Original Article

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Investigation of antiaromatase activity using hepatic microsomes of Nile tilapia (*Oreochromis niloticus*)

Tanongsak Sassa-deepaeng[1], Wasana Chaisri[2], Surachai Pikulkaew[2,*], Siriporn Okonogi[3,*]

[1] *Nanoscience and Nanotechnology Program, the Graduate School, Chiang Mai University, Chiang Mai, Thailand;*
[2] *Department of Food Animal Clinic, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand;*
[3] *Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand.*

Summary **Microsomal aromatase enzymes of humans and rats have been used in antiaromatase assays, but enzyme activity is species-specific. The current study extracted hepatic microsomes of Nile tilapia (*Oreochromis niloticus*) to investigate and compare the antiaromatase activity of chrysin, quercetin, and quercitrin. This activity was evaluated using a dibenzylfluorescein (DBF) assay. Results revealed that the age and body weight of Nile tilapia affected the yield of extracted microsomes. Extraction of hepatic microsomes of Nile tilapia was most effective when using a reaction medium with a pH of 8.0. A DBF assay using Nile tilapia microsomes revealed significant differences in levels of antiaromatase activity for chrysin, quercetin, and quercitrin. Chrysin was the most potent aromatase inhibitor, with an IC_{50} of 0.25 mg/mL. In addition, chrysin is an aromatase inhibitor that also inhibits the proliferation of cancer cells. Hepatic microsomes of Nile tilapia can be used to investigate and compare the antiaromatase activity of different compounds.**

Keywords: Aromatase inhibitor, Nile tilapia, microsome, chrysin, pH effect

1. Introduction

Aromatase, a cytochrome P-450 enzyme consisting of a polypeptide chain of 503 amino acids, is key to estrogen biosynthesis (*1,2*). Aromatase is an essential component of the process of conversion of C-19 androgens to C-18 estrogens. Excessive aromatase activity can cause excessive synthesis of estrogen in breast tissue and lead to the development of breast cancer and increase the ability of immature breast tissue cells to strongly bind to carcinogens, decreasing their DNA repair capacity (*3*). Several recent studies have reported that a high concentration of estrogen promotes breast cancer (*4-6*). Therefore, inhibition of aromatase activity is a potential approach to the treatment of breast cancer (*7,8*).

Aromatase is involved in sex differentiation and ovarian development in many species of animals (*9,10*) since the enzyme plays a key role in estrogen biosynthesis. Estrogen influences ovarian differentiation in non-mammalian vertebrates, including amphibians (*11*), reptiles (*12*), birds (*13*), and fish (*14*). In aquaculture, aromatase is involved in sex control. Sex control in fish is important because one sex may grow faster than the other (*15*). Efforts to control unwanted breeding have focused on male fish rather than female fish because of their faster growth rate and more distinctive coloration. Therefore, research on mechanisms of fish reproduction has sought to develop agents with potent anti-aromatase activity, i.e. aromatase inhibitors, in order to decrease estrogen synthesis and masculinize fish to complete the sex shift to males (*16*).

Many aromatase inhibitors used in the treatment of breast cancer (*17*) and in the process of producing only male fish in aquaculture (*18*) have different levels of potency. Antiaromatase activity can be evaluated in order to search for potent aromatase inhibitors using a dibenzylfluorescein assay; microsomal aromatase enzymes are usually extracted from human placenta (*19,20*), the rat liver (*21*), or the rat ovaries (*22*).

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**Address correspondence to:*
Dr. Siriporn Okonogi, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand.
E-mail: okng2000@gmail.com

Dr. Surachai Pikulkaew, Department of Food Animal Clinic, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand.
E-mail: surapikulkaew@gmail.com

However, the applications of those enzymes are limited by animal species because the microsomes of each species are specific to certain animals. Nile tilapia (*Oreochromis niloticus*) is an economic teleost fish cultured in Thailand, and Nile tilapia has garnered attention worldwide as a food source. Male fish of this species have received greater attention than female fish because of their faster growth rate and larger size at the same age. Aromatase from Nile tilapia microsomes is therefore an essential component of a search for potent aromatase inhibitors. To the extent known, no study has used Nile tilapia microsomes for this purpose. The current study extracted microsomal aromatase from the liver of Nile tilapia and it tested compounds for their anti-aromatase activity. The effects of body weight and age of Nile tilapia on microsome yield and the effect of pH on the aromatase activity of the microsomes obtained were investigated. Three active compounds, chrysin, quercetin, and quercitrin, were previously reported to have antiaromatase activity (*23,24*). However, the antiaromatase activity of these compounds has yet to be compared. The current study is the first to examine the antiaromatase potency these compounds and to investigate their inhibition of the proliferation of breast cancer cells.

2. Materials and Methods

2.1. *Materials*

Chrysin, quercetin, quercitrin, dibenzylfluorescein, and thiazolyl blue tetrazolium blue (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium chloride (KCl) and methanol were obtained from Merck (Darmstadt, Germany). Acetic acid was obtained from RCI Labscan (Bangkok, Thailand). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from AMRESCO (Solon, OH, USA). Nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from Tocris (Ellisville, MO, USA). Antibiotic-antimycotic Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL Life Technologies (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) was obtained from Amresco (Salon, OH, USA). All solvents were of analytical grade. Ninety-six-well plates were obtained from Costar (Cambridge, MA, USA). Ultra-clear centrifuge tubes were purchased from Beckman Coulter Inc. (Brea, CA, USA).

2.2. *Microsome preparation*

This study was approved by the Animal Experimentation Committee, Faculty of Veterinary Medicine, Chiang Mai University, Thailand. Hepatic microsomes from Nile tilapia were prepared as previously described (*25*) with some modifications. Briefly, female fish were anesthetized by hypothermic shock in an ice bucket and then killed by decapitation. The liver was quickly removed and placed in 1.15% KCl solution. The liver was then chopped into small pieces and flash frozen in liquid nitrogen. The three volumes (w/v) of chilled 1.15% KCl solution was added to a homogenizer for 30 min. Homogenized liver samples were pooled and centrifuged at 10,000× g, 4°C for 30 min. The lipid layer was removed and the supernatant was subjected to ultracentrifugation at 105,000× g, 4°C for 1 h. The liquid solution containing microsomes was lyophilized for further experiments.

2.3. *Scanning electron microscopy (SEM)*

SEM was performed to observe the morphology of fish microsomes after lyophilization. Samples were placed in a standard sample holder and examined under a scanning electron microscope (Phenom Pure Desktop SEM, Phenom-World BV, Eindhoven, Netherlands).

2.4. *Protein determination*

Total protein content of the obtained microsomes was determined using NanoDrop™ 2000 (Thermo Scientific, USA). The instrument was first calibrated using distilled water and then 2 µL of a 2-fold serial dilution of the sample was placed on the pedestal. The proteins contain aromatic side chains that influence light absorption at 280 nm. The protein concentration was automatically calculated with the NanoDrop™ 2000 software.

2.5. *Effect of pH on the aromatase activity of fish microsomes*

The aromatase activity of hepatic Nile tilapia microsomes was quantified by measuring the fluorescent intensity of fluorescein, a dibenzylfluorescein product, in black 96-well microplates as described previously (*26*) with some modifications. Briefly, Nile tilapia microsomes were resuspended in water and adjusted with HEPES at a pH of 7.2, 7.4, 7.6, 7.8, or 8.0 to obtain a microsomal protein concentration of 350 µg/mL. Precisely 100 µL of the mixture was added to a 96-well plate (Corning™ 96-Well Black, Costar, Cambridge, MA, USA), and the plate was incubated at room temperature for 60 min. Afterwards, 10 µL of dibenzylfluorescein was added, and the plate was further incubated for 30 min at room temperature. Precisely 10 µL of 0.3 mM NADPH was added to the mixture and dibenzylfluorescein was allowed to generate a fluorescent product. The signal was measured at 485 nm (excitation) and 530 nm (emission). The experiment was performed in triplicate.

2.6. *Antiaromatase activity assay*

An antiaromatase activity assay was performed in the

similar manner as described in Section 2.5. Briefly, Nile tilapia microsomes were resuspended in water and adjusted to the desired microsomal protein concentration with HEPES at a pH of 8.0. Precisely 10 µL of the test compound (chrysin, quercetin, or quercitrin) at the desired concentration was added to 96-well plates pre-incubated with 100 µL of 350 µg/mL microsomal protein for 60 min at room temperature. Afterwards, 10 µL of dibenzylfluorescein was added and plates were further incubated for 30 min at room temperature. Precisely 10 µL of 0.3 mM NADPH was added to the mixture and dibenzylfluorescein was allowed to generate a fluorescent product. The signal was measured at 485 nm (excitation) and 530 nm (emission). The experiment was performed in triplicate. Concentrations of the aromatase inhibitors used were varied to generate dose response curves and IC_{50} values were calculated.

2.7. *Anti-proliferative activity assay*

Two cancer cell lines, HepG2 human hepatoma and MCF-7 human breast cancer cells from the American type Culture Collection (Rockville, MD, USA), were cultured in a 75-cm^2 sterile flat-bottomed bottle containing DMEM supplemented with 10% FBS and 10% of antibiotic-antimycotic in a humidified atmosphere of 5% CO_2 at 37°C until confluent. An anti-proliferative activity assay was performed using the method previously described (*27*) with some modifications. Cells (5×10^5 cells/well) were seeded in 96-well plates (Thermo Scientific™ Nunc™ MicroWell™ Plates with Nunclon™ Delta Surface, Nunc™, Nalge Nunc, Denmark). Various concentrations of the tested sample were added and the plates were incubated at 37°C for 20 h. DMSO was used as a vehicle control. Afterwards, the medium was drained and 100 µL of (5 mg/mL) MTT was added to each well. Plates were further incubated at the same temperature for 4 h. Finally, the cells were dissolved by adding DMSO before measuring the absorbance of the resulting purple solution at 570 nm with a microplate reader (Tecan Sunrise, Software Magellan V.5.00, Switzerland). Absorbance at 630 nm served as a reference. All treatments were done in quadruplicate.

2.8. *Statistical analysis*

The experiment was performed in triplicate. Data are presented as the mean ± S.D. Statistical analysis was performed using ANOVA and p values less than 0.05 were considered significant.

3. Results

3.1. *Microsome preparation and SEM images*

The outer appearance of the liver of Nile tilapia and the homogenized liver is shown in Figure 1(a) and 1(b), respectively. After the first centrifugation at 10000× g, the fractions of unbreakable cells, nuclei, and mitochondria were precipitated while the soluble enzymes and fragmented endoplasmic reticulum containing microsomes remained in the supernatant. After ultracentrifugation at 105,000× g, the microsomes appeared as a gel-like mass at the bottom of the tube that was reddish color, as shown in Figure 1(c). Under SEM, fish microsomes had an irregular shape as shown in Figure 1(d). The yields of microsomes are shown in Table 1. Results suggested that a higher yield of fish microsomes can be extracted from adult fish with a heavier weight.

3.2. *Effect of pH on the aromatase activity of Nile tilapia microsomes*

The aromatase activity of Nile tilapia microsomes at different pHs was examined. As shown in Figure 2, results indicated that fish microsomes dissolved in a

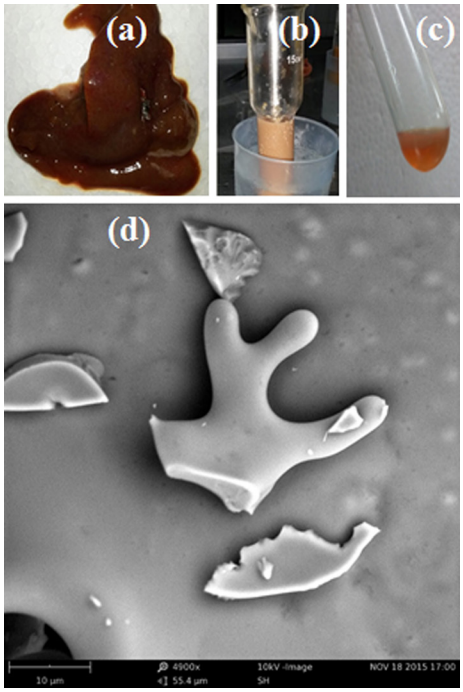

Figure 1. The outer appearance of (a) the liver of Nile tilapia, (b) homogenized liver of Nile tilapia, (c) extract from the liver of Nile tilapia microsomes, and (d) SEM image of Nile tilapia microsomes.

Table 1. Effects of age and body weight of Nile tilapia on the yield of microsomes (*n* = 3)

Body weight (g)	Age (month)	Extracted microsomes (mg)
150-160	3-4	0 ± 0
170-180	3-4	0 ± 0
190-200	3-4	0 ± 0
220-230	5-6	27 ± 6
240-250	5-6	41 ± 16
300-320	5-6	198 ± 33

medium with a pH of 7.2-7.8 had significantly less aromatase activity than microsomes dissolved in a medium with a pH of 8.0. At microsome concentrations of 21.9-175 µg/mL, the activity of the enzyme was independent of its dose. Dose-dependent activity was noted only when the enzyme was in medium with a pH of 8.0.

3.3. *Antiaromatase activity*

Three active compounds, chrysin, quercetin, and quercitrin, were used in the current study because they have similar structures, as shown in Figure 3. Results indicated that all 3 assayed compounds inhibited the aromatase activity of Nile tilapia microsomes in a dose-dependent manner. However, that level of inhibition differed, as shown in Figure 4. Chrysin had the most potent antiaromatase activity, with an IC_{50} of 0.25 mg/mL. This activity was approximately 2 times that of quercetin (0.44 mg/mL). Quercitrin had the lowest activity, with an IC_{50} that could not be detected.

3.4. *Anti-proliferative activity*

Chrysin was chosen to determine whether it inhibited the proliferation of HepG2 human hepatoma and MCF-7 human breast cancer cells. Results indicated that chrysin significantly inhibited the proliferation of both types of cells in a dose-dependent manner. As shown in Figure 5, treatment with chrysin for 20 h dramatically decreased the viability of cancer cells as the concentration of chrysin increased. The anti-proliferative activity of chrysin against both types of cancer cells differed slightly. The IC_{50} of chrysin with respect to HepG2 cells was 8.76 ± 0.79 µg/mL while its IC_{50} with respect to MCF-7 cells was 9.77 ± 1.21 µg/mL.

4. Discussion

During sex differentiation, overexpression of estrogens or androgens in genetically male or female individuals, respectively, is known to transform the sexual phenotype to the opposite gender (*9,10*). In fish, aromatase enzymes can irreversibly convert androgens into estrogens

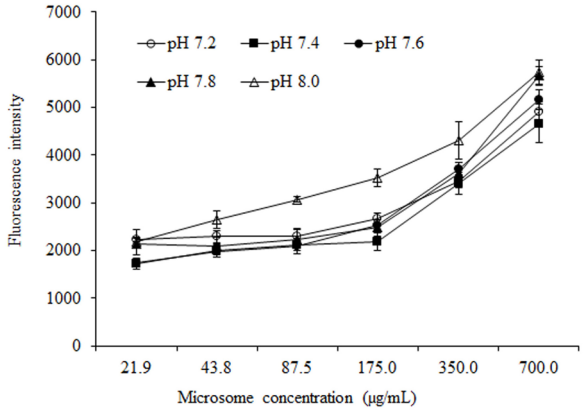

Figure 2. Effect of pH on aromatase activity of Nile tilapia microsomes measured with fluorescence intensity.

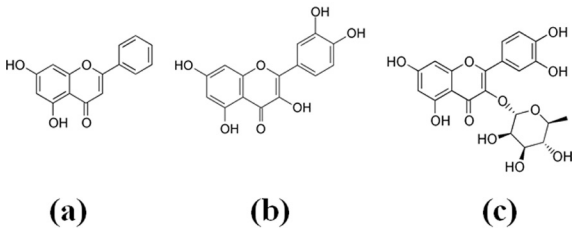

Figure 3. Chemical structure of (a) chrysin, (b) quercetin, and (c) quercitrin.

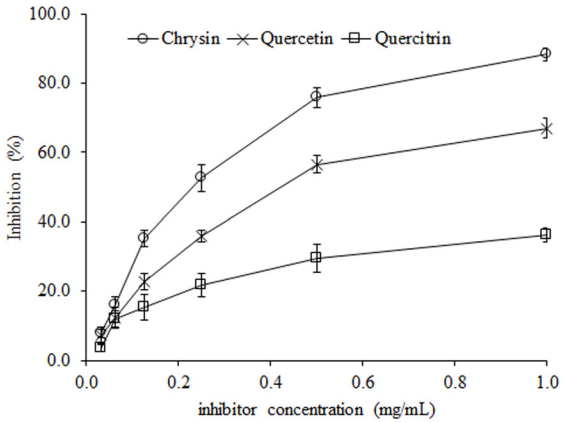

Figure 4. Inhibition of aromatase by the tested compounds.

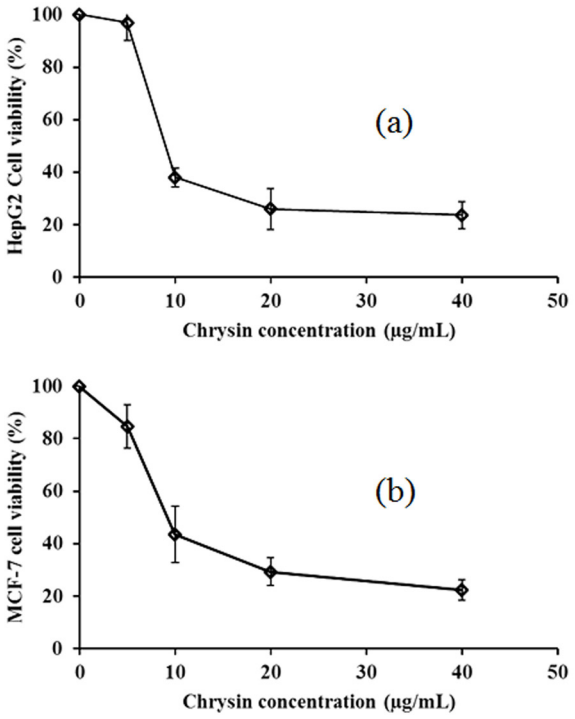

Figure 5. Anti-proliferative activity of chrysin against (a) HepG2 and (b) MCF-7 cancer cells.

(*14*). Hence, aromatase is essential to establishing the final sex phenotype of fish. A high level of expression of aromatase enzymes is associated with ovarian differentiation while a low level is associated with testicular differentiation. Male Nile tilapia grow at a rate 20% faster than female fish (*15*). Therefore, male fish need to be produced when aquaculturing Nile tilapia. For years, aromatase inhibitors have been used as sex inversion agents to produce all-male populations of fish since they can significantly reduce the biosynthesis of estrogen (*16,18*). The antiaromatase activity of different compounds should be compared in order to search for the most potent aromatase inhibitor. Microsomes of some fish species such as rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*), and seabream (*Sparus aurata*) have previously been used to assay antiaromatase activity (*28,29*). However, Nile tilapia microsomes have not been used for this purpose. The current study therefore sought to investigate whether the hepatic microsomes of Nile tilapia could be a source of aromatase enzymes and whether they would be suitable for testing or comparing the antiaromatase activity of different compounds.

Microsomes are vesicle-like fractions of the endoplasmic reticulum (ER) found in healthy living cells. Microsomes can feasibly be isolated when eukaryotic cells are dissolved and centrifuged (*30*). Many organs of fish have been used to extract microsomes. However, the parts that are normally rich in microsomes are the liver (*28*), brain (*31*), and ovaries (*32*). The liver was selected for the source of microsomes in the current study because of its larger size and ease of isolation. To the extent known, the current study is the first to extract hepatic microsomes of Nile tilapia for use in an assay of antiaromatase activity.

As expected, results indicated that the hepatic microsomes of Nile tilapia obtained were reddish in color due to the presence of heme (*33*). Fish of two age groups, 3-4 months and 5-6 months, were compared. Results clearly indicated that the older fish yielded more microsomes. Moreover, fish in the same age group but with different weights yielded different amounts of microsomes. Fish with a heavier weight yielded a larger amount of microsomes. Based on the fish body weight, the percent yield from fish with the lightest average weight (225 g) was only 0.12% whereas that from the heaviest average weight (310 g) was 0.63%. Thus, the percent yield was almost three times greater. The yield of hepatic microsomes from Nile tilapia was influenced by the age and body weight of fish.

The pH of fish blood is reported to fluctuate due to species variation, environment, electrolytes, temperature, and stress (*34*). Moreover, the pH of a reaction medium occasionally plays a key role in many reactions (*35,36*). The current study found that pH played an important role in the aromatase activity of Nile tilapia microsomes. The pH of the medium was adjusted to 8.0, which was the most effective pH, since aromatase enzymes behaved in a dose-dependent manner. Therefore, this pH was chosen for further study of the antiaromatase activity of different compounds.

Three compounds, chrysin, quercetin, and quercitrin, were tested for their antiaromatase activity. These compounds have the same core structure of 5,7 dihydroxy flavone as is found in chrysin. Addition of two hydroxyl groups to chrysin at C3' and C4' yields quercetin, which is the aglycone part of many important flavonoid glycosides. Substitution of deoxy sugar rhamnose at C3 of quercetin yields quercitrin, a glycoside. Comparison of the antiaromatase activity of these three compounds in hepatic microsomes from Nile tilapia revealed different levels of antiaromatase activity. Results also indicated the effects that the structure-activity relationship of the compounds had. Substitution of OH group to chrysin, such as in quercetin molecule, caused significant decrease in antiaromatase activity. In addition, consideration between a flavonoid quercetin and its glycoside formed from deoxyrhamnose substitution, quercitrin, resulted that the aglycone quercetin possessed higher activity than its corresponding glycoside, quercitrin. Since the active site of aromatase is highly hydrophobic (*37*), the compounds with alkyl or higher hydrophobic aromatic groups could have higher affinity to this enzyme leading to a higher efficiently block enzyme activity. Substituting OH groups or sugar moieties in chrysin can decrease its hydrophobicity. Chrysin had the highest hydrophobicity and most potent antiaromatase activity, so chrysin was investigated for its inhibition of the proliferation of cancer cells.

A study has reported that estrogen-dependent breast carcinogenesis can be treated by blocking estrogen activity using aromatase inhibitors (*38*). Recent studies have indicated that some aromatase inhibitors inhibit the proliferation of cancer cells as well (*39,40*). Chrysin had the most potent antiaromatase activity among the compounds tested in the current study, so chrysin was selected to investigate its ability to inhibit the proliferation of MCF-7 human breast cancer cells. To confirm a compound's ability to inhibit the proliferation of cancer cells, HepG2 human hepatoma cells were also used. Results indicated that chrysin efficiently inhibited the growth of both cancer cell lines but at different levels. Chrysin has been reported to have anti-inflammatory action (*41*). The current study used hepatic microsomes from Nile tilapia as a novel source of test enzymes. Results indicated that chrysin inhibited the proliferation of MCF-7 and HepG2 cancer cells and that it had potent antiaromatase activity.

In conclusion, the current study indicated that hepatic microsomes from Nile tilapia are a high specific means of investigating the activity of aromatase inhibitors *in vitro*. The age and body weight of Nile tilapia significantly affected the yield of the extracted microsomes. The aromatase activity of Nile tilapia

microsomes was influenced by the pH of the reaction medium. A pH of 8.0 is the most effective pH at which to assay the antiaromatase activity of different compounds. The antiaromatase activity of chrysin, quercetin, and quercitrin differed. Chrysin was the most potent aromatase inhibitor. Results confirmed that chrysin is an aromatase inhibitor and indicated that chrysin inhibits the proliferation of cancer cells as well.

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