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ANTIOXIDATIVE, ANTIMICROBIAL AND CYTOTOXIC PROPERTIES OF ISATIS FLORIBUNDA BOISS. EX BORNM. EXTRACTS

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ABSTRACT

The *Isatis* species has antibacterial, anticancer and antiviral properties and these important endemic plants grow widely in various parts of Anatolian-Turkey. In the present study, the antioxidant activities of *Isatis floribunda* Boiss. ex Bornm. flower and root extracts were determined with total antioxidant activity, free radical scavenging activity, ferric ion reducing power, and cupric ion reducing antioxidant capacity assay. The total phenolic compounds and flavonoids were also examined for the extracts. The antimicrobial activities of the extracts were investigated by using the disc diffusion and microdilution-broth methods against human and fish pathogen microorganisms. The phenolic composition of *I. floribunda* root methanolic extract was analyzed by high performance liquid chromatography (HPLC). The major component in the extract was chlorogenic acid (1980.20 µg/g). The cytotoxic effect of the methanolic root extract was also tested on human breast cell (MCF12A). The results showed that *I. floribunda* could be used as a natural source in the food and feed industry and clinical and food chemistry, and that the antimicrobial agents could be used against human and fish pathogens.

Keywords: cytotoxic activity, HPLC, fish pathogens, clinical/food borne pathogens

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs has been isolated from natural sources, many based on their use in traditional medicine. Various medicinal plants have been used for years in daily life to treat disease all over the world (Nair et al., 2005). Traditional medicine using plant extracts continues to provide health coverage for over 80 % of the world's population, especially in the developing world (WHO,

2002). Similarly, natural antioxidants and their chemical properties are currently the subject of intensive research (Mavi et al., 2011). Plants are potential sources of natural antioxidants. They produce various antioxidative compounds to counteract reactive oxygen species (ROS) in order to survive (Lu and Foo, 1995). Today, we know that radicals cause molecular transformations and gene mutations in many types of organisms. Oxidative stress is well-known to cause many diseases (Storz and Imlay, 1999). Antioxi-

dant-based drug formulations are used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer (Devasagayam et al., 2004).

As the use of antibiotics and chemical substances increases, their application is becoming restricted not only due to the emergence of drug-resistant forms but also because of their adverse effects on the ecosystem (Aoki, 1992). A decreased efficacy of antibiotics, regardless of their mechanism of action, has led to the need for suitable alternatives. Turkey is an important floristic center internationally because of its geographic location, climate and the presence of nearly 10.000 natural plant species (Ateş and Erdoğrul, 2003).

Brassicaceae (Cruciferae) is a cosmopolitan family of about 350 genera and 3000 species, occurring mainly in North Temperate Zone, particularly in the Mediterranean region (Mabberley, 1987). The family species are used as antidiabetic, antibacterial (Radwan et al., 2008), antifungal, anticancer (Vang, 1994) and antirheumatic (Kirtikau and Basu, 1984) treatments, and show a potent insecticidal effect (Malik et al., 1983). *Isatis*, one of the most common plant species, are biennial, herbaceous shrubs belonging to the family Brassicaceae. It is represented by 40 taxa, of which 24 are endemic to Turkey (Davis, 1965, 1988) with representation of 31 species and 14 subspecies in Eastern and South-eastern Anatolia (Misirdali, 1985). The chemical compounds found in the leaves of the *Isatis* species have antibacterial, anticancer, antiviral, astringent and febrifuge properties. These compounds are also used to treat a wide range of disorders including meningitis, encephalitis, mumps, influenza, erysipelas, heat rash etc. Similarly, the roots of these plants are rich in antibacterial and anticancer chemical compounds (Bown, 1995).

The objectives of this study were (i) to investigate antioxidant activities using different tests, (ii) to quantify the main phenolic and flavonoid content and (iii) to estimate the antimicrobial capacities against a set of human clinical and food borne and fish patho-

gens in *I. floribunda* Boiss. ex Bornm. flower and root extracts and (iv) to determine the content of phenolic acid compounds (HPLC) and the cytotoxic effect on human breast cell in the methanolic root extract.

MATERIALS AND METHODS

Plant materials and extraction procedure

I. floribunda plants were collected during the flowering stage in July from the area between Aksaray city and Genç Osman village (an altitude of ca. 1100 m, Tekşen 2599 & Karaman) in Turkey. The authenticated specimens of the plants were deposited at the herbarium of the Biology Department, Faculty of Arts and Sciences, Aksaray University. The flowers and roots were washed thoroughly 2-3 times with running water and once with distilled water. The materials were then air-dried and homogenized to fine powder and stored in airtight bottles. Fifteen grams of powdered plant materials were extracted separately with ethanol (E), methanol (M), water (W), n-hexane (H) and dichloromethane (DCM) by using Soxhlet equipment for 24 hours. The extracts were filtered and evaporated using a rotary evaporator (Heidolph, Laborota 4000, Schwabach, Germany) and stored in the dark at 4 °C. After determining the yield, the extract was dissolved in methanol, ethanol, water or dimethyl sulfoxide (DMSO) for further study.

Determination of extraction yield

The yield of evaporated dried extracts based on dry weight was calculated from the equation shown below:

$$\text{Yield (\%)} = (W1 * 100) / W2$$

where W1 was the weight of extract after evaporation of solvent and W2 was the dry weight of the sample.

Antioxidant assay

Assay for total phenolics

Total soluble phenolics were determined using the Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (1997) and gallic acid as an internal standard.

Briefly, the extracts were dissolved in methanol, and 0.2 ml of extract solution (1 mg/ml) was introduced into the test tube containing 1 ml of Folin-Ciocalteu's reagent and 2 ml of Na₂CO₃ (7.5 %). The final volume was adjusted to 7 ml with deionized water. After incubation for 2 h at room temperature, the absorbance against blank was measured at 765 nm with an UV-Vis Spectrophotometer (HITACHI U-2000). The total phenolic content was expressed as mg gallic acid equivalents (GAE)/g dry extracts. All tests were done in duplicate.

Assay for total flavonoid analysis

The total flavonoid content in root and flower extracts was determined spectrophotometrically according to Arvouet-Grand et al. (1994). Briefly, 1 ml of 2 % aluminium trichloride (AlCl₃) methanolic solution was mixed with the same volume of extract solution (1 mg/ml). Absorption readings at 415 nm were taken after 10 min against a blank sample consisting of a 1 ml extract solution with 1 ml methanol without AlCl₃. Quercetin was used as the standard and the total flavonoid content was expressed as mg quercetin equivalent (QE)/g of dry extract.

Determination of total antioxidant capacity

The total antioxidant capacity was measured using Prieto et al. (1999) phosphomolybdenum method. The extracts were dissolved in methanol (2 mg/ml), and 0.3 ml of each extract was added to 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate mixture). The tubes were incubated for 90 min at 95 °C. The mixture was cooled to room temperature and the absorbance was read at 695 nm against blank. Ascorbic acid equivalents were calculated using the standard graph of ascorbic acid. The experiment was conducted in duplicates and values are expressed as equivalents of ascorbic acid in mg per g of extract.

Ferric ion reducing power

The ferric reducing power method was applied with slight modifications to the method of Oyaizu (1986). Various concentrations of the extracts (2.5 ml) were mixed with 2.5

ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1 % potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After 20 min of incubation, the reaction mixture was acidified with 2.5 ml of trichloroacetic acid (10 %). 2.5 ml of the reaction mixture was mixed with 2.5 ml distilled water and 0.5 ml of 0.1 % ferric chloride. The solution absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates greater reduction capability (Büyükokuroğlu et al., 2001). The same procedure was applied with butylated hydroxytoluene (BHT).

Scavenging activity on DPPH (2,2-diphenyl-1-picrylhydrazyl) radical

Scavenging free radical potentials were tested in solution of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) according to the method described by Sarikurkcu et al. (2008) with some modifications. The antioxidant reaction was initiated by transferring 0.5 ml of plant extract into a sample cavity containing 3.5 ml of freshly prepared methanol solution of DPPH. After 30 min of incubation in the dark at room temperature, the absorbance was measured at 517 nm using a spectrophotometer. Inhibition of DPPH in percent (I%) of each extract sample was calculated from the decrease of absorbance according to the formula:

$$I\% = 100 \times (A_0 - A_1) / A_0$$

where A₀ is the absorbance of the control, and A₁ is the absorbance of the extract/standard. BHT was used as positive controls.

CUPRAC (cupric ion reducing antioxidant capacity) assay

For determination of the reducing ability of the cupric ions (Cu²⁺), the reducing power method was also used (Apak et al., 2006), with a slight modification. This assay is based on utilizing the copper (II)-neocuproine [Cu (II)-Nc] reagent as the chromogenic oxidizing agent. To the mixture of 1 ml CuCl₂ (10 mM), 1 ml neocuproine (7.5 mM), and 1 ml NH₄Ac buffer (1 M, pH 7.0) solution, 0.5 ml different concentrations of extract and water were added to make the final volume of

4.1 ml. The absorbance at 450 nm was recorded against a reagent blank after 30 min. BHT was used as positive controls.

Determination of antimicrobial activity

Microbial strains

Antimicrobial studies were carried out on four gram-positive bacteria (*Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* RSKK 863, *Micrococcus luteus* NRRL B-4375), seven gram-negative bacteria (*Escherichia coli* ATCC 11229, *Escherichia coli* ATCC 35218, *Escherichia coli* O157:H7, *Salmonella enteritidis* ATCC 13076, *Pseudomonas aeruginosa* ATCC 27853, *Shigella sonnei* Mu:57, *Yersinia enterocolitica* NCTC 11175), and one yeast (*Candida albicans* ATCC 10231). Nutrient agar (NA) and Tryptic Soy Agar (TSA) were used for the cultivation of bacteria while YPD medium was used to culture the yeast. All bacterial cultures were incubated at 37 °C for 24 h whereas the yeast cultures were incubated at 30 °C for 48 h.

In vitro antibacterial studies were also carried out on two gram-positive bacteria (*Lactococcus garviae* and *Streptococcus agalactiae* Pasteur Institute 55118) and four gram-negative bacteria (*Yersinia ruckeri*, *Vibrio anguillarum* M1 and A4 strains, from two different companies, *Vibrio alginolyticus* and *Aeromonas hydrophila* ATCC 19570). *V. anguillarum* and *V. alginolyticus* were cultured in TSA supplemented with 2 % NaCl. *Y. ruckeri*, *L. garviae*, *A. hydrophila* and *S. agalactiae* were grown on TSA without additional NaCl. *V. anguillarum* and *V. alginolyticus*, *Y. ruckeri* and *L. garviae* cultures were incubated at 25 °C for 24 h. *A. hydrophila* and *S. agalactiae* cultures were grown at 30 °C and 37 °C respectively for 24 h.

Disc-diffusion assay

The disc diffusion method was employed for the determination of the antimicrobial activity (Murray et al., 1995). The culture suspensions were adjusted by comparing with 0.5 McFarland. One hundred microlitres of suspension of the test microorganisms were

spread on solid media plates. Filter paper discs (6 mm in diameter) were impregnated with 10 µl of the extracts and then placed on the inoculated plates. Afterwards, they were kept for 2 h in a refrigerator to enable prediffusion of the extracts into the agar. Then, the inoculated plates were incubated for 24 h and 48 h for bacterial and yeast strains, respectively. Antibiotic discs of Ampicillin (Amp, 10 µg/disc), Gentamicin (CN, 10 µg/disc), and Amikacin (AK, 30 µg/disc) were also used as positive controls. Absolute solvents were used as negative controls. The diameters of inhibition zones (mm) were used as a measure of antimicrobial activity, and each assay was repeated twice.

Minimal Bactericidal (MBC)/Fungicidal (MFC) Concentration

The MBC/MFC values of the extracts were determined by the micro-dilution method using serially diluted (2 folds) plant extracts according to Chandrasekaran and Venkatesalu (2004). Some modifications were made to the method. The extracts were studied for microorganisms which are sensitive to the extracts in the disc diffusion assay. The serial dilutions of the extract were made in a concentration range from 1.41 to 180 mg/ml in the tubes. The inoculums of microorganisms were prepared by using 12 h cultures, and the suspensions were adjusted to 0.5 McFarland standard turbidity. The final volume in each tube was 100 µl. 2.5 µl of standardized suspension of each tested microorganism (at 0.5 McFarland standard turbidity) was transferred to each tube. A positive control (containing 2.5 µl inoculum and 100 µl growth medium) and a negative control (containing 2.5 µl of extract, 100 µl growth medium without inoculum) were included in each micro tube. The contents of the tubes were mixed by pipetting and were incubated for 24 h. 5 µl samples from clear tubes were then plated on solid growth medium to confirm microbial growth because the plant extracts tested in this study were colored (Şahin et al., 2003). Minimum inhibitory concentrations (MIC) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight

incubation, and minimum bactericidal/fungicidal concentrations (MBC/MFC) as the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic-free media (Andrews, 2001). So, the concentrations of the extracts that prevent the growth of a microorganism on the solid media were evaluated as MBC/MFC values in this study. Each test was repeated twice.

High performance liquid chromatography (HPLC) analysis conditions

HPLC analysis was performed by Süleyman Demirel University (Isparta, Turkey). The HPLC system consisted of a Shimadzu LC-10ADVP pump, a 20 µl sample loop, SCL-10A VP UV-DAD detector, and an Agilent Eclipse XDB-C18 column (4.6 x 250 mm, 5 µm). The mobile phase was 3 % acetic acid/methanol at a flow rate of 0.8 ml/min and UV detection was at 278 nm. The amount of each phenolic acid is expressed as micrograms per gram of dry extract.

Cytotoxicity assay

Cytotoxicity assay was performed by the Molecular Biology Research and Application Center, Gazi University (Ankara, Turkey). The extract of *I. floribunda* was evaluated on human breast cell (MCF12A) in order to examine its cytotoxic effects on normal cells. The cells were harvested (2×10^5 cells/well) and inoculated in 96 well micro titer plates. Cell proliferation was analyzed 48 h after MCF12A cells had been cultured with an extract of 250, 500, 1000 µg/ml in the final concentration using trypan blue dye assay. After 48 h incubation, 0.4 % trypan blue was added to each well and incubated for 15 min. The dye was removed with phosphate buffered saline. Cells were lysis with 1 % SDS (Sodium Dodecil Sulphate), and absorbance was read at 590 nm with micro-plate reader. The concentration that Gazi University utilized to determine the anticarcinogenic activity was chosen at the same values as the concentration that determines the cytotoxic activity in healthy human breast cells.

RESULTS AND DISCUSSION

The yield of extracts

The yield in different solvent extracts obtained from the flower and root of the plant are shown in Table 1. The extract yields for flower and root extracts of *I. floribunda* ranged from 4.53 to 47.53 % and 0.63 to 7.28 % (w/w), respectively. A wide range of the yields among the extracts was observed depending on the extraction solvent and plant part used. Among all the plant extracts, water extract was found to have maximum extractive yield followed by the methanol and ethanol extract (Table 1). Water is a universal solvent and is generally used in traditional settings to prepare plant decoctions for health remedies. It has been reported that many natural products including pigments, enzymes and bioactive components are soluble in water, which explains the highest yield of extract (Majorie, 1999). However, our earlier research and other studies indicated that the plant extracts extracted in organic solvents showed profoundly distinct antibacterial activity from aqueous extract (Asan Ozusaglam et al., 2013; Parekh and Chanda, 2007). Hence, in the present work, in addition to water, methanol, ethanol, n-hexane and DCM were used for the extraction.

Table 1: The flower and root extracts yields of *Isatis floribunda*

Extract Yield % (w/w)					
Part	Solvents				
	Methanol	Ethanol	Water	n-Hexane	DCM
Flower	37.93	23.30	47.53	4.53	4.86
Root	3.88	2.90	7.28	0.63	0.84

Table 2: Total phenolics, flavonoid and antioxidant capacities of *Isatis floribunda* flower and root extracts

Part	Solvent	Total phenolic content ^a (mg GAE/g extract)	Total flavonoid content ^a (mg QE/g extract)	Total antioxidant capacity ^a (mg AAE/g extract)
Flower	Methanol	37.09±0.96	12.65±0.05	46.08±12.51
	Ethanol	25.50±5.14	4.86±0.92	40.69±8.16
	Water	93.91±0.32	25.90±0.12	33.38±2.18
	n-Hexane	- ^b	-	-
	DCM	6.86±4.50	-	4.54±0.54
Root	Methanol	98.23±5.79	-	128.38±20.13
	Ethanol	68.91±4.18	-	151.85±27.20
	Water	62.77±0.64	-	16.08±1.63
	n-Hexane	1.64±0.32	-	24.54±1.63
	DCM	57.77±0.64	-	129.15±19.04

^a: values are reported as means ± S.D. of two separate experiments

^b: not determined

Antioxidant capacity

Total phenolics (TPC) and flavonoids (TFC) content

The total phenolic and flavonoid content of the extracts are given in Table 2. The amount of total phenolics is measured by Folin–Ciocalteu method. In the flower extracts, the highest level of phenolics (93.91 mg GAE/g extract) was found in water extract, while the lowest (6.86 mg GAE/g extract) was in the DCM extract, but the flower n-hexane extract did not have TPC in this study. In the root extracts, the total content of phenolic compounds varied from 1.64 to 98.23 mg GAE/g extract. The highest content of phenolic compounds was found in the methanolic extract (98.23 mg GAE/g extract), followed by the ethanol extract (68.91 mg GAE/g extract). The lowest level of TPC was found in the n-hexane (1.64 mg GAE/g extract) in the root extracts. As a result, the methanolic root extract showed the highest TPC value in the flower and root extracts of *I.*

floribunda. The phenolic composition of plant extracts is affected by different factors – variety, climate, and storage, processing etc. (Tomsone et al., 2012).

Wang (2012) determined that the phenol amount in the *Isatis indigotica* Fort. plant was 2.34 mg GAE/g in methanol extract, 1.82 mg GAE/g in ethanol extract and 5.94 mg GAE/g in water extract. As regards the phenol content in flower extracts in our study, the methanol extract was 37.09 mg GAE/g extract, the ethanol extract was 25.50 mg GAE/g extract and the water extract was 93.91 mg GAE/g extract. In the root extracts, methanol, ethanol and water extract were 98.23 mg GAE/g extract, 68.91 mg GAE/g extract and 62.77 mg GAE/g extract, respectively.

The flavonoids content was only determined for some flower extract quercetin equivalents per gram of extract (mg QE/g extract). Total flavonoid contents the flower extracts of *I. floribunda* decreased in the following order: W > M > E. However, in this study the root extracts had no flavonoid content.

Determination of total antioxidant capacity (TAC)

This assay is based on the reduction of Mo (VI) to Mo (V) in presence of the antioxidant compounds and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH; this is measured at 695 nm (Prieto et al., 1999). TAC was reported as ascorbic acid equivalents and is shown in Table 2. The total antioxidant capacity in plant extracts varied from 4.54 to 151.85 mg AAE/g extract. In the flower extracts, the two highest total antioxidant capacities were found methanol and ethanol extracts of *I. floribunda*. The flower n-hexane extract did not contain TAC in our study. The maximum TAC value in the root extracts was found in ethanol extract, followed by DCM extract. Wang (2012) reported total antioxidant capacity in the *I. indigotica* Fort. plant as 6.93 mg AAE/g in methanol extract, 6.15 mg AAE/g in ethanol extract and 5.56 mg AAE/g in water extract. Regarding the total antioxidant capacity in the flower extracts in the present study, methanol extract was 46.08 mg AAE/g extract, ethanol extract was 40.69 mg AAE/g and water extract was 33.38 mg AAE/g. In the root extracts, methanol, ethanol and water extracts were 128.38 mg AAE/g, 151.85 mg AAE/g and 16.08 mg AAE/g, respectively.

DPPH free radical scavenging assay

The antioxidant activity of the above-mentioned plant extracts was also determined by employing DPPH. Figure 1a-b show that the results of the assay are expressed in percentage (%) of inhibition of DPPH free. The analysis shows that the radical scavenging activity of the flower extracts increases with concentration and follows the given orders W > M > E > DCM > H. The water extract of the flower of *I. floribunda* showed the highest DPPH scavenging activities: 89.58 % (IC₅₀ > 1000) (at 2000 µg/ml concentration). In the root extracts, the methanol and ethanol extracts were the most effective in the DPPH assay: 87.49 % (IC₅₀ 747.45) and 81.84 % (IC₅₀ 943.69), respectively. The radical scavenging activity of the root extracts was found

to decrease in the order of M > E > W > DCM > H. From the present results it may be postulated that the extracts of *I. floribunda* reduce the DPPH radical to corresponding hydrazine when they react with hydrogen donors in antioxidant principles. The radical scavenging of BHT at 200 µg/ml concentration was determined as 91.19 %. Wang (2012) found DPPH scavenging activity in the methanol, ethanol and water extract at 2.0 mg dw/ml of the *I. indigotica* Fort. and determined only 34.85% in the water extract. In our study, DPPH scavenging activity was found in flower and root methanol, ethanol and water extracts at 2.0 mg/ml concentration and the highest DPPH scavenging activity was determined in the flower water extract as 89.58 %.

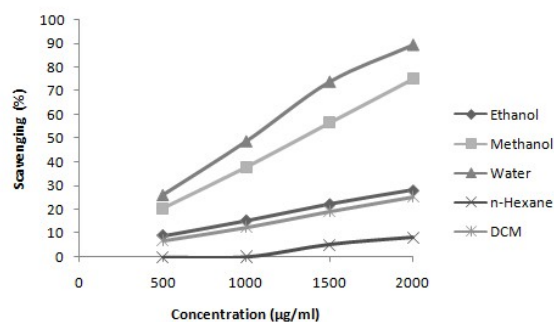


Figure 1a: DPPH scavenging activity of *Isatis floribunda* flower extracts

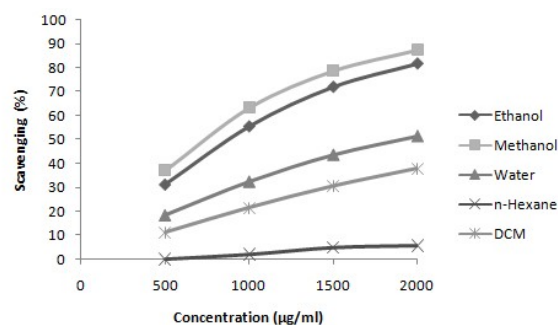


Figure 1b: DPPH scavenging activity of *Isatis floribunda* root extracts

Cupric reducing antioxidant capacity (CUPRAC) assay

CUPRAC assays have been used by many researchers to determine the reducing power of antioxidant compounds (Apak et al., 2006; Prior et al., 2005; Cakmak et al., 2012). The ion (Cu²⁺) reducing ability of the *I. floribunda* extracts is shown in Figure 2a-b. Among the flower extracts, the lowest Cu²⁺

reducing power (at concentration 800 $\mu\text{g/ml}$) had $\text{H} < \text{DCM} < \text{M} < \text{E} < \text{W}$. The highest Cu^{2+} reducing power ($A_{450\text{nm}}$ 0.39) was found in the flower water extract. The Cu^{2+} reducing power of the root extracts was found to decrease in the order of $\text{M} > \text{E} > \text{W} > \text{DCM} > \text{H}$. Also, the highest Cu^{2+} reducing power was noticed in the root methanol extract ($A_{450\text{nm}}$ 0.61). BHT, at 31.25 $\mu\text{g/ml}$ the concentration, exhibited remarkably higher cupric ion reducing power ($A_{450\text{nm}}$ 0.39) than the extracts.

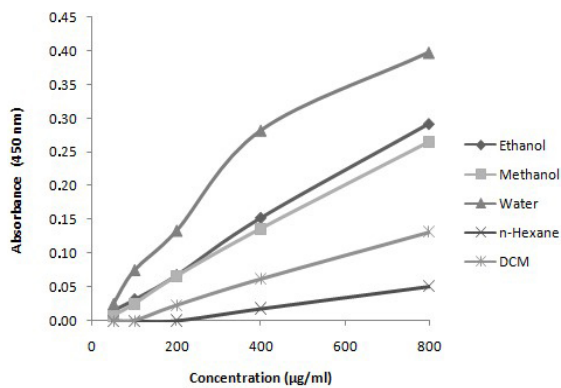


Figure 2a: Cupric ion reducing power of *Isatis floribunda* flower extracts

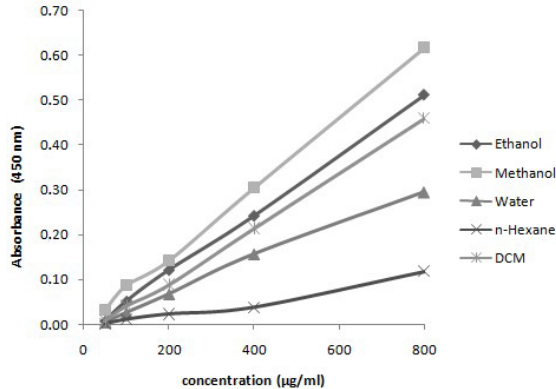


Figure 2b: Cupric ion reducing power of *Isatis floribunda* root extracts

Ferric ion reducing power

The *I. floribunda* flower and root extracts' ferric ion reducing power values varied from 0.12 to 0.74 at the concentration 1000 $\mu\text{g/ml}$ (Figure 3a-b). The highest result for ferric ion reducing power ($A_{700\text{nm}}$ 0.50) was found in the water extract while the lowest values for ferric ion reducing power were found in the n-hexane extract ($A_{700\text{nm}}$ 0.11) in the flower extracts. Of all the root extracts, the highest ferric ion reducing power, the methanol extract of *I. floribunda* was found

as $A_{700\text{nm}}$ 0.74, while the lowest ferric ion reducing power was determined as $A_{700\text{nm}}$ 0.15 in the n-hexane extract. BHT, at 31.25 $\mu\text{g/ml}$ the concentration, exhibited remarkably higher reducing power ($A_{700\text{nm}}$ 0.61) than the extracts.

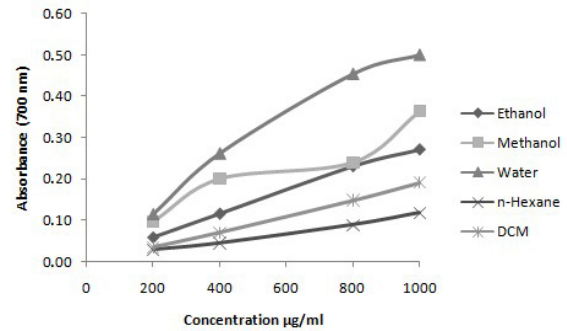


Figure 3a: Ferric ion reducing power of *Isatis floribunda* flower extracts

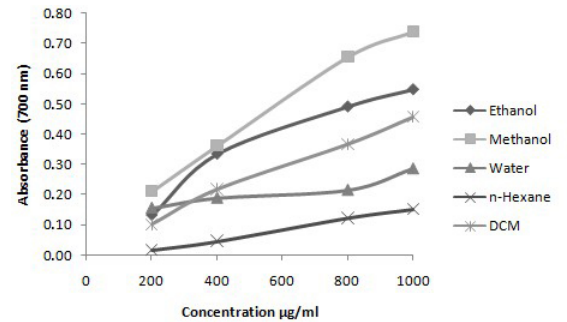


Figure 3b: Ferric ion reducing power of *Isatis floribunda* root extracts

Antimicrobial activity

Antimicrobial assay with disc diffusion against food borne and clinical human pathogens

The antimicrobial activities of the flower and root extracts obtained from the plant under study by the disc diffusion method are shown in Table 3. Screenings were performed of the flower and root extracts' antimicrobial activities against clinical and food borne pathogenic microorganisms. The flower and root extracts showed different degrees of inhibition against 12 food borne and clinical pathogens. In the present study, the range of inhibition of the microorganisms varied from 13.64-7.87 mm and 14.31-7.68 mm, respectively for the flower and root extracts and no activity was found in aqueous extract. In the flower extracts of *I. floribunda*, the two maximum inhibition zones were observed against

S. enteritidis (ATCC 13076, 13.64 mm) and *B. cereus* (RSKK 863, 13.00 mm) in the ethanolic extract, and the minimum inhibition zone was noticed against *S. sonnei* (Mu:57, 7.87 mm) in the DCM extract. Among the root extracts, the methanolic extract showed high inhibiting activity (14.31 mm) against *B. cereus* (RSKK 863) while the DCM extract showed minimum inhibition zone against *M. luteus* (NRRL B-4375, 7.68 mm). Some of these plant extracts were more effective than traditional antibiotics (Ampicillin, Amikacin and Gentamicin) at combating the pathogenic microorganism strains studied (Table 3).

The disc diffusion test alone is not enough to decide whether the activity type is lethal or static. In order to identify the type of activity, the disc diffusion test should be followed by MIC and MBC/MFC tests (Altuner et al., 2011). However, the tested plant extracts in the study were colored, and the visible growth could not be observed and so MBC/MFC values were determined. The results of the MBC/MFC are shown in Table 4. The plant extracts of MBC/MFC values varied from 2.82 to 90.00 mg/ml. The lowest MBC value of 5.63 mg/ml was found in flower methanol extract against *B. cereus* (RSKK 863). Among the flower extracts, n-hexane extract showed the highest MBC value of 90.00 mg/ml against *E. coli* (ATCC 35218) and *S. enteritidis* (ATCC 13076). The lowest MBC values of 2.82 mg/ml were found in the DCM root extract against *B. cereus* (RSKK 863). The root extracts showed the highest MBC value of 22.50 mg/ml against various microorganisms. The results of the MBC show that the root extracts seemed to be more effective than the flower extracts against the test microorganisms used in this study.

The antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. In their study, Faiyaz et al. (2012) used the agar well diffusion method and found that the methanol and ethanol seed extracts (at the concentration of 40 mg) of the *Raphanus sativus* Linn. (Brassicaceae) plant had an antimicrobial effect on *S. aureus* (ATCC 25923, 13.50 and 19.00

mm), *E. coli* (ATCC 25922, 12.50 and 14.50 mm) and *P. aeruginosa* (ATCC 27853, 14.60 and 21.3 mm). In our study, the methanol and ethanol flower extracts (at the concentration of 2.5 mg/disc) showed antimicrobial activity against the pathogens of *S. aureus* (ATCC 25923, 10.49 and 12.92 mm), *E. coli* (ATCC 35218, 12.74 mm for only the ethanol extract) and *P. aeruginosa* (ATCC 27853, 11.60 mm for the methanol extract).

In one of their studies, Emam and El-Moaty (2009) researched the antimicrobial activity of ethanol and water extracts of *Isatis microcarpa* J. Gay ex Boiss. against pathogen microorganisms (*E. coli*, *S. aureus*, *B. subtilis*, *Pseudomonas sp.*, *Salmonella spp.*). The results showed that the extracts had various antimicrobial activity, while in our study; water extract had no activity against these pathogens. Brasileiro et al. (2006) studied the antimicrobial activity of ethanol extract of *Nasturtium sp.* (Brassicaceae) against *S. aureus* (ATCC 25985) and *E. coli* (ATCC 25922). While there was no activity of the ethanol extract against these pathogens, in our study antimicrobial activity was recorded from flower and root ethanol extracts against *S. aureus* (ATCC 25923; 12.92 mm and 12.95 mm, respectively) and against *E. coli* (O157:H7; 13.51 mm and 11.93 mm, respectively). In the study by Kıvanç and Kunduhoğlu (1997), fresh juice of *Raphanus sativus* L. var. *radicula* (Brassicaceae) and *R. sativus* L. (Brassicaceae) was tested for antimicrobial activity against *B. cereus*, *E. coli*, *S. aureus*, and *P. aeruginosa*. In Kıvanç and Kunduhoğlu's study, these extracts only showed antimicrobial activity against *B. cereus* (13 mm and 12 mm, respectively). In our study, flower and root extracts showed various antimicrobial activities against the same microorganisms.

Table 3: Antimicrobial activity of *Isatis floribunda* flower and root extracts against food and human pathogen microorganisms

		Inhibition zone diameter ^a (mm)											
		863 ^b	O:157 ^c	Mu:57 ^d	4375 ^e	11175 ^f	11229 ^g	27853 ^h	25923 ⁱ	35218 ^j	13076 ^k	10231 ^l	7644 ^m
FLOWER	M	12.49±0.02	9.77±0.35	12.22±0.01	-	-	11.63±0.02	11.60±0.45	10.49±0.32	-	11.11±0.65	-	11.81±0.03
	E	13.00±0.56	13.51±0.41	-	-	-	-	-	12.92±0.71	12.74±0.03	13.64±0.21	12.32±0.32	-
	W	- ⁿ	-	-	-	-	-	-	-	-	-	-	-
	H	-	9.65±0.25	9.23±0.06	-	-	8.88±0.08	8.52±0.23	9.45±0.03	9.52±0.22	9.20±0.42	-	8.52±0.05
	DCM	8.68±0.75	8.88±0.06	7.87±0.49	-	-	-	8.75±0.01	8.77±0.09	9.40±0.07	9.69±0.41	-	8.03±0.06
ROOT	M	14.31±0.03	-	-	-	-	-	-	9.94±0.23	-	-	-	11.69±0.01
	E	9.34±0.01	11.93±0.06	12.38±0.55	-	-	12.39±0.78	12.92±0.07	12.95±0.54	-	9.50±0.01	-	10.10±0.06
	W	-	-	-	-	-	-	-	-	-	-	-	-
	H	10.12±0.05	-	-	-	-	9.40±0.42	10.20±0.51	-	9.50±0.40	-	-	-
	DCM	8.61±0.02	8.69±0.05	9.12±0.21	7.68±0.65	8.62±0.94	9.27±0.05	7.87±0.15	9.36±0.10	8.90±0.27	8.16±0.33	9.13±0.80	-
Inhibition zone diameter ^a (mm) Antibiotics	Amp	37.68±0.03	25.92±0.15	38.43±0.16	34.65±0.12	11.58±0.09	27.99±0.14	-	34.82±0.06	25.78±0.19	29.49±0.15	-	25.13±0.06
	CN	18.02±0.11	18.37±0.17	19.49±0.05	13.48±0.22	16.17±0.11	14.98±0.12	15.89±0.05	15.52±0.14	12.17±0.21	16.38±0.17	-	20.63±0.16
	AK	18.72±0.07	22.58±0.09	27.07±0.04	19.55±0.14	21.19±0.07	19.81±0.13	19.71±0.08	19.46±0.16	20.03±0.09	17.27±0.11	-	20.52±0.21

^a: Diameter of the inhibition zone including disc diameter. Values are reported as means ± SD of two separate experiments.

^b: *B. cereus* RSKK 863, ^c: *E. coli* O157:H7, ^d: *S. sonnei* Mu:57, ^e: *M. luteus* NRRL B-4375, ^f: *Y. enterocolitica* NCTC 11175, ^g: *E. coli* ATCC 11229, ^h: *P. aeruginosa* ATCC 27853, ⁱ: *S. aureus* ATCC 25923, ^j: *E. coli* ATCC 35218, ^k: *S. enteritidis* ATCC 13076, ^l: *C. albicans* ATCC 10231, ^m: *L. monocytogenes* ATCC 7644

ⁿ: indicates no antimicrobial activity

Table 4: MBC/MFC values of *Isatis floribunda* flower and root extracts against clinical and food borne human pathogenic microorganisms

		MBC/MFC (mg/ml) values ^a											
		863 ^b	O:157 ^c	Mu:57 ^d	4375 ^e	11175 ^f	11229 ^g	27853 ^h	25923 ⁱ	35218 ^j	13076 ^k	10231 ^l	7644 ^m
FLOWER	M	5.63	45.00	22.50	-	-	45.00	22.50	22.50	-	45.00	-	45.00
	E	22.50	45.00	-	-	-	-	-	22.50	22.50	22.50	22.50	-
	W	-	-	-	-	-	-	-	-	-	-	-	-
	H	-	45.00	45.00	-	-	45.00	45.00	45.00	90.00	90.00	-	45.00
	DCM	22.50	45.00	22.50	-	-	-	22.50	22.50	45.00	45.00	-	45.00
ROOT	M	5.63	-	-	-	-	-	-	11.25	-	-	-	22.50
	E	5.63	22.50	22.50	-	-	22.50	11.25	22.50	-	22.50	-	22.50
	W	-	-	-	-	-	-	-	-	-	-	-	-
	H	5.63	-	-	-	-	-	11.25	-	22.50	-	-	-
	DCM	2.82	22.50	11.25	11.25	11.25	11.25	11.25	11.25	22.50	11.25	22.50	-

^a: Minimal Bactericidal Concentration (MBC) values, Minimal Fungicidal Concentration (MFC) values

^b: *B. cereus* RSKK 863, ^c: *E. coli* O157:H7, ^d: *S. sonnei* Mu:57, ^e: *M. luteus* NRRL B-4375, ^f: *Y. enterocolitica* NCTC 11175, ^g: *E. coli* ATCC 11229, ^h: *P. aeruginosa* ATCC 27853, ⁱ: *S. aureus* ATCC 25923, ^j: *E. coli* ATCC 35218, ^k: *S. enteritidis* ATCC 13076, ^l: *C. albicans* ATCC 10231, ^m: *L. monocytogenes* ATCC 7644

Antimicrobial assay with disc diffusion against fish pathogens

The antibacterial activity of plant *I. floribunda* extracted in different solvents against the six different fish bacterial pathogens is shown in Table 5. These bacterial pathogens commonly occur in the aquaculture sector and cause serious infectious diseases and mortality in fish (Buller, 2004). The flower extracts of *I. floribunda* showed various antimicrobial activities (7.69-15.52 mm) against the tested microorganisms. The ethanol extract showed the two highest inhibitions against *A. hydrophila* (ATCC 19570) and *L. garviae* (15.52 mm and 14.28 mm respectively). The two weakest inhibitory activities were determined against *A. hydrophila* (ATCC 19570, 7.69 mm) and *V. anguillarum* (M1, 8.08 mm) from flower DCM and n-hexane extracts, respectively. In our study, the flower water extract showed no antimicrobial activity against food borne and clinical human pathogens, while the antimicrobial activities of water flower extracts against fish pathogens *V. anguillarum* (M1), *V. anguillarum* (A4) and *A. hydrophila* (ATCC 19570) were determined as 8.48 mm, 8.10 mm and 13.28 mm, respectively. In the root extracts, the n-hexane extract demonstrated the highest activity (16.66 mm), followed by the dichloromethane extract (16.00 mm) against *S. agalactiae* (Pasteur Inst. 55118). The water extracts demonstrated the least activity (7.87 mm) against *V. anguillarum* (A4). The n-hexane root extract, which had a higher antimicrobial activity zone against fish pathogens in the all-plant extract, also showed better antibacterial activities against *S. agalactiae* (Pasteur Inst. 55118) when compared with standard AK (Table 5). In the flower extracts, the DCM extract was found to have the highest inhibitory activity against *S. agalactiae* (Pasteur Inst. 55118, 2.82 mg/ml), while the lowest inhibitory activity was against *Y. ruckeri* (90.00 mg/ml) (Table 5). Similarly, the MBC values of the root extracts varied from 1.41 mg/ml for DCM (*A. hydrophila* ATCC 19570) to 45.00 mg/ml for water (*V. anguillarum*) extract. The results indicated that the flower and root extracts showed various anti-

bacterial activities against different fish pathogens. The extracts could therefore be used to source antibiotic substances for possible treatment of bacterial infections and in the feed industry for fish.

Türker et al. (2009) used the disc diffusion method to study the antimicrobial activity of aerial parts of water, methanolic and ethanolic extracts (at the concentration of 1.5 mg/disc) of *Capsella bursa-pastoris* (L.) Medik. (Family Brassicaceae) against fish pathogens. There was no activity of the water, methanolic and ethanolic extract of *C. bursa-pastoris* against *Y. ruckeri*, *L. garviae*, *A. hydrophila* (ATCC 19570) and *S. agalactiae* (Pasteur Inst. 55118) pathogens. In our study the flower and root water, methanol and ethanol extracts (at the concentration of 2.5 mg/disc) of *I. floribunda* various antimicrobial activity with using the same method was recorded against *Y. ruckeri*, *L. garviae*, *A. hydrophila* (ATCC 19570) and *S. agalactiae* (Pasteur Inst. 55118).

HPLC analysis

In the present study we are, to our knowledge, reporting the phenolic composition of the methanolic root extract of *I. floribunda* for the first time. Ten components were analyzed in the methanolic root extract and eight phenolic components were determined among them (Table 6). The HPLC analysis of the methanolic root extract was examined because the extract had the highest phenolic content and the antioxidant value was in general higher than the others. The HPLC analysis results showed that chlorogenic acid was the major component (1980.20 µg/g), followed by quercetin (150.60 µg/g), p-coumaric acid (136.30 µg/g), caffeic acid (112.20 µg/g) and p-hydroxy benzoic (106.90 µg/g) acid. Benzoic acid and rutin were not determined among these components. Chromatograms for standards and *I. floribunda* methanol root extract eluted by HPLC are represented in Figures 4a and 4b, respectively.

Table 5: Antibacterial activity of *Isatis floribunda* flower and root extracts against different bacterial fish pathogens

Test microor-ganisms	Diameter of zone of inhibition ^a (mm)										MBC ^b (mg/ml)										Inhibition zone diameter ^a (mm) Antibiotics		
	FLOWER					ROOT					FLOWER					ROOT					Amp	CN	AK
	M	E	W	H	DCM	M	E	W	H	DCM	M	E	W	H	DCM	M	E	W	H	DCM			
<i>V. anguillarum</i> M1	11.82 ±0.02	13.71 ±0.03	8.48 ±0.04	8.08 ±0.04	8.18 ±0.07	11.37 ±0.71	13.09 ±0.25	9.04 ±0.72	9.87 ±0.08	10.31 ±0.52	45.00	22.50	22.50	45.00	45.00	11.25	22.50	45.00	11.25	11.25	9.02 ±0.04	12.38 ±0.09	9.46 ±0.12
<i>V. anguillarum</i> A4	8.31 ±0.05	12.61 ±0.21	8.10 ±0.29	9.44 ±0.05	10.50 ±0.14	10.72 ±0.36	12.27 ±0.06	7.87 ±0.65	9.79 ±0.01	14.72 ±0.12	45.00	22.50	22.50	45.00	45.00	11.25	22.50	45.00	11.25	5.63	9.40 ±0.11	15.13 ±0.15	12.07 ±0.13
<i>V. alginolyticus</i>	- ^c	-	-	9.97 ±0.62	9.77 ±0.32	-	9.84 ±0.45	-	12.63 ±0.03	9.85 ±0.37	-	-	-	11.25	11.25	-	11.25	-	45.00	5.63	13.57 ±0.09	15.06 ±0.07	15.03 ±0.03
<i>L. garviae</i>	11.03 ±0.56	14.28 ±0.09	-	8.77 ±0.09	9.24 ±0.18	10.45 ±0.22	-	-	12.86 ±0.29	9.87 ±0.45	45.00	45.00	-	45.00	45.00	22.50	-	-	11.25	22.50	33.10 ±0.12	15.19 ±0.10	10.30 ±0.08
<i>Y. ruckeri</i>	13.06 ±0.41	11.83 ±0.15	-	8.20 ±0.22	8.16 ±0.36	12.60 ±0.90	12.68 ±0.23	-	10.10 ±0.22	8.95 ±0.05	45.00	45.00	-	45.00	90.00	22.50	22.50	-	22.50	22.50	32.30 ±0.15	18.85 ±0.05	18.69 ±0.12
<i>A. hydrophila</i> ATCC 19570	10.84 ±0.44	15.52 ±0.65	13.28 ±0.02	8.76 ±0.36	7.69 ±0.47	11.67 ±1.15	12.90 ±0.18	-	9.36 ±0.26	9.99 ±0.74	11.25	11.25	22.50	45.00	22.50	5.63	5.63	-	5.63	1.41	11.20 ±0.41	19.03 ±0.12	30.57 ±0.33
<i>S. agalactiae</i> Pasteur Ins. 55118	11.24 ±0	10.33 ±1.73	-	12.05 ±1.06	9.20 ±0.38	13.84 ±0.06	12.32 ±0.55	-	16.66 ±0.77	16.00 ±0.14	22.50	22.50	-	5.63	2.82	5.63	5.63	-	1.41	1.41	37.46 ±0.45	19.72 ±0.18	16.15 ±0.02

^a: Diameter of the inhibition zone including disc diameter. Values are reported as means ± SD of two separate experiments.

^b: MBC: Minimal Bactericidal Concentration (MBC)

^c: indicates no antimicrobial activity.

Table 6: Phenolic compositions of *Isatis floribunda* methanolic root extract

Phenolic compounds	Composition (µg/g)
Catechin	93.80
p-hydroxy benzoic acid	106.90
Chlorogenic acid	1980.20
Caffeic acid	112.20
p-coumaric acid	136.30
Ferulic acid	89.90
Benzoic acid	- ^a
Rutin	-
Cinnamic acid	47.70
Quercetin	150.60

^a: not determined

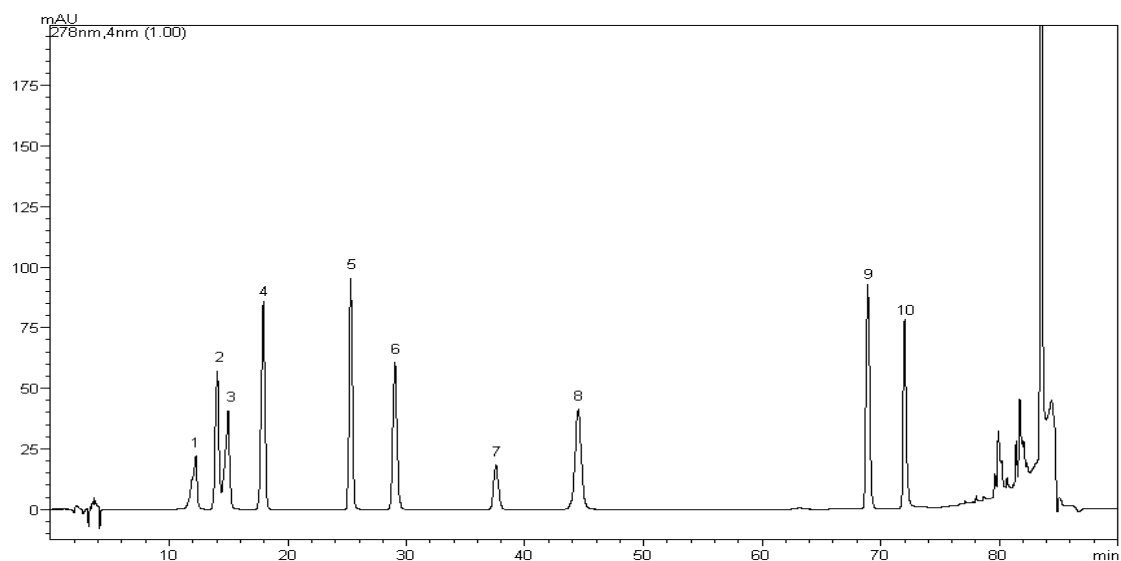


Figure 4a: Standard chromatogram; 1 = catechin, 2 = p-hydroxy benzoic acid, 3 = chlorogenic acid, 4 = caffeic acid, 5 = p-coumaric acid, 6 = ferulic acid, 7 = benzoic acid, 8 = rutin, 9 = cinnamic acid, 10 = quercetin

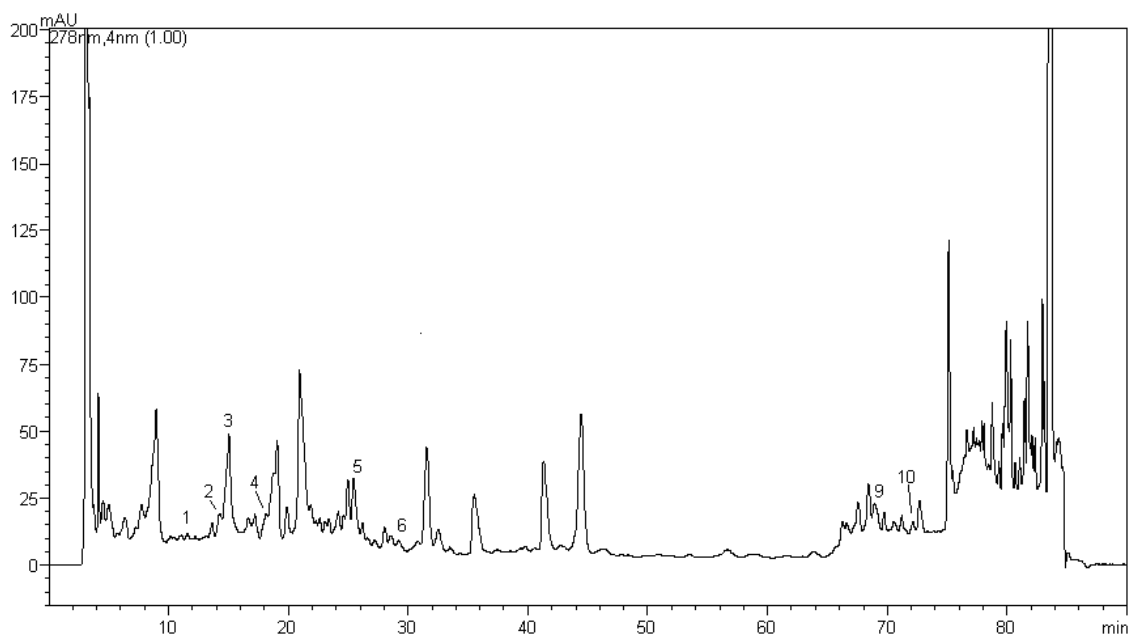


Figure 4b: HPLC chromatogram of *Isatis floribunda* methanolic root extract

Chlorogenic acid and many other polyphenol compounds are extensively used in medicine and industries such as the consumer chemicals and food industries (Kweon et al., 2001). Chlorogenic acid is used as various additives in beverage, cosmetics, tea products and foods as well as in medical substances (Jiang et al., 2000; Jin et al., 2005). A series of health benefits has been associated with the consumption of chlorogenic acid in the

last few years, such as reduction of the relative risk of cardiovascular disease, diabetes type 2, and Alzheimer's disease (Ranheim and Halvorsen, 2005; Salazar-Martinez et al., 2004; Lindsay et al., 2002), chlorogenic acid has also antibacterial and antiviral properties, and it is a natural antioxidant and anticancer agent (Jiang et al., 2001). It is also a promising precursor compound for the development of medicine that can resist the AIDS virus

HIV (Ma and Liang, 2003). Therefore, this potentially provides an alternative commercial source for chlorogenic acid and other valuable polyphenol compounds (Chen et al., 2004).

Emam and El-Moaty (2009) indicated the phenolic acid compounds of aqueous ethanol extracts of *Isatis microcarpa* Boiss. and *Pseuderucaria clavate* Boiss. & Reut. belong to the family Brassicaceae. As a result of this study, the phenolic acid compounds of *I. microcarpa* and *P. clavata* have been identified as ferulic acid, gallic acid, while the compound caffeic acid was identified in *I. microcarpa* only. Meanwhile chlorogenic acid and 2, 3-dihydroxy benzoic acid were detected and identified in *P. clavata* only.

Cytotoxicity analysis

In this study, the cytotoxic activity of an *I. floribunda* extract was investigated. The methanolic extract prepared from the root of *I. floribunda* was tested for cytotoxic activity on human breast cell (MCF12A). The methanolic root extract of *I. floribunda* had the highest phenolic content of all the extracts. The result of the cytotoxic activity is summarized in Figure 5. The methanolic root extract at a concentration of 250, 500, and 1000 $\mu\text{g/ml}$ showed 5.88, 6.27, and 13.25 % cytotoxic effect on human breast cell (MCF12A), respectively. These values show that the methanolic root extract has no cytotoxic effect.

El-Sharabasy and Naima (2013) examined the cytotoxic effect of chloroform aerial parts extract of *Zilla spinosa* (Brassicaceae) against human tumor cell lines culture HCT116 (colon carcinoma human cell line) and HepG2 (liver carcinoma human cell line). *Zilla spinosa* among these plants had an IC_{50} value of 16.10 $\mu\text{g/ml}$ on HCT116 and 15.70 $\mu\text{g/ml}$ on HepG2 cell line. In summary, the *I. floribunda* extract investigated in this study appears to show potential for cytotoxic activity. Future studies will investigate in greater detail the action of *I. floribunda* substances, and synthesize new and possibly more active derivatives for their pharmaceutical application.

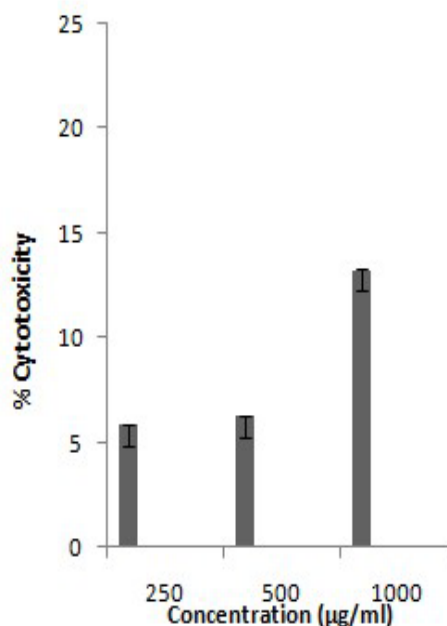


Figure 5: Cytotoxicity of the methanolic root extract from *Isatis floribunda* on human breast cell (MCF12A)

CONCLUSIONS

Due to their high antioxidant, antibacterial and antifungal activities, the plant flower and root extracts have promising potential as natural antioxidants in the food industries, in the preservation of food stuffs against a range of food related bacterial and fungal species or in the pharmaceutical and cosmetic industries and as an antibacterial-antifungal agent for human and fish. From this study it can be concluded that the tested *I. floribunda* root and flower extracts have potential antioxidant and antimicrobial properties. In general, the root extracts of *I. floribunda* showed better antimicrobial and antioxidant activities than the flower extracts. The methanolic root extract had the highest total phenolic content and showed better antioxidant activity than all the other extracts. This study clearly indicated that it is important to consider both the associated antioxidant activity and the phenolic content. Our data indicate that the plant extracts possess a good antioxidant activity and also show a broad spectrum of antimicrobial activity against food borne/clinical human and fish pathogens.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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