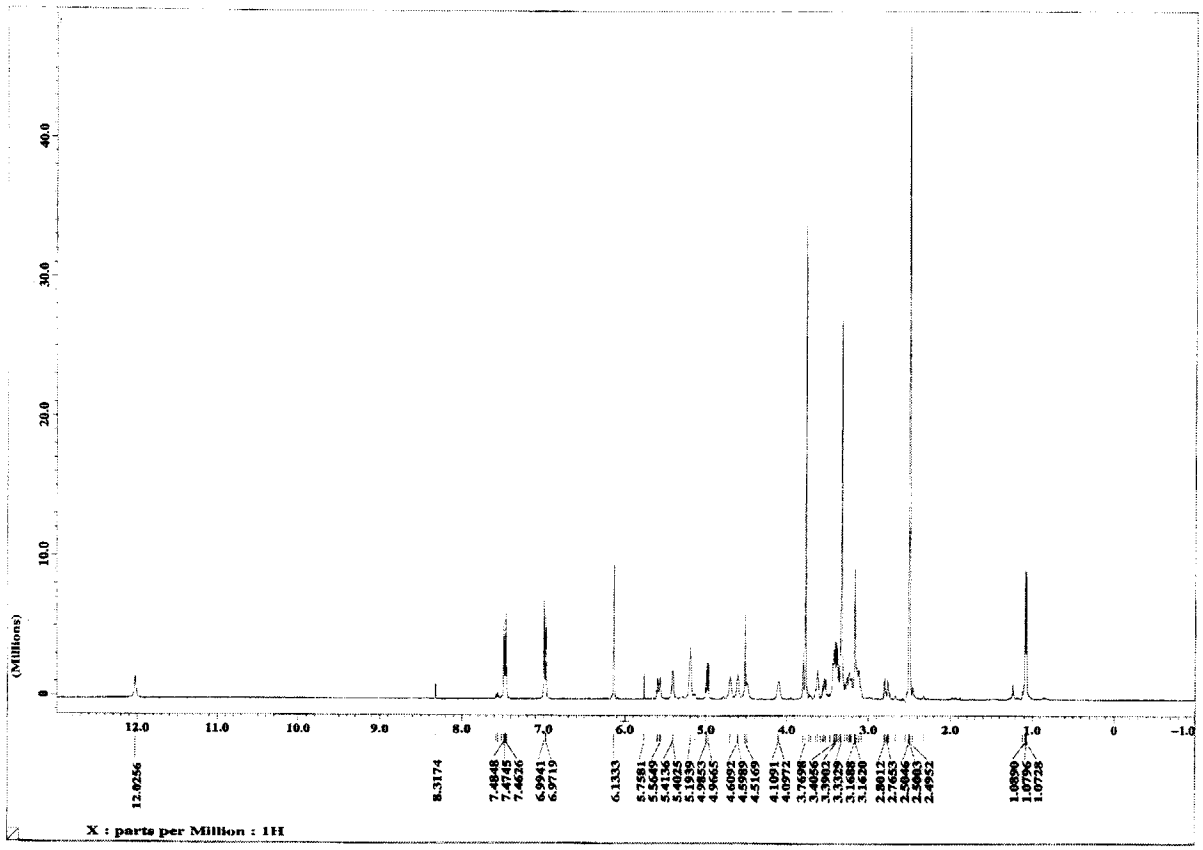


Figure 12. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound-1

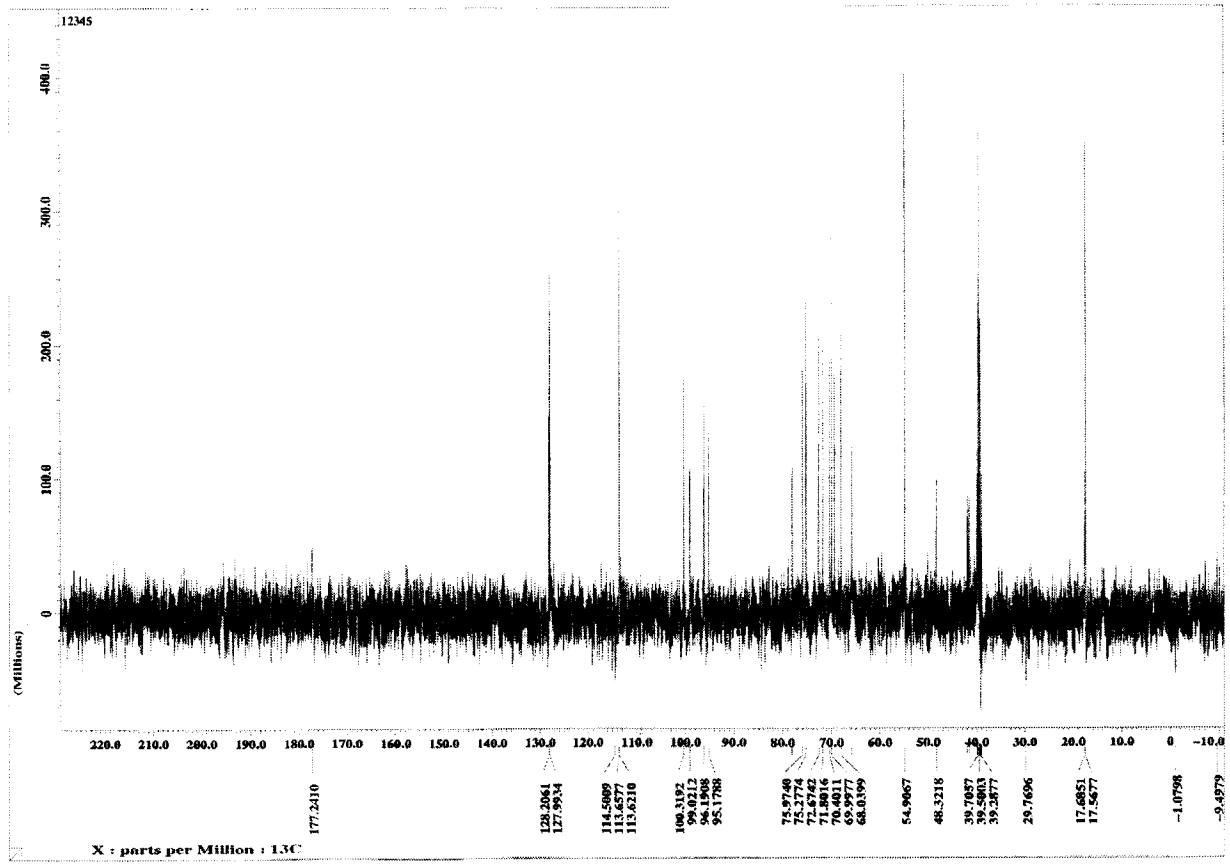


Figure 13. DEPT (45°) spectrum of compound-1

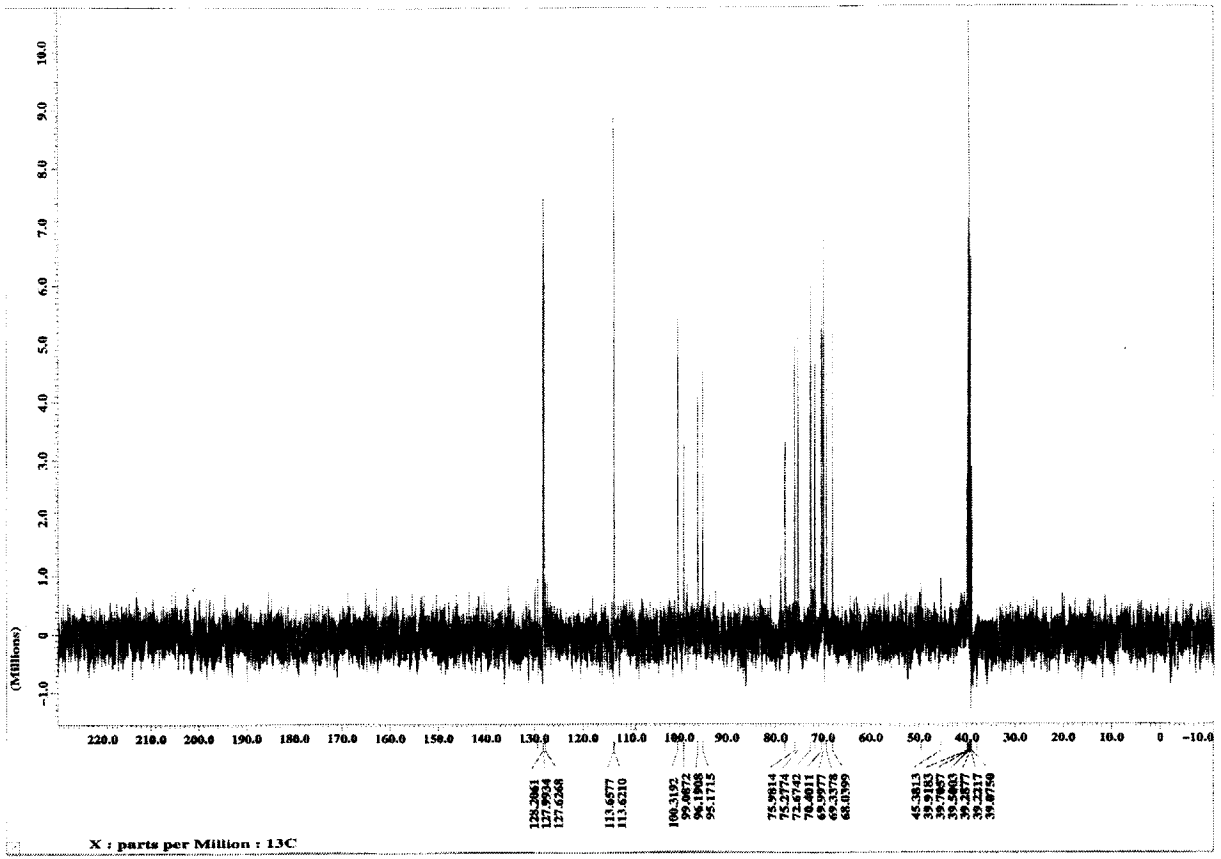


Figure 14. DEPT (90°) spectrum of compound-1

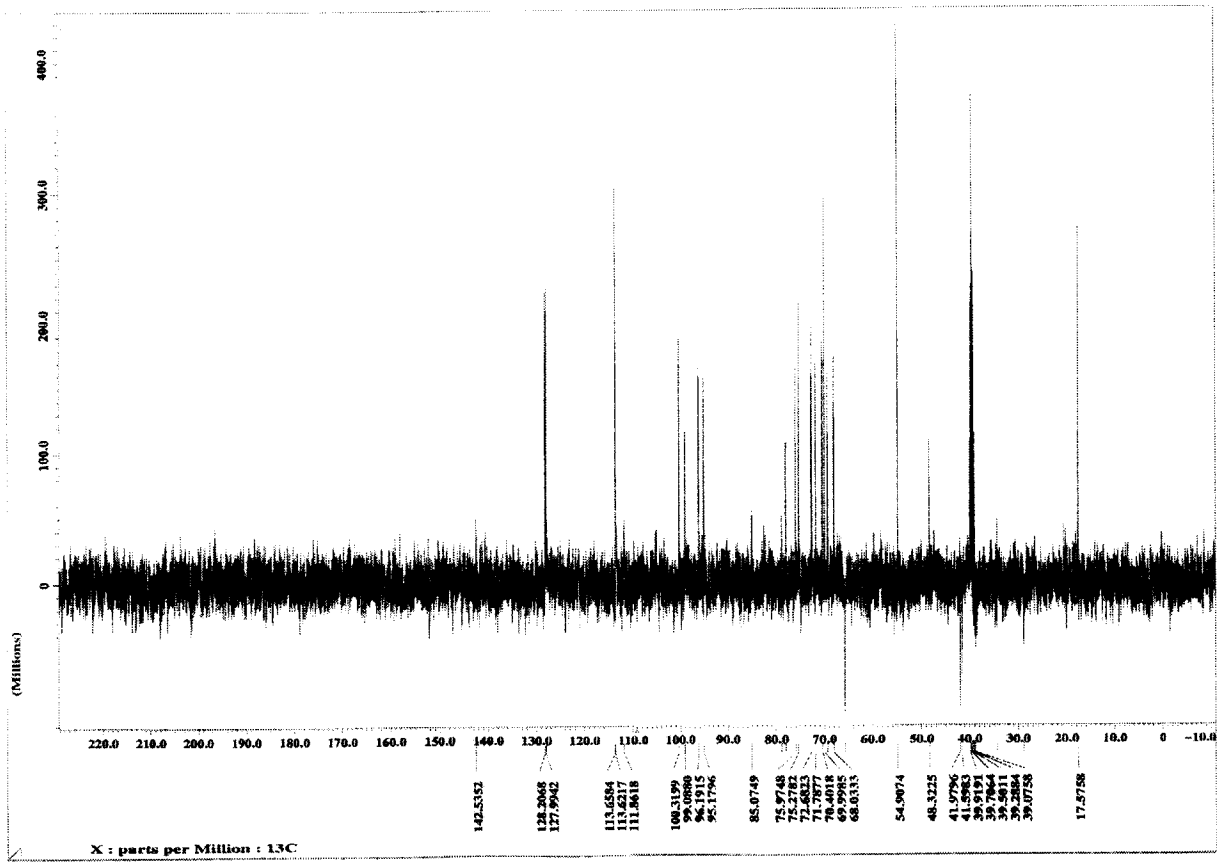


Figure 15. DEPT (135°) spectrum of compound-1

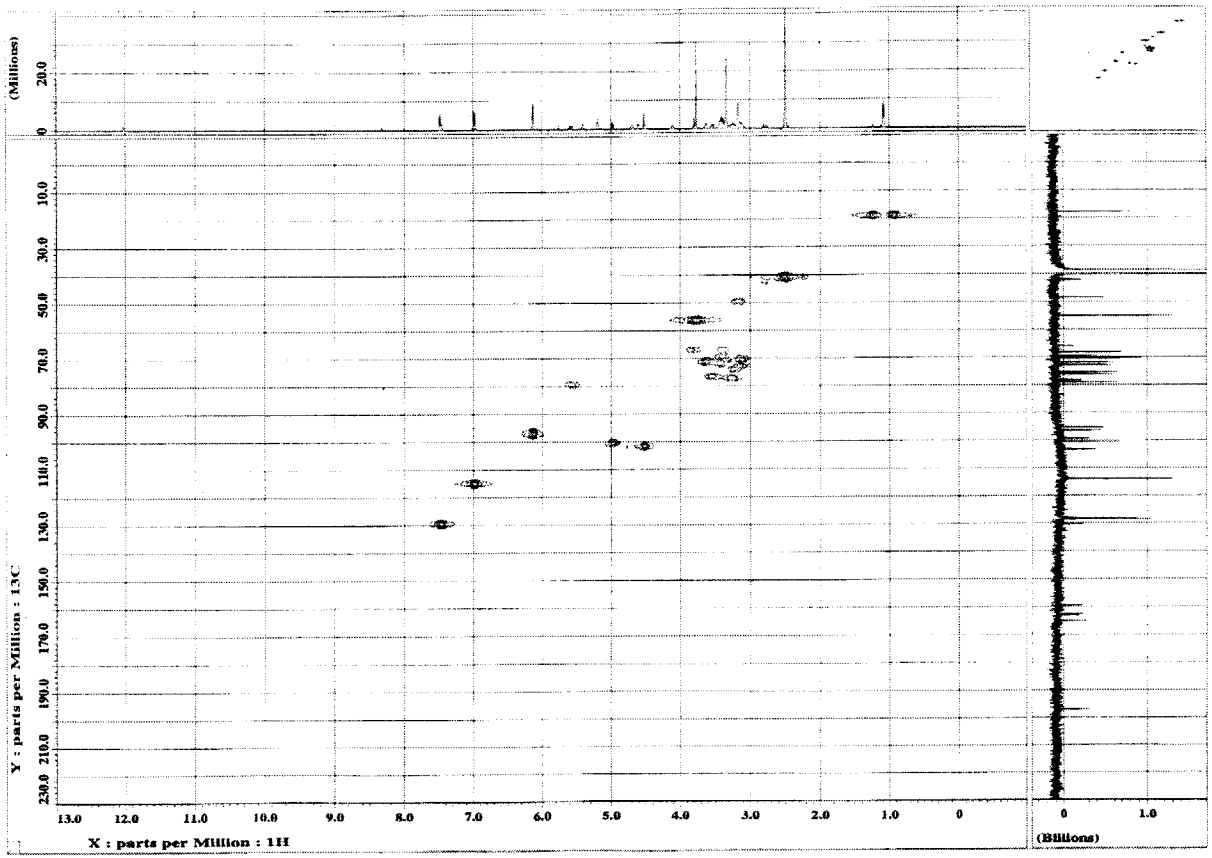


Figure 16. HMQC spectrum of compound-1 in DMSO-d_6

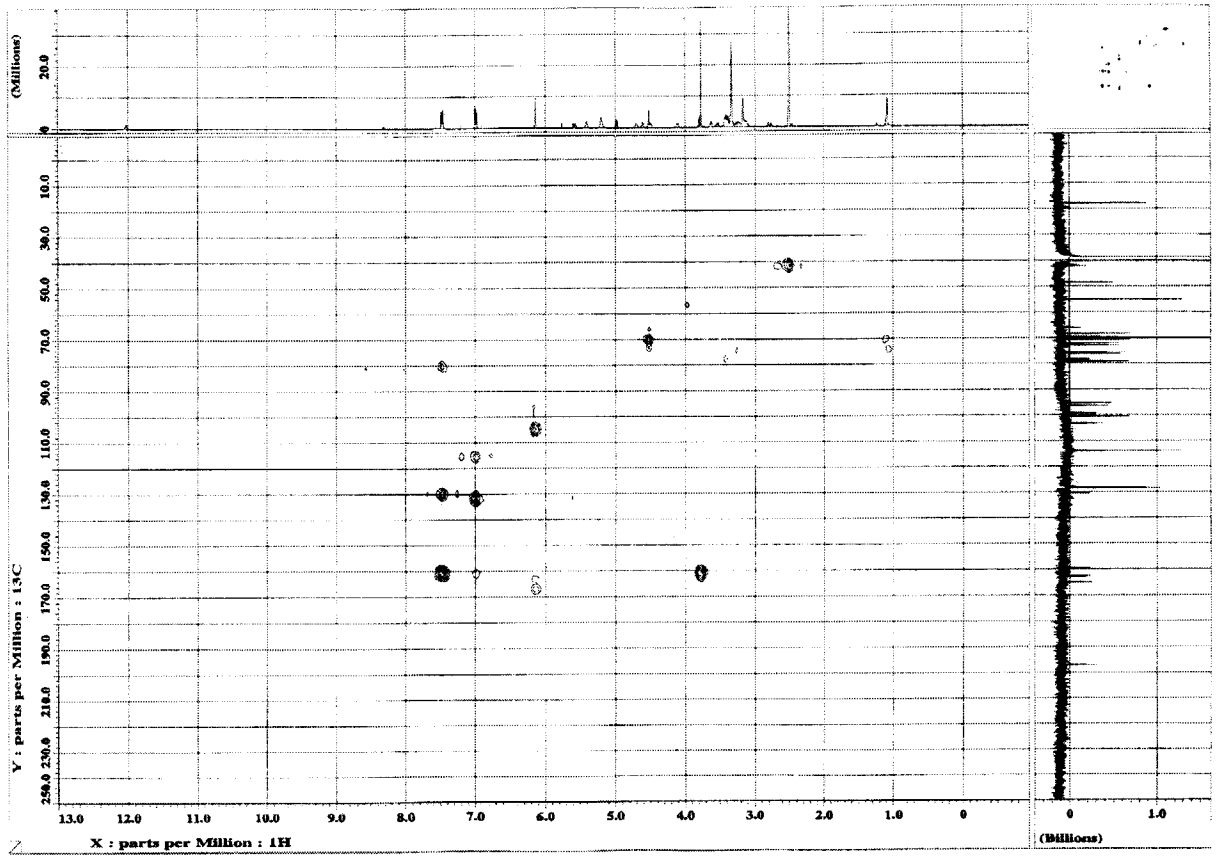


Figure 17. HMBC spectrum of compound-1 in DMSO-d_6

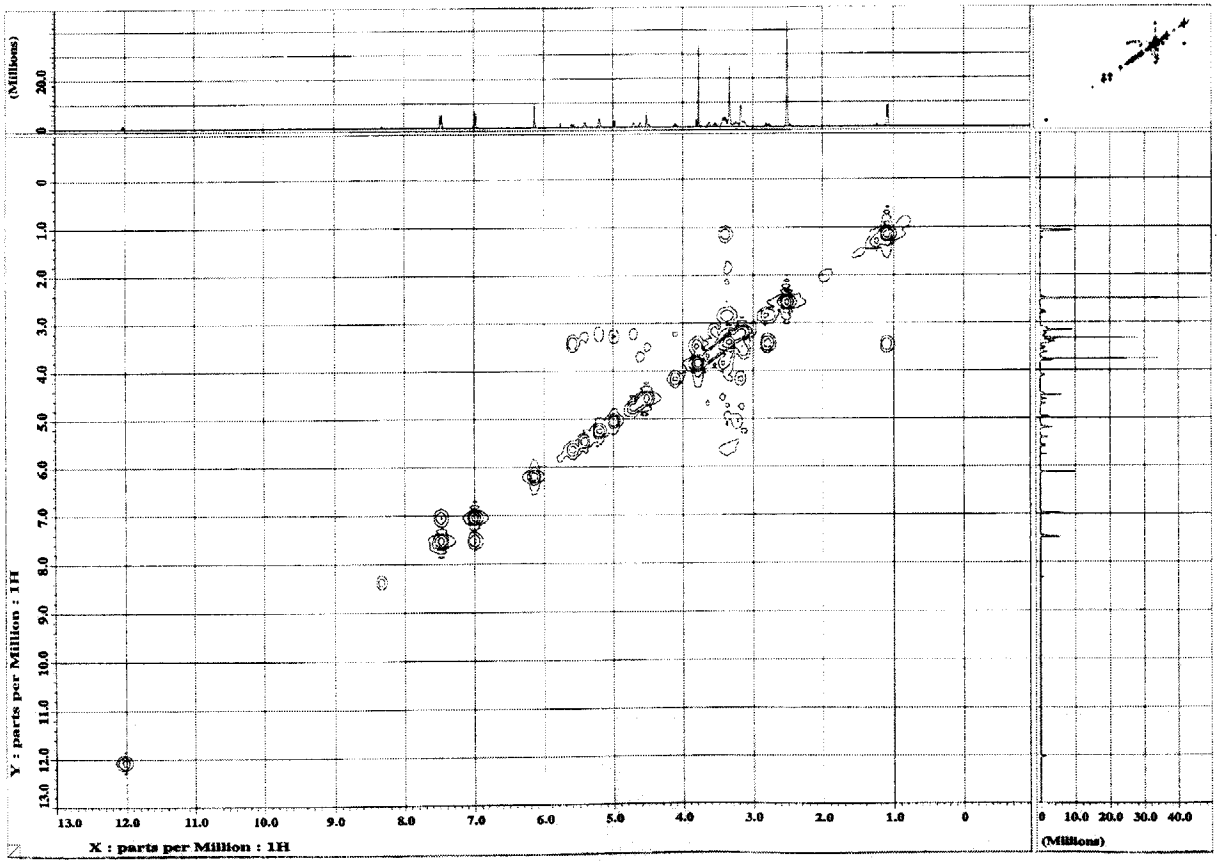


Figure 18. COSY spectrum of compound 1 in DMSO-d_6

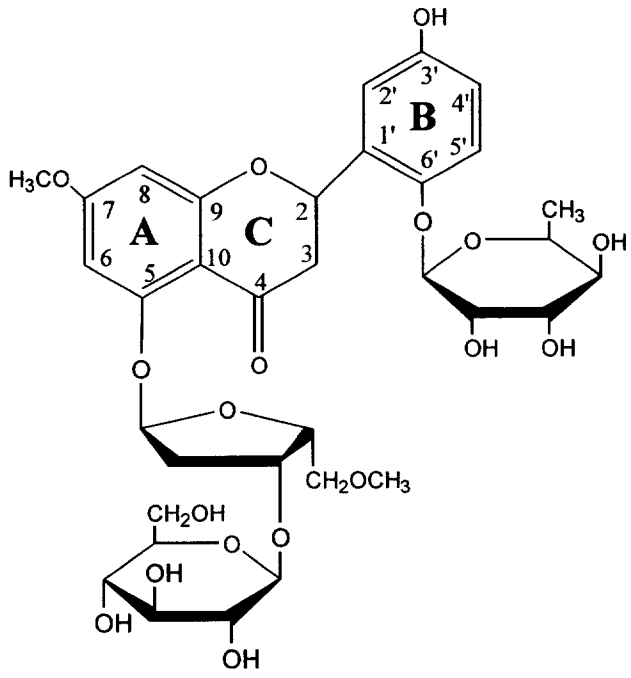


Figure 19. A proposed chemical structure of compound-1

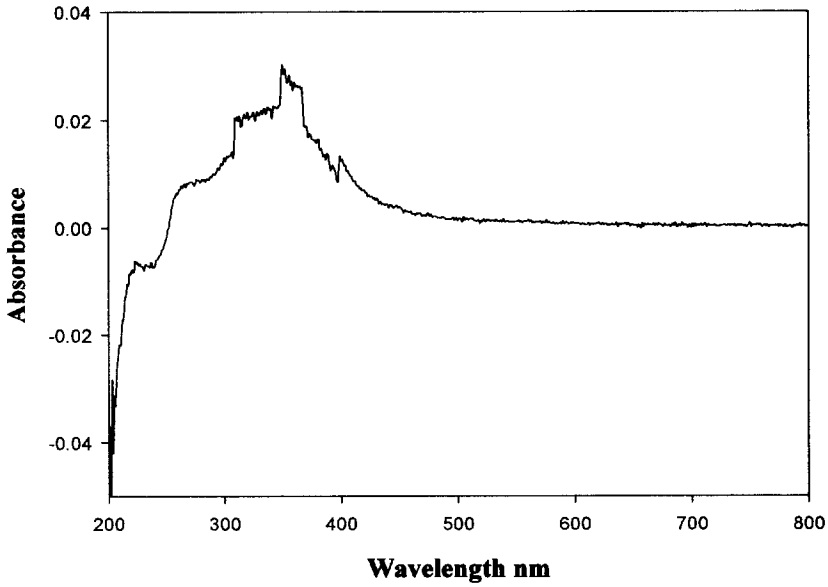


Figure 20. UV spectrum of compound-1

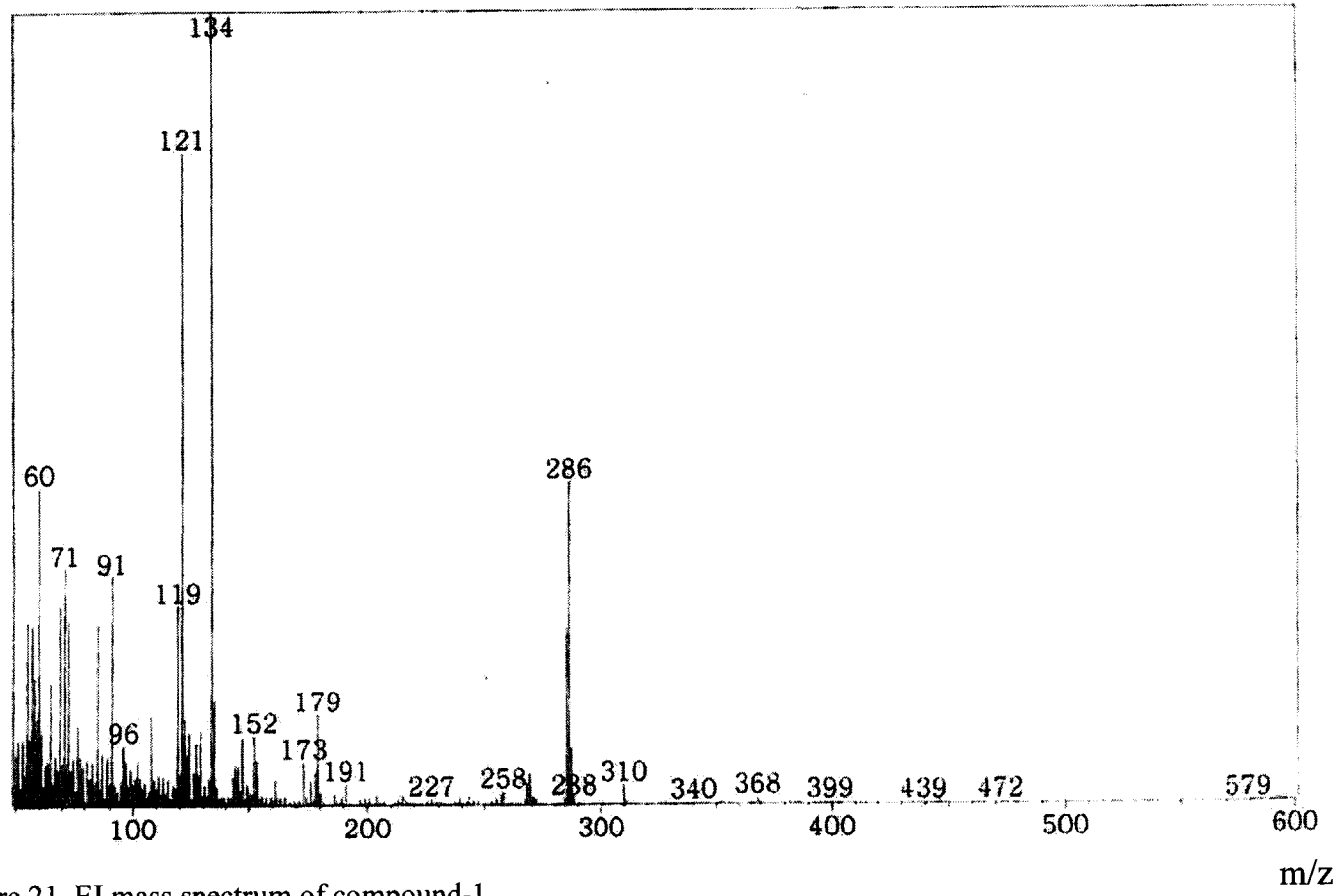


Figure 21. EI mass spectrum of compound-1

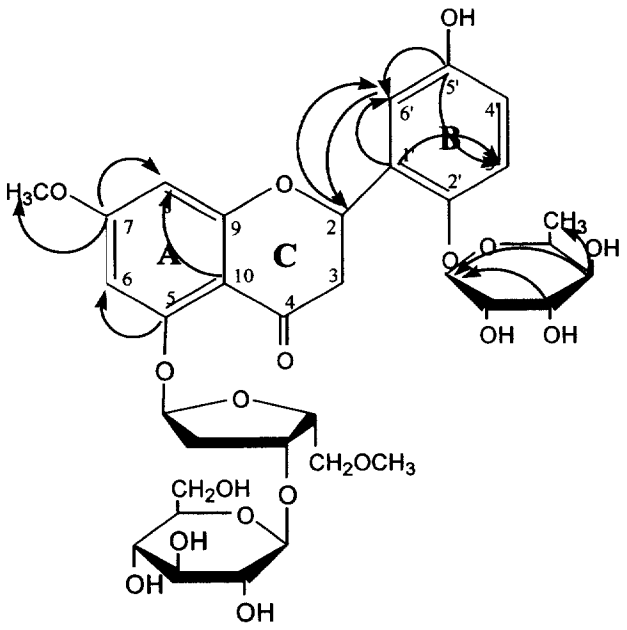


Figure 22. Heteronuclear multiple-bond correlations of compound-1

3.5. Tyrosinase inhibitory activity of compound-1

Bioassay of the purified compound-1 indicated that it exhibited potent mushroom inhibitory activity and dose dependent effect on the oxidation of L-tyrosine. Figure 6 illustrates dose-dependent responses of the compound-1, crude, methanol extract of *Hizikia fusiformis* and a potent tyrosinase inhibitor, kojic acid on tyrosinase inhibitory activity. Concentrations of 1, 0.8, 0.6, 0.4 and 0.2 mg/ml of compound-1 were tested for enzyme inhibitory activity and observed that it could inhibit tyrosinase at IC_{50} of 0.4 mg/ml (0.52 mM). The results showed that compound-1 could exhibit tyrosinase activity by approximately 50% higher than that of crude methanol extract of *Hizikia fusiformis* (IC_{50} of 0.8 mg/ml). This indicated that during the purification process the total inhibitory activity has doubled. Comparison made with kojic acid revealed that activity of compound-1 was approximately 30-folds lower than that of kojic acid, where IC_{50} value of kojic acid was 0.013 mg/ml (0.09 mM) Chen et al, 1991 and Ha et al, 2001 have reported similar tyrosinase inhibition of kojic acid on the oxidation of L-tyrosine. But it was difficult to compare the reported activities of kojic acid as activity tests were conducted with different enzyme concentrations, different substrates (L-tyrosine or L-DOPA) and different assay methods. Also, most inhibitory activity tests of kojic acids have been carried out using L-DOPA as a substrate (Huang et al., 2005, Kubo and Kinst-Hori, 1999).

3.6. Structure-activity relationship of compound-1

Flavonoids are a large family of polyphenolic compounds synthesized by plants and widespread plant secondary metabolites (Hempel and Bohm, 1996). Though they constitute one of the most characteristic classes of compounds in higher plants, presence of flavonoids is reported even in lower classes of plants, such as algae (Markham and Porter, 1969). The basic structure of compound-1

isolated from methanol extract of edible brown algae, *Hizikia fusiformis* is similar to that of basic flavonoid skeleton and it can be categorized as a flavonoid glycoside (Fig. 19). These extend the range of plants in which flavonoids are known to exist. Flavonoids are regularly contained in human food as components of vegetables and fruits (Hempel and Bohm, 1996). It has revealed that flavonoids consumed in the human diet and their synthetic analogues, display a variety of biological effects including anticarcinogenic, antiinflammatory, antioxidant and antiallergenic activities (Glusker and Russi, 1986). Some flavonoids affect the function of enzyme systems involved in immune response and generation of the inflammatory process (Middleton and Kandaswami, 1993). In addition to these findings, their abundance in nature and structural diversity have driven the attention towards isolation of plant-derived flavonoids from various plant sources including algae. So far, hundreds of flavonoids have been isolated and studied for various biological activities. It has found out that many of these phenolic compounds are bound with sugars in living plants and formed sugar conjugates. Earlier reported that these sugar conjugates of phenolic aglycons may decrease the toxicity or reactivity and increase solubility of the compounds to make it easier for them to be transported or stored without harm to the plant producing them (Vickery and Vickery, 1981). However, bioactivities of sugar conjugates such as anticomplementary (Shahat et al 2003), antioxidantive (Gamez et al, 1998, Yesilada, 2000), immunomodulatory (Akbay et al, 2003), antiallergic (Inoue, 2002), enzyme inhibitory (Mizushina, 2003), and antimicrobial (Arima and Danno, 2003) activities revealed that, their capability to play key roles in biological systems. Presence of sugar moieties attached to the basic flavonoid skeleton in compound-1 is helpful to recognize it under the category of flavonoid glycoside and its tyrosinse inhibition also revealed that the capacity of sugar conjugates of phenolic compounds to possess biological activities.

It is well known fact that flavonoids consist of diverse range of compounds and their reactivity is based on their chemical structures. Over the years, tyrosinase inhibitory activities of flavonoids have been studied well and some specific structure-activity relationships have been established. It has found out that most flavonoids react with tyrosinase as substrates, cofactors, or metal chelators (Kubo et al, 2000) where, metal chelation found to be playing a major role in tyrosinase inhibition.

Oxidation of monophenolic substrates, such as L-tyrosine, to L-DOPA undergoes a characteristic lag phase (Sanchez-Ferrer et al, 1995). Presence of reducing agents (hydrogen donors), especially diphenols can shorten or eliminate this lag phase (Ros et al, 1993). Kubo et al, 2000 have reported that a flavonoid, quercetin (*o*-diphenol) can initiate this hydroxylase (monophenolase) activity due to presence of two vicinal hydroxyl groups and eliminate the lag phase. Further, they have shown that absence of two vicinal hydroxyl groups in a flavonoid (eg. kaemferol, Fig. 24) does not suppress this lag phase. Since, these two flavonoid compounds suppress, *o*-diphenolase activity and act as potent tyrosinase inhibitors, quercetin is considered as a cofactor, but not as a substrate. This phenomenon led to suggest that compound-1 is not behaved as a cofactor as there are no vicinal two-hydroxyl groups attached to its flavonoid skeleton. Similarly, it can be presumed that it is not act as a substrate either (as a monophenol or diphenol), because it inhibited *o*-monophenolase activity considerably.

In previous studies some flavonoids were described to chelate copper (Thompson et al, 1976, Hudson and Lewis, 1983). Comparisons made among various flavonoids on their tyrosinase inhibitory activity and their structures have found out that 3-hydroxy-4-keto moiety plays an important role in eliciting tyrosinase inhibitory activity (Kubo and Kinst-Hori, 1999). The inhibition exerted by kojic acid is well established to come from its ability to chelate copper in the

enzyme (Kahn, et al, 1995). The 3-hydroxy-4-keto moiety present in some flavonoids (eg. quercetin) is clearly analogous to kojic acid. Therefore, Kubo and Kinst-Hori, 1999 have suggested that their tyrosinase inhibition was due to chelation of copper. This has been proven by using characteristic bathochromic shifts of particular flavonoid compounds. Addition to this, presence of 5-hydroxy-4-keto moiety is also found out to chelate copper in the enzyme (Kubo et al, 2000). Therefore, it is clear that absence of either 3-hydroxy-4-keto moiety or 5-hydroxy-4-keto moiety would not favor compound-1 to chelate copper in the enzyme.

Figure 23 illustrates the inhibition kinetics of compound-1 analyzed by a Lineweaver-Burk plot. The three lines obtained from uninhibited enzyme and two different concentrations of compound-1, intersected on the horizontal axis. This result indicates that compound-1 was a noncompetitive inhibitor for the oxidation of L-tyrosine by mushroom tyrosinase and it indicated that compound-1 did not inhibit tyrosinase by metal chelation. This observation is agreeable with the structural features of compound-1 as absence of vicinal hydroxyl groups and absence of hydroxyl groups vicinity to the C-4 keto group. Kubo et al, 2000 have hypothesized that non-competitive inhibitor of luteolin could have disrupted the tertiary structure of enzyme through intermolecular hydrogen bonding and reduced the affinity of the substrate with the enzyme. Also, it has found out that some flavonoid glycosides can react with proteins and change their structures (Roseiro et al, 2005). Therefore, we presumed that compound-1 could have inhibited enzyme through changing the structure of protein. This can be further elaborated, as compound-1 is soluble in water-based media. Because of that, there is a possibility of compound-1 to reach and react with enzyme easily in order to form intermolecular hydrogen bonds. Although, the mechanism of tyrosinase inhibition has not explained, kurarinone, a flavanone derivative, found out to be a non-competitive tyrosinase inhibitor (Ha, et al, 2001). Chemical structure of

kurarinone also revealed that its inability to inhibit the enzyme in a competitive way as it has no structural criteria, which support to inhibit tyrosinase in competitive manner. The sequence in which hydrogen atoms dissociate from hydroxyl groups in flavonoid glycosides is to be known as 7-OH > 4'-OH > 3'-OH > 5-OH (Mielczarek, 2005). Therefore, it can be predicated that presence of free 3'hydroxyl group of compound-1 would yield negative charge, hence increase the reactivity with tertiary structure of enzyme especially with positively charged amino groups. However, it would be difficult to establish proper mechanism, in which how compound-1 inhibit the enzyme non-competitively as the tertiary structure of tyrosinase has not been established yet (Soloman, Sundaram and Machonkin, 1996).

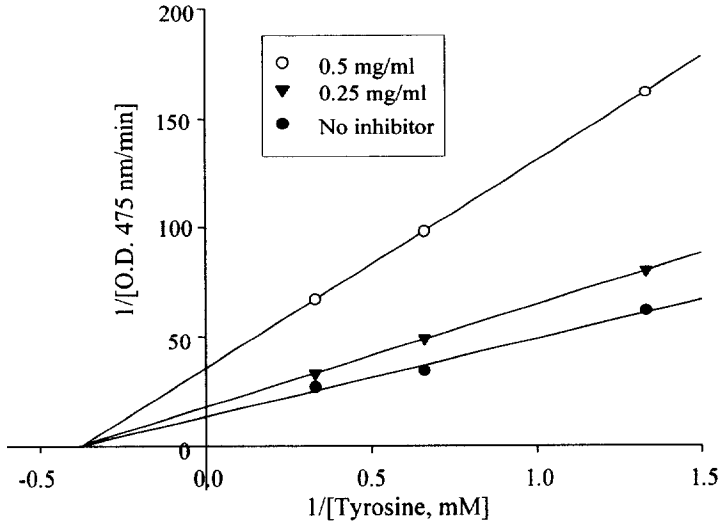


Figure 23. Lineweaver–Burk plot for the determination of inhibition pattern of tyrosinase by compound 1. Tyrosinase inhibitory activity was determined in the presence (0.5 and 0.25 mg/ml) or absence of inhibitor, compound 1 as described in the text using L-tyrosinse as the enzyme substrate. Three concentration of L-tyrosinse were used during the test as 0.75, 1.5 and 3.0 mM, respectively. K_m and V_{max} calculated were of 2.65×10^{-3}M and 0.07 Δ OD_{475}/min respectively.

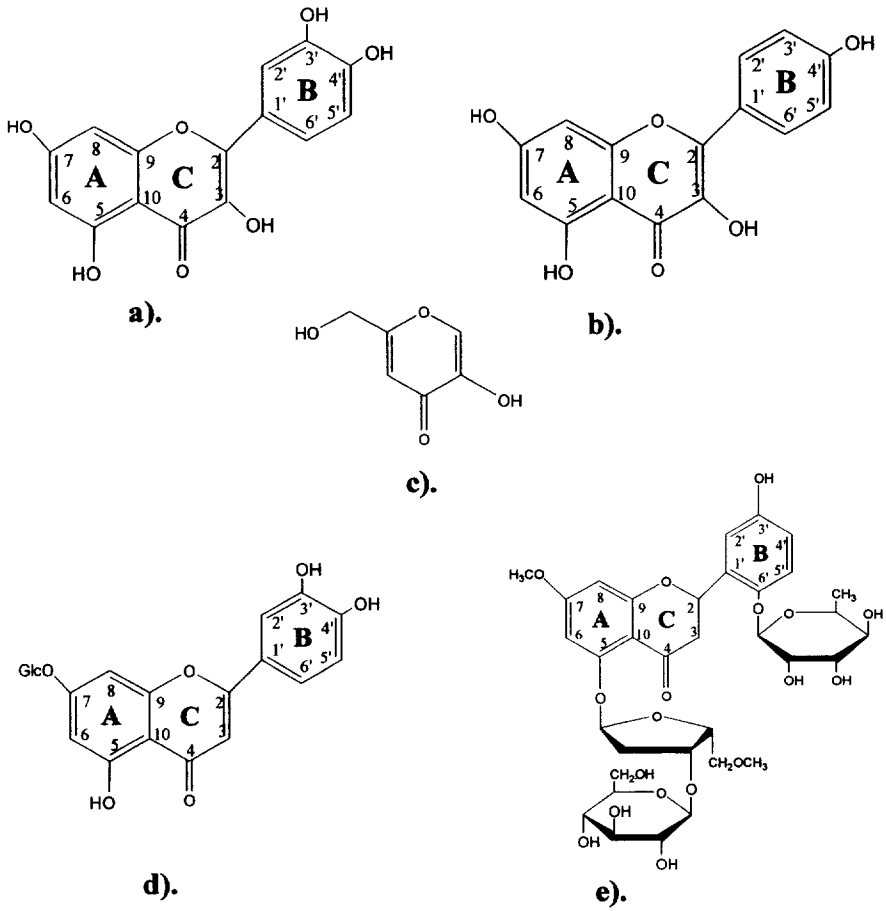


Figure 24. Chemical structures of tyrosinase inihibitory flavonoids and kojic acid. Competitive inhibitors, a). Quercetin and b). Kaemferol (Kubo et al 2000. c). Kojic acid. Non competitive inhibitors, d). Luteolin 7-*O*-glucoside and e). compound-1.

Summary

Hizikia fusiformis was selected as the best species through the preliminary screening of tyrosinase inhibitor among fifteen marine plant species. Components in methanol extract of *Hizikia fusiformis* were purified using assay guided, polarity based fractionation and high performance liquid chromatography. Purity of isolated tyrosinase inhibitor (compound-1) was confirmed using thin layer chromatography. Structural characterization with ^{1}H, NMR, ^{13}C NMR, FAB-MS, IR and UV-Vis spectroscopic techniques revealed that compound-1 as a flavanoid glycoside and estimation of chemical structure of compound-1 was comparable with the reported literature. Comparison showed that activity of the purified compound was 50% more potent than that of the crude methanol extract of *Hizikia fusiformis* and its estimated IC_{50} value was 0.52 mM. However, compound-1 expressed approximately 30 fold less tyrosinase inhibitory activity compared to a potent tyrosinase inhibitor, kojic acid. Enzyme kinetic analysis using Lineweaver-Burk plots revealed that compound-1 inhibits tyrosinase in non-competitive manner. Structural features of compound-1 were agreeable with characteristics of reported flavonoid tyrosinase inhibitors. Therefore, these findings demonstrate that compound-1 derived from marine alga, *Hizikia fusiformis* may be potentially used in industries.

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