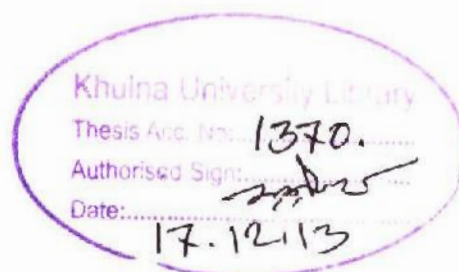


**AVAILABILITY OF ANTIOXIDANT IN FISHES OF DIFFERENT
FEEDING HABITS AVAILABLE IN BANGLADESH**



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Fisheries and Marine Resource Technology Discipline

Khulna University

Khulna, Bangladesh

April, 2011

**AVAILABILITY OF ANTIOXIDANT IN FISHES OF DIFFERENT
FEEDING HABITS AVAILABLE IN BANGLADESH**

A Thesis

By

MD. ABIR HASAN

Examination Roll No: MS-100614

Session: 2009-2010

A project thesis submitted in partial fulfillment of the requirements
for the degree of Masters of Science in Fisheries and Marine Resource Technology Discipline

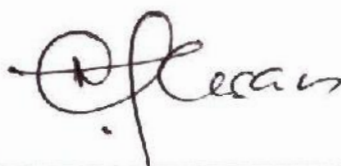
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April, 2011

DECLARATION

AVAILABILITY OF ANTIOXIDANT IN FISHES OF DIFFERENT FEEDING HABITS AVAILABLE IN BANGLADESH

The results presented in the thesis with the above mentioned title are entirely of the candidate's own investigations and no part of the results has been accepted for any degree nor is it being concurrently submitted for any degree.

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PULOK & PROSEN

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Abstract

Free radical scavenging activity in the muscle of fifteen species of different feeding habit fishes were evaluated using both qualitative and quantitative method. Thin layer Chromatography (TLC) method was used for qualitative analysis. DPPH scavenging analysis reveals IC₅₀ of about 1266.02 µg/ml (*Rhinomugil corsula*), 963.46 µg/ml (*Hypophthalmichthys molitrix*), 267.70 µg/ml (*Puntius ticto*), 965.75 µg/ml (*Labeo bata*), 1678.96 µg/ml (*Puntius gonionotus*), 327.69 µg/ml (*Channa striatus*), 346.32 µg/ml (*Channa punctatus*), 2138.52 µg/ml (*Notopterus chitala*), 1757.17 µg/ml (*Notopterus notopterus*), 1170.29 µg/ml (*Mystus aor*), 600.89 µg/ml (*Pampus chinensis*), 872.19 µg/ml (*Scatophagus argus*), 397.72 µg/ml (*Anabas testudineus*), 154.45 µg/ml (*Ctenopharyngodon idella*) and 530.69 µg/ml (*Mackerel sp*). In both the qualitative and quantitative method, ascorbic acid was used as positive control. Generally, herbivore fishes are supposed to get high antioxidant component as they consume mainly plant material and plants are the main source of antioxidant. But, in this experiment, no strong relationship was found among the feeding habit of fish and antioxidant activity. Two herbivore, two carnivore and one omnivorous fishes showed high antioxidant activity in this test. Being very common and rich in antioxidant, *Channa striatus*, *Puntius ticto*, *Anabas testudineus*, *Ctenopharyngodon idella* and *Channa punctatus* can be used in our daily diet.

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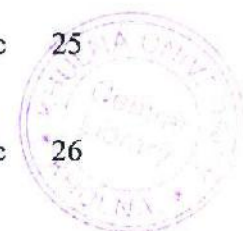
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List of Abbreviations and non English word

Abbreviation	Elaboration
ATF	A-tocopherol
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CD	Crohn disease
DHA	Docosaehaenoic acid
DPPH	2,2-diphenl-1-picryl hydrazyl
EPA	Eicosapentaenoic acid
<i>et al.</i>	et alli: Latin word meaning “and another”
IC	Inhibitory concentration
P	Page
PBMC	Peripheral blood mononuclear cell
PG	Propyl gallate
PUFA	Polyunsaturated fattyacid
ROS	Reactive oxygen species
TBHQ	Terf-butylhydroquinone
TMAO	Trimethylamine <i>N</i> -oxide
TLC	Thin layer chromatography
µg	Micro-gram
nm	nanometer
°C	Degree centigrade

CHAPTER 1

INTRODUCTION



Chapter One

Introduction

All aerobic organisms require protection against deleterious effect of oxygen radical produced from metabolic oxidation reaction in the cell. These reactive oxygen species (ROS) are capable of damaging lipids, DNA, nucleic acid and protein (Di Giulio, 1991; Tyrrell, 1991). Unsaturated membrane lipids are the main cellular targets of ROS damage, oxidation of which impairs normal metabolic functions (Slater, 1984). Living organisms are usually protected from reactive oxygen species by several defense mechanisms, including antioxidant enzymes and low molecular weight antioxidants (Pascual *et al.*, 2003). Biochemical defenses against reactive oxygen species include hydrophilic (glutathione, ascorbic acids and uric acids) and lipophilic compounds (vitamin E, ubiquinol and retinol) that scavenge radical species. In contrast to these low molecular weight scavengers, antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase can specifically remove active species leading to the initiation of lipid per-oxidation and oxidation of other cellular biomolecules (Ahmad, 1995).

Current sources of natural lipophilic antioxidants include vegetables, plants and animal tissues (Ruperez *et al.*, 2001). The most commonly used synthetic antioxidants to preserve food are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), *tert*-butylhydroquinone (TBHQ) and α -tocopherol (ATF). However, synthetic antioxidants have been implicated for several diseases such as liver damage and carcinogenesis (Gulcin *et al.*, 2004). Therefore, the utilization of more effective antioxidants of natural origin is needed.

Foods high in antioxidants are the healthiest and the most nutritious. 'Antioxidant' is a name given to a group of substances that protect human cells from damage, usually caused by the formation of free radicals. The free radicals can impair our immune system considerably. They are the atoms that are formed during the natural metabolic activities of our body.

Antioxidant is the type of molecule that neutralizes harmful compounds called free radicals that damage living cells, spoil food, and degrade materials such as rubber, gasoline and lubricating oils. Antioxidant can take the form of enzymes in the body, vitamin supplements

or industrial additives. They are routinely added to metals, oils, food stuff and other materials to prevent free radical damages.

Free radicals are produced under certain environmental conditions and during normal cellular functions in the body. These molecules are lack of an electron, giving them a net negative charge. To neutralize this charge, free radicals try to steal an electron from or donate an electron to a neighboring molecule. This process called oxidation creates a new free radical from the neighboring molecule. The newly created free radical, in turn, searches out another molecule and steals or donates an electron, setting off a chain reaction that can damage hundreds of molecules.

Free radicals are atoms or groups of atoms with an odd (unpaired) number of electrons and can be formed when oxygen interacts with certain molecules. Once formed, these highly reactive radicals can start a chain reaction, like dominoes. Their chief danger comes from the damage they can do when they react with important cellular components such as DNA, or the cell membrane. Cells may function poorly or die if this occurs. To prevent free radical damage, body has a defense system of antioxidants.

It is ironic that oxygen, an element indispensable for life can, under certain situations, have severely deleterious effects on the human body. Most of the potentially harmful effects of oxygen are due to the formation and activity of a number of chemical compounds, known as reactive oxygen species, which have a tendency to donate oxygen to other substances. Many such reactive species are free radicals and have a surplus of one or more free-floating electrons rather than having matched pairs and are, therefore, unstable and highly reactive. Types of free radicals include the hydroxyl radical (OH.), the superoxide radical (O₂), the nitric oxide radical (NO.) and the lipid per-oxyl radical. Free radicals and other reactive oxygen species are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants and industrial chemicals.

Free radical formation occurs continuously in the cells as a consequence of both enzymatic and non-enzymatic reactions. Free radicals also arise in non-enzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing radiations.

Some internally generated sources of free radicals are (Langseth, 1996):

- mitochondria
- phagocytes
- xanthine oxidase
- reactions involving iron and other transition metals
- arachidonate pathways
- peroxisomes
- exercise
- inflammation and
- ischaemia/reperfusion.

Some externally generated sources of free radicals are (Langseth, 1996):

- cigarette smoke
- environmental pollutants
- radiation
- ultraviolet light
- certain drugs, pesticides, anesthetics and industrial solvents and
- Ozone.

A well accepted fact is that in human being the incidence of diseases increases with the advancing age. A plausible explanation for the association of age and disease is based on the implications of free radical reactions in the pathogenesis of several disorders.

Free radical reactions are expected to produce progressive adverse changes that accumulate with age throughout the body. Such "normal" changes with age are relatively common to all. However, superimposed on this common pattern are patterns influenced by genetics and environmental differences that modulate free radical damage. These are manifested as diseases at certain ages determined by genetic and environmental factors. Cancer and atherosclerosis, two major causes of death, are due to salient "free radical" diseases. Cancer

initiation and promotion is associated with chromosomal defects and oncogene activation. It is possible that endogenous free radical reactions, like those initiated by ionizing radiation, may result in tumour formation. The highly significant correlation between consumption of fats and oils and death rates from leukaemia and malignant neoplasia of the breast, ovaries and rectum among persons over 55 years may be a reflection of greater lipid per-oxidation (Lea, 1966). Studies on atherosclerosis reveal the probability that the disease may be due to free radical reactions involving diet-derived lipids in the arterial wall and serum to yield peroxides and other substances (Harman, 1992).

The results of recent epidemiological studies have suggested that several carotenoids may be associated with reduced cancer risk. The evidence is much stronger in the case of β -carotene. Numerous studies have reported reduced risk of certain cancers with a high dietary intake of β -carotene. The β -carotene may alter the way cancer cells communicate with normal cells. Cancer cells multiply rapidly when growth regulating signals from adjoining normal cells cease. Carotenoids act as "arbitrators" by enhancing the communication between the two cells and helping the cancer cells to act more like normal cells. Furthermore, if cancer cells receive the growth regulating signals, tumor growth may be suppressed.

Several clinical trials showed regression in precancerous lesions of the cervix and the lung as well as the oral cavity with the administration of β -carotene. Studies by Zheng (1995) and Negri (1996) have reported that dietary antioxidants, especially β -carotene, may be important in the prevention of stomach and breast cancer. Whether the protective effects are attributable to the antioxidant nutrients themselves or to other substances found in the same foods, however, needs to be verified.

Eating antioxidants regularly ensures healthy eyesight. It can also slow down the aging process, as it protects the cells from free radical damage, which will be considered to be the main cause of aging.

Free radicals are harmful to important molecules, such as proteins, enzymes and even DNA. Free radicals damage cells, which causes the body's organs and systems to lose functional capacity. Free-radical damage also impairs protein synthesis, which is vital to tissue regeneration and repair.

To minimize the oxidative effect of free radicals enough antioxidants are needed that may come from day to day intake of food. Supplements can also be used to replenish the increasing demand of antioxidant at our middle and later part of life. Along with vegetables and fruits, fish could be a good source of antioxidant. Consumption of fish having the antioxidant potential can certainly fulfill the requirement of antioxidant.

Crohn disease (CD) is associated with osteoporosis and other extra-intestinal manifestations that might be mediated by cytokines from circulating (peripheral blood) mononuclear cells (PBMCs). Fish oil rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reduces disease activity in patients with CD with raised laboratory markers of inflammation and in healthy subjects alters PBMC function. Crohn disease (CD) is an inflammatory condition of the gastrointestinal tract associated with extra-intestinal manifestations (Greenstein *et al.*, 1976) that include altered bone turnover (Robinson *et al.*, 1998 and Bjarnason *et al.*, 1997) and poor nutritional status (Harries *et al.*, 1982). The mechanism through which localized inflammation in the CD-affected gastrointestinal tract is associated with systemic manifestations remains unconfirmed but can involve activated mononuclear cells that migrate by way of the peripheral blood (PBMCs) to bone, muscle, liver, and other tissues. PBMCs release inflammatory mediators (Ambrosone, 1995; Stephens, 1996), including tumor necrosis factor α (TNF- α), prostaglandin E₂ (PGE₂), and interferon γ (IFN- γ), with independent effects on bone turnover (Katagiri *et al.*, 2002), (Horowitz *et al.*, 2001) or nutritional status (Beisel and Herman, 1995). Altered production of cytokines by PBMCs is noted in patients with CD when compared with healthy subjects (Elsasser-Beile *et al.*, 1994; Sasaki *et al.*, 1992). Fish oil is a rich source of EPA and DHA and, in healthy subjects, alters the n-3 PUFA composition of PBMCs (Yaqoob *et al.*, 2000) and their production of inflammatory mediators, including TNF- α , IFN- γ , and PGE₂ (Calder, 2001).

Dietary supplementation with fish oil plus antioxidants is associated with modified PBMC composition and lower production of PGE₂ and IFN- γ by circulating monocytes or macrophages.

Science has been looking at antioxidants and their role in everything from preventing cancer and heart disease to boosting the immune system and slowing the aging process. Antioxidants provide a layer of protection for the cells and tissues of the body, just as a thick

coat of wax helps protect a car's finish. Specifically, antioxidants protect against free radical damage. Antioxidant-rich food may help prevent various cancers, heart disease, and diseases of aging. Vitamins C and E, carotenoids (including beta-carotene), and the mineral selenium are all powerful antioxidants found in food. Vitamin C, a water-soluble vitamin, is also known as ascorbic acid. Most of the vitamin C in the diet (90%) comes from fruits and vegetables. However, since vitamin C is water soluble, cooking can destroy the vitamin C in a food. Vitamin E, also known as alpha tocopherol, is a fat. Because vitamin E is found in oils, people who follow a low-fat diet may not get enough. Typically, beta-carotene is a conditionally essential nutrient, but when one's intake of vitamin A is low, beta-carotene becomes an essential nutrient, meaning that it must be obtained from food and cannot be manufactured by the body. Selenium (Fish & shellfish, red meat, grains, eggs, chicken and garlic) is an essential trace mineral (trace minerals are needed only in small amounts). The amount of selenium found in food is directly related to the amount of selenium in the soil in which the food was grown.

Fish and sea foods may contain several other antioxidative compounds than hitherto mentioned. Seaweeds are known to contain antioxidative compounds (Ramarathnam *et al.*, 1995) and some of the active compounds have been identified as compounds related to chlorophyll a and bromophenols (Sakata *et al.*, 1994). Fish and marine animals feeding directly or indirectly on algae may also contain these antioxidants. The edible part of short necked clam was thus found to contain several antioxidative compounds related to chlorophyll a, pyropheophorbide A and purpurin (Sakata *et al.*, 1994). Scallop, oyster and abalone have also been shown to contain compounds related to chlorophyll a (Sakata *et al.*, 1994). Antioxidative compounds have also been found in shrimp. Pasquel and Babitt (1991) isolated and characterized an antioxidative compound in shrimp and suggested that it was a polyhydroxylated derivative of an aromatic amino acid. Seymour *et al.* (1996) isolated and characterized a natural antioxidant from shrimp shell waste, which results indicated a phenolic compound with a primary amino group.

Few studies on the lipophilic antioxidants in fish species were reported in the previous studies particularly in the marine fish species (Giardina *et al.*, 1997; Erickson, 1992). However, no study has been carried out before on the antioxidant activity of the target fish

species used in this study except *Anabas testudineus*, *Channa striatus* and *Puntius gonionotus*. (Lokman, 2006).

In this study, the antioxidant activity of fifteen different species of fish of different feeding habits has been compared. The fishes were collected and extracts of fish muscle samples were prepared with the help of ethanol. Then, qualitative and quantitative analysis was done by Thin Layer Chromatography (TLC) and DPPH free radical scavenging way respectively to ensure the presence of antioxidants and their inhibitory concentration (IC_{50}) was determined.

The five potential fish species with high antioxidant activities identified in this study were *Anabas testudineus*, *Puntius ticto*, *Channa striatus*, *Channa punctatus* and *Ctenopharyngodon idella*.

Fish species, examined, which were found to have high antioxidant activities can be recommended as part of the diet as they may be able to protect the human body from free radicals and retard the progress of many chronic diseases. The fish extracts found to have antioxidants in this study also can be used as accessible source of natural antioxidants to replace synthetic antioxidants, as possible food supplement as well as in pharmaceutical applications.

Therefore, the objective of this study was:

- To determine the antioxidant activity in the fish muscle of different feeding habits.

CHAPTER 2

MATERIALS AND METHODS

Chapter Two

Materials and Methods

Research Area and Period

This research was carried out in the Biochemistry and molecular genetics lab of Fisheries and Marine Resource Technology Discipline, Khulna University from December, 2010 to March, 2011.

Sample collection

Antioxidant activity of fifteen different fishes depending on their feeding habits was measured in this study. Five herbivore, five carnivore and five omnivore fishes were collected from different local fish market of Khulna. For this research, the following fish samples were used.

Herbivore	Carnivore	Omnivore
<i>Ctenopharyngodon idella</i> (Grass Carp)	<i>Channa striatus</i> (Shol)	<i>Pampus chinensis</i> (Rupchanda)
<i>Hypophthalmichthys molitrix</i> (Silver carp)	<i>Channa punctatus</i> (Taki)	<i>Scatophagus argus</i> (Bishtara)
<i>Puntius ticto</i> (Titputi)	<i>Notopterus chitala</i> (Chital)	<i>Mackerel sp</i> (Konkon)
<i>Puntius gonionotus</i> (Thai Shorputi)	<i>Notopterus notopterus</i> (Foli)	<i>Anabas testudineus</i> (Koi)
<i>Labeo bata</i> (Bata)	<i>Mystus aor</i> (Ayre)	<i>Rhinomugil corsula</i> (Khorsula)

Test methods for Antioxidant activity

Antioxidant activity of the selected fish species were done by the following methods:

- Qualitative test (by TLC method)
- Quantitative test (by DPPH free radical scavenging method)

Apparatus used in this study for qualitative test:

- a. TLC jars with closure
- b. TLC plates containing fluorescent material
- c. Fine capillary tube as spotter
- d. Filter paper
- e. Glass rod
- f. Glass watch
- g. Spatula
- h. Volumetric flask
- i. Spirit lamp
- j. UV detector with short (254 nm) and long (360 nm) wavelength
- k. Spray gun
- l. Hot plate and Magnetic Stirrer; model: VS-130SH
- m. Filter paper
- n. Dryer

Reagents for qualitative test:

1. Chloroform (CHCl_3)
2. Methanol (CH_3OH)
3. N-hexane
4. Acetone
5. DPPH (0.04 % w/v) solution in ethanol
6. Distilled water.

Apparatus used for quantitative test (DPPH Scavenging capacity method):

- Test tubes
- Beakers
- Pipette

- UV spectrophotometer; model: HITACHI, U-2910
- °C • Electronic balance; model: SHIMADZU-AUY220
- Conical flask
- Shaking water bath; model: VS-1205SW1
- °C • Homogenizer; model: WiseTis-HG-15D

Reagent used for quantitative test:

- Ethanol
- 0.004% DPPH ethanolic solution
- Ethanolic extract of selected fishes
- Ascorbic acid (as '+' ve control)

Extract preparation

The fresh muscle of 100-150 gram was homogenized in ethanol and then filtered it. The filtrate was kept in the shaking water bath at 40°C for drying.

Procedure for Qualitative test**TLC plate preparation**

Commercially available TLC plate was cut into appropriate size.

Sample application

A fine capillary tube was used as spotter for sample application in the TLC plates. A very little amount of fish extract was taken in a small vial and diluted suitably with ethanol.

The sample was spotted in uniform size on TLC plates.

The sample was applied several times in each spot to get better chromatogram.

Each spot was dried before applying another volume of solution to the same spot.

Solvent front was scratched by a knife.

Development of chromatogram:

- The chromatogram was developed by ascending technique.
- A little amount of ethanolic extract of fish was dissolved in ethanol and diluted suitably and was applied on the TLC plate by spotter. Three plates were spotted in such a manner.
- Chromatogram were developed by using three solvent system comprising polar (CHCl_3 : CH_3OH : H_2O =40:10:1), medium polar (CHCl_3 : CH_3OH =5:1) and non-polar (n-Hexane: Acetone=2:1).
- These plates were then kept in three different jars containing three different solvent systems.
- A Filter paper was kept into the jar by wetting with solvent systems to keep the jar saturated and the jar was closed tightly.
- The plates were placed in the jar in such a manner that the sample spots were just above the solvent surface.
- The solvents were allowed to move up the plates by capillary action until they have traveled a distance of about 7-8 cm from the point of application of the sample on a 10 cm plate.
- The plates were then removed from the jar and dried with a current of air suitably.
- The three plates from three different solvent systems were then observed visually under UV light and various regions were marked.
- After these plates were sprayed with 0.02% DPPH and dried on hot plate at 110-120°C for some time.

For quantitative (DPPH scavenging capacity assay method):

The anti-oxidant potential of the ethanolic extract was determined on the basis of their scavenging activity of the stable 2, 2-diphenyl-1-picryl hydrazyl (DPPH) free radical. DPPH is a stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis. The aliquots of the different concentrations (1-500 $\mu\text{g/ml}$) of the extract were added to 3 ml of a 0.004% EtOH solution of DPPH. Absorbance at 517 nm was determined after 30 min, and IC_{50} (Inhibitory conc. 50%) was determined. IC_{50} value denotes the concentration of sample required to scavenge 50% of the DPPH free radicals (Gupta *et al.*, 2003).

The formula used for % inhibition ratio is-

$$\% \text{ inhibition} = (\text{Blank OD} - \text{Sample OD} / \text{Blank OD}) \times 100$$



- Fish extracts and ascorbic acid were weighed 2 times and dissolved in ethanol to make the required concentrations by dilution technique. Here ascorbic acid was taken as '+ve control.
- DPPH was weighed and dissolved in ethanol to make 0.004% (w/v) solution. To dissolve homogeneously magnetic stirrer was used.
- After making the desired concentrations 3 ml of 0.004% DPPH solution was applied on each test tube by pipette.
- The room temperature was recorded and kept the test tubes for 30 minutes in dark to complete the reactions.
- DPPH was also applied on the blank test tubes at the same time where only ethanol was taken as blank.
- After 30 minutes, absorbance of each test tube was determined by UV spectrophotometer at 517 nm.
- IC_{50} was determined from % inhibition vs conc. graph.

Data analysis

The results obtained in the experiment were subjected to statistical analysis using Windows Excel 2007.

CHAPTER 3

RESULTS

Chapter Three

Results

3.1 Qualitative analysis

Qualitative analysis was done by TLC (Thin Layer Chromatography) method. After applying DPPH on the TLC plates, yellow or whitish color on purple background was observed which indicates the presence of antioxidant components in the ethanolic extracts of fishes.

TLC plates of carnivore fishes compared with ascorbic acid

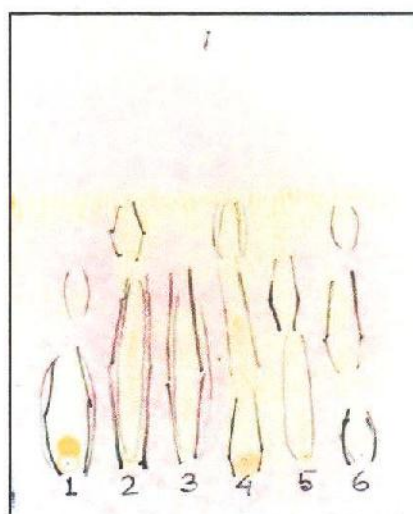


Figure 3.1.1. TLC plates for carnivore fishes along with ascorbic acid (1. ascorbic acid, 2. *Notopterus chitala*, 3. *Channa punctatus*, 4. *Channa striatus*, 5. *Mystus aor*, 6. *Notopterus notopterus*) in polar solvent (CHCl_3 : CH_3OH : H_2O =40:10:1).

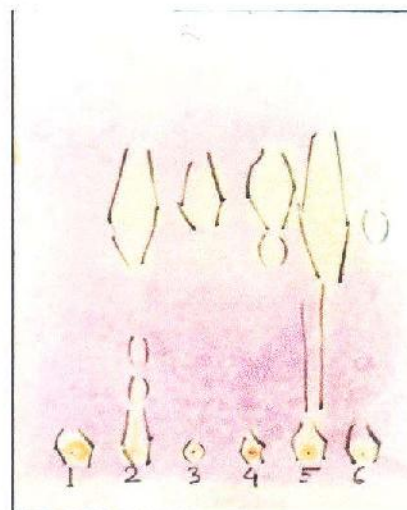


Figure 3.1.2. TLC plates for carnivore fishes along with ascorbic acid (1. ascorbic acid, 2. *Notopterus chitala*, 3. *Channa punctatus*, 4. *Channa striatus*, 5. *Mystus aor*, 6. *Notopterus notopterus*) in medium polar solvent (CHCl_3 : CH_3OH =5:1)

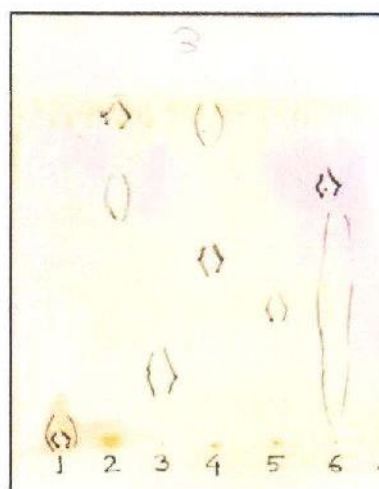


Figure 3.1.3: TLC plates of carnivore fishes along with ascorbic acid (1. Ascorbic acid, 2. *Notopterus chitala*, 3. *Channa punctatus*, 4. *Channa striatus*, 5. *Mystus aor*, 6. *Notopterus notopterus*) in non-polar solvent (n-Hexane: Acetone=2:1).

TLC plates of omnivore fishes compared with ascorbic acid

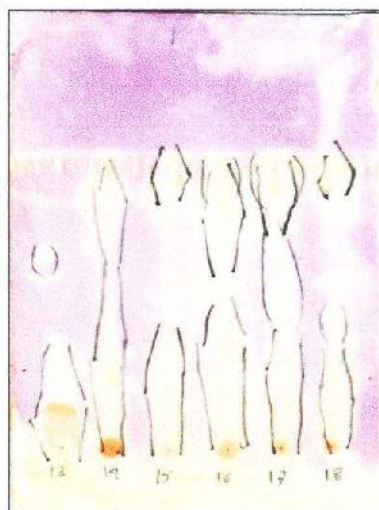


Figure3.1.4. TLC plates for omnivore fishes along with ascorbic acid (13.ascorbic acid, 14. *Mackerel sp*, 15. *Anabas testudineus*, 16. *Rhinomugil corsula*, 17. *Pampus chinensis* & 18. *Scatophagus argus*) in polar solvent ($\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}=40:10:1$)

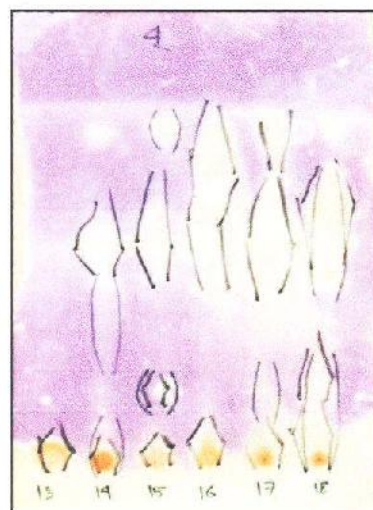


Figure3.1.5. TLC plates for omnivore fishes along with ascorbic acid(13.ascorbic acid, 14. *Mackerel sp*, 15. *Anabas testudineus*, 16. *Rhinomugil corsula*, 17. *Pampus chinensis* & 18. *Scatophagus argus*) in medium polar solvent ($\text{CHCl}_3:\text{CH}_3\text{OH}=5:1$)

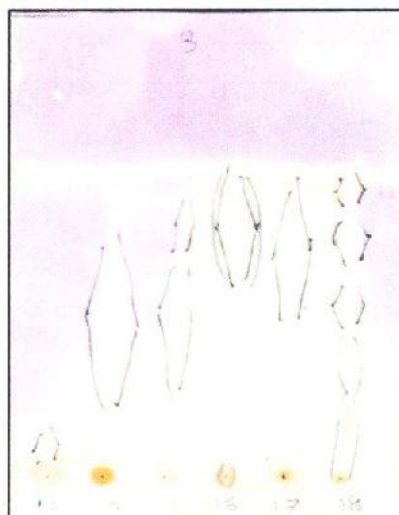


Figure 3.1.6. TLC plates of omnivore fishes along with ascorbic acid (13.ascorbic acid, 14. *Mackerel sp*, 15. *Anabas testudineus*, 16. *Rhinomugil corsula*, 17. *Pampus chinensis* & 18. *Scatophagus argus*) in non-polar solvent (n-Hexane: Acetone=2:1).

TLC plates of herbivore fishes compared with ascorbic acid

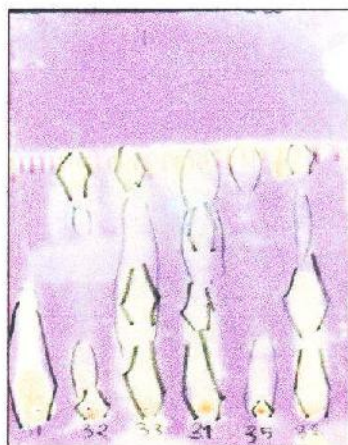


Figure 3.1.7. TLC plates of herbivore fishes (31.ascorbic acid, 32. *Labeo bata*, 33. *Hypophthalmichthys molitrix*, 34. *Puntius ticto*, 35. *Puntius gonionotus*, 36. *Ctenopharygodon idella*) along with ascorbic acid in polar solvent (CHCl_3 : CH_3OH : H_2O =40:10:1).

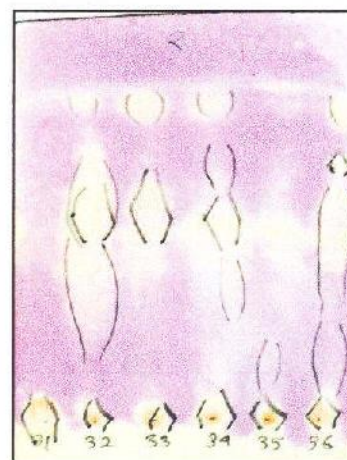


Figure 3.1.8. TLC plates of herbivore fishes along with ascorbic acid (31.ascorbic acid, 32. *Labeo bata*, 33. *Hypophthalmichthys molitrix*, 34. *Puntius ticto*, 35. *Puntius gonionotus*, 36. *Ctenopharygodon idella*) in medium polar solvent (CHCl_3 : CH_3OH =5:1)

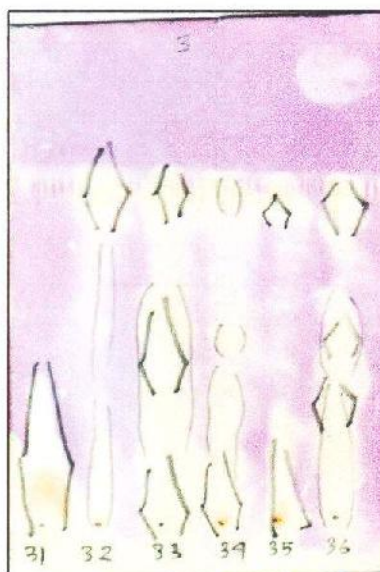


Figure 3.1.9. TLC plates in non polar solvent (n-Hexane: Acetone=2:1) for, 31.ascorbic acid, 32. *Labeo bata*, 33. *Hypophthalmichthys molitrix*, 34. *Puntius ticto*, 35. *Puntius gonionotus*, 36. *Ctenopharygodon idella*.

3.2 Quantitative analysis

3.2.1 DPPH free radical scavenging test of different herbivore fishes

***Labeo bata*:** The IC_{50} of *L. bata* was 965.7 $\mu\text{g/ml}$ which was determined from the inhibition versus concentration curve although it was not found within the tested concentration (0 to 500 $\mu\text{g/ml}$).

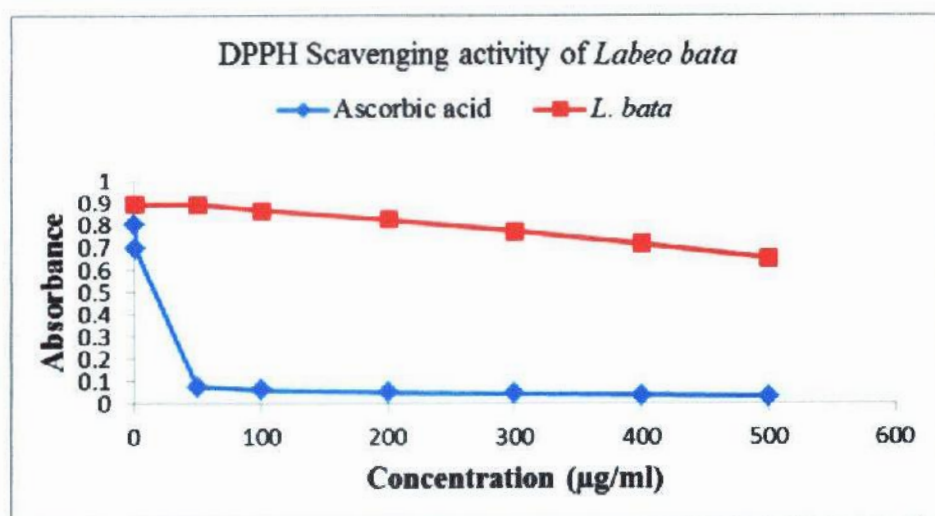


Figure3. 2.1.1: Absorbance versus concentration curve of ascorbic acid and ethanolic extract of *Labeo bata*.

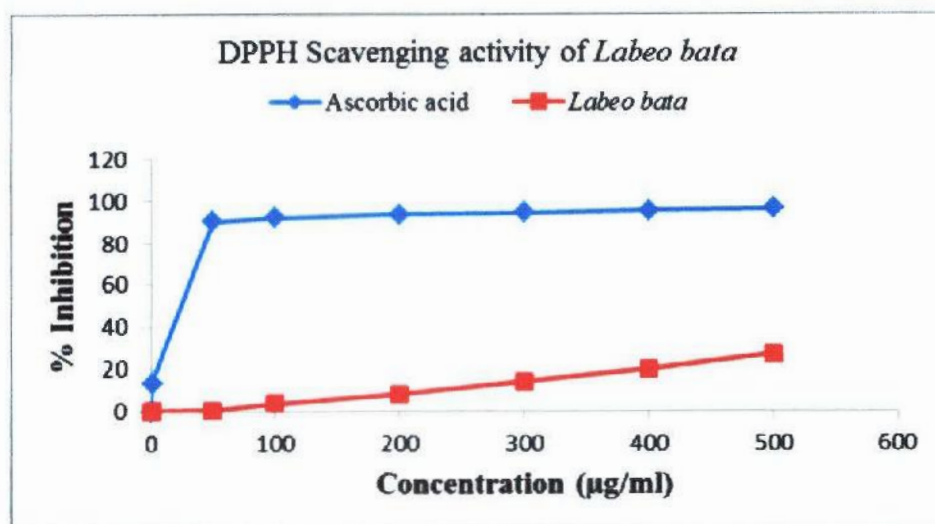


Figure3. 2.1.2: %Inhibition versus concentration curve of ascorbic acid and ethanolic extract of *Labeo bata*.

Ctenopharyngodon idella : The data of *C. idella* species was not potent. The IC_{50} value of *C. idella* was 154.45 $\mu\text{g/ml}$ which was determined from the inhibition versus concentration curve although there is no evidence of IC_{50} in the tested concentration (0 to 500 $\mu\text{g/ml}$).

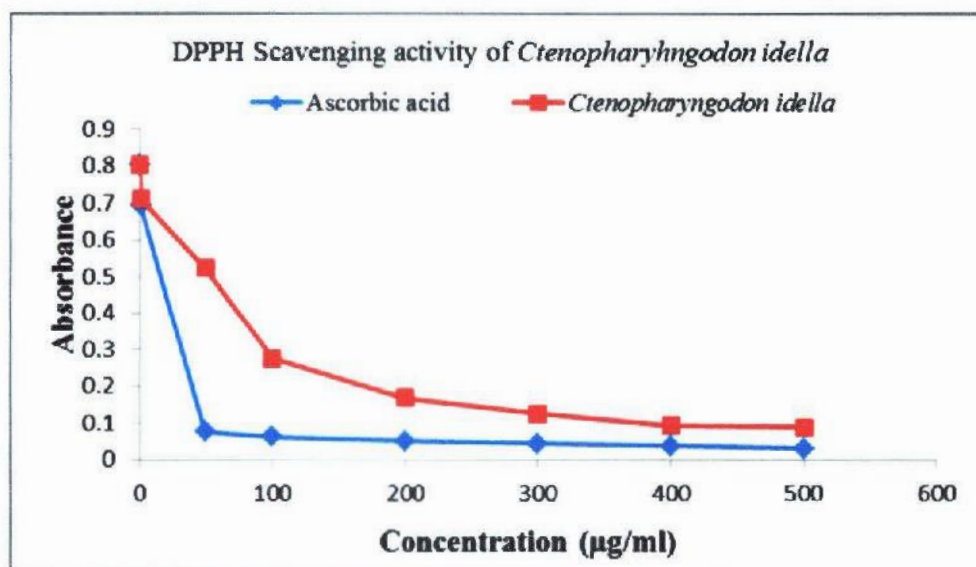


Figure 3.2.1.3: Absorbance versus concentration curve of ascorbic acid and ethanolic extract of *Ctenopharyngodon idella*.

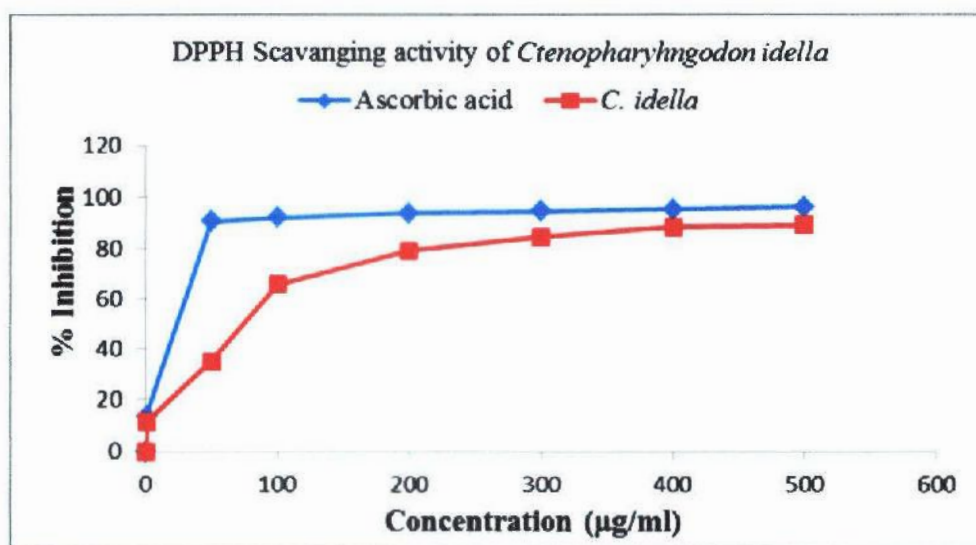


Figure 3.2.1.4: % Inhibition versus concentration curve of ascorbic acid and ethanolic extract of *Ctenopharyngodon idella*.

***Puntius ticto*:** The IC_{50} of *Puntius ticto* was 267.70 $\mu\text{g/ml}$ which was determined from the inhibition versus concentration curve which is in the range of tested concentration (0 to 500 $\mu\text{g/ml}$).

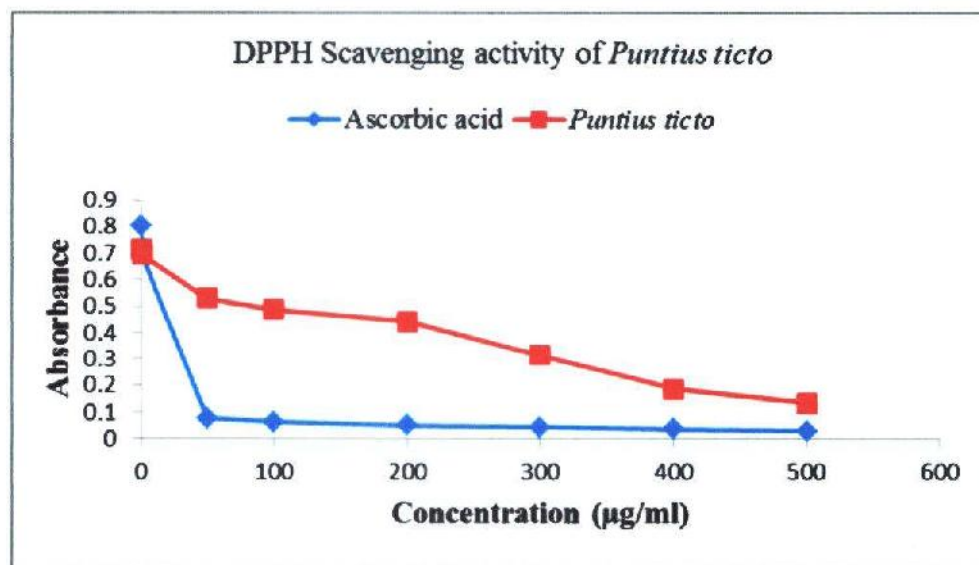


Figure 3.2.1.5: Absorbance versus concentration curve of ascorbic acid and ethanolic extract of *Puntius ticto*.

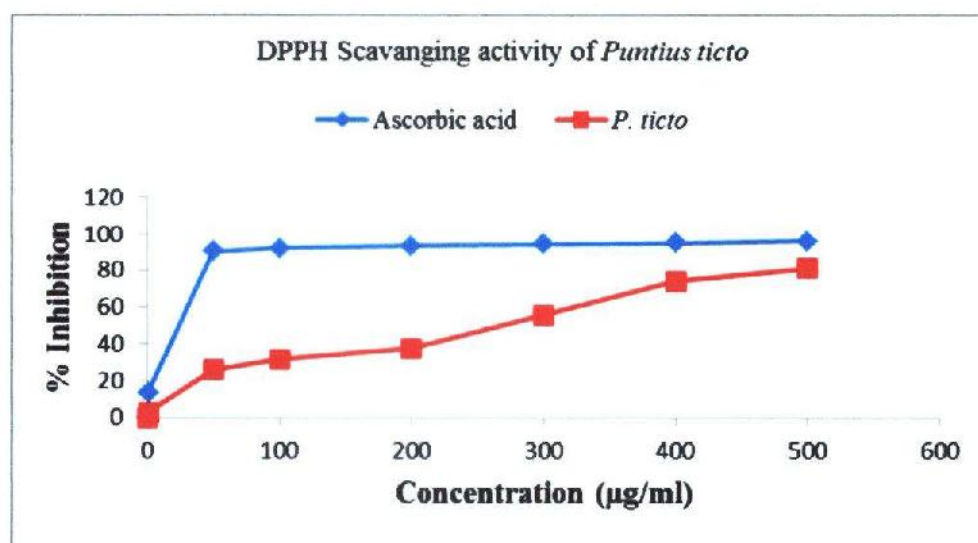


Figure 3.2.1.6: % Inhibition versus concentration curve of ascorbic acid and ethanolic extract of *Puntius ticto*.

Puntius gonionotus: : From the inhibition versus concentration curve, the IC₅₀ value of *Puntius gonionotus* was found 1678.96 µg/ml which is not present in the tested concentration (0 to 500 µg/ml).

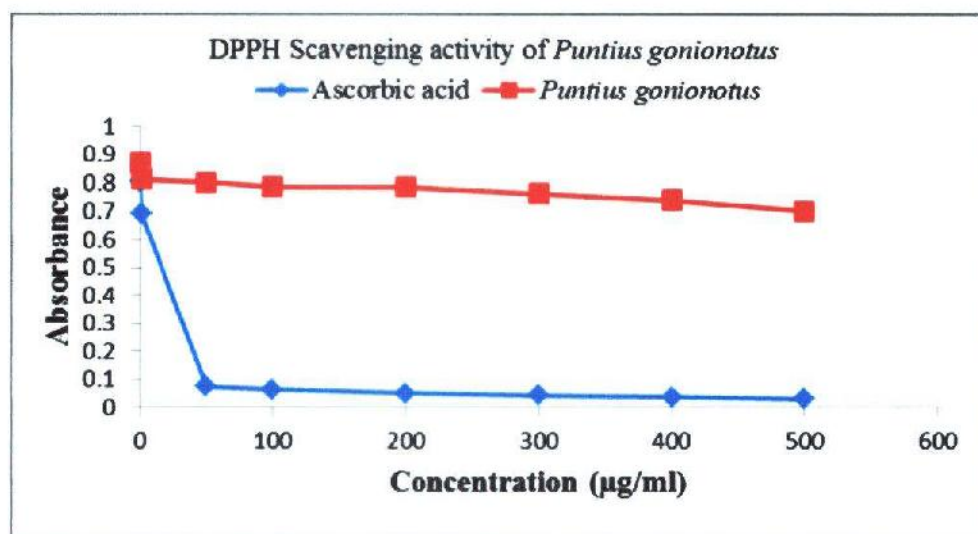


Figure 3.2.1.7: Absorbance versus concentration curve of ascorbic acid and ethanolic extract of *Puntius gonionotus*.

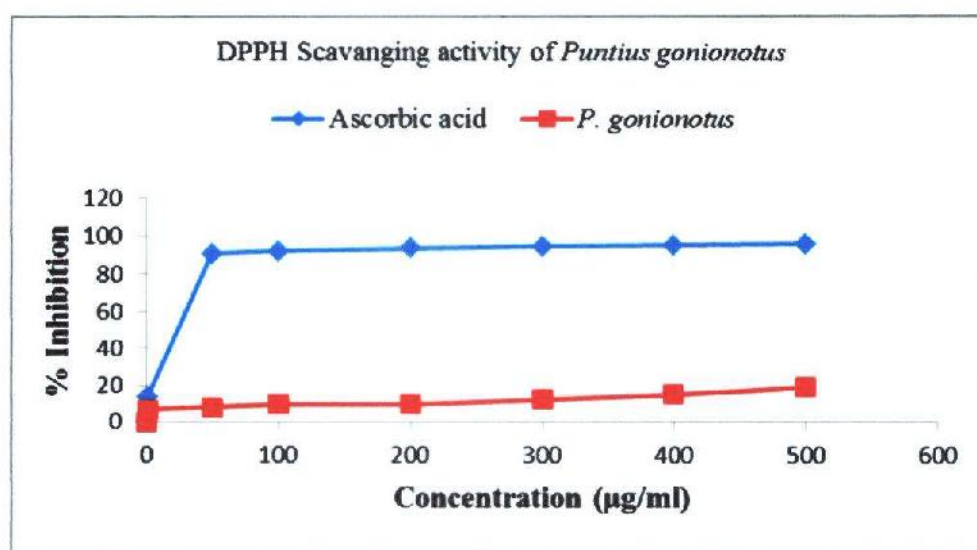


Figure 3.2.1.8. % Inhibition versus concentration curve of ascorbic acid and ethanolic extract of *Puntius gonionotus*

***Hypophthalmichthys molitrix*:** In this experiment, the IC_{50} value of *H. molitrix* was found 963.46 $\mu\text{g/ml}$ from the inhibition versus concentration curve which is out of the tested concentration (0 to 500 $\mu\text{g/ml}$) range.

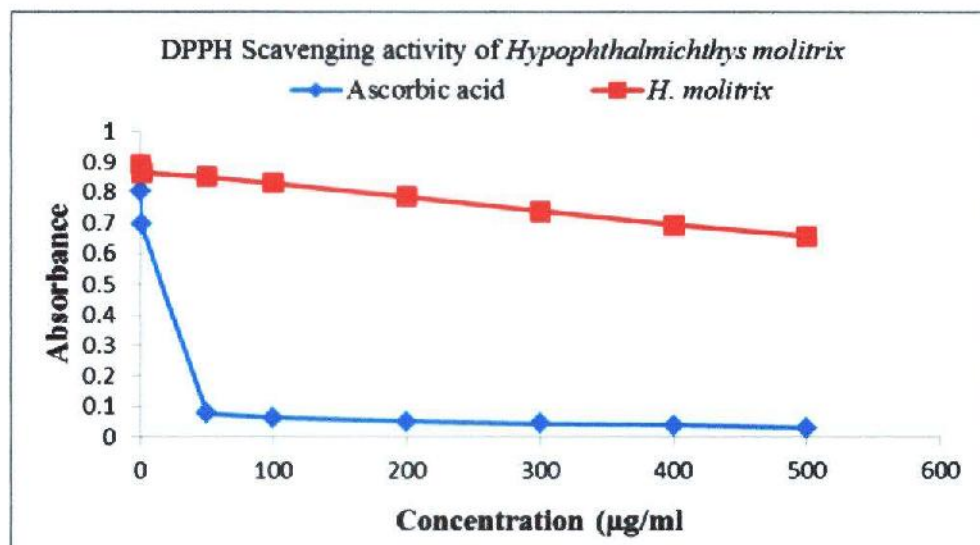


Figure 3.2.1.9: Absorbance versus concentration curve of ascorbic acid and ethanolic extract of *Hypophthalmichthys molitrix*.

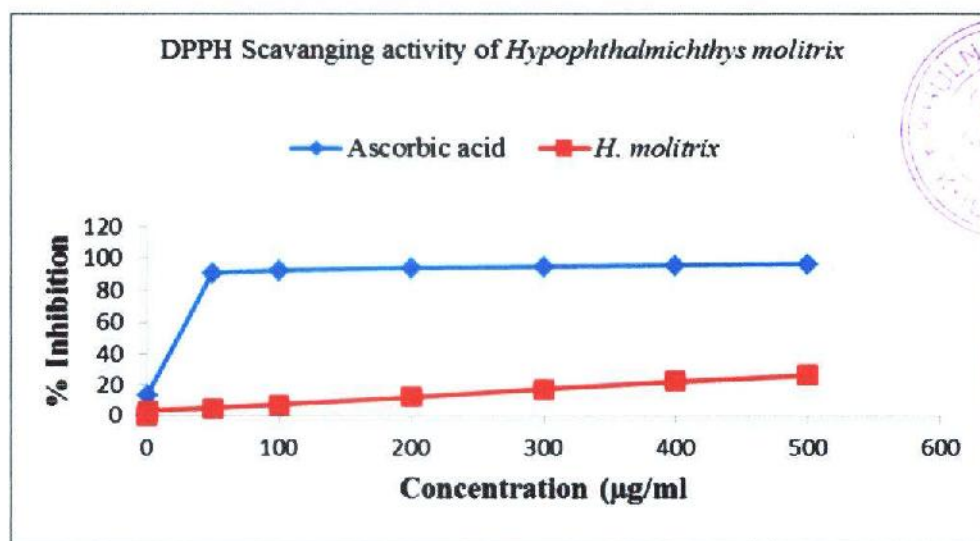


Figure 3.2.1.10: %Inhibition versus concentration curve of ascorbic acid and ethanolic extract of *Hypophthalmichthys molitrix*.

3.2.2 DPPH free radical scavenging test of different carnivore fishes

Channa striatus : *Channa striatus* showed potent data in this experiment. The IC_{50} value of *Channa striatus* was 327.69 $\mu\text{g/ml}$ which was determined from the inhibition versus concentration curve concentration (0 to 500 $\mu\text{g/ml}$).

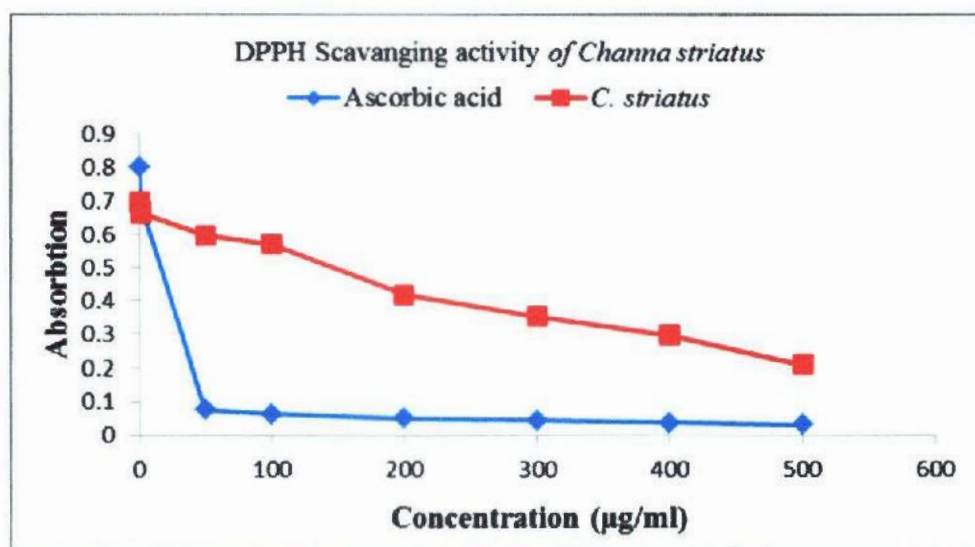


Figure 3.2.2.1: Absorbance versus concentration curve of ascorbic acid and ethanolic extract of *Channa striatus*.

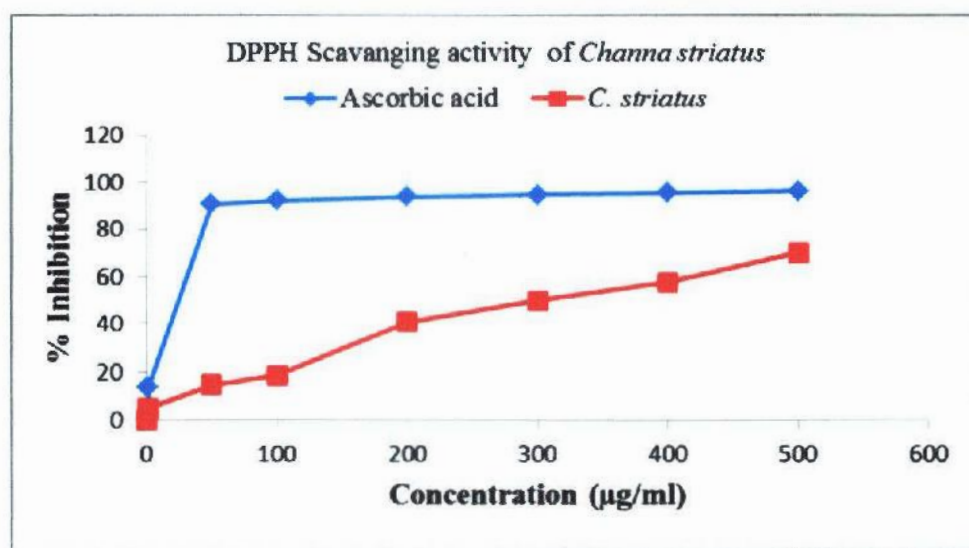


Figure 3.2.2.2: %Inhibition versus concentration curve of ascorbic acid and ethanolic extract of *Channa striatus*.

***Channa punctatus*.** Like *C. striatus*, the data of *C. punctatus* was also potent. From the inhibition versus concentration curve, the IC_{50} value of *C. striatus* was found 327.69 $\mu\text{g/ml}$ which is within the tested concentration (0 to 500 $\mu\text{g/ml}$) value.

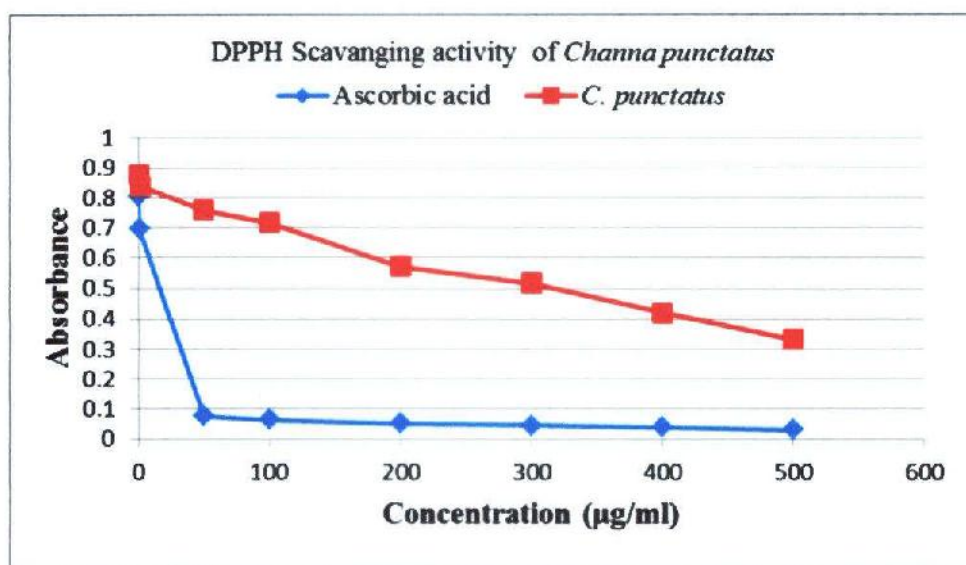


Figure 3.2.2.3: Absorbance versus concentration curve of ascorbic acid and ethanolic extract of *Channa punctatus*.

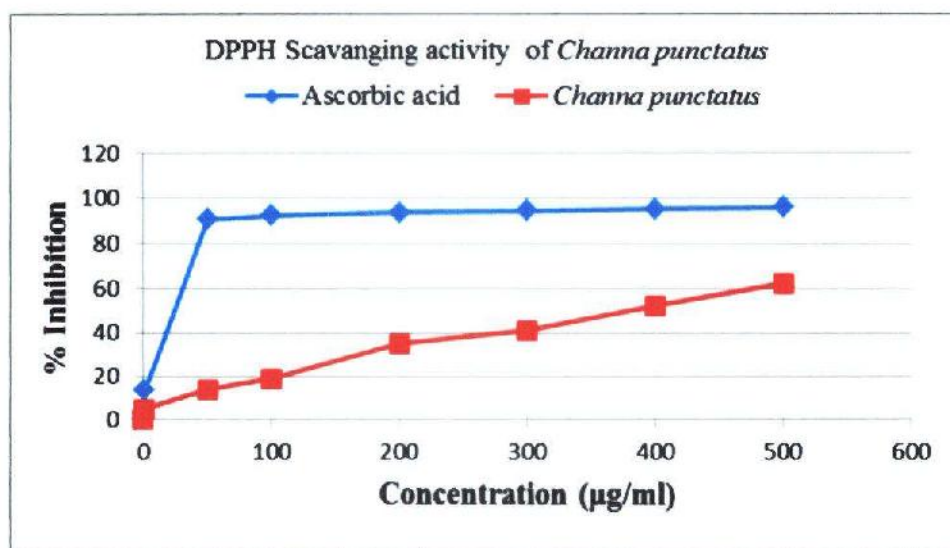


Figure 3.2.2.4: %Inhibition versus concentration curve of ascorbic acid and ethanolic extract of *Channa punctatus*.

Notopterus notopterus The IC₅₀ value of *N. notopterus* was 1757.17 µg/ml which was determined from the inhibition versus concentration curve.

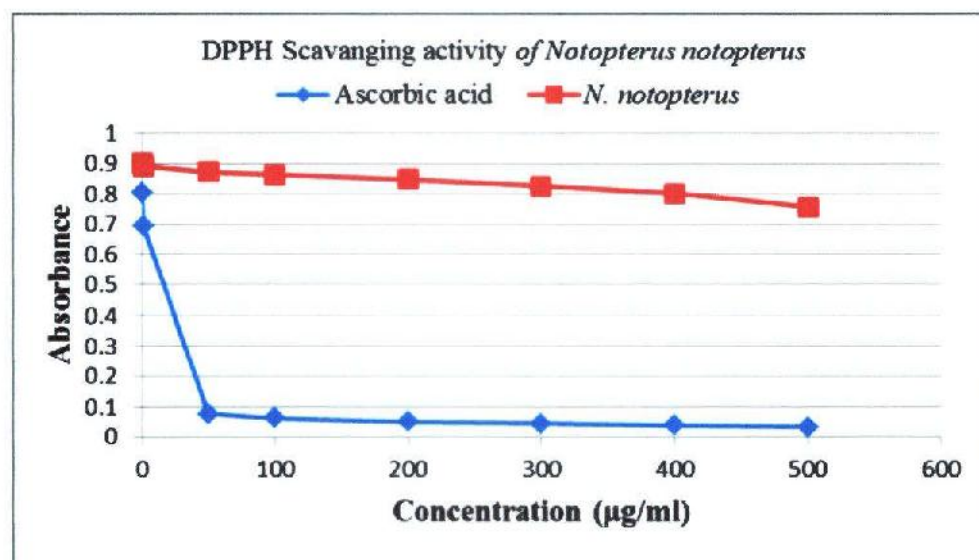


Figure 3.2.2.5: Absorbance versus concentration curve of ascorbic acid and ethanolic extract of *Notopterus notopterus*.

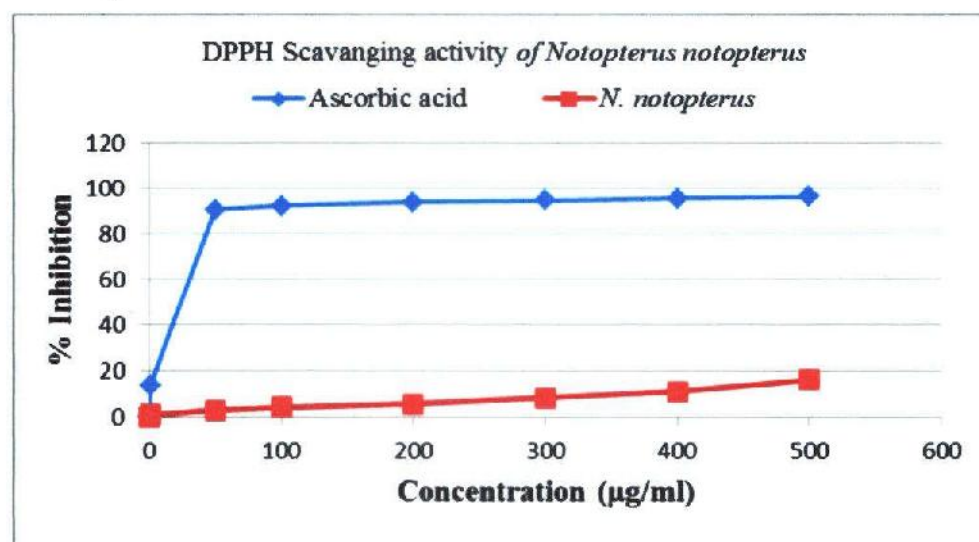


Figure 3.2.2.6: %Inhibition versus concentration curve of ascorbic acid and ethanolic extract of *Notopterus notopterus*.

***Notopterus chitala*:** In this experiment, the IC_{50} value of *Notopterus chitala* was found 1757.17 $\mu\text{g/ml}$ from the inhibition versus concentration curve which is out of the tested concentration (0 to 500 $\mu\text{g/ml}$) range.

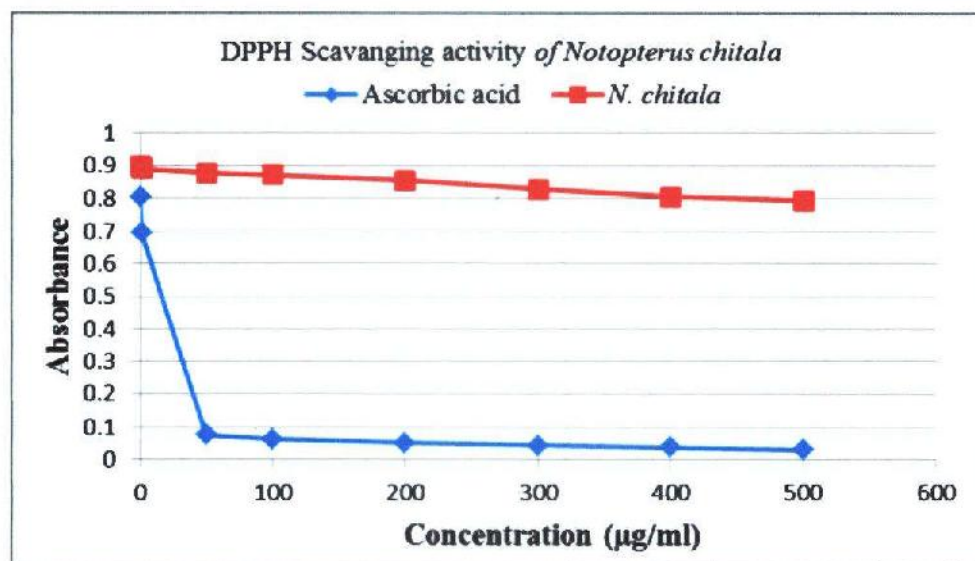


Figure 3.2.2.7: Absorbance versus concentration curve of ascorbic acid and ethanolic extract of *Notopterus chitala*.

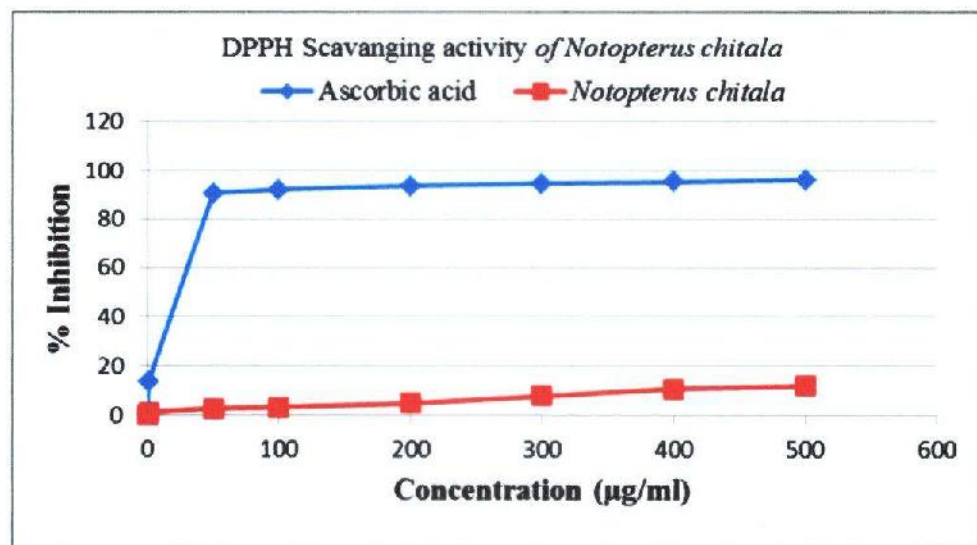


Figure 3.2.2.8: %Inhibition versus concentration curve of Ascorbic acid and ethanolic extract of *Notopterus chitala*.

***Mystus aor*:** From the inhibition versus concentration curve, the IC_{50} value of *Mystus aor* was found 1170.29 $\mu\text{g/ml}$ which is lacking in the tested concentration (0 to 500 $\mu\text{g/ml}$).

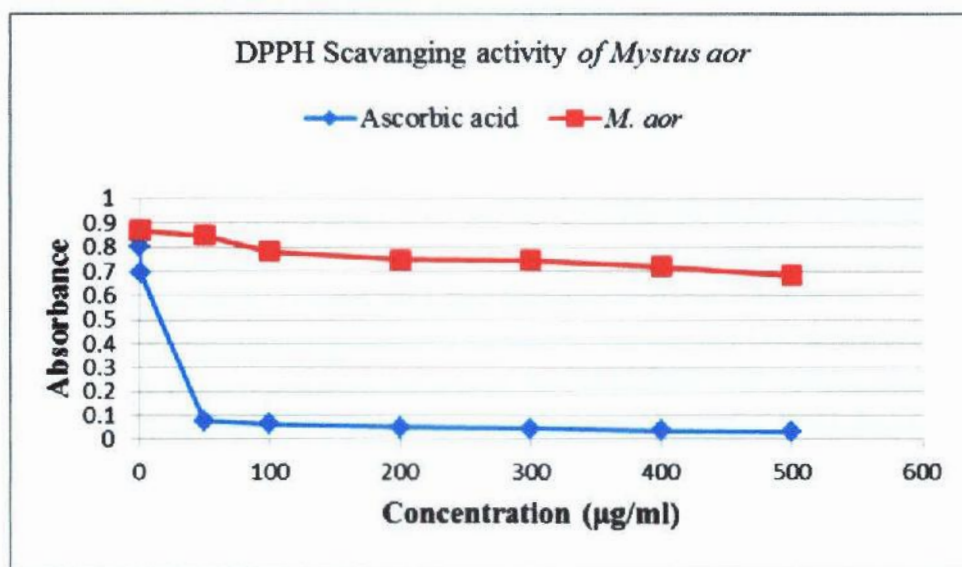


Figure 3.2.2.9: Absorbance versus concentration curve of ascorbic acid and ethanolic extract of *Mystus aor*.

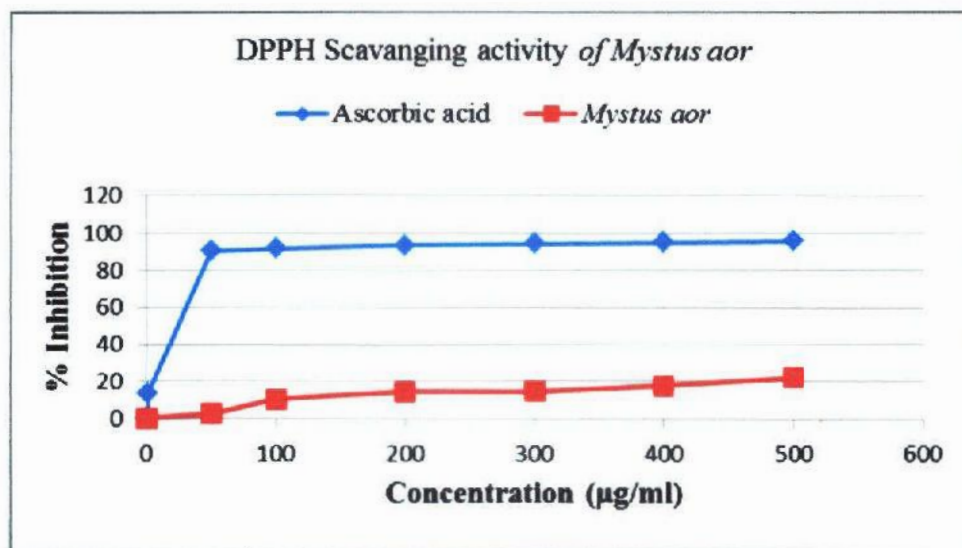


Figure 3.2.2.10: %Inhibition versus concentration curve of ascorbic acid and ethanolic extract of *Mystus aor*.

3.2.3 DPPH free radical scavenging test of different omnivore fishes

***Rhinomugil corsula*:** *Rhinomugil corsula* showed IC_{50} value about 1266.02 $\mu\text{g/ml}$ which was determined from the inhibition versus concentration curve of this sample.

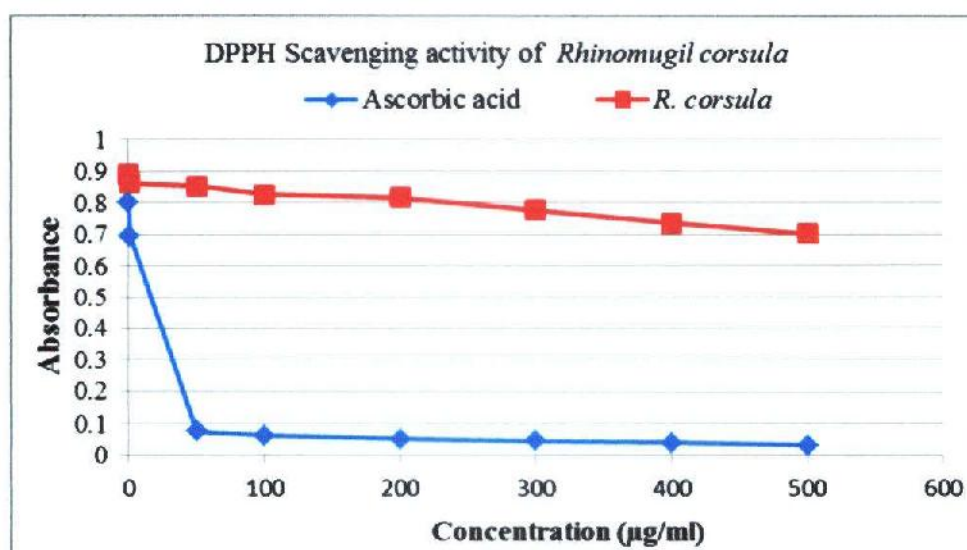


Figure 3.2.3.1: Absorbance versus concentration curve of ascorbic acid and ethanolic extract of *Rhinomugil corsula*.

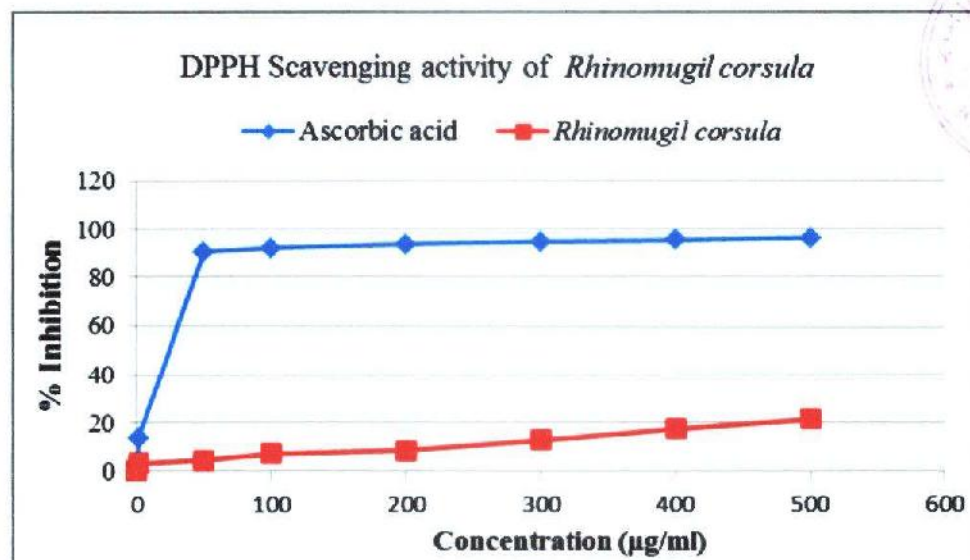


Figure 3.2.3.2: %Inhibition versus concentration curve of ascorbic acid and ethanolic extract of *Rhinomugil corsula*.

***Pampus chinensis*:** The IC_{50} value of *Pampus chinensis* was 600.89 $\mu\text{g/ml}$ which was determined from the inhibition versus concentration curve although there is no evidence of IC_{50} in the tested concentration (0 to 500 $\mu\text{g/ml}$).

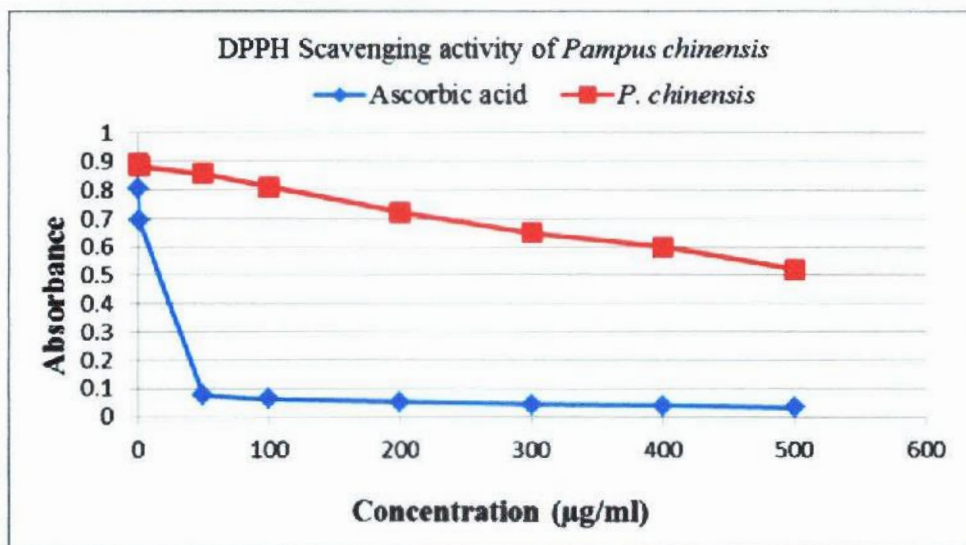


Figure 3.2.3.3: Absorbance versus concentration curve of ascorbic acid and ethanolic extract of *Pampus chinensis*.

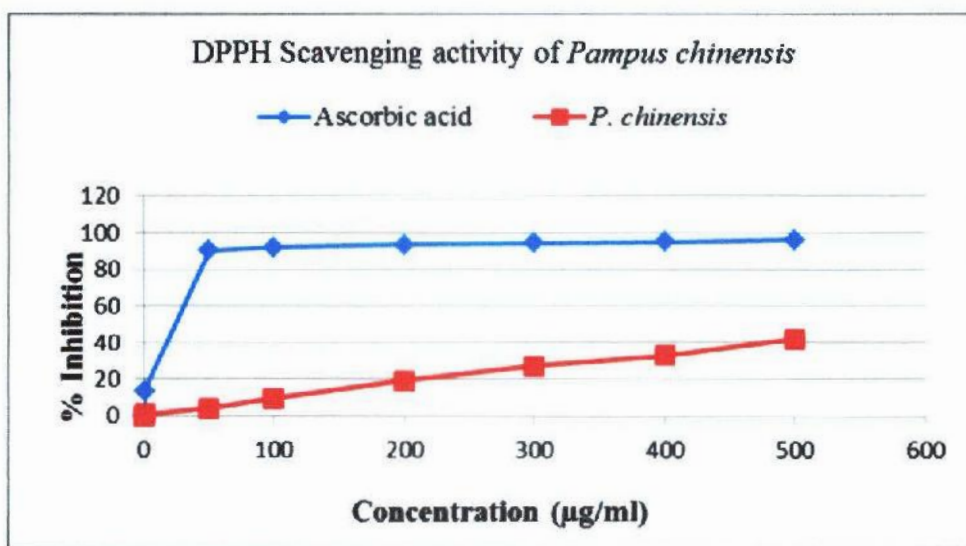


Figure 3.2.3.4: %Inhibition versus concentration curve of ascorbic acid and ethanolic extract of *Pampus chinensis*.

Anabas testudineus: *Anabas testudineus* showed potent data in this test and the IC₅₀ value of this fish species was found 397.72 µg/ml which was determined from the inhibition versus concentration curve.

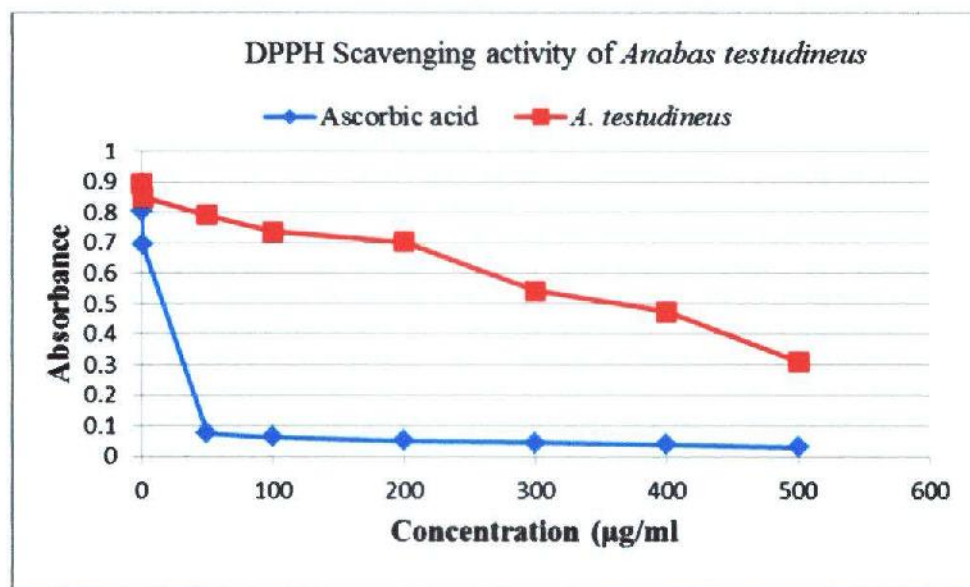


Figure 3.2.3.5: Absorbance versus concentration curve of ascorbic acid and ethanolic extract of *Anabas testudineus*.

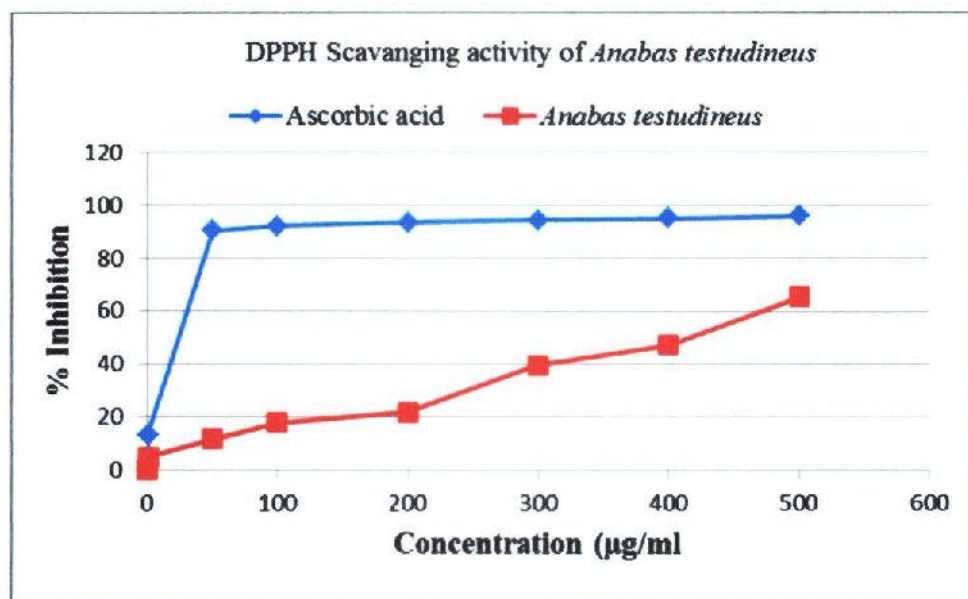


Figure 3.2.3.6: %Inhibition versus concentration curve of ascorbic acid and ethanolic extract of *Pampus chinensis*.

***Scatophagus urgus*:** From the inhibition versus concentration curve, the IC_{50} of *S. urgus* was determined 872.19 $\mu\text{g/ml}$ which is not present in the tested concentration range (0 to 500 $\mu\text{g/ml}$).

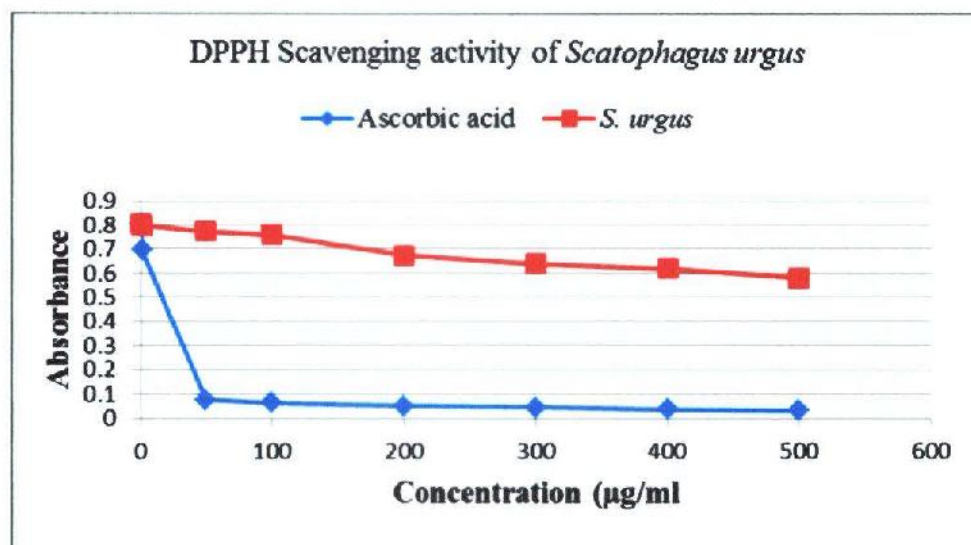


Figure 3.2.3.7: Absorbance versus concentration curve of ascorbic acid and ethanolic extract of *Scatophagus urgus*.

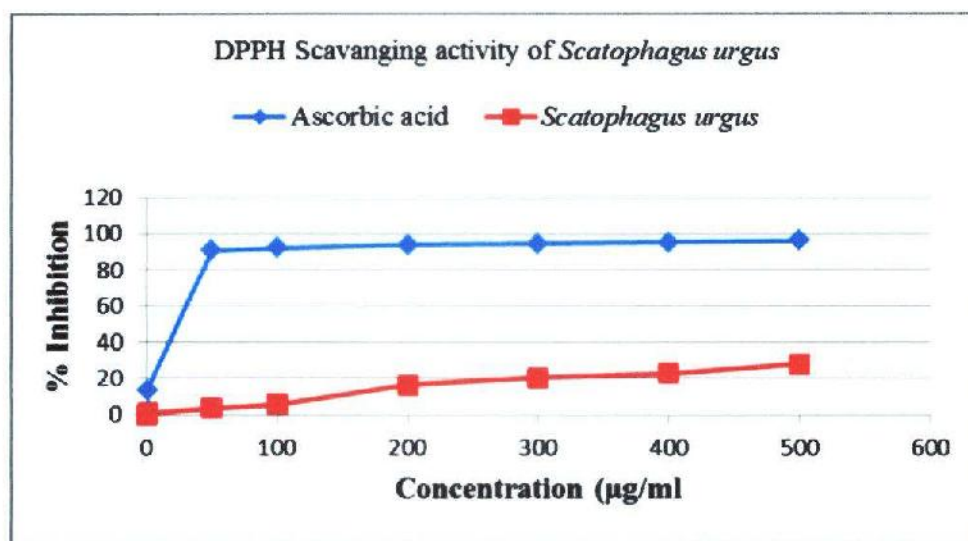


Figure 3.2.3.8: %Inhibition versus concentration curve of ascorbic acid and ethanolic extract of *Scatophagus urgus*.

Mackerel sp. The IC_{50} value of *Mackerel sp* was 496.66 $\mu\text{g/ml}$ which was determined from the inhibition versus concentration curve although there is no evidence of IC_{50} in the tested concentration (0 to 500 $\mu\text{g/ml}$).

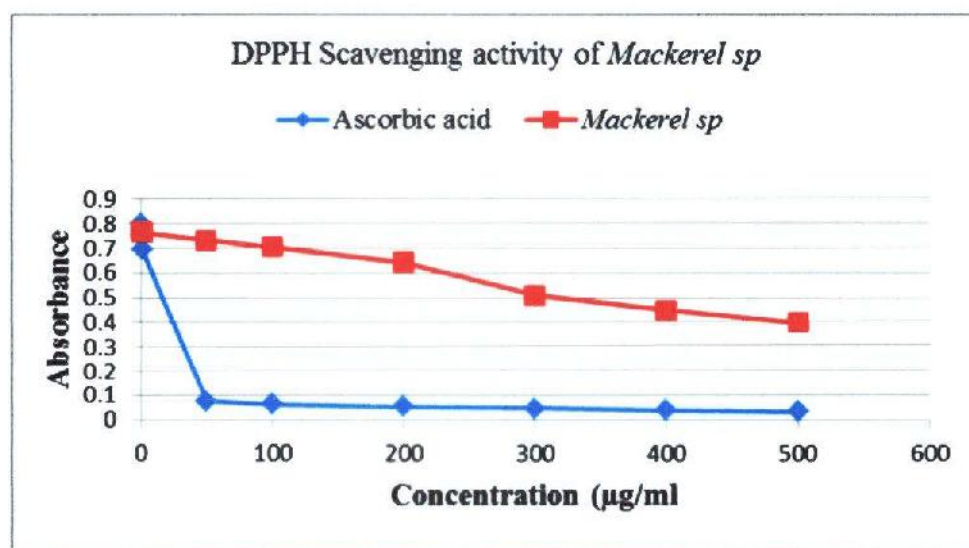


Figure 3.2.3.9: Absorbance versus concentration curve of ascorbic acid and ethanolic extract of *Mackerel sp*.

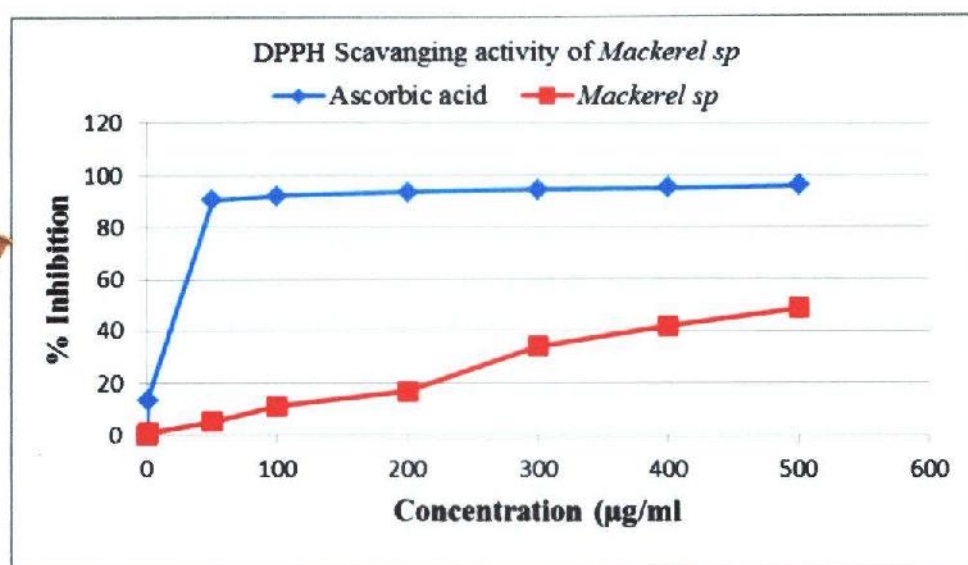


Figure 3.2.3.10: %Inhibition versus concentration curve of ascorbic acid and ethanolic extract of *Mackerel sp*.

3.2.4. DPPH free radical scavenging test of different herbivore fishes compared with ascorbic acid

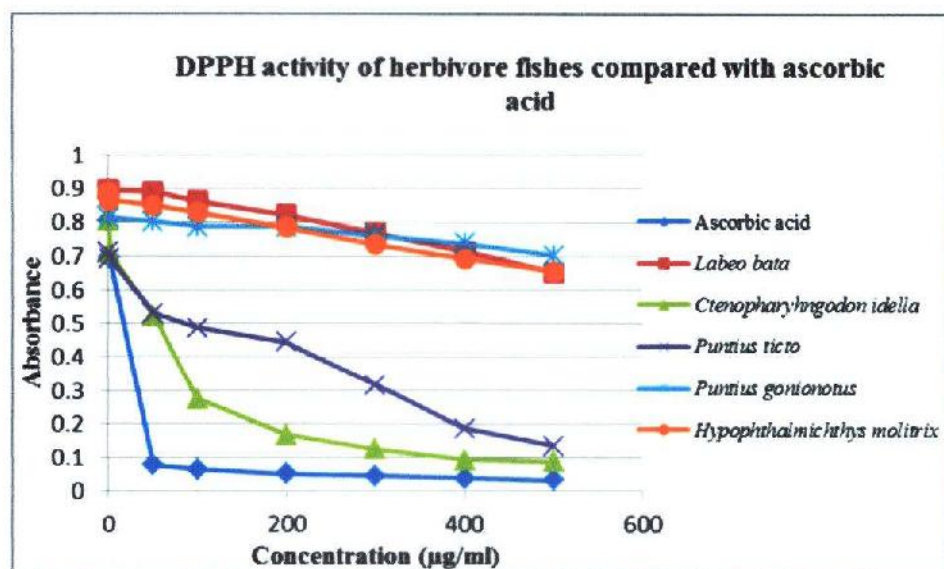


Figure 3.2.4.1: Absorbance versus concentration curves of different herbivore fishes compared with ascorbic acid.

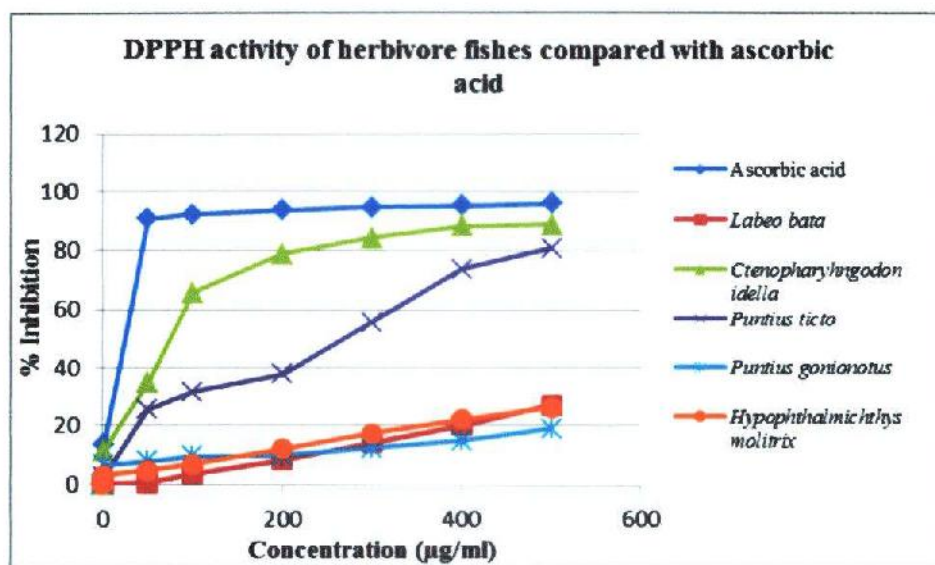


Figure 3.2.4.2: % Inhibition versus concentration curves of different herbivore fishes compared with ascorbic acid.

3.2.5. DPPH free radical scavenging test of different carnivore fishes compared with ascorbic acid.

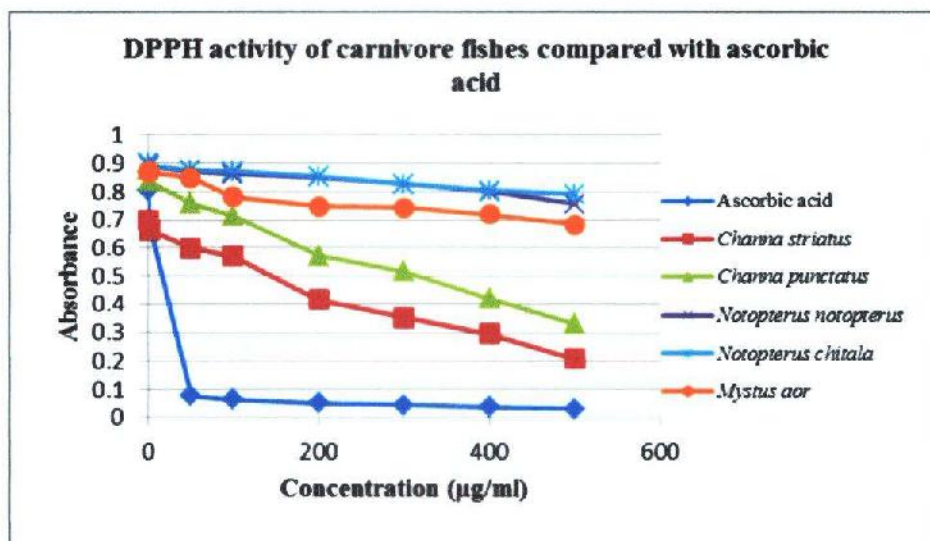


Figure 3.2.5.1: Absorbance versus concentration curves of different carnivore fishes compared with ascorbic acid.

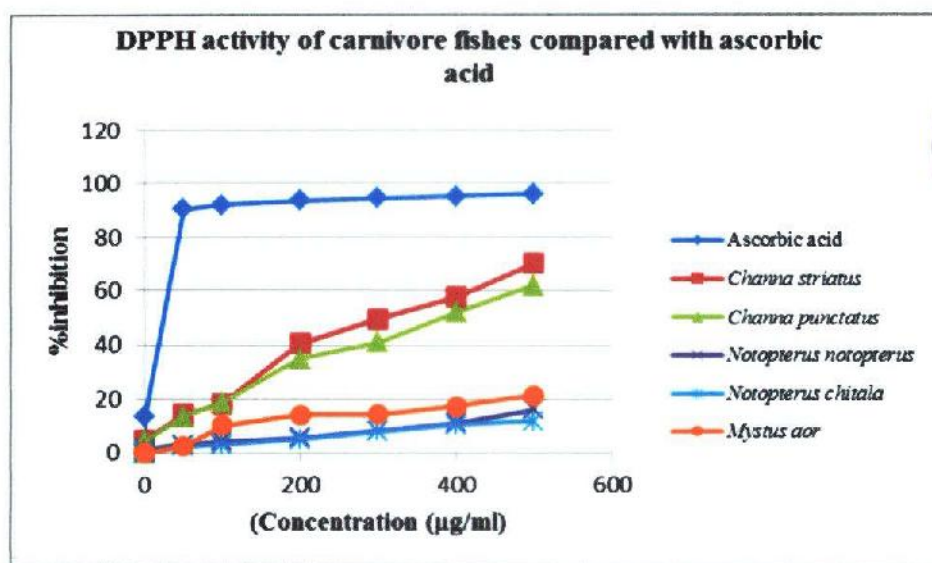


Figure 3.2.5.2: % Inhibition versus concentration curves of different carnivore fishes compared with ascorbic acid.

3.2.6. DPPH free radical scavenging test of different omnivore fishes compared with ascorbic acid.

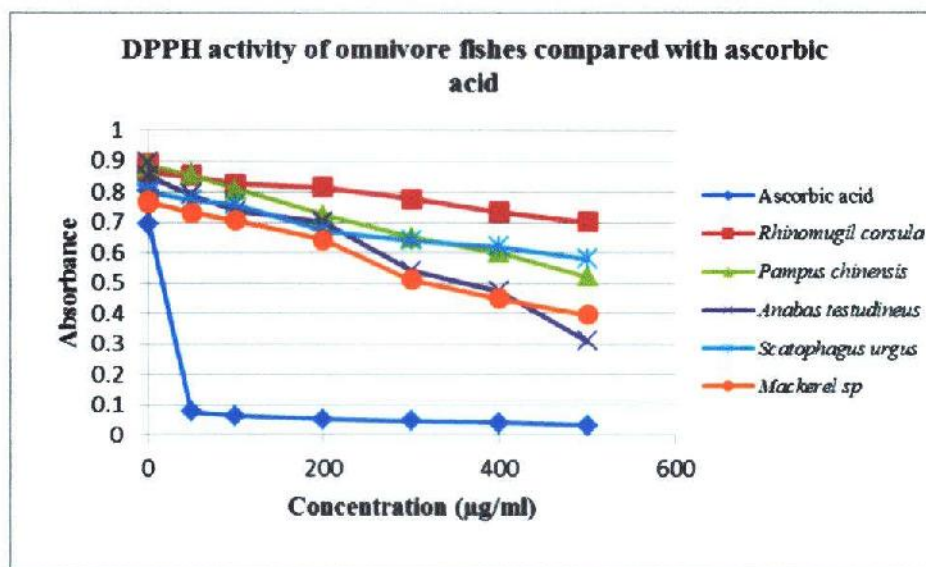


Figure 3.2.6.1: Absorbance versus concentration curves of different omnivore fishes compared with ascorbic acid.

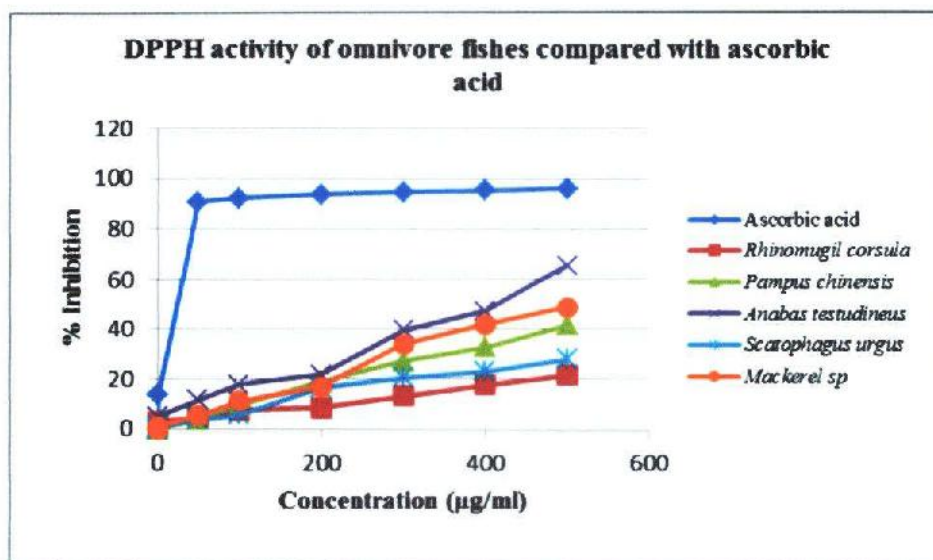


Figure 3.2.6.2: % Inhibition versus concentration curves of different carnivore fishes compared with ascorbic acid.

CHAPTER 4

DISCUSSION

Chapter Four

Discussion

Antioxidant compounds in food play an important role as a health-protecting factor. Scientific evidence suggests that antioxidants reduce risk for chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables that are mentioned before. Foods containing antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. Some compounds, such as gallates, have strong antioxidant activity, while others, such as the mono-phenols are weak antioxidants. The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases.

There are a number of clinical studies suggesting that the antioxidants in fruits, vegetables, tea and red wine are the main factors for the observed efficacy of these foods in reducing the incidence of chronic diseases including heart disease and some cancers. The free radical scavenging activity of antioxidants in foods have been substantially investigated and reported in the literature by Miller *et al.*, (2000a and 2000b).

An increased concentration of end products of lipid per-oxidation is the evidence most frequently quoted for the involvement of free radicals in human diseases. Several studies support the hypothesis that lipid oxidation products ingested with food or produced endogenously represent a health risk (Esterbauer, 1993). It is well established that free radicals and reactive oxygen species are continuously produced *in vivo*. Numerous detoxifying mechanisms in the body protect the cells from the toxic effects of free radicals and the associated per-oxidation of lipids. These include protective enzymes (superoxide dismutase, glutathione per-oxidase, glutathione reductase, glutathione transferase, catalase, etc.) and several low molecular weight water and lipid soluble antioxidants (reduced

glutathione, uric acid, ubiquinol-10, etc). Apart from these endogenous antioxidants, there are several essential antioxidants which include vitamins E, A, C and carotenoids. There is increasing evidence that under certain circumstances these protective systems may be overwhelmed. Free radical mediated oxidation of lipids particularly affects the polyunsaturated fattyacids (PUFA). The primary products of free radical oxidation undergo rapid and spontaneous fragmentation. Some have considerable lethal potential. The peroxidation of lipids has gained significance in recent times because of its implication in the causation of a number of diseases (Sies, 1991).

Epidemiological studies all over the world have shown that vegetable-rich diets are associated with higher life expectancy. The reason for this could be due to the higher antioxidants present in such diets (Gey *et al.*, 1993). Higher fruit and vegetable intake was associated with a lower risk of myocardial infarction and other cardiovascular diseases (Liu *et al.*, 2000), cancers of the mouth, pharynx, larynx, stomach, colon, etc (Hirayama, 1985; Paganini *et al.*, 1987). Habitual consumption of fish on the other hand is also associated with reduced mortality from coronary heart diseases (Kromhout *et al.*, 1985; Shekelle *et al.*, 1985). The beneficial effects, namely, lowered plasma lipids, diminished thrombogenicity and decreased blood pressure have been attributed to the accumulation of n-3 fatty acids over time in the individuals consuming fish regularly. The PUFAs account for 30-50% of the total fatty acids in fishes from Indian waters (Reena *et al.*, 1996).

In contradiction to these beneficial effects of a fish- enriched diet it is found that high PUFA content in the diet increases the ratio of the PUFAs to the saturated fatty acids in all biological membranes. Hence it can be expected that an increase in the PUFA content of the biological membranes enhances their susceptibility to oxidation.

Fish with its high content of unsaturated fatty acids (Ackman, 1980; 1989a; 1989b; Morris and Culkin, 1989) is highly susceptible towards lipid oxidation (Ackman, 1988; Hardy, 1980; Khayat and Schwall, 1983). The resulting development of rancidity in fish leads to undesirable changes in flavor (Durnford and Shahidi, 1998; Karahadian and Lindsay, 1989), texture (Castell, 1971; Sikorski *et al.*, 1976), color (Haard, 1992; Jensen *et al.*, 1998, Smith and Hole, 1991) and nutritional value (Opstvedt, 1971 and 1975; Watanabe, 1982). In fresh fish the balance between the prooxidative and antioxidative factors which control oxidative reactions is maintained by numerous systems (Hultin, 1992a; 1992b and 1994; Undeland,

1997). With processing and prolonged storage time the control of oxidation is lost and the onset of lipid oxidation can no longer be prevented (Flick *et al.*, 1992). How long this will take is highly dependent on the type of handling the fish is subjected to and the level of antioxidants present in the fish tissue (Decker, 1998; Petillo *et al.*, 1998).

There are several natural compounds that participate in the antioxidative defense mechanism of fish (Undeland, 1997). These include enzymes (catalase, peroxidase, glutathione and superoxide dismutase), carotenoids, peptides, amino acids and phenolic compounds (tocopherols, ubiquinones). These compounds are found in the cell plasma, mitochondria of cell membranes.

The basic TLC procedure has largely remained unchanged over the last fifty years. It involves the use of a thin, even sorbent layer, usually about 0.10 to 0.25 mm thick, applied to a firm backing of glass, aluminium or the sorbent layer approximately 1 cm from the edge. An eluent (single solvent or solvent mixture) is allowed to flow by capillary action through the sorbent starting at a point just below the applied samples. Most commonly this is achieved by using a glass rectangular tank in which the eluent is poured to give a depth of about 5 mm. The plate is placed in the tank or chromatography chamber and the whole covered with a lid. As the eluent front migrates through the sorbent, the components of the sample also migrate, but at different rates, resulting in separation. When the solvent front has reached a point near the top of the sorbent layer, the plate or sheet is removed and dried. The spots or bands on the developed layer are visualized, if required, under UV light or by chemical treatment or derivatisation. For quantitative determinations, zones can be removed or eluted from the layer, or the plate can be scanned at pre-determined wavelengths without disturbing the layer surface. The modern use of TLC has seen a strong move in the direction of plate scanning and video imaging as a means of providing sensitive and reliably accurate results and a more permanent record of the chromatogram. This is in addition to its obvious labour saving aspect and chemically "clean" approach. Although TLC is an analytical method in its own right, it is also complimentary to other chromatographic techniques and spectroscopic procedures. Results obtained with TLC can often be transferred to HPLC or vice versa with some adjustment in eluting solvent conditions. For multi-component samples (e.g. pesticides in water), fractions of interest from an HPLC separation can be collected and subsequent re-chromatography of these on HPTLC can give a "fine tuned" separation of the

components of the fractions (Burger, 1989; Kaiser, 1989; Jönchen and Issaq, 1988). Thin-layer chromatography has been successfully hyphenated with high performance liquid chromatography (HPLC), mass spectroscopy (MS), Fourier transform infra-red (FTIR), and Raman spectroscopy, to give far more detailed analytical data on separated compounds. Even the UV/visible diode array technique has been utilized in TLC to determine peak purity or the presence of unresolved analytes. (Jönchen, 1996).

Although column chromatography can be traced to its discoverer, the Russian botanist, Tswett in 1903, it was not until 1938 that separations on thin-layers were achieved when Izmailov and Shraiber, (1938) looking for a simpler technique, which required less sample and sorbent, separated plant extracts using aluminium oxide spread on a glass plate. The sorbent was applied to a microscope slide as slurry, giving a layer about 2 mm thick. The sample (plant extracts) was applied as droplets to the layer. The solvent (methanol) was then added dropwise from above on to the applied spots and a series of circular rings were obtained of differing colours on the layer. Circular TLC was born, and Izmailov and Shraiber named this new technique “drop chromatography”.

Meinhard and Hall (1949) used a starch binder to give some firmness to the layer, in order to separate inorganic ions, which they described as “surface chromatography”. Further advances were made in by Kirchner *et al.*, (1951) who used the now conventional ascending method, with a sorbent composed of silicic acid, for the separation of terpene derivatives, describing the plates used as “chromatostrips”. Reitsema (1954) used much broader plates and was able to separate several mixtures in one run. Surprisingly it was some time before the advantages of this development were recognized.

Up to this time quantitative TLC was fraught with experimental error. However, the introduction of commercial spectrodensitometric scanners enabled the quantification of analytes directly on the TLC layer. Initially peak areas were measured manually, but later integrators achieved this automatically.

In recent years TLC/HPTLC research has entered the chiral separation field using a number of chiral selectors and chiral stationary phases. Only one type of chiral pre-coated plate is presently commercially available, which is based on a ligand exchange principle and is produced commercially either as a TLC or HPTLC plate. Günther (1988) has reported

results with amino-acids and derivatives on the TLC plate and Mack *et al.*, (1988) similarly with their HPTLC equivalent.

Thin layer chromatographic (TLC) method, in this experiment was used using three different solvent system that has mentioned in the methodology portion of this paper. The solvents were polar, non-polar and medium polar. According to previous journals and research work, various antioxidants are soluble in different polar solvent system. As the antioxidant components present in fish muscle are unknown whether its polar, medium polar or non-polar, different solvents were used. Only to identify the presence of antioxidant component in fish sample, this process was applied. But this technique is failed to quantify the amount of antioxidant. For determination of the amount of antioxidant present in the fishes, DPPH free radical scavenging method has been used in this experiment.

In this experiment, ascorbic acid was used as positive control. That means ascorbic acid is wellknown for its antioxidative effect. Vitamin C or L-ascorbic acid is chemically simple, although its ene-diol structure provides it with a highly complex chemistry, which has been reviewed by Buettner and Jurkiewicz (1996). Vitamin C is water-soluble and is present in its deprotonated state as ascorbate under most physiologic conditions (Sies and Stahl 1995). It has redox chemistry with radical intermediates and it is modified by the acidic properties of the molecule. The chemical structure of ascorbate is α -keto lactone with a five-membered ring. Ascorbate (I) is an excellent reducing agent. The loss of an electron by ascorbate gives the stable semidehydroascorbate radical (II) due to the delocalized unpaired electron, which can be further oxidized to give dehydroascorbate (III). Ascorbic acid is a diacid. The dissociation constants are $pK_1 = 4.17$ (at the 3-OH) and $pK_2 = 11.57$ (at the 2-OH).

The history of vitamin C is associated with the prevention of scurvy and it was named ascorbic acid due to its antiscorbutic properties. Scurvy is caused by deficiency of ascorbate from the human diet. Ascorbic acid is required as a cofactor for proline hydroxylase and lysine hydroxylase, involved in the biosynthesis of collagen. Collagen synthesized in the absence of ascorbic acid is insufficiently hydroxylated and does not form fibers properly, giving rise to the scurvy symptoms, which are poor wound-healing and fragility of blood vessels (Halliwell and Gutteridge, 1989). Nearly all species of animals synthesize vitamin C and do not require it in their diets. However, humans, other primates, some birds and fish such as Coho salmon, rainbow trout, and carp cannot synthesize the vitamin because of the

loss of a liver enzyme, Lglucono- γ -lactone oxidase, during the course of evolution (Deshpande *et al.*, 1996). Ascorbate is required as a cofactor for several enzymes and for the action of the copper enzyme dopamine- β -hydroxylase which converts dopamine into noradrenalin (Halliwell and Gutteridge, 1989). Ascorbic acid is an essential factor in several hydroxylation reactions, primarily because of its ability to act as a redox couple, but most physiological functions of ascorbic acid are related to its ability to act as an electron donor (Deshpande *et al.*, 1996).

Vitamin C is found throughout the plant and animal kingdom and its occurrence in all living tissues, both animal and vegetable gives an indication of its vital biological role (Klăui and Pongracz, 1981). Fish muscle is generally low in Vitamin C content and the levels may be considered as negligible (Nettleton and Exler, 1992). Levels of ascorbic acid in the edible portion of fish and fish products have been reported by Gordon and Martin (1982). The ascorbic acid found in white fish muscle averaged 0.33 ± 0.19 mg/100 g with slightly more in oysters and 1.7 mg/100 g in salmon. Sidwell and coworkers (1978) reported somewhat higher values; 7.1 ± 1.9 in salmon, 1.1 ± 1.1 in trout, 2.6 ± 1.2 in tuna, 1.5 ± 0.6 in shrimp and prawns, and 9.0 ± 3.8 mg/100 g in herring. Nettleton and Exler (1992) measured ascorbic acid in edible portion of wild and cultivated channel catfish, coho salmon, rainbow trout, red swamp crayfish and White River crayfish. The vitamin C content of these fish was generally below 1 mg/100 g, except for rainbow trout with 1.8 mg/100 g in wild and 2.9 mg/100 g in cultivated fish, and eastern oysters with 3.1 in wild and 3.8 mg/100 g in cultivated oysters. Cultivated fish did not in general contain higher amounts of vitamin C. Nettleton and Exler (1992) did also determine the vitamin C content in the relevant fish species after cooking. Vitamin C values for all cooked samples were greater than those in raw samples. The authors proposed that these findings were due to moisture loss during cooking and that vitamin C was well retained after cooking. Other processing of fish like hot smoking has also been found to have little effect on vitamin C in fish. Thus Bhuiyan and coworkers (1993) found 4.7 mg/100 g of ascorbic acid in fresh and 4.5 mg/100 g in hot-smoked fillets of Atlantic fall mackerel. Storage time will probably affect the content of vitamin C in fish. Brannan and Erickson (1996) measured the effect of frozen storage on the stability of ascorbic acid in fillets and mince of channel catfish (*Ictalurus punctatus*). The initial ascorbic acid concentration in fillets and mince was around 1.47 mg/100 g and decreased by a first order

regression equation for both fillets and mince during frozen storage at -6°C . The loss of vitamin C occurred at a faster rate in the mince, especially through the first four months. The ascorbic acid values for the fillets and mince at the end of the storage experiment (6 months) were 1.19 and 0.63 mg/100 g, respectively. Large losses of ascorbic acid in the initial phases of accelerated frozen storage (temperature fluctuations) have also been reported in minced muscle of cultivated channel catfish (*Ictalurus punctatus*) (Erickson 1993a) and striped bass (*Morone saxatilis*) (Erickson 1993b).

The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by half. In other words, it is the half maximal (50%) inhibitory concentration (IC) of a substance (50% IC, or IC_{50}). It is commonly used as a measure of antagonist drug potency in pharmacological research. Sometimes, it is also converted to the pIC_{50} scale ($-\log \text{IC}_{50}$), in which higher values indicate exponentially greater potency. According to the FDA, IC_{50} represents the concentration of a drug that is required for 50% inhibition *in vitro*. It is comparable to an EC_{50} for agonist drugs. EC_{50} also represents the plasma concentration required for obtaining 50% of a maximum effect *in vivo*.

The scientific community has devised a means of evaluating antioxidant efficiencies, known as IC_{50} . This is defined as Scavenging Effect=Inhibitory Concentration or IC. The IC_{50} is the concentration of an antioxidant at which 50% inhibition of free radical activity is observed. The lower the IC_{50} number, the greater the overall effectiveness of the antioxidant in question.

According to Ronald (2004), IC_{50} of ascorbic acid is 1.96 ± 0.013 ($\mu\text{g/ml}$). In this experiment, IC_{50} of ascorbic acid was found about $16.50\mu\text{g/ml}$.

IC_{50} of fifteen fishes were tested in this experiment. Fishes were categorized according to their feeding habit. The IC_{50} number of herbivore fishes found from this experiment is about $154.45 \mu\text{g/ml}$ (*Ctenopharygodon idella*), $963.46 \mu\text{g/ml}$ (*Hypophthalmichthys molitrix*), $267.70 \mu\text{g/ml}$ (*Puntius ticto*), $965.75 \mu\text{g/ml}$ (*Labeo bata*) and $1678.96 \mu\text{g/ml}$ (*Puntius gonionotus*).

Channa striatus and *Channa punctatus* showed comparatively lower IC₅₀ value than other three carnivore fishes in this experiment. DPPH free radical scavenging assay revealed the IC₅₀ of about 327.69 µg/ml (*Channa striatus*), 346.32 µg/ml (*Channa punctatus*), 2138.52 µg/ml (*Notopterus chitala*), 1757.17 µg/ml (*Notopterus notopterus*) and 1170.29 µg/ml (*Mystus aor*).

Among five omnivore fishes, *Anabas testudineus* showed lower IC₅₀ value than other fishes of this category. The value of IC₅₀ for *Pampus chinensis*, *Scatophagus argus*, *Anabas testudineus*, *Rhinomugil corsula* and *Mackerel sp.* were found 600.89 µg/ml, 872.19 µg/ml, 397.72 µg/ml, 512.66 µg/ml and 1266.02 µg/ml respectively. Lokman (2006) found higher antioxidant activity in *Anabas testudineus* and *Labeo rohita*.

Normally, herbivore fishes are supposed to get high antioxidant component because of their feeding habit as they consume mainly plant material and plants are the main source of antioxidant. Weeds have been reported to contain high levels of TMAO and there is a possibility of direct incorporation of TMAO by aquatic animals feeding on seaweed (Ikeda 1980). Balerine has been detected only in small quantities in a limited number of species of tuna and sharks, but it makes up the major part of the extractive components of baleen whales which is plankton eater, amounting to more than 1500 mg (Konosu and Yamaguchi 1982). According to the experiment, relationships found among the feeding habit of fish and antioxidant was less significant. High antioxidant activity was found in two herbivores, two carnivores and only one omnivore fishes although herbivore fishes showed comparatively higher antioxidant activity (*Puntius ticto*, *Ctenopharyngodon idella*) and lowest IC₅₀ value.

Each fish species has characteristic tocopherol levels in its tissue, which, since fish are unable to synthesize the vitamin, are related to diet (Ackman and Cormier 1967). The size or the age of the fish may also influence the tocopherol concentration. Thus, López *et al.* (1995) found highest values for α-tocopherol in the youngest rainbow trout, but no significant difference was found between sexes in adult samples. Some antioxidant components level in fish varies with the season, size and age of fish, as well as environmental conditions to which the animal is subjected (Hebard *et al.*, 1982). In addition white-fleshed fishes have higher TMAO in ordinary muscle than in the dark muscle, but the opposite is true for dark-fleshed fish such as sardine, mackerel, tuna and skipjack (Tokunaga 1970).

Seasonal variations in tocopherol content of fish species may be considerable. Syväoja and Salminen (1985) measured tocopherols and tocotrienols in Finnish fish and fish products and found that fish caught in the spring, the spawning season of most species, had a higher tocopherol content and lower fat content, than those caught in the autumn. Others have reported the same situation. Hardy and Mackie (1969) found a decrease in fat content, followed by an increase in tocopherol content in the period from October to March in sprats. Ackman (1974) found a corresponding inverse relationship between tocopherol content and fat content in commercial sole fillets. The tocopherol content was as high as 500-600 mg/kg lipid in the lean period of the year, and as low as 100 to 200 mg/kg lipid when lipid was at its highest in September and October. The results of Syväoja and Salminen (1985) showed that the tocopherol content of for example Baltic herring was 360 mg/kg lipid in the spring, but the values for the autumn herring was 200 mg/kg lipid, or about 55% that of the spring herring. The reason for this seasonal variation in tocopherol content has been suggested to be a consequence of the sexual maturation of the fish, reaching their peak during the spawning season (Syväoja and Salminen, 1985). In this experiment, no seasonal variation was examined but thought to have seasonal effects on the tested fishes.

Fish species, examined, which were found to have high antioxidant activities can be recommended as part of the diet as they may be able to protect the human body from free radicals and retard the progress of many diseases. The fishes found to have antioxidants in this study also can be used as accessible source of natural antioxidants to replace synthetic antioxidants, as possible food supplement.

CHAPTER 5

CONCLUSION

Chapter Five

Conclusion

Fish contains various antioxidants for protection of their unsaturated lipids and other nutrients from reactive oxygen species. Oxidative protection of fish includes various water-soluble and lipid soluble antioxidant systems. The function of these antioxidants is to control pro-oxidants, scavenge free radicals and inactivate reactive oxygen species. The chemistry and function of well established food antioxidants such as ascorbic acid and tocopherol that also occur naturally is fairly well understood. Recently research on antioxidants has gained much interest due to increased evidence of the importance of antioxidants in human health. This interest has brought about considerable research on defining and finding components with antioxidative activity in humans and in biological systems. Relatively few studies on the various antioxidants in fish are currently available. Studies on the interaction between the various endogenous antioxidants in fish are also very few and may be difficult to rationalize because of the multiplicity of the antioxidant system in fish. However, the occurrence of the most abundant endogenous antioxidants found in fish such as tocopherol and carotenoids is fairly well established. The relative concentration of antioxidants varies with the fish species as well as the type of fish muscle tissue. The quantity of these antioxidants may also fluctuate with storage time and handling post mortem. Control of lipid oxidation in fish involves utilizing processing and storage techniques that do not greatly decrease the activity of endogenous antioxidants. However, the fish species examined which were found to have high antioxidant activities are recommended as part of the diet as they may be able to protect the human body from free radicals and retard the progress of many chronic diseases. The fish extracts found to have antioxidants in this study also can be used as accessible source of natural antioxidants to replace synthetic antioxidants, as possible food supplement as well as in pharmaceutical applications.

CHAPTER 6

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Chapter Six

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APPENDIX

Chapter Seven

Appendix

Table 6.1: Absorbance and %inhibition data of tested fishes

ASCORBIC ACID

Conc.(µg/ml)	0	1	50	100	200	300	400	500
AB1	0.801	0.695	0.075	0.063	0.052	0.046	0.039	0.032
AB2	0.806	0.696	0.077	0.064	0.051	0.043	0.038	0.029
AVG	0.803	0.695	0.076	0.063	0.051	0.044	0.038	0.031
%INHIBITION	0	13.44	90.53	92.15	93.64	94.52	95.26	96.13

Labeo bata

Conc.(µg/ml)	0	1	50	100	200	300	400	500
AB1	0.895	0.896	0.892	0.862	0.819	0.767	0.716	0.650
AB2	0.898	0.894	0.890	0.864	0.825	0.770	0.716	0.656
AVG	0.896	0.895	0.891	0.863	0.822	0.769	0.716	0.653
%INHIBITION	0	0.11	0.55	3.68	8.26	14.18	20.08	27.12

Puntius gonionotus

Conc.(µg/ml)	0	1	50	100	200	300	400	500
AB1	0.869	0.808	0.798	0.786	0.790	0.763	0.739	0.711
AB2	0.872	0.816	0.803	0.788	0.782	0.763	0.743	0.699
AVG	0.870	0.812	0.801	0.787	0.786	0.763	0.741	0.705
%INHIBITION	0	6.67	7.93	9.54	9.66	12.30	14.88	18.97

Hypophthalmichthys molitrix

Conc.(µg/ml)	0	1	50	100	200	300	400	500
AB1	0.894	0.868	0.848	0.829	0.787	0.742	0.693	0.655
AB2	0.888	0.861	0.851	0.832	0.782	0.733	0.694	0.659
AVG	0.891	0.864	0.849	0.830	0.784	0.737	0.693	0.657
%INHIBITION	0	3.03	4.71	6.85	12.01	17.28	22.22	26.26

Ctenopharyngodon idella

Conc.(µg/ml)	0	1	50	100	200	300	400	500
AB1	0.806	0.710	0.523	0.277	0.169	0.124	0.091	0.088
AB2	0.801	0.712	0.524	0.271	0.168	0.125	0.095	0.088
AVG	0.803	0.711	0.523	0.274	0.168	0.125	0.093	0.088
%INHIBITION	0	11.45	34.86	65.87	79.07	84.44	88.41	89.04

Puntius ticto

Conc.(µg/ml)	0	1	50	100	200	300	400	500
AB1	0.716	0.695	0.527	0.483	0.441	0.316	0.188	0.138
AB2	0.711	0.694	0.533	0.491	0.447	0.315	0.185	0.131
AVG	0.713	0.694	0.530	0.487	0.444	0.315	0.186	0.134
%INHIBITION	0	2.66	25.67	31.69	37.73	55.82	73.91	81.20

Channa striatus

Conc.(µg/ml)	0	1	50	100	200	300	400	500
AB1	0.696	0.650	0.599	0.569	0.414	0.350	0.298	0.211
AB2	0.703	0.679	0.596	0.571	0.417	0.353	0.295	0.207
AVG	0.699	0.664	0.598	0.570	0.415	0.351	0.296	0.209
%INHIBITION	0	5.00	14.49	18.45	40.63	49.78	57.65	70.11

Notopterus notopterus

Conc.(µg/ml)	0	1	50	100	200	300	400	500
AB1	0.904	0.887	0.878	0.867	0.852	0.828	0.806	0.751
AB2	0.901	0.896	0.871	0.860	0.848	0.827	0.797	0.762
AVG	0.902	0.891	0.874	0.863	0.850	0.827	0.801	0.756
%INHIBITION	0	1.22	3.10	4.32	5.76	8.31	11.20	16.19

Channa punctatus

Conc.(µg/ml)	0	1	50	100	200	300	400	500
AB1	0.875	0.838	0.758	0.717	0.565	0.516	0.422	0.334
AB2	0.883	0.836	0.759	0.712	0.578	0.519	0.417	0.330
AVG	0.879	0.837	0.758	0.715	0.571	0.517	0.420	0.332
%INHIBITION	0	4.78	13.77	18.71	35.03	41.19	52.21	62.22

Notopterus chitala

Conc.(µg/ml)	0	1	50	100	200	300	400	500
AB1	0.901	0.893	0.878	0.871	0.850	0.822	0.806	0.791
AB2	0.897	0.886	0.875	0.869	0.858	0.832	0.802	0.794
AVG	0.899	0.889	0.876	0.870	0.854	0.827	0.804	0.792
%INHIBITION	0	1.11	2.55	3.22	5.00	8.00	10.57	11.90

Mystus aor

Conc.(µg/ml)	0	1	50	100	200	300	400	500
AB1	0.869	0.867	0.844	0.778	0.745	0.742	0.713	0.678
AB2	0.872	0.870	0.853	0.784	0.750	0.747	0.723	0.687
AVG	0.870	0.868	0.848	0.781	0.747	0.744	0.718	0.682
%INHIBITION	0	0.23	2.52	10.22	14.13	14.48	17.47	21.61

Rhinomugil corsula

Conc.(µg/ml)	0	1	50	100	200	300	400	500
AB1	0.894	.863	.850	.823	.815	.777	.731	.699
AB2	0.889	0.866	.857	0.830	.817	.776	.739	.703
AVG	0.891	0.864	0.853	0.826	0.816	0.776	0.735	0.701
%INHIBITION	0	3.03	4.26	7.30	8.41	12.90	17.50	21.34

Pampus chinensis

Conc.(µg/ml)	0	1	50	100	200	300	400	500
AB1	0.894	0.884	0.858	0.809	0.730	0.651	0.611	0.513
AB2	0.889	0.881	0.856	0.809	0.717	0.662	0.589	0.530
AVG	0.891	0.882	0.857	0.809	0.723	0.651	0.600	0.521
%INHIBITION	0	1.01	3.82	9.20	18.85	26.94	32.66	41.53

Anabus testudineus

Conc.(µg/ml)	0	1	50	100	200	300	400	500
AB1	0.893	0.850	0.795	0.735	0.698	0.545	0.479	0.310
AB2	0.898	0.852	0.787	0.737	0.705	0.538	0.465	0.318
AVG	0.895	0.851	0.791	0.736	0.701	0.541	0.472	0.309
%INHIBITION	0	4.92	11.62	17.76	21.68	39.55	47.26	65.47

Scatophagus argus

Conc.(µg/ml)	0	1	50	100	200	300	400	500
AB1	0.801	0.795	0.773	0.753	0.677	0.647	0.622	0.575
AB2	0.806	0.799	0.774	0.764	0.669	0.633	0.617	0.584
AVG	0.803	0.797	0.773	0.758	0.673	0.640	0.619	0.579
%INHIBITION	0	0.75	3.74	5.60	16.19	20.30	22.91	27.90

Mackerel sp

Conc.(µg/ml)	0	1	50	100	200	300	400	500
AB1	0.767	0.771	0.736	0.700	0.638	0.509	0.445	0.394
AB2	0.775	0.758	0.726	0.711	0.647	0.510	0.450	0.396
AVG	0.771	0.764	0.731	0.705	0.642	0.509	0.448	0.395
%INHIBITION	0	0.91	5.19	11.10	16.73	33.98	41.89	48.77

Table 6.2: IC₅₀ value of different fishes determined from %inhibition vs. concentration graph

Species	R ² value	IC ₅₀ value (µg/ml)
<i>Labeo bata</i>	$y = 0.053x - 1.185$ $R^2 = 0.988$	965.75
<i>Ctenopharyngodon idella</i>	$y = 0.164x + 24.67$ $R^2 = 0.748$	154.45
<i>Puntius ticto</i>	$y = 0.155x + 8.506$ $R^2 = 0.953$	267.70
<i>Puntiu gonionotus</i>	$y = 0.027x + 4.668$ $R^2 = 0.843$	1678.96
<i>Hypophthalmichthys molitrix</i>	$y = 0.050x + 1.827$ $R^2 = 0.991$	963.46
<i>Rhinomugil corsula</i>	$y = 0.038x + 1.891$ $R^2 = 0.975$	1266.02
<i>Pampus chinensis</i>	$y = 0.082x + 0.727$ $R^2 = 0.995$	600.89
<i>Scatophagus argus</i>	$y = 0.056x + 1.157$ $R^2 = 0.970$	872.19
<i>Anabas testudineus</i>	$y = 0.118x + 3.068$ $R^2 = 0.979$	397.72
<i>Mackerel sp</i>	$y = 0.100x + 0.334$ $R^2 = 0.987$	530.69
<i>Channa striatus</i>	$y = 0.135x + 5.761$ $R^2 = 0.973$	327.69
<i>Channa punctatus</i>	$y = 0.118x + 5.594$ $R^2 = 0.978$	346.32
<i>Notopterus notopterus</i>	$y = 0.028x + 0.799$ $R^2 = 0.971$	1757.17
<i>Mystus aor</i>	$y = 0.041x + 2.018$ $R^2 = 0.909$	1170.29
<i>Notopterus chitala</i>	$y = 0.023x + 0.814$ $R^2 = 0.986$	2138.52

PHOTO PLATES

Photoplates



Plate 1: Measurement of muscle weight
by electronic balance

Plate 2: Homogenization of Fish
muscle by homogenizer



Plate 3: Ethanolic extract of fishes are
being placed into shaking water-bath for
drying

Photoplates

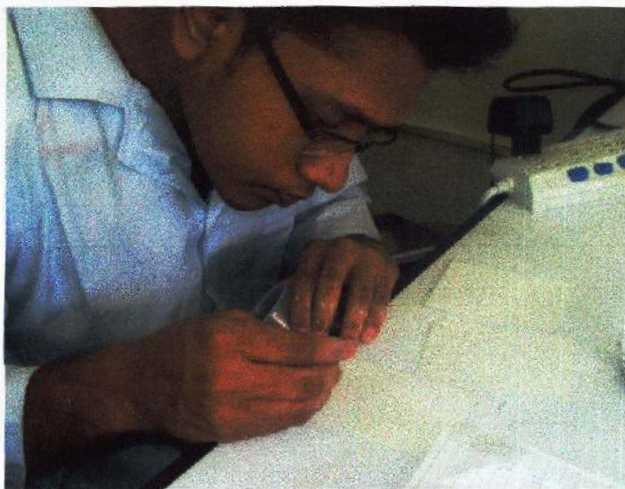


Plate 4: Application of ethanolic extract of fish on TLC plate by spotter

Plate 5: Glass jar with TLC plate



Photoplates

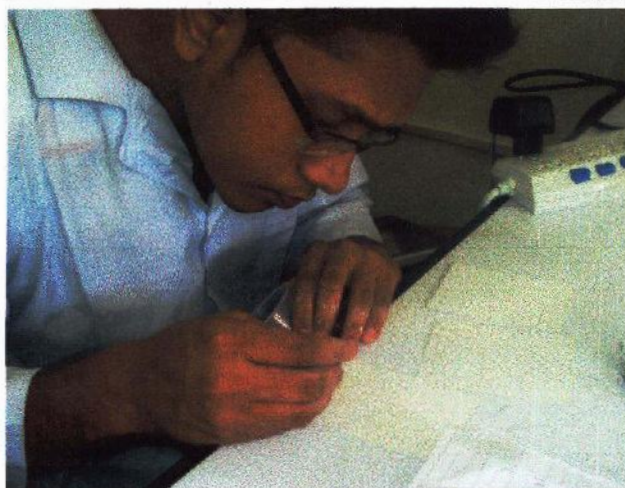


Plate 4: Application of ethanolic extract of fish on TLC plate by spotter

Plate 5: Glass jar with TLC plate



Plate 6: DPPH are being sprayed on TLC plate by spray gun

Photoplates

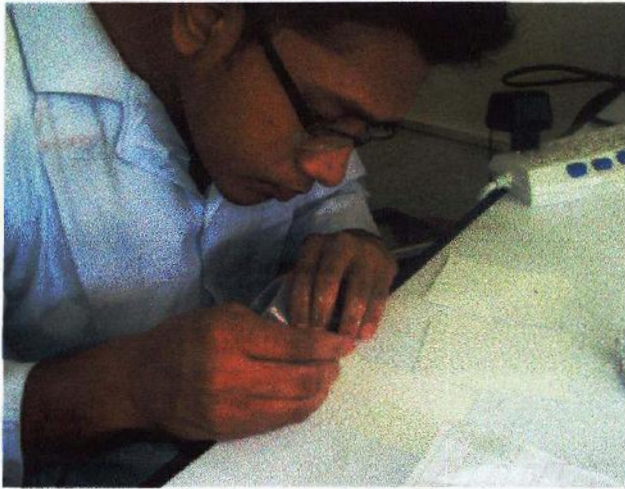


Plate 4: Application of ethanolic extract of fish on TLC plate by spotter

Plate 5: Glass jar with TLC plate



Photoplates

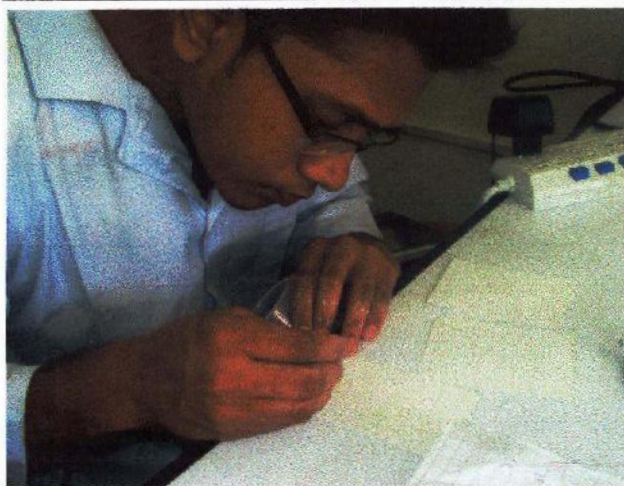


Plate 4: Application of ethanolic extract of fish on TLC plate by spotter

Plate 5: Glass jar with TLC plate



Plate 6: DPPH are being sprayed on TLC plate by spray gun

Photoplates



Plate 7: Observation of TLC plate under UV light

Plate 8: Solutions of desired concentration are being prepared for quantitative analysis



Plate 9: Determination of absorbance of the fish sample by spectrophotometer