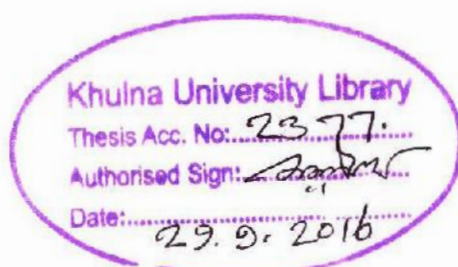


**Profiling of Different Bioactive Compounds in Water Spinach
by High Performance Liquid Chromatography**



SAJAL KRISHNA RAY

MS-130604



Fisheries and Marine Resource Technology Discipline

Khulna University

Khulna, Bangladesh

April, 2014

Profiling of Different Bioactive Compounds in Water Spinach by High Performance Liquid Chromatography

A Thesis

By

Sajal Krishna Ray

Student ID: MS-130604

Session: 2012-2013



**The thesis submitted to Fisheries and Marine Resource Technology
Discipline in partial fulfillment of the requirements for the Degree
of
Masters of Science in Fish Genetics and Biotechnology**

**Fisheries and Marine Resource Technology Discipline
Life Science School, Khulna University
Khulna-9208, Bangladesh**

April, 2014

**Profiling of Different Bioactive Compounds in Water Spinach
by High Performance Liquid Chromatography**

Approved as to the style and content

By



Professor Dr. Md. Ayaz Hasan Chisty

Head

Fisheries and Marine Resource Technology Discipline

Khulna University

Khulna-9208, Bangladesh

Fisheries and Marine Resource Technology Discipline

Life Science School, Khulna University

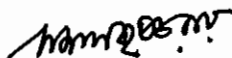
Khulna-9208, Bangladesh

April, 2014

Declaration

Profiling of Different Bioactive Compounds in Water Spinach by High Performance Liquid Chromatography

The results presented in this 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.



(Signature of the candidate)

Sajal Krishna Ray

Student ID: MS-130604

Session: 2012-2013



(Signature of Supervisor)

Dr. Md. Golam Sarower

Professor

Fisheries and Marine Resource Technology Discipline

Khulna University, Khulna-9208, Bangladesh

April, 2014

DEDICATED

TO

MY BELOVED PARENTS

Acknowledgement

My gratification is for being able to complete this thesis for the degree of M.Sc. in Masters of Science in Fish Genetics and Biotechnology from Fisheries and Marine Resource Technology Discipline, so first of all, my appreciation and indebtedness go to God.

No word is appropriate for expressing my sincere gratitude, heartfelt appreciation and profound respect with due solemnity to my honorable supervisor **Dr. Md. Golam Sarower**, Professor, Fisheries and Marine Resource Technology (FMRT) Discipline, Khulna University, Khulna. His kind and continuous inspiration, scholastic guidance, constructive criticism, constant encouragement and providence of all necessary facilities help me to complete thesis paper successfully.

I am so grateful with sincere thanks to my honorable teacher **Dr. Shaikh Jamal Uddin**, Assistant Professor, Pharmacy Discipline for his inspiration, advice and valuable technical information regarding my thesis work.

My endless thanks to Hemayet vi and Ismet vi, Bangladesh Council of Scientific and Industrial Research (BCSIR) Laboratories, Dhaka, for their reporting help during my thesis work.

My special thanks to Rabiul vi, Lab Registrar Assistant, Pharmacy laboratory, Pharmacy Discipline and Ashik vai, for their continuous help during laboratory work.

Special thanks to Zilani, Kushi, Akhe and Siraj. I would like to express my deepest gratitude to my beloved parents and my family for standing all the way by my side during the preparation of the thesis.

Sajal Krishna Ray

Fisheries and Marine Resource Technology Discipline,
Khulna University, Khulna

April, 2014

Abstract

Water spinach (*Ipomoea aquatica*) is popularly known as kalmi shak which has played an important role as an energy and a phytochemical source in human nutrition and fish feeding. Consumption of vegetables has been shown to be effective in the prevention of diseases because of its bioactive compounds. The current study was conducted to evaluate the antioxidant, antimicrobial activities, phytochemical and bioactive compounds from the plant extract of water spinach (*I. aquatica*). The yield of ethanolic extract was found 2.146 % w/w. TLC plates analysis showed antioxidant effect in the water spinach (*I. aquatica*). The phytochemical screening showed positive results for alkaloid, tannins, glycoside and steroid. The crude extract was partitioning into two fractions of n-Hexane and aqueous ethanol using solvent-solvent partition methods. n-Hexane fraction from the ethanolic extract of water spinach (*I. aquatica*) was separated into thirty-seven sub-fractions using silica column chromatography and a n-Hexane-acetone (3:1, v/v). Five same spots of n-Hexane sub-fractions, n-Hexane fraction, aqueous ethanol fraction and crude ethanolic extract were analyzed by HPLC for profiling bioactive compounds. No antimicrobial activities of these extract showed against three bacterial strains of *Escherichia coli*. In this study, HPLC technique was used to detect bioactive compounds in a single run by using an ultra-deactivated C18 column. Five, one and five polyphenolic compounds namely catechin, vanillic acid, p-coumaric acid, rutin hydrate, ellagic acid, gallic acid, catechin, caffeic acid, p-coumaric acid, rutin hydarte and ellagic acid were detected from crude ethanolic extract, n-Hexane fraction and aqueous ethanol fraction respectively by HPLC analysis following indication of the presence antioxidant compounds.



List of Contents

Title	Title of the contents	Page No
Acknowledgement		i
Abstract		ii
List of Contents		iii-v
List of Tables		vi
List of Figure		vi-vii
Chapter 1	1.0 Introduction	1
	1.1 Background of the study	1-3
	1.2 Objectives of the study	4
Chapter 2	2.0 Literature review	5-8
	2.1 Plant preview	8
	2.1.1 Botanical Features	8
	2.1.2 Plant Morphology	9
	2.1.3 Distribution	9
	2.1.4 Uses of water spinach (<i>I. aquatica</i>)	9-10
Chapter 3	3.0 Materials and Methods	11
	3.1 Materials	11
	3.1.1 Materials used for cold extraction	11
	3.1.2 Materials used for solvent-solvent partition	11
	3.1.3 Materials used for sub-fractionation column chromatography	11
	3.1.4 Materials used for TLC analysis	12
	3.2.5 Reagents used for phytochemical group test	12
	3.2.6 Materials used for HPLC analysis	12
	3.2.7 Materials used for anti-microbial activity test	13
	3.2.7.1 Microorganisms used for the test	13
	3.2.7.2 Composition of Nutrient agar media	14
	3.2.7.3 Composition of Nutrient broth media	14

	3.2.8 Materials used for qualitative anti-oxidant test	14
	3.2.9 Source of chemicals and reagents	15
	3.2 Methods	15
	3.2.1.1 Collection	15
	3.2.1.2 Drying & Grinding	15
	3.2.1.3 Cold extraction (Ethanol extraction)	15-16
	3.2.1.4 Evaporation of the solvent	16
	3.2.1.5 Extraction at a glance	16
	3.2.2 Solvent-solvent partition	16
	3.2.2.1 Selection of solvent system	16
	3.2.2.2 Separation Process	16
	3.2.3 Methods used for sub-fractionation column chromatography	17
	3.2.3.1 Adsorbent	18
	3.2.3.2 Solvent system	18
	3.2.3.3 Process of column chromatography	18-19
	3.2.4 Methods used for TLC analysis	19
	3.2.4.1 TLC plate preparation	19
	3.2.4.2 Sample application	19
	3.2.4.3 Development of chromatogram	20-21
	3.2.5 Phytochemical group test	21
	3.2.5.1 Preparation of The reagents	21
	3.2.5.2 Tests procedure for identifying different chemical groups	22
	3.2.6 Methods used for HPLC analysis	23
	3.2.6.1 High performance liquid chromatography (HPLC) system	23
	3.2.6.2 Chromatographic conditions	23-24
	3.2.6.3 Standard and sample preparation	24
	3.2.6.4 Peak characterization and quantification	24
	3.2.7 Methods used for antimicrobial activity test	25
	3.2.7.1 Preparation of agar media	25
	3.2.7.2 Sterilization of different equipments and media	25
	3.2.7.3 Preparation of sub-culture	25

	3.2.7.4 Preparation of Nutrient broth media	25-26
	3.2.7.5 Preparation of the seeded test plates	26
	3.2.7.6 Preparation of test sample	26
	3.2.7.7 Preparation of disc	26
	3.2.7.8 Sample discs	27
	3.2.7.9 Standard discs	27
	3.2.7.10 Blank discs	27
	3.2.7.11 Application of discs	27
	3.8 Methods used for qualitative anti-oxidant test	28
	3.8.1 Solvent selection	28
	3.8.2 Thin Layer Chromatography (TLC) Technique	28
	3.8.3 Antioxidant positive components detection	28
	3.8.4 UV positive components detection	28
Chapter 4	4.0 Results	29
	4.1 Yield from the extraction of water spinach (<i>Ipomoea aquatica</i>)	29
	4.2 Results of solvent-solvent partition	29
	4.3 Sub-fraction of n-Hexane fraction by using column chromatography	29
	4.4 TLC plates analysis	29-31
	4.5 Investigation of phytochemical group test	32-33
	4.6 HPLC analysis	33
	4.6.1 Method validation	33-34
	4.6.2 Method development	34-36
	4.6.3 Analysis of the plant extract	37
	4.7 Antimicrobial activity test	39
	4.8 In-vitro qualitative anti-oxidant test	40
Chapter 5	5.0. Discussion	41-43
Chapter 6	6.0. Conclusion	44
Chapter 7	7.0. References	45-52
	List of abbreviations	53

8	The High Performance Liquid Chromatography (HPLC) system	23
9	TLC plates for water spinach (<i>I. aquatica</i>) of crude ethanolic extract after applying 10% H ₂ SO ₄	30
10	TLC plates for water spinach (<i>I. aquatica</i>) of n-Hexane fraction and aqueous ethanol fraction after applying 10% H ₂ SO ₄ .	30
11	TLC plates for water spinach (<i>I. aquatica</i>) of sub-fractionation of n-Hexane fraction after applying 10% H ₂ SO ₄	31
12	HPLC chromatogram of a standard mixture of polyphenolic compounds	35
13	HPLC chromatogram of crude ethanolic extract of water spinach (<i>I. aquatica</i>)	35
14	HPLC chromatogram of n-Hexane fraction of water spinach (<i>I. aquatica</i>)	36
15	HPLC chromatogram of aqueous ethanol fraction of water spinach (<i>I. aquatica</i>)	36
16	Identification of antioxidant compounds of water spinach (<i>I. aquatica</i>) extract	38
17	Chemical structures of seven antioxidant compounds in water spinach (<i>I. aquatica</i>)	39
18	TLC plates for <i>I. aquatica</i> of crude ethanolic extract after applying 0.02%DPPH	40
19	TLC plates for <i>I. aquatica</i> of aqueous ethanol fraction and n-Hexane fraction after applying 0.02% DPPH	40



Chapter One

INTRODUCTION

Chapter 1

1.0 Introduction

1.1 Background of the study

Vegetables and fruits could be a good source of antioxidant (Shahid, 2011). Consumption of plant having the antioxidant potential can certainly fulfill the requirement of antioxidant. Aquatic weeds are known to contain antioxidative compounds (Ramarathnam *et al.*, 1995). 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 antioxidant compounds related to chlorophyll a and purpurin. There are other lower marine animal which also contain chlorophyll-a (Sakata *et al.*, 1994).

Fish is a source of omega-3 fats. These richest sources of omega-3 fats come from aquatic plants which are helping to keep the heart healthy (Buttriss, 1999).

Water spinach (*Ipomoea aquatica*) has been considered native to Africa, Asia, and the southwestern Pacific Islands. The herbs have been a medicinal vegetable in southern Asia since at least A.D. 300, and perhaps since 200 B.C. (Austin, 2007).

I. aquatica is also fodder for animals in limited quantity as it is somewhat laxative. These herbs are often grown in fish ponds by Chinese, particularly as food for their pigs (Ly *et al.*, 2002; Westphal, 1993). Although they are also fed to cattle and fish (Edie and Ho, 1969). This plant provides food, shelter, reproductive habitat or breeding ground of numerous fish and other aquatic animals (Sarower *et al.*, 2008).

Fresh plants have from 1.9-4.6% proteins and carbohydrates average ca. 4.3% (Yamaguchi *et al.*, 2006). In the Philippines a mouthwash made of water spinach was experimentally shown to be effective against *Escherichia coli* and *Staphylococcus aureus* (Castillo, 1982).

In China, fish ponds with static water and earthen-bottom were mostly open or semi-open farm system. In order to maximize fish production, a great deal of formulated feed was applied. But fish culture only retained 20–50% of feed nitrogen (N) and 15–65% of feed phosphorus (P) (Schneider *et al.*, 2005).

Large amount of uneaten feed and fish feces made water quality bad and turnover of algae was rapid in such ponds dominated by cyanobacteria (Van Rijn, 1996). Research has indicated that stigmasterol may be useful in prevention of certain cancers, including ovarian, prostate, breast, and colon cancers. Studies have also indicated that a diet high in phytoesters may inhibit the absorption of cholesterol and lower serum cholesterol levels by competing for intestinal absorption. It was demonstrated that it inhibits several proinflammatory (Gabay *et al.*, 2010).

The leaves contain adequate quantities of most of the essential amino acids, e.g., aspartic acid, glycine, alanine and leucine, in accordance with the WHO-recommended pattern for ideal dietary protein (WHO, 1973). This is comparable to conventional foodstuffs such as soybean or whole egg, indicating the potential of *I. aquatica* for utilisation as a food supplement (Rangan *et al.*, 1990).

Green leafy vegetables form a rich source of dietary fibres, nutraceuticals and vitamins, e.g., carotene (a precursor of vitamin A), vitamin C, riboflavin and folic acid, and minerals, e.g., calcium, iron and phosphorus. Leafy vegetables have been extensively investigated as new sources of natural antioxidants as well as other bioactive compounds of human health benefits (Lakshmi *et al.*, 2000).

Increased consumption of vegetables containing high levels of phytochemicals has been recommended to prevent chronic diseases related to oxidative stress in the human body (Chu *et al.*, 2002). Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of certain diseases such as cancer, heart diseases and stroke (Prior and Cao, 2000). *I. aquatica* Forsk (IA), commonly called water spinach, belongs to the family Convolvulaceae. It is cultivated commercially as an edible green leafy vegetable in Hong Kong, Taiwan and China (Rao *et al.*, 2002).

Natural products of plant origin have been proposed as a potential source of natural antioxidants with strong activity. Antioxidants play an important role in nutraceuticals by extending the shelf life of food and reducing nutritional losses and formation of harmful substances. The antioxidants occurring naturally are present in all foods and herbal medicines. Nowadays food scientists and nutrition specialists agree that food antioxidants, consumed daily contribute to the conservation of good health (Monic *et al.*, 2012). Hence the dietary intake of antioxidant compounds becomes important. Currently, there is a growing interest toward natural antioxidants of herbal resources (Sivasankari *et al.*, 2011). In much of southeastern Asia, *I. aquatica* is considered a tonic. The species contains several vitamins, including A, B, C, E, and U (S-methyl-methionine), and is used to treat gastric and intestinal disorders (Watt, 1972; Westphal, 1993). The species also contains aliphatic pyrrolidine amides, carotenoids, hentriacontane, β -sitosterol and its glycosides, prostaglandin, leukotrine, N-trans- and N-cis feruloyltyramines (Chen & Chen, 1992; Snyder *et al.*, 1981; Sundar Rao, 1990; Tofern *et al.*, 1999; Van Valkenburgh & Bunyaphrathasara, 2001; Wills & Ranga, 1996).

Now-a-days, the consumers demand of new functional foods including those enriched with vitamins is clearly rising. Vitamins are structurally heterogeneous group of essential food constituents that are required in small amounts for fish growth and maintenance of human health. They are generally divided in two main groups: lipid- and water-soluble vitamins (Kledjus *et al.*, 2004). The simultaneous determination of water and fat-soluble vitamins in pharmaceutical preparations and food samples was achieved after a careful optimization of all the factors influencing the separation such as type of column, pH, temperature, gradient elution and so on. Even considering the enormous interest of vitamins as micronutrients in food, there exist other compounds with known biological activity that can have greater contribution in the functional activity of a food and/or vegetable. These compounds (e.g., polyphenols, carotenoids, chlorophylls, etc) can play an important role in the associated bioactivity of a food product and, therefore, their determination can also be of great importance for food and nutrition studies. (Fukumoto and Mazza, 2000; Villano *et al.*, 2007; Jaime *et al.*, 2005). On the other hand, some biological effects have also been associated to chlorophylls (Schoefs, 2005). The analysis of carotenoids and chlorophylls is commonly done by HPLC and several methods have been developed to simultaneously determine these type of compounds (Mendiola *et al.*, 2005; Montero *et al.*, 2005).

1.2 Objectives of the study

Bangladesh has a good source of aquatic plants in fresh water. This aquatic plants are a good source of antioxidant (Shahid, 2011). Aquatic plants are also used as a good source of food for fish. These fish contains omega-3 fats which are helpful for keeping the heart healthy of human being (Buttriss, 1999). The prime interest of the current study was screening the sources of bioactive compounds responsible for antioxidant, phytochemical and antibacterial activities from Water spinach (*I. aquatica*). Although numerous studies in the literature exist with respect to plant-derived antioxidants. but no report on bioactive compounds of water spinach (*I. aquatica*) has published yet. HPLC is a major tool for separation of individual bioactive compounds of the plant extract. Specific objectives of this study are:-

- To know antioxidant components in water spinach.
- To evaluate antibacterial activity against *E. coli* strains (Enteropathogenic *E. coli*, Enterotoxigenic *E. coli* and *E. coli*).
- To identify bioactive compounds by using HPLC technique in water spinach.

Chapter Two



LITERATURE REVIEW

Chapter 2

2.0 Literature Review

Before conducting an experiment, it is important to know the information of the previous related works. The following information was reviewed in favor of the present study that was performed elsewhere in the world and relevant to the study. The following information was reviewed in favor of the present study which was done around the world.

Chen and Chen (1992) studied the carotenoids and chlorophylls in water convolvulus (*Ipomoea aquatica*) by liquid chromatography. An extensive study on the identification of these pigments using high performance liquid chromatography (HPLC) showed that it contains 12 components.

Mendiola *et al.* (2007) studied on Profiling of Different Bioactive Compounds in Functional Drinks by HPLC. The goal of this work was to develop an HPLC method (using diode array detection) for the simultaneous separation, detection and quantification of water and fat-soluble vitamins, phenolic compounds, chlorophylls and carotenes in a single run. The developed method should allow obtaining a “bioactive profile” of different food matrices. The usefulness of the HPLC method was corroborated through its application to the analysis of several commercial drinks, some of them fortified with vitamins.

Choi *et al.* (2010) studied on quantitative determination of bioactive compounds in some *Artemisia capillaries* by high performance liquid chromatography. A simple reversed phase HPLC method was developed for extracting pharmacologically active compounds scopolin, chlorogenic acid, 2,4-dihydroxy-6-methoxyacetophenone 4-glycoside, hyperoside, isorhamnetin 3-O-robinobioside, and scoparone from *A. capillaris* using a binary gradient of acetonitrile : 0.1% trifluoroacetic acid with UV detection at 254 nm. The developed assay has been applied successfully to quantify the six compounds in fifteen batches of the *Artemisia* genus collected from different locations.

Prasad *et al.* (2006) studied on isolation of a free radical-scavenging antioxidant from water spinach.

Phytochemicals and antioxidant constituents in plant material have raised interest among scientists, food manufacturers, producers, and consumers for their roles in the maintenance of human health (Milner, 1999).

Chang *et al.* (2013) studied on carotenoids retention in leafy vegetables based on cooking methods. Green leafy vegetables are good sources of carotenoids. Many vegetables and fruits contain significant amounts of β -carotene and other pro-vitamin A carotenoids that can be absorbed and converted to vitamin A in the human body.

Kulkarni *et al.* (2011) studied on the serious side effects associated with these drugs, namely rebound insomnia, sedation, muscle relaxation, withdrawal and tolerance, sexual dysfunction, anticholinergic, antihistaminic effects have limited their use in patients.

Yadav *et al.* (2008) studied on anxiety affects one-eighth population worldwide and has become an important research area in the field of psychopharmacology.

Many epidemiological studies indicate that bioactive compounds may have a protective role against several diseases, although the results are seldom unambiguous. Observational studies have shown associations between a low intake of ascorbic acid, carotenoids and flavonoids and an increased risk of cardiovascular disease (Knekt *et al.*, 1996; Kritchevsky, 1999; Khaw *et al.*, 2001; Arts and Hollman, 2005). Few intervention trials have studied the effects of vitamin C and flavonoids on cardiovascular disease (Christen *et al.*, 2000; Manach *et al.*, 2005).

Sivaraman *et al.*, (2010) studied on nootropic effect of *Ipomoea aquatic* forsk in rat hippocampus. They studied the acetylcholine enhancing activity of methanol leaf extract of *Ipomoea aquatic* forsk in rat hippocampus. Acetylcholine plays a central role in basic nerve transmission, concentration, memory and learning. Preliminary phytochemical investigations of MEIA revealed the presence of carbohydrates, flavonoids, sterols, saponins, phenols and terpenes.

Phokrai *et al.* (2010) studied on sulfate phytoremediation through Transgenic *Ipomoea aquatica* forsk. Harbouring Rice (*Oryza sativa* L.) Cysteine Synthase Gene.

Alkiyumi *et al.* (2012) conducted a research on *Ipomoea aquatica* extract shows protective action against thioacetamide-induced hepatotoxicity. Phytochemical investigations of this plant have revealed the presence of carotenes such as β -carotene, cryptoxanthin, lutein, lutein

epoxide, violoxanthin and neoxanthin, flavonoids such as mycertin, quercetin, luteolin and apigenin and some alkaloids.

Baysa *et al.* (2006) studied on lead and cadmium contents in *Ipomoea aquatica* forsk grown in laguna de Bay. This study was conducted to determine the concentrations of lead (Pb) and cadmium (Cd) in the top, middle, and bottom of the edible portions, and in the different organs of *I. aquatica* grown in Laguna de Bay. Pb and Cd concentrations in the edible portions (leaves and stems) of the upper 36cm of the shoot were far below the maximum tolerable daily intake for man set by World Health Organization (WHO). The bottom of the edible portion of the plant should be removed to minimize Pb intake.

Hamid *et al.* (2011) studied on evaluation of the Leaves of *Ipomoea aquatica* for its Hypoglycemic and Antioxidant Activity. Leafy vegetables are extensively investigated as a newer source of natural antioxidants and other bioactive compounds of human health benefits. *I. aquatica* is one of the richest sources of carotenoids and chlorophylls. An extensive study on the identification of these pigments was carried out using high performance liquid chromatography (HPLC) revealed that it contains 12 components. its leaf extract can be used to reduce blood sugar levels and as an antibiotic against *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus Subtilis*.

Khan *et al.* (2011) studied on anxiolytic activity of *Ipomoea aquatica* leaves. The total flavonoids contents were determined as 200 mg/kg of dried plant material. The extract also increased duration of ketamine-induced sleep. The observations suggest anxiolytic activity of *Ipomoea aquatica* leaves. It may be concluded that the methanol: acetone extract of rich in flavonoids and possesses anxiolytic principles.

Huang *et al.* (2005) studied on antioxidant and antiproliferative activities of water spinach (*Ipomoea aquatica* forsk) constituents. DPPH staining, total phenolic compounds, total flavonoid content, DPPH radical, reducing power method, FTC method, and inhibition of cancer cell proliferation were employed. Ethanol extract of stem demonstrated a positive effect in DPPH staining when it was diluted to 6.25 mg dry matter/mL while all other fractions showed no effect at the same dilution. This fraction also had the highest content of the total phenolic compounds, as well as the highest reducing power and FTC activity.

Lako *et al.* (2005) studied on phytochemical flavonols, carotenoids and the antioxidant properties of a wide selection of Fijian fruit, vegetables and other readily available foods.

Foods with the richest sources of b-carotene in descending order are *Moringa oleifera* leaves, *Amaranthus viridis*, *Ipomoea aquatica*, *Ipomoea batatas* leaves and *Colocasia esculenta* leaves. The richest source of quercetin and kaempferol in green leafy vegetable is *Moringa oleifera* followed by *Ipomoea batatas* leaves.

Shah *et al.* (2010) studied on Estimation of two Bioactive Compounds from *Azadiracta india* leaves using HPLC. Sensitive, simple, and accurate high-performance liquid chromatographic method has been established for determination of rutin and quercetin both simultaneously in *Azadiracta indica* leaf powder. The medicinal and industrial uses of various parts of Neem tree and the compounds isolated from it have been reviewed. More than 135 compounds of diverse structure have been isolated from various parts of Neem but few of them have been studied for their biological and pharmacological actions.

2.1 Plant preview

2.1.1 Botanical Features

Common name: Water-spinach, Chinese water-spinach or morning-glory, water bindweed, Chinese spinach, Chinese convolvulus, swamp cabbage or Kangkong, river spinach, and water convolvulus.

Local name: Kalmi

Scientific name: *Ipomoea aquatica*

Scientific classification

Kingdom:	Plantae
(unranked):	Angiosperms
(unranked):	Eudicots
(unranked):	Asterids
Order:	Solanales
Family:	Convolvulaceae
Genus:	<i>Ipomoea</i>
Species:	<i>I. aquatica</i>

2.1.2 Plant Morphology

Leaves: The leaves vary from typically sagittate (arrow head-shaped) to lanceolate, 5–15 cm (2–6 in) long and 2–8 cm (0.8–3 in) broad.

Flowers: The flowers are trumpet-shaped, 3–5 cm (1–2 in) in diameter, and usually white in colour with a mauve centre. Propagation is either by planting cuttings of the stem shoots that will root along nodes or planting the seeds from flowers that produce seed pods.

2.1.3 Distribution

Now considered native to China, but widely cultivated and naturalized in Africa, Asia, Australia, the Pacific Islands, and South America.

Australian Distribution: Grown in tropical areas like Darwin – Cairns and also in temperate areas like the Sydney basin.



Fig.1 Ariel parts of water spinach (*I. aquatica*)

2.1.4 Uses of water spinach (*I. aquatica*)

Human food

Water spinach is grown as a green vegetable crop in many tropical countries. The highly nutritious young stems and leaves are eaten raw, boiled, stir-fried, steamed or pickled as a vegetable throughout Asia. The percentage of protein in the leaves is high as well as vitamin-A, iron, calcium and phosphorus.

Medicinal uses

- Leaves and juice are used as a mild purgative (laxative).
- A chemical resembling insulin is present in the buds of pigmented variety and is recommended as a food for diabetics.
- The plant is used as an antidote for opium and arsenic poisoning.
- Buds are used in the treatment of ringworm.
- The plant are used as a poultice in febrile delirium.
- Increased consumption of the vegetable is protective.

Fish culture

Plants are often planted against dikes of fish ponds so that the floating stems can be eaten by fish.

Water purification

I. aquatica can scavenge some organic and inorganic components including some heavy metals from waste water. The weed absorbs and incorporates dissolved materials into its own system. *I. aquatica* could be useful for removing nitrates from contaminated water such as farm drainage and municipal. The weed shows maximum capacity in water containing about 15-20 % effluents.

Soil conservation

In India, the plants prevent erosion on the periphery of farm ponds and percolation tanks.

Biomass as a substrate for food production

The edible mushroom *pleurotus sajor-caju* (Oyster mushroom) grows readily on it. With an enhanced nitrogen content after the mushroom growth, these aquatic weeds could be better bio-fertilizer than a direct application as green manure.

Counteractive to viruses

I.aquatica deactivates some viruses and minimizes the virulence of abaca mosaic when the virus passes through it as an intermediate host.

Chapter Three



MATERIALS AND METHODS

Chapter 3

3.0 Materials and Methods

3.1 Materials

3.1.1 Materials used for cold extraction

- 95% ethanol
- A glass jar
- Aluminium foil

3.1.2 Materials used for solvent-solvent partition

Apparatus

- ❖ Separatory funnel
- ❖ Beaker

Reagents

- ❖ Ethanol
- ❖ n-Hexane
- ❖ Distilled water

3.1.3 Materials used for sub-fractionation column chromatography

Apparatus

- ❖ Test tube
- ❖ Sand
- ❖ Silica gel 60
- ❖ Conical flask

Reagents

- ❖ Acetone
- ❖ Ethanol
- ❖ n-Hexane

3.1.4 Materials used for TLC analysis

Apparatus

- ❖ TLC plates.
- ❖ TLC jars with closure.
- ❖ Fine capillary tube.
- ❖ Filter paper
- ❖ Dryer

Reagents

- ❖ Chloroform
- ❖ Methanol
- ❖ n-Hexane
- ❖ Ascorbic acid
- ❖ 10% H₂SO₄
- ❖ Distilled water

3.2.5 Reagents used for phytochemical group test

- ❖ Mayer's Reagent
- ❖ Dragendroff's Reagent
- ❖ Fehling's Solution A
- ❖ Fehling's Solution B

3.2.6 Materials used for HPLC analysis

Apparatus

- ❖ Vials
- ❖ volumetric flask
- ❖ Ultrasonic bath
- ❖ Nylon syringe filter
- ❖ Vortex mixing

Chemicals

Gallic acid (GA), (+)-catechin hydrate (CH), vanillic acid (VA), caffeic acid (CA), (-)-epicatechin (EC), *p*-coumaric acid (PCA), rutin hydrate (RH), ellagic acid (EA), myricetin (MC), kaempferol (KF), and quercetin (QU). Acetonitrile (HPLC), methanol (HPLC), acetic acid (HPLC), and ethanol.

3.2.7 Materials used for anti-microbial activity test

Apparatus and chemicals

Petri dishes	Blank discs
Electronic balance	Sterile forceps
volumetric flask	Water bath
Nutrient agar medium	Standard antibiotic discs (Kanamycin)
Autoclave	Vials
Laminar air flow unit	Nose masks and hand gloves
Inoculating loop	Ethanol
Incubator	Sterile cloth
Test tubes	Beaker
Sprit lamp	Distilled water
Micropipette	Calibrated scale
Hot plate	Indelible Marker

3.2.7.1 Microorganisms used for the test

Gram-negative bacterial strains are taken for the test. The bacterial strains used for the investigation are listed in Table-1. These organisms were collected from the Biochemistry and Molecular Genetics Lab, Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna-9208.

Table.1 List of bacteria strain used for screening of antimicrobial activity

Gram negative
<ol style="list-style-type: none">1. <i>Enterococcus coli</i> strain Enteropathogenic <i>E. coli</i> (EPEC)2. <i>Enterococcus coli</i> strain Enterotoxigenic <i>E. coli</i> (ETEC)3. <i>Enterococcus coli</i>

3.2.7.2 Composition of nutrient agar media

Ingredients	Amounts (gram/liter)
Peptone	5
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5
Agar	15
pH	7.4 ± 0.2 (at 25° C)

3.2.7.3 Composition of nutrient broth media

Ingredients	Amounts (gram/liter)
Beef infusion form	300
Casein hydrolysate (acid)	17.50
Starch	1.50
pH	7.4 ± 0.2 (at 25° C)

3.2.8 Materials used for qualitative anti-oxidant test

Apparatus

- UV detector
- Spray gun
- Hot plate
- Spreading trough
- Pipette
- Beaker

Reagents used

- DPPH (0.02 % w/v) solution in ethanol
- Distilled water

Table.2 Solvent system used

Groups	Solvent system	Ratio
Medium polar	CHCl ₃ : CH ₃ OH	5:1
Polar	CHCl ₃ : CH ₃ OH : H ₂ O	20:5:0.5
Non-polar	C ₆ H ₁₄ : CH ₃ COCH ₃	3:1

3.2.9 Source of chemicals and reagents

Chloroform, acetone (Loughborough); ethanol, methanol, n-Hexane, Sulphuric acid, hydrochloric acid (Darmstadt, Germany); potassium iodide, mercuric iodide, potassium dichromate (England); lead acetate, copper sulphate, silica gel 60, potassium hydroxide, Ferric chloride, sodium potassium tartarate, bismuth nitrate (India); Gallic acid, (+)-catechin hydrate, vanillic acid, caffeic acid, (-)-epicatechin, *p*-coumaric acid, rutin hydrate, ellagic acid, myricetin, kaempferol, and quercetin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC), methanol (HPLC), acetic acid (HPLC), and ethanol was obtained from Merck (Darmstadt, Germany).

3.2 Methods

3.2.1.1 Collection

The plant water spinach (*Ipomoea aquatica*) was collected from Brozola college area, Daulatpur, Khulna, Bangladesh, on 10th November, 2013 on the day time.

3.2.1.2 Drying & Grinding

The collected plant parts were separated from undesirable materials or plants or plant parts. They were air dried for four weeks. The plant parts were ground into a fine powder with the help of a suitable grinder (Capacitor start motor, Wuhu motor factory, China). The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced.

3.2.1.3 Cold extraction (Ethanol extraction)

A glass jar with plastic cover was taken and washed thoroughly. The jar was rinsed with ethanol and dried. Then 300 gm of the dried powder was taken in the jar. After that 95% ethanol (950 ml) was poured into the jar up to 1 inch height above the sample surface as it can sufficiently cover the sample surface. The plastic cover with aluminium foil was closed

properly to resist the entrance of air into the jar. This process was performed for 8 days. The jar was shaken and stirring several times during the process to get better extraction.

3.2.1.4 Evaporation of the solvent

The filtrate (ethanolic extracts) obtained was evaporated by Rotary Evaporator and after that it was kept under ceiling fan to evaporate the ethanol completely. It rendered a gummy concentrate (6.44 gm) of greenish black color. The gummy concentrate was designated as crude extract of ethanol. Therefore, yield value (%) = $6.44 \text{ gm}/300 \times 100\% = 2.146\%$

3.2.1.5 Extraction at a glance

Whole plant collection → Drying → Grinding → Maceration with ethanol for 8 days → Filtration by cotton plug → Filtration by Whatman filter paper → Evaporation → Crude ethanol extract of *Ipomoea aquatic*.

3.2.2 Solvent-solvent partition

Any plant extract may contain hundreds of compounds of various polarities. Partitioning of plant extract of water spinach (*I. aquatic*) was subjected to separate the polar, moderately polar and non polar compounds. The methodology is described below.

3.2.2.1 Selection of solvent system

The first requirement in the extraction process is to select two immiscible solvents. Water was chosen as one solvent. So, second solvent should be non polar n-Hexane which is immiscible with water. The solvents are-

- n-Hexane
- Water

3.2.2.2 Separation Process

3.09 g of ethanolic extract was obtained by cold extraction and evaporation of solvent. This extract was dissolved into 10% ethanol of 100 ml water and 100 ml of n-Hexane solvent were added to the funnel. This solution was transferred to the separating funnel. The two solutions were mixed together too vigorously. The funnel was then inverted and the tap was carefully opened to release excess vapor pressure. The separating funnel was set aside to allow for the complete separation of the phases. The non polar compounds are more soluble in n-Hexane than in the water. So, they would be dissolved in n-Hexane. The top and the bottom tap are then opened and the two phases are released by gravitation. Water part was again taken to the separating funnel and this time 50 ml n-Hexane was added. At last 50 ml n-Hexane was taken and same procedure was followed.

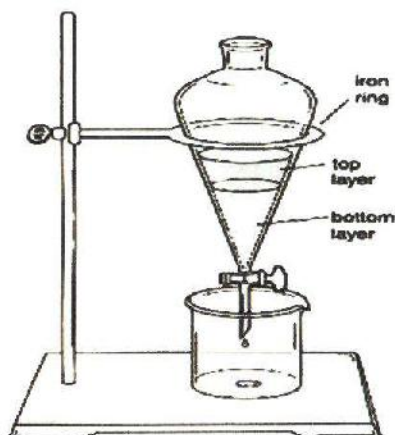


Fig.2 Illustration of partitioning

3.2.3 Methods used for sub-fractionation column chromatography

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids when carrying out small-scale experiments. The separation can be liquid/liquid (partition) in column chromatography. The stationary phase, a solid adsorbent, is usually placed in a vertical glass column and the mobile phase, is added from the top and let flow down through the column by either gravity external pressure.

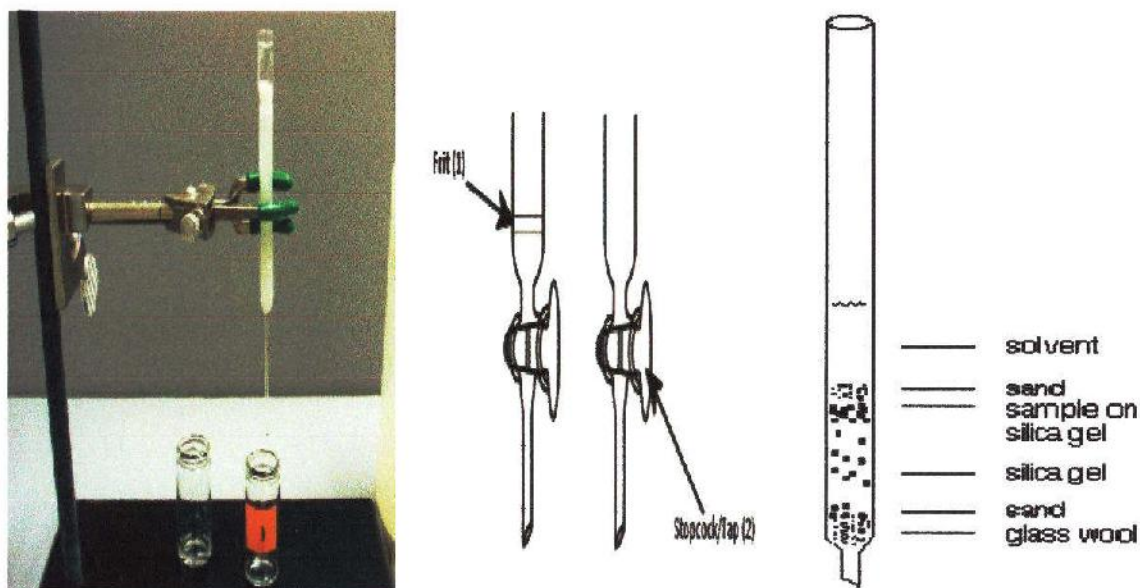


Fig.3 sub-fractionation of n-Hexane fraction by using column chromatography

3.2.3.1 Adsorbent

Silica gel (SiO_2) is commonly used by organic chemists for column chromatography. This adsorbent is sold in different mesh sizes, indicated by a number on the bottle label: “silica gel 60” or “silica gel 230-400”. This number refers to the mesh of the sieve used to size the silica, specifically, the number of holes in the mesh or sieve through which the crude silica particle mixture is passed in the manufacturing process. If there are more holes per unit area, those holes are smaller, thus only smaller silica particles are allowed to pass the sieve. The larger the mesh size, the smaller the adsorbent particles.

3.2.3.2 Solvent system

Polar solvents can more effectively compete with the polar molecules of a mixture for the polar sites on the adsorbent surface and will also better solve the polar constituents. On the other hand, if a solvent is not polar enough, no compounds will elute from the column. Proper choice of an eluting solvent is thus crucial for a successful application of column chromatography as a separation technique since compounds interact with the silica due to polar interactions. The solvent are:-

- Ethanol
- Acetone
- n-Hexane : Acetone =3:1



3.2.3.3 Process of column chromatography

- ❖ 5% n-Hexane extract of 20 ml silica gel was mixed with ethanol and quickly but carefully passed into the column. Immediately to maximize the amount of silica that goes into the column instead of remaining behind in the beaker then silica was solid form in some times. Surface on the side of the column with a rubber stopper or tubing to help the silica settle uniformly.
- ❖ To allow 60ml ethanol solvent was passed into the column to collect into a clean flask. The same procedure was followed for acetone and n-Hexane : acetone solvent.
- ❖ A small amount of sand was added to surface of the silica gel 60. Sand is heavier than silica. If the silica has not settled, the sand may sink into the silica instead of forming a layer on top of it. 0.98 gm of n-Hexane extract was added to surface of the sand.
- ❖ 40 ml of n-Hexane : acetone solvent was passed into the column to collect into thirty seven test tube of 2 ml, respectively.

- ❖ Further, same procedure was followed for washing of column as n-Hexane: acetone, acetone and ethanol, respectively.

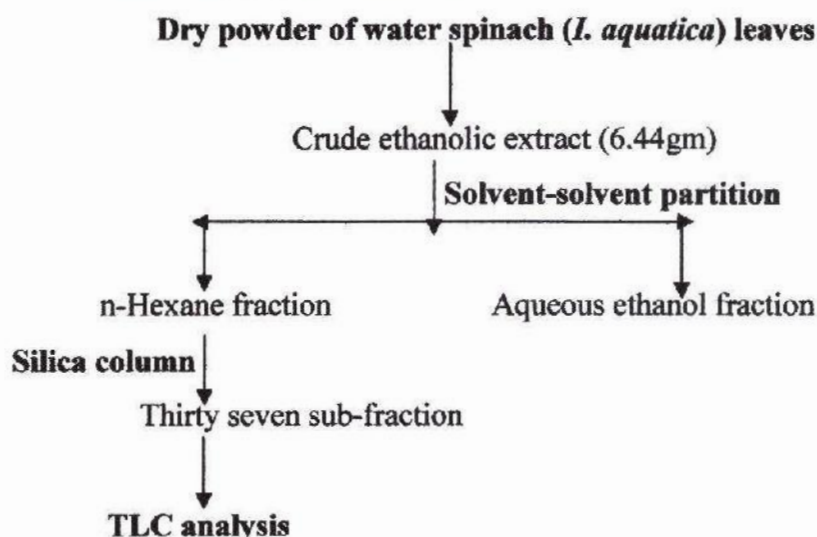


Fig.4 Sub-fractionation of n-Hexane fraction

3.2.4 Methods used for TLC analysis

3.2.4.1 TLC plate preparation

Commercially available TLC plate is cut into appropriate size of 10 cm long and 8 cm wide.

3.2.4.2 Sample application

- A fine capillary tube was used as spotter for sample application in the TLC plates.
- A very little amount of plant extract was taken in a small vial and diluted suitably with ethanol. The sample was spotted in uniform size (about 0.3 cm) 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.



Fig.5 Capillary tube as sample spotter

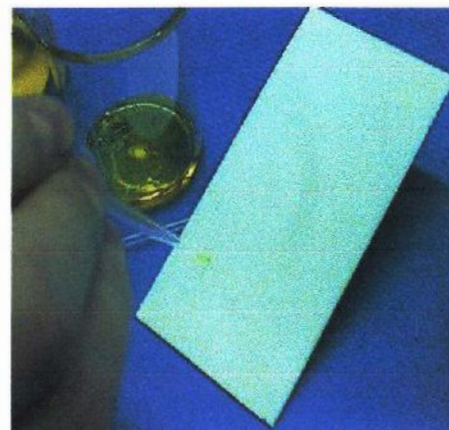


Fig.6 Spotting of sample

3.2.4.3 Development of chromatogram

A. By polar solvent system

- The chromatogram was developed by ascending technique.
- A little amount of ethanolic extract of plant was dissolved in ethanol and diluted suitably and was applied on the TLC plate by spotter. A little amount of standard ascorbic acid was also dissolved in ethanol and diluted suitably and was applied on the same TLC plate by spotter. Two plates were spotted in same way.

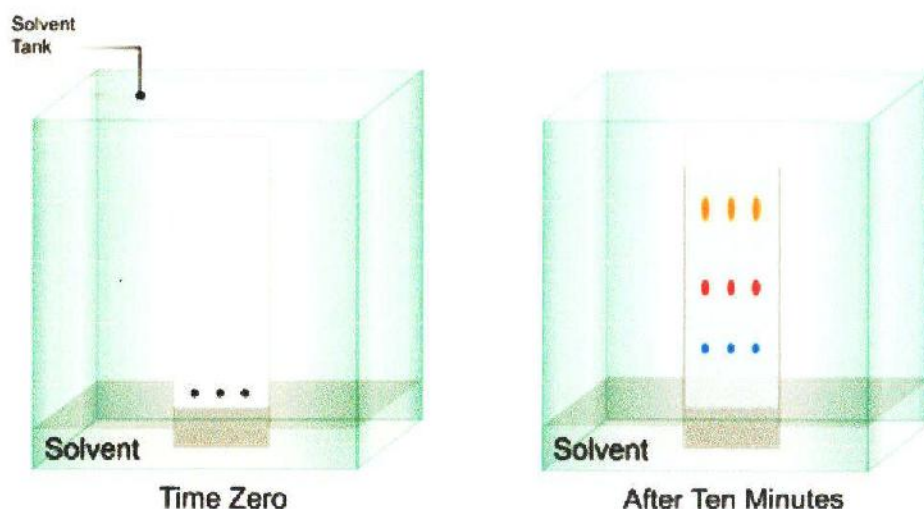


Fig.7 A typical chromatogram by ascending technique

- These plates were then kept in a jar containing a solvent system of chloroform and methanol and water (20:5:0.5).
- Filter papers were kept into jar by wetting with solvent systems to keep those jars saturated and the jar was closed tightly.
- The plates were placed in the jar in such a way that the sample and standard 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 and standard on a 10 cm plate.
- The plates were then removed from the jar and dried with a current of air suitably.
- After developing the chromatogram one of the plate was sprayed with 0.02 % DPPH solution of ethanol on it by a spray gun and the other plate was sprayed with 10% H_2SO_5 .

B. By medium polar solvent

Again two plates were spotted by the same process mentioned above and chromatogram was run in solvent system chloroform and methanol (5:1). After developing the chromatogram one of the plate was sprayed with 0.02 % DPPH solution of ethanol on it by a spray gun. And the other plate was sprayed with 10% H_2SO_5 .

C. By non polar solvent

Again two plates were spotted by the same process mentioned above and chromatogram was run in solvent system n-Hexane and acetone (3:1). After developing the chromatogram one of the plate was sprayed with 0.02 % DPPH solution of ethanol on it by a spray gun. And the other plate was sprayed with 10% H_2SO_5 .

3.2.5 Phytochemical group test

3.2.5.1 Preparation of the reagents

The following reagents were used for the different chemical group test (Trease *et al.*, 1983; Ali, 1998).

i) Mayer's reagent (40ml)

0.68gm mercuric iodide (HgI_2) in 30 ml of water was mixed with a solution contains 2.5 gm of potassium iodide (KI) in 10 ml of water.

ii) Dragendorff's Reagent (60ml)

0.85 gm basic bismuth nitrate and 10 gm tartaric acid were dissolved in 40 ml water. This solution was mixed with a solution contains 8 gm potassium iodide and 20 ml water.

iii) Fehling's solution A (100ml)

6.92 gm copper sulphate was dissolved in a mixture of 0.10 ml of sulfuric acid and sufficient water to produce 100 ml.

iv) Fehling's solution B (100ml)

35.2 gm of sodium potassium tartarate and 15.4 gm of sodium hydroxide were dissolved in sufficient water to produce 100 ml.

3.2.5.2 Tests procedure for identifying different chemical groups

The following tests were performed for identifying different chemical groups (Trease *et al.*, 1983; Ghani, 2003).

Test for Flavonoids

A few drops of concentrated hydrochloric acid was added to a small amount of an alcoholic extract of the plant material. Immediate development of a red color indicates the presence of Flavonoids.

Test for Saponins

1 ml solution of the extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. One centimeter layer of foam indicates the presence of saponins.

Test for alkaloids

➤ Mayer's test

2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of Mayer's reagent was added. Yellow color precipitate was formed indicating the presence of alkaloids.

➤ Dragendroff's test

2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of Dragendroff's reagent was added. Orange brown precipitate was formed indicating the presence of alkaloids.

Tests for tannins

➤ Ferric Chloride Test

4 ml solution of the extract was taken in a test tube. Then 1 ml of 5% Ferric chloride solution was added. Greenish black precipitate was formed and indicated the presence of tannins.

➤ Potassium dichromate test

5 ml solution of the extract was taken in a test tube. Then 1 ml of 10% Potassium dichromate solution was added. A yellow precipitate was formed in the presence of tannins.

Test for Steroids

➤ Sulphuric acid test

1 ml solution of chloroform extract was taken to add 1ml Sulphuric acid. Red color indicates the presence of steroid.

3.2.6 Methods used for HPLC analysis

3.2.6.1 High performance liquid chromatography (HPLC) system

Chromatographic analyses were carried out on a Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC (RSLC) systems (Thermo Fisher Scientific Inc., MA, USA), coupled to a quaternary rapid separation pump (LPG-3400RS), Ultimate 3000RS autosampler (WPS-3000) and rapid separation diode array detector (DAD-3000RS). Phenolic compounds were separated on a Acclaim® C18 (4.6 x 250 mm; 5µm) column (Dionex, USA) which was controlled at 30°C using a temperature controlled column compartment (TCC-3000). Data acquisition, peak integration, and calibrations were performed with Dionex Chromeleon software (Version 6.80 RS 10).

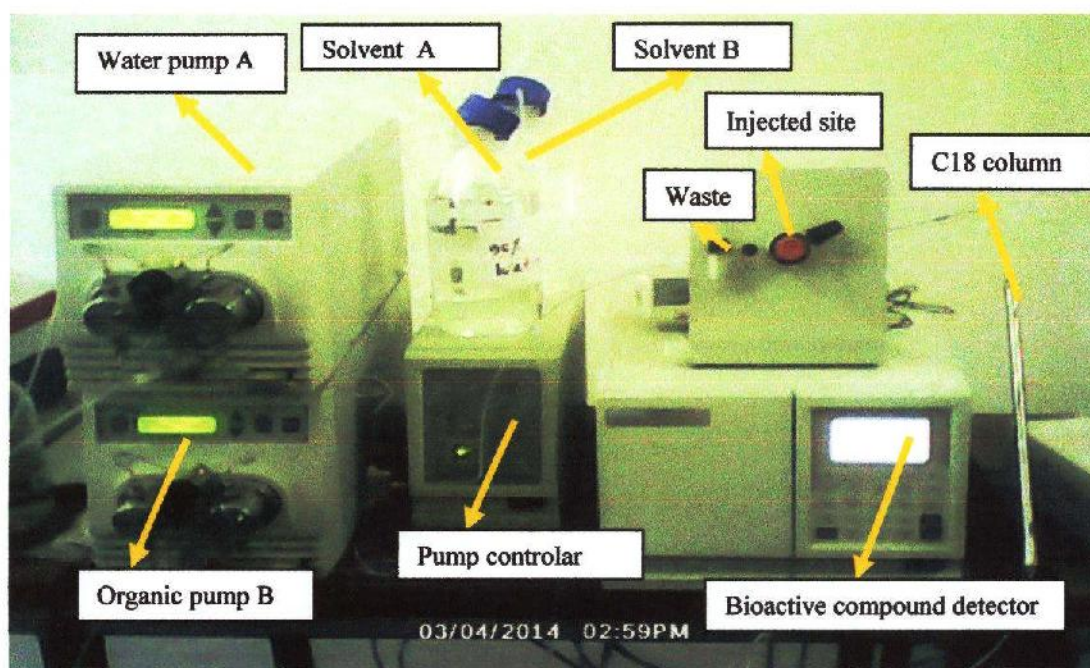


Fig.8 The High Performance Liquid Chromatography (HPLC) system

3.2.6.2 Chromatographic conditions

The phenolic composition of the crude ethanolic extract, n-Hexane fraction and aqueous ethanol fraction of *I. aquatica* were determined by HPLC as described by Sarunya & Sukon (2006) with some modifications. The mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B), and methanol (solvent C). The system was run with the following gradient elution program: 0 min, 5%A/95%B; 10 min, 10%A/80%B/10%C; 20 min, 20%A/60%B/20%C and 30min, 100%A. There was a 5 min post run at initial conditions for equilibration of the column. The flow rate was kept constant throughout the analysis at 1 ml/min and the injection volume was 20 µl. For UV detection, the wave length program was

optimized to monitor phenolic compounds at their respective maximum absorbance wavelengths as follows: λ 280 nm held for 18.0 min, changed to λ 320 nm and held for 6 min, and finally changed to λ 380 nm and held for the rest of the analysis and the diode array detector was set at an acquisition range from 200 nm to 700 nm. The detection and quantification of GA, CH, VA, CA, and EC was done at 280 nm, of PCA, RH, and EA at 320 nm, and of MC, QU, and KF at 380 nm, respectively.

3.2.6.3 Standard and sample preparation

A stock standard solution (100 $\mu\text{g/ml}$) of each phenolic compound was prepared in methanol by weighing out approximately 0.0050 g of the analyte into 50 ml volumetric flask. The mixed standard solution was prepared by dilution of the mixed stock standard solutions in methanol to give a concentration of 5 $\mu\text{g/ml}$ for each polyphenols except (+)-catechin hydrate, caffeic acid, rutin hydrate (4 $\mu\text{g/ml}$) and quercetin (3 $\mu\text{g/ml}$). All standard solutions were stored in dark at 5°C and were stable for at least three months. The calibration curves of the standards were made by a dilution of the stock standards (five set of standard dilutions) with methanol to yield 1.0 - 5.0 $\mu\text{g/ml}$ for GA, CH, VA, EC, PCA, EA, MC, KF; 0.5 - 4.0 $\mu\text{g/ml}$ for CH, CA, RH, and 0.25 - 3.0 $\mu\text{g/ml}$ for QU. The calibration curves were constructed from chromatograms as peak area vs. concentration of standard. A solution of the crude ethanol extract, n-Hexane fraction and aqueous ethanol fraction of *I. aquatica* at a concentration of 10 mg/ml was prepared in ethanol by vortex mixing (Branson, USA) for 30 min. The samples were stored in dark at low temperature (5°C). Spiking the sample solution with phenolic standards was done for additional identification of individual polyphenols. Prior to HPLC analysis, all solutions (mixed standards, sample, and spiked solutions were filtered through 0.20 μm nylon syringe filter (Sartorius, Germany) and then degassed in an ultrasonic bath (Hwashin, Korea) for 15 min.

3.2.6.4 Peak characterization and quantification

The compounds were identified by comparing with standards of each identified compound using the retention time, the absorbance spectrum profile and also by running the samples after the addition of pure standards. Quantification was performed by establishing calibration curves for each compound determined, using the standards. Linear calibration curves for standards (peak area vs concentration) were constructed with R^2 exceeding 0.995. Data are reported as means $\pm$ standard deviations of triplicate independent analyses.

3.2.7 Methods used for antimicrobial activity test

3.2.7.1 Preparation of agar media

Nutrient agar media was prepared by adding water to a dehydrated product that contains all the ingredients. Practically all media are available commercially in powdered form (Roland, 1982). The following steps were involved in the preparation of bacteriological media

- Definite amount of nutrient agar (7 gm) was accurately weighed.
- It was taken in volumetric flask (250 ml) containing 200 ml distilled water. A clear medium was obtained by thorough dissolving agar over a hot plate with occasional shaking.
- Then the final volume was adjusted to 250 ml.

3.2.7.2 Sterilization of different equipments and media

Media, petridishes and other glassware were sterilized by autoclaving at a temperature of 121°C and a pressure of 15-lbs/sq inch for 15 minutes. Blank discs kept in a covered petridishes. loop and forceps were subjected to dry heat sterilization at 160°C for 1 hour. Later they were kept in a laminar hood under UV light for 15 minutes. UV light was switched on one hour before working in a laminar hood to avoid accidental contamination.

3.2.7.3 Preparation of sub-culture

- ✓ These slants were used for fresh culture of micro-organisms which in turn would be used for sensitivity tests.
- ✓ With the help of an inoculating loop, the test organisms from the pure cultures were transferred to the agar slants in an aseptic condition using laminar air hood.
- ✓ The inoculated slants were then incubated at 37 c for 18-24 hours to assure the growth of test organisms. This culture was used within two days.

3.2.7.4 Preparation of Nutrient broth media

Nutrient broth media was prepared by adding water to a dehydrated product. Practically all media are available commercially in powdered form.

The following steps were involved in the preparation of bacteriological media

- Definite amount of Nutrient broth (2.1 gm) was accurately weighed.
- It was taken in volumetric flask (250 ml) containing 100 ml distilled water.

- A clear medium was obtained by thorough dissolving broth with vigorous shaking.
- The medium was then autoclaved at temperature of 121°C and a pressure of 15 lbs/square inch for 15 minutes.

3.2.7.5 Preparation of the seeded test plates

1. Each of the test organisms were transferred from the subculture to the test tube containing 16 ml autoclaved media with the help of the sterilized inoculating loop at 45°C in an aseptic area.
2. The test tubes were shaken by rotation to get a uniform suspension of organism. The bacterial suspensions were immediately transferred to the sterile petridishes aseptically.
3. The petridishes were rotated several times, first clockwise and then anticlockwise, to assure homogeneous distribution of the test organisms.
4. The medium was poured into petridishes in such a way as to give a uniform layer of depth of approximately 4 mm. after the medium became cooled to room temperature, it was stored in a refrigerator (4°C).
5. In this way 16 discrete media in petridishes were prepared for 4 bacterial strains used for screening & was properly marked.

3.2.7.6 Preparation of test sample

1000, 500 mg and 250 mg of the of ethanolic extracts of the leaves of *I. aquatica* was accurately measured by the electronic balance & taken in a 10 ml volumetric flask. Then small amount of ethanol was added & was triturated in unidirectional manner, after proper mixing the volume (10 ml) was adjusted by ethanol & the concentration became 100 µg/µl, 50 µg/µl and 25 µg/µl.

3.2.7.7 Preparation of disc

Three types of discs were used for antibacterial screening:

- I. Sample discs
- II. Standard discs and
- III. Blank discs

3.2.7.8 Sample discs

Sterile filter paper discs (5 mm in diameter) were taken in a blank petridish. 10 μ l of the test sample solution was applied on the discs (1000, 500 and 250 μ g/disc) with the help of a micropipette in an aseptic condition under the laminar air flow. These discs were left for several hours (4-6) in aseptic condition under the laminar air flow for complete removal of solvent as the solvent (ethanol) has some antimicrobial activity.



3.2.7.9 Standard discs

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that produced by test samples. In this investigation Kanamycin (30 μ g/disc) standard discs was used as the reference.

3.2.7.10 Blank discs

These were used as negative control. They ensure that the residual solvents (left over the discs even after air drying) and the filter paper were not active themselves.

3.2.7.11 Application of discs

Sample impregnated discs, standard antibiotic discs (Kanamycin discs) and negative control discs (blank discs) were placed gently on the 16 discrete solidified agar plates, freshly seeded with the test organisms with the help of a sterile forceps to assure complete contact with medium surface. The spatial arrangement of the discs was such that the discs were no closer than 15 mm to the edge of the plate and far enough apart to prevent overlapping the zones of inhibition. The plates were then inverted and kept in refrigeration for about 4 hours at 4°C. This was sufficient time for the material to diffuse into a considerable area of the medium. Finally the plates were incubated upside down at 37°C for 12-18 hours.

3.8 Methods used for qualitative anti-oxidant test

3.8.1 Solvent selection

The applied sample was run properly with different solvent systems and observed on which solvent system separated the sample well.

3.8.2 Thin Layer Chromatography (TLC) Technique

TLC technique is a method of analysis in which the stationary phase (a finely divided solid) is spread as film layer on a rigid supporting plate and a liquid (mobile phase) is allowed to migrate across the surface of the plate.

TLC technique is simple, easy, rapid, versatile and effective primary tool for identification of antioxidant compounds. (Sharma *et al.*, 2012; Badarinath *et al.*, 2010; Santwani *et al.*, 2011).

This method is used

- To observe the characteristics of the plant extract
- To identify polar, non-polar and medium polar groups present in the plant extract using suitable solvent system under UV light at short (254 nm) and long (366 nm) wave lengths.

3.8.3 Antioxidant positive components detection

After spraying DPPH solution on the chromatogram, it was visually observed that the pink color of DPPH solution was turned into yellow. This indicated the presence of antioxidant in the plant extract.

3.8.4 UV positive components detection

After applying DPPH on the TLC plate, yellow color on purple background was observed which indicated the presence of antioxidant components in the plant extract. Then these plates were dried on hot plate at 110-120°C for some time. The observed plates were given below which were viewed under UV detector both in short (254 nm) and long (360 nm) wave length. When the plates were viewed under UV detector a lot of colored and fluorescent positive components were found in short and long wave lengths respectively which indicate the presence of UV positive materials in the plant extract and they were marked. Spots which were observed at longer wave length were manifested by the symbol () and spots which were observed at short wave length were manifested by the symbol < > .

Chapter Four

RESULTS

Chapter 4

4.0 Results

4.1 Yield from the extraction of water spinach (*Ipomoea aquatica*)

Ethanollic extracts were filtrated to keep under ceiling fan to evaporate the ethanol completely. Therefore, yield in the extraction process was 2.146 % w/w.

4.2 Results of solvent-solvent partition

The solvents were evaporated to obtain the separated plant extract according to the polarity of the compound. Water was evaporated by using a fan. After evaporation the following amount of different compounds were obtained:-

Partition	Amount (gm)
n-Hexane fraction	1.38
Aqueous ethanol fraction	1.5

4.3 Sub-fraction of n-Hexane fraction by using column chromatography

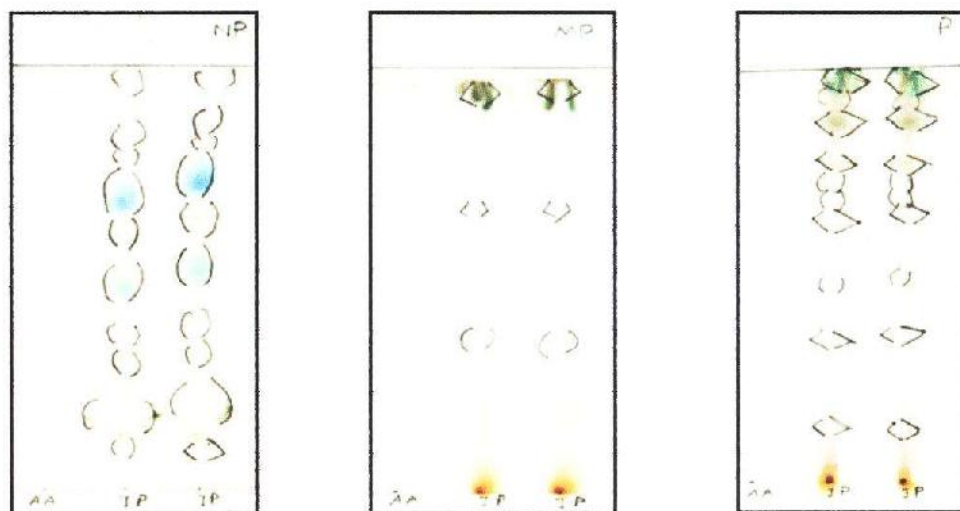
After solvent-solvent partition, TLC analysis was performed where n-Hexane fraction exhibit better chromatogram than aqueous ethanol fraction. So, 5% n-Hexane extract of 20ml silica gel that means 0.98g n-Hexane extract was used in column chromatography. After column separation, thirty sub fractions were obtained and TLC analysis was performed on each sub – fraction.

4.4 TLC plates analysis

After spraying 10% H_2SO_4 , the TLC plates were viewed under UV detector both in short (257 nm) and long (364 nm) wavelength. When plates were viewed under UV detector a lot of colored and fluorescent positive components were found in short and long wavelengths respectively which indicated the presence of UV positive materials in the plant extract and they were marked by the sign () and materials viewed in longer wavelength are marked by < >.

As shown in Fig. 9, after applying 10% H_2SO_4 on the TLC plate that was run in non- polar solvent system exhibits better chromatogram than the other two TLC plates run in medium polar and polar solvent systems respectively. The TLC plate that run in medium polar solvent system exhibit poor chromatogram than the other two TLC plates. After solvent-solvent

partition, crude ethanolic extract has been partitioned in two parts-n-Hexane fraction and aqueous ethanol fraction.



(n-Hexane : Acetone = 3:1 as non polar solvent), (CHCl_3 : CH_3OH = 5: 1 as medium polar solvent) and (CHCl_3 : CH_3OH : H_2O =40: 10: 1 as polar solvent).

Fig. 9 TLC plates for water spinach (*I. aquatica*) of crude ethanolic extract after applying 10% H_2SO_4 . Here, Aa=Ascorbic acid, Ia=*Ipomoea aquatica*.

As shown in **Fig. 10**, the chromatogram of n-Hexane fraction has far more spots than the chromatogram of aqueous ethanol fraction which means n-Hexane fraction has more UV positive compound. So, n-Hexane fraction was used for further fractionation by column chromatography.

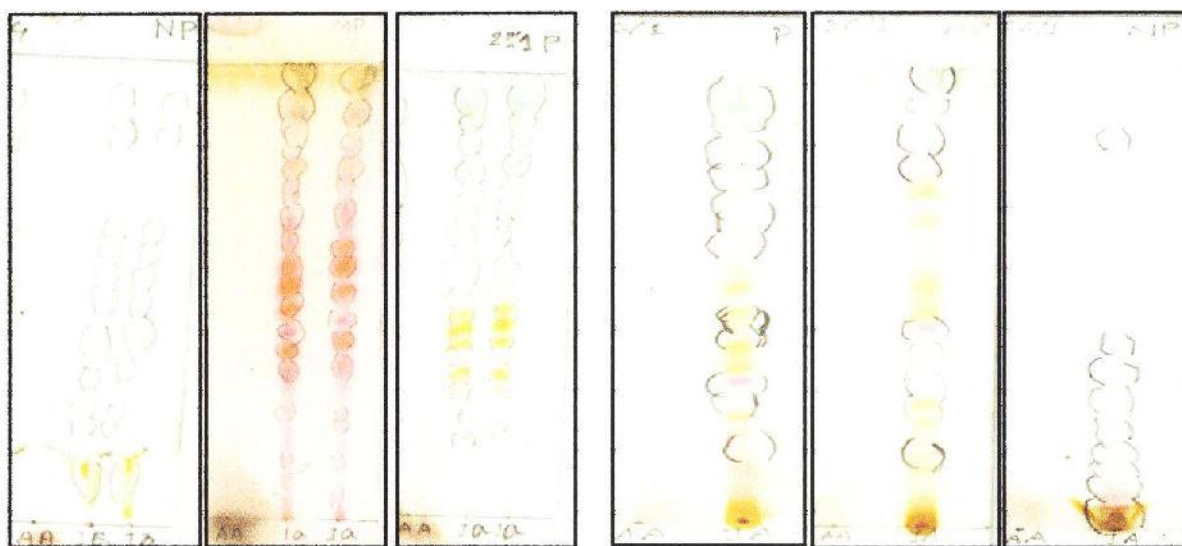


Fig. 10 TLC plates for water spinach (*I. aquatica*) of n-Hexane fraction and aqueous ethanol fraction after applying 10% H_2SO_4 . Here, Aa=Ascorbic acid, Ia= *Ipomoea aquatica*.

As showed in Fig. 11, there was no spots at the lower region from sub fraction 1 to 11. But lower region spots become increased gradually from sub fraction 12 to 36 while upper region spots gradually disappear. It is indicated that the compounds from later sub fractions were more polar than the compounds from earlier sub fractions. Subfraction-7,8,11 has similar 2 spots which indicates same compounds are present. Subfraction-9,10,13,14,15,16,17,18 has similar 3 distinct spots at middle region. Subfraction-19,21,22,26,27,28,29 has similar 3 joined spots at lower region. Subfraction-23,24,25,30 has similar joined 2 spots. Subfraction-31,32,33,34,35,36 has similar one distinct spots. Its contain lesser spots (means lesser compounds) are easy to be used for further separation. Subfraction- 1 to 6, 20 has dissimilar which indicates didn't better seperation. Next, five same spots of n-Hexane subfraction was analyzed by HPLC. The chromatograms of 37 sub fractions of n-Hexane fraction are given below-

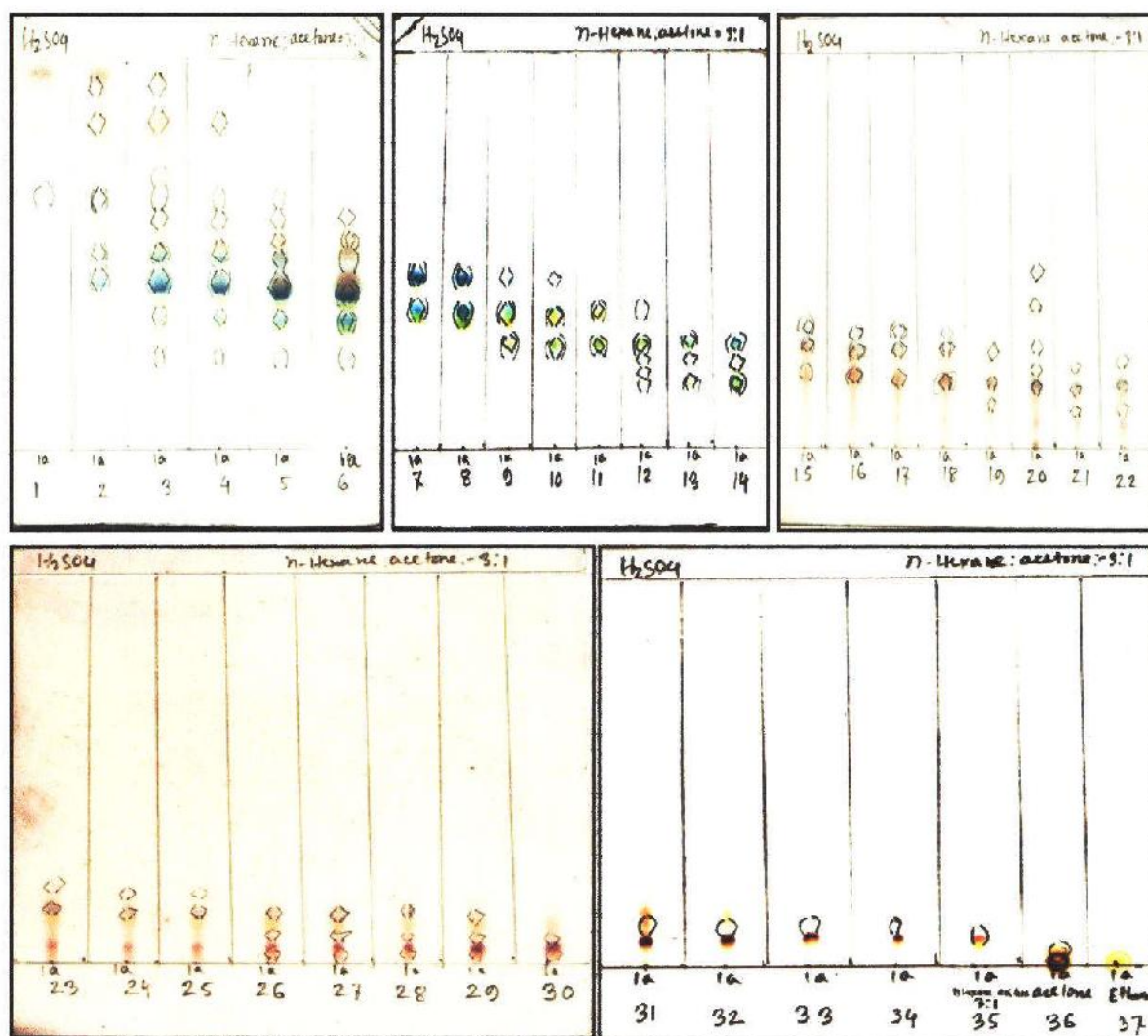


Fig. 11 TLC plates for *I. aquatic* of sub-fractionation of n-Hexane fraction after applying 10% H₂SO₄, n-Hexane : acetone = 3:1 as non polar solvent. Here, 1a= *Ipomoea aquatica*

4.5 Investigation of phytochemical group test

The crude ethanolic extract of water spinach (*I. aquatica*) was subjected for chemical results of different group tests are showed in Table 3.

Table. 3 Results of different group tests are given bellow:

Sample	Reagent	Observation	Inference
Test for Alkaloids			
2ml solution of the extract and 0.2 ml of dilute hydrochloric acid	Mayer's reagent	Precipitation of yellowish buff colored	Alkaloid present
	Dragendroff's reagent	Precipitation of orange brown	Alkaloid present
Test for Glycosides			
A small amount of the extract in water & alcohol.	Boiled with Fehling's solution	Precipitation of brick-red	Glycoside present
Test for Steroids			
Dissolved 10 mg extract in 1 ml CHCl_3	1 ml Sulphuric acid	No observable change	Steroid present
Test for Saponins			
Distilled 1 ml solution of the extract with 20 ml distilled water.	Shaken in a graduated cylinder for 15 minutes.	No foam formation.	Saponins absent.
Test for Flavonoids			
A little amount of extract.	Few drops of concentrated HCl	No colored development.	Flavonoids absent.
Test for tannins			
5 ml solution of extract.	1 ml of 10% potassium dichromate solution.	Precipitation of yellow.	Tannins present.

As shown in **Table. 4**, phytochemical studies showed that *I. aquatica* has alkaloid, glycoside, tannin and steroid was present in ethanolic extract. The plant extract didn't show the existence of saponins and flavonoid.

Table. 4 Qualitative screening of phytochemicals in water spinach (*I. aquatica*) plant

No. of tests	Alkaloid	Glycoside	Steroid	Tannin	Saponin	Flavonoid
Crude ethanolic extract	+	+	+	+	-	-
n-Hexane fraction	-	+	+	+	-	-
aqueous ethanol fraction	+	-	+	+	-	-

(+) indicates the presence of chemical constituents and (-) indicates the absence of chemical constituents.

4.6 HPLC analysis

A new HPLC method to analyze eleven polyphenolic compounds (gallic acid, catechin, vanillic acid, caffeic acid, (-)-epicatechin, *p*-coumaric acid, rutin hydrate, ellagic acid, myricetin, quercetin and kaempferol simultaneously has been developed and validated for linearity, accuracy, stability and precision. This HPLC procedure provided excellent identification and quantification of these phenolic compounds were presented in the crude ethanolic extract (0.2gm), n-Hexane fraction (0.2gm) and aqueous ethanol fraction (0.2gm) of water spinach (*I. aquatica*) within a short analysis time (30 min). Since the phenolic compounds have been of interest of health benefits, the present HPLC study could be a potential application to identify and quantify the polyphenolic compounds in any medicinal plant extracts.

4.6.1 Method validation

The validation of this developed HPLC method was followed according to the ICH guidelines (Anonymous, 1995). Linearity ranges, correlation coefficients, detection limits, quantitation limits, and recovery were determined using standard polyphenolics (**Table. 5**). A series of the standard mixture solutions of nine phenolic compounds were tested to determine the linearity between the standard mixture concentrations and peak areas. Linearity was evaluated on five calibration points (1.0 – 5.0 µg/ml for all standards except 0.5 - 4.0 µg/ml for CH, CA, RH and 0.25 - 3.0 µg/ml for QU) with three measurements for each calibration point. All the standard polyphenolics exhibited good linearity over the evaluated range with

correlation coefficients (r^2) between 0.995 and 1.000. The limits of detection and quantification were calculated as signal-to-noise ratios with nominal values of 3:1 and 10:1, respectively. Detection and quantitation limits of these compounds were in the range of 0.10 - 0.32 and 0.21 – 6.8 $\mu\text{g/ml}$, respectively. In recovery assay, the samples were fortified with all standards at 2.5, and 5.0 $\mu\text{g/ml}$. The recovery tests of all compounds range of 95.0-104%.

Table. 5 Parameters of calibration graphs for the eleven phenolic standards in this study.

Peak no.	Polyphenolic compound	Linearity range ($\mu\text{g/ml}$)	Correlation coefficients (r^2)	Detection limit ($\mu\text{g/ml}$) ^a	Quantitation Limit ($\mu\text{g/ml}$) ^a	Recovery (%) ^b
1	GA	1.0 – 5.0	0.9951	0.20	0.65	97.3 $\pm$ 1.99
2	CH	0.5 – 4.0	0.9972	0.10	0.38	97.5 $\pm$ 1.81
3	VA	1.0 – 5.0	0.9948	0.21	0.72	96.4 $\pm$ 1.04
4	CA	0.5 – 4.0	0.9950	0.14	0.47	97.9 $\pm$ 1.02
5	EC	1.0 – 5.0	0.9959	0.28	0.85	98.2 $\pm$ 2.84
6	PCA	1.0 – 5.0	0.9982	0.26	0.90	102.9 $\pm$ 2.65
7	RH	0.5 – 4.0	0.9976	0.13	0.45	101.3 $\pm$ 2.90
8	EA	1.0 – 5.0	0.9990	0.29	0.92	97.2 $\pm$ 2.08
9	MC	1.0 – 5.0	0.9981	0.29	0.92	98.2 $\pm$ 3.01
10	QU	0.25–3.0	0.9972	0.07	0.24	100.2 $\pm$ 3.13
11	KF	1.0 – 5.0	0.9991	0.27	0.86	101.5 $\pm$ 3.54

^a Data were expressed as mean of triplicate measurements.

^b Recovery are expressed as mean $\pm$ standard deviation.

4.6.2 Method development

For method development the method of Sarunya & Sukon (2006) was followed with some modifications. The HPLC system was developed and run the mobile phase with the following gradient elution program: 0 min, 5%A/95%B; 10 min, 10%A/80%B/10%C; 20 min, 20%A/60%B/20%C and 30min, 100%A for perfect separation of polyphenolic compounds. 280, 320 and 380 nm wavelength were selected for the appropriate wave length for the detection of polyphenolic compounds.

As shown in Fig. 12, symmetrical, sharp and well-resolved peaks were observed for the eleven polyphenolic standards. A good separation can be achieved within 30 min using the above condition described. The elution order and the retention times for GA, CH, VA, CA, EC, PCA, RH, EA, MC, QU, and KF were 6.25, 13.69, 15.95, 16.24, 16.69, 19.89, 21.07, 21.79, 24.54, 26.12, and 27.13 min respectively.

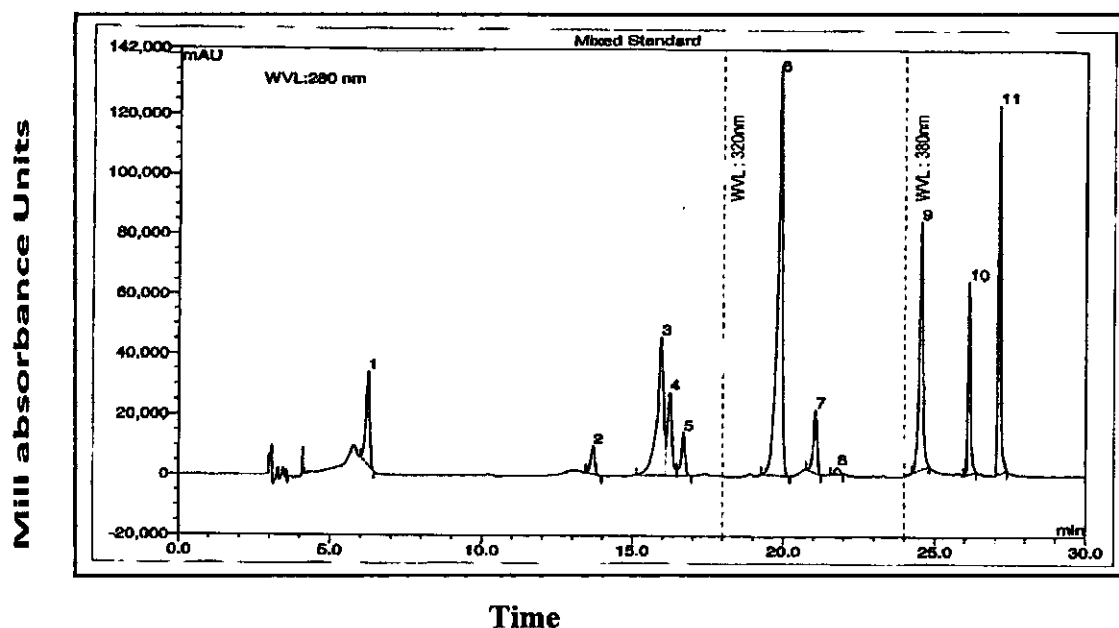


Fig. 12 HPLC chromatogram of a standard mixture of polyphenolic compounds. Peaks:1. gallic acid (GA), 2. (+)-catechin (CH), 3. vanillic acid (VA), 4. caffeic acid (CA), 5. (-)-epicatechin (EC), 6. *p*-coumaric acid (PCA), 7. rutin hydrate (RH), 8. ellagic acid (EA), 9. myricetin (MC), 10. quercetin (QU) and 11. kaempferol (KF).

As shown in Fig. 13, the chromatographic separations of two and three polyphenolic compounds (catechin, vanillic acid, *p*-coumaric acid, rutin hydrate and ellagic acid) peaks in crude ethanolic extract of water spinach (*I. aquatica*) were observed at 280nm and 320nm wave length with mAU for 13.69, 15.95, 19.89, 21.07 and 21.79 min respectively.

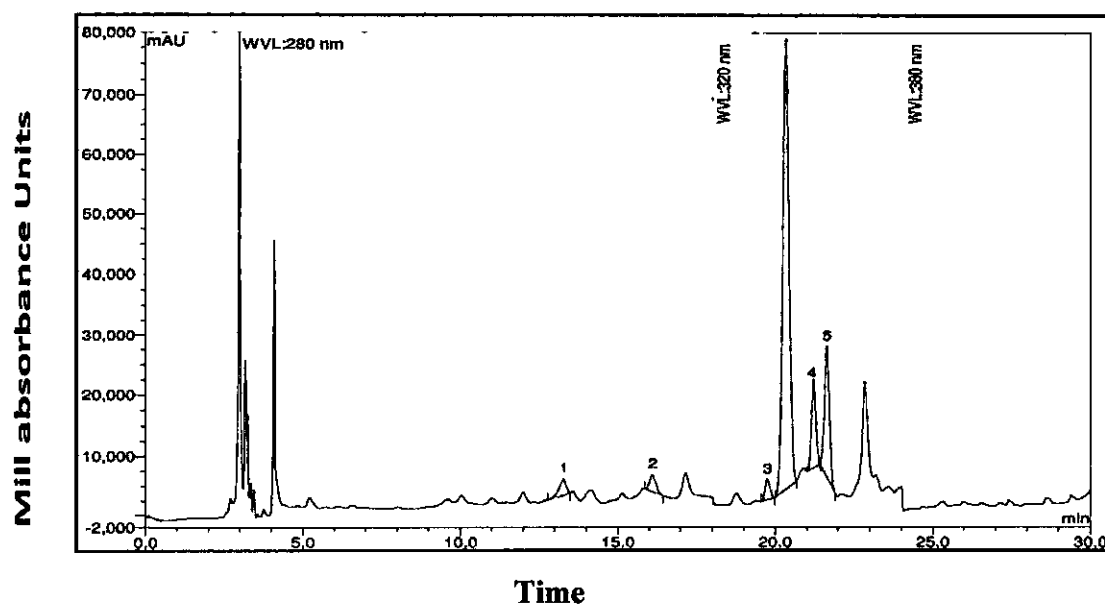


Fig.13 HPLC chromatogram of crude ethanolic extract of water spinach (*I. aquatica*). Peaks: 1. (+)-catechin, 2. vanillic acid, 3. *p*-coumaric acid, 4. rutin hydrate and 5. ellagic acid.

As shown in **Fig. 14**, the chromatographic separation of gallic acid peak in n-Hexane fraction of water spinach (*I. aquatica*) was observed at 280nm wave length with mAU for 6.25 min.

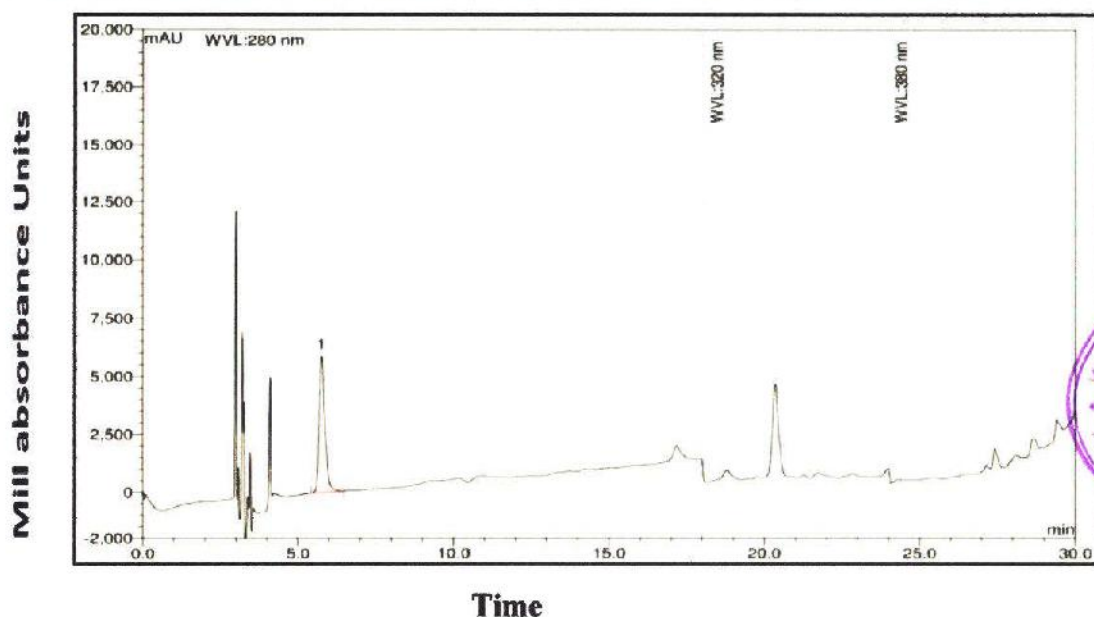


Fig. 14 HPLC chromatogram of n-Hexane fraction of water spinach (*I. aquatica*). Peaks:1. gallic acid.

As shown in **Fig. 15**, the chromatographic separations of three and two polyphenolic compounds (catechin, caffeic acid, *p*-coumaric acid, rutin hydrate and ellagic acid) peaks in aqueous ethanol fraction of water spinach (*I. aquatica*) were observed at 280nm and 320nm wave length with mAU for 13.69, 16.24, 19.89, 21.07 and 21.79 min respectively.

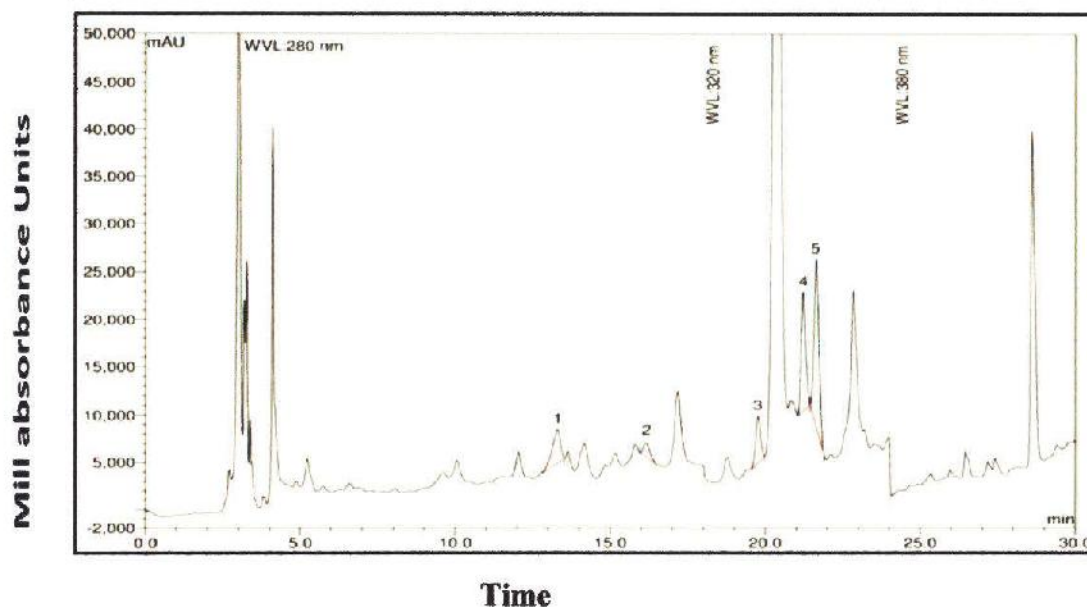


Fig. 15 HPLC chromatogram of aqueous ethanol fraction of water spinach (*I. aquatica*). Peaks:1. (+)- catechin, 2. caffeic acid, 3. *p*-coumaric acid, 4. rutin hydrate and 5.ellagic acid.

4.6.3 Analysis of the plant extract

The content of each phenolic compounds of ethanolic extract, n-Hexane fraction and aqueous ethanol fraction of water spinach (*I. aquatica*) were calculated from the corresponding calibration curve and presented as the mean of determinations as shown in Table. 6, 7.

Table.6 Used concentration and obtained average area from mixed standards and crude ethanolic extract, n-Hexane fraction and aqueous ethanol fraction of *I. aquatica* (n=3).

Polyphenolic compounds	Mixed standards		Crude ethanolic extract of <i>I. aquatica</i>		n-Hexane fraction of <i>I. aquatica</i>	Aqueous ethanol fraction of <i>I. aquatica</i>
	Conc ⁿ (µg/ml)	Avg.Area	Conc ⁿ (µg/ml)	Avg.Area	Avg.Area	Avg.Area
GA	5.0	4538.196	10000	---	1275.142	---
CH	4.0	1390.189		679.237	---	1084.388
VA	5.0	11479.90		659.231	---	---
CA	4.0	4910.547		---	---	368.979
EC	5.0	2152.048		---	---	---
PCA	5.0	26059.00		624.432	---	893.223
RH	4.0	3033.851		2398.845	---	2042.971
EA	5.0	424.195		3011.857	---	2501.138
MC	5.0	11393.0		---	---	---
QU	3.0	6330.564		---	---	---
KF	5.0	11342.70		---	---	---

Table. 7 Contents of polyphenolic compounds in ethanol extract, n-Hexane fraction and aqueous ethanol fraction of water spinach (*I. aquatica*) (n=3).

Polyphenolic compounds	Crude ethanolic extract		n-Hexane fraction		Aqueous ethanol fraction	
	Content (mg/100g of dry extract)	% RSD	Content (mg/10 g of dry extract)	%RSD	Content (mg/100 g of Dry extract)	%RSD
CH	19.35	0.42	---	---	30.88	0.46
VA	2.84	0.09	---	---	---	---
PCA	1.16	0.03	---	---	1.70	0.05
RH	31.31	0.92	---	---	26.67	0.31
EA	337.26	6.57	---	---	280.06	5.82
GA	---	---	13.91	0.86	---	---
CA	---	---	---	---	2.98	0.09

As shown in **Fig. 16**, identification of antioxidant compounds of water spinach (*I. aquatica*) extract was observed in flow chart.

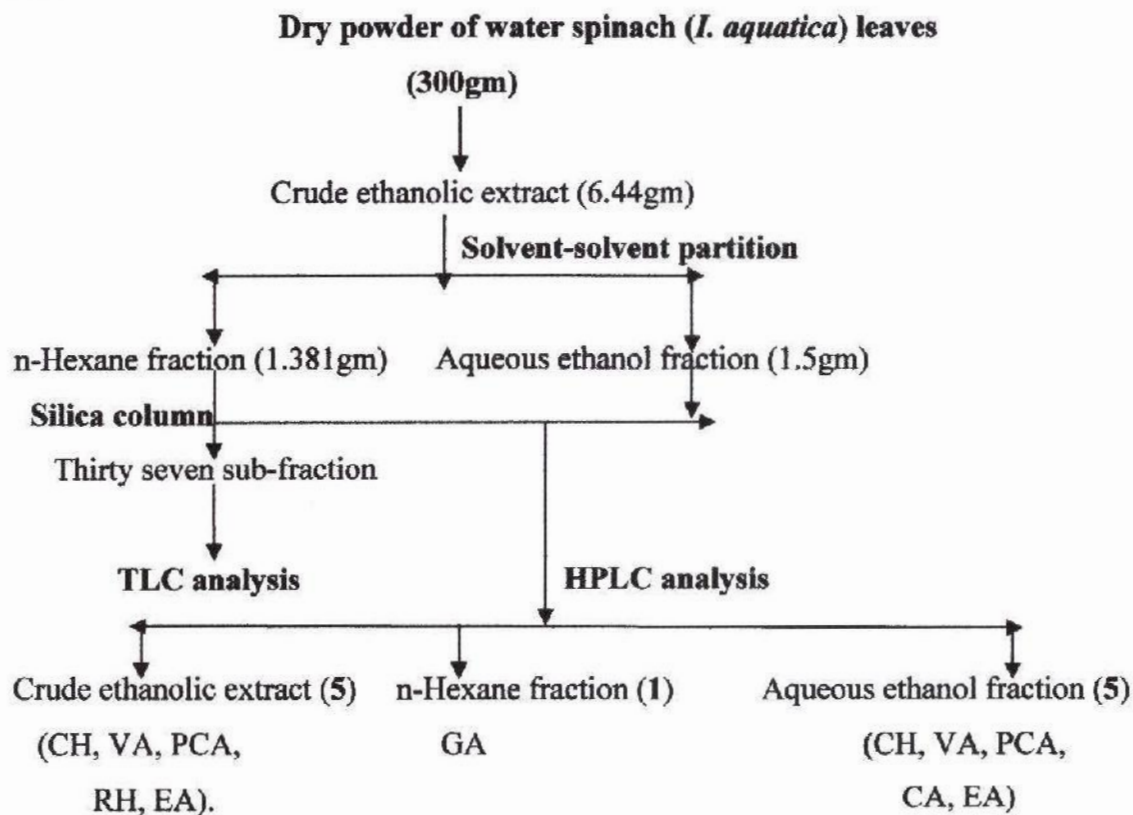
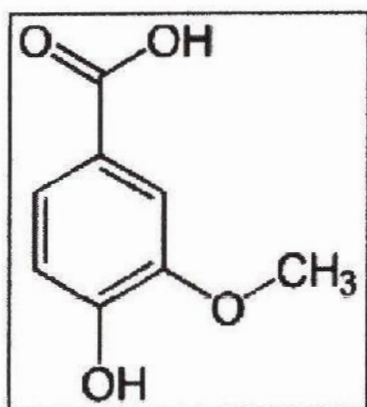
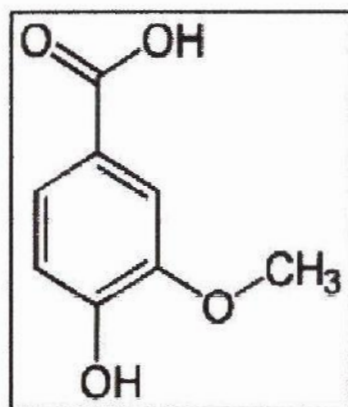


Fig.16 Identification of antioxidant compounds of water spinach (*I. aquatica*) extract. (+) catechin (CH), vanillic acid(VA), *p*-coumaric acid (PCA), rutin hydarte (RH), caffeic acid (CA), ellagic acid (EA) and gallic acid (GA).

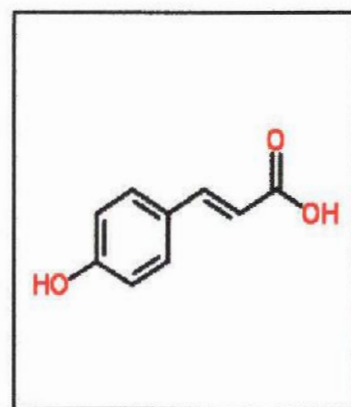
As shown in **Fig. 17**, the chemical structures of seven antioxidant compounds identified by HPLC analysis has given below:-



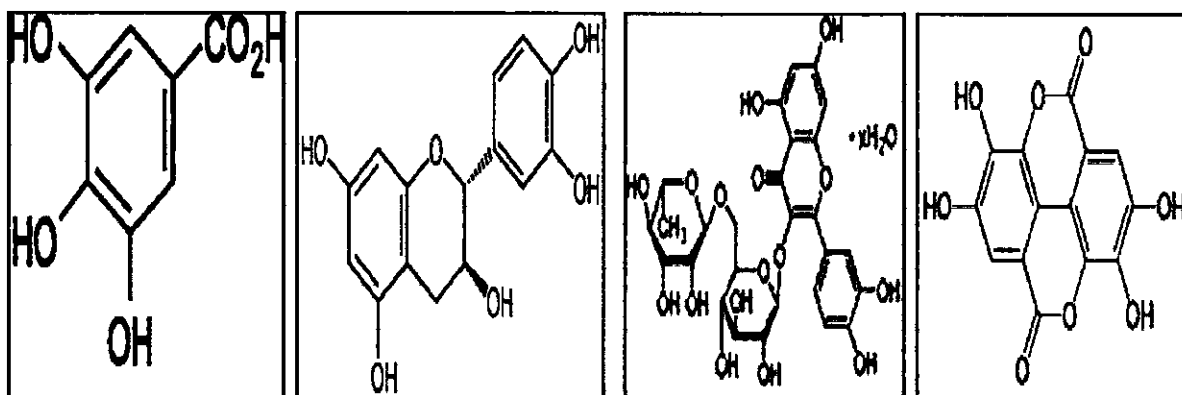
1. (+) Catechin (CH)



2. Vanillic acid (VA)



3. *p*-Coumaric acid (PCA)



4. Gallic acid (GA) 5. Epicatechin (EA) 6. Rutin hydrate (RH) 7. Ellagic acid (EA)

Fig.17 Chemical structures of seven antioxidant compounds in water spinach (*I. aquatica*).

4.7 Antimicrobial activity test

As shown in Table. 8, the antibacterial activity of the test agent was determined by measuring the diameter of zone of inhibition in term of millimeter with a calibrated scale.

Table. 8 Determination of zone of inhibition

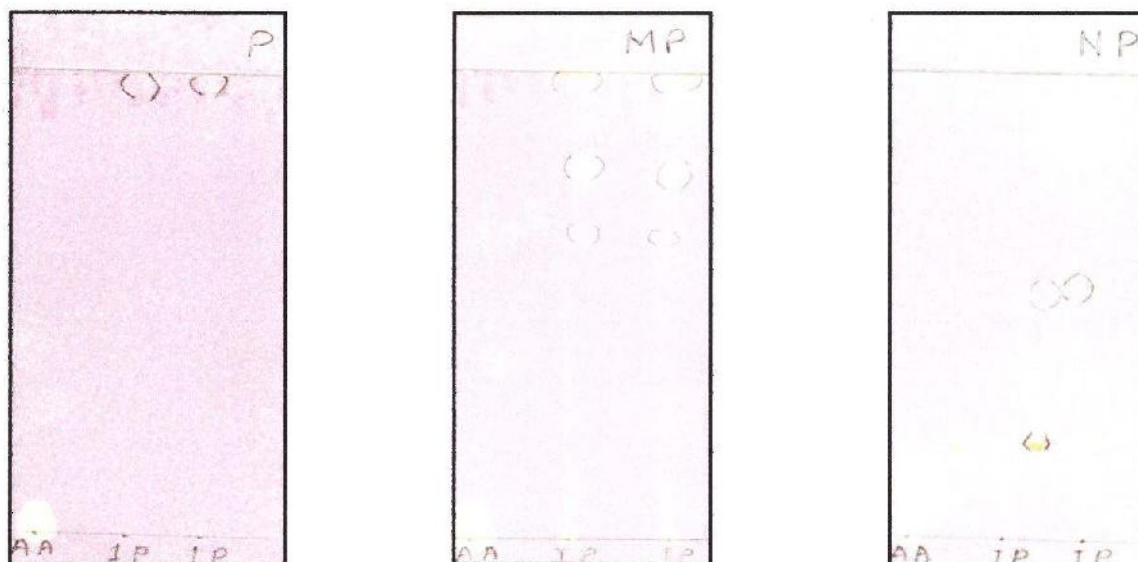
S. N	Bacterial Strains	Type of Bacterial Strains	Diameter of Zone of Inhibition in mm				
			Blank	Kanamycin (30µg/disc)	Extract (250µg/disc)	Extract (500µg/disc)	Extract (1000µg/disc)
1	<i>E.coli</i> Strains EPEC	Gram(-)	-	25.82	-	-	-
2	<i>E.coli</i> Strains ETEC	Gram(-)	-	23.88	-	-	-
3	<i>E. coli</i>	Gram(-)	-	22.50	-	-	-

EPEC= Eteropathogenic *E. coli*; ETEC=Eterotoxigenic *E. coli*; Gram (-):-Gram Negative Bacteria; (-):- No inhibition

Although it is widely established that the plant water spinach (*I. aquatica*) has anti-bacterial activity against *Escherichia coli* but the results of antibacterial screening indicate that the plant has no antibacterial activity against these particular three *Escherichia coli* strains. Again the experiment was conducted only with four strains of *Escherichia coli* as test species which not at all indicate the total inactivity against other micro-organisms. Therefore further research is essential with other species of bacteria, viruses or other micro-organisms.

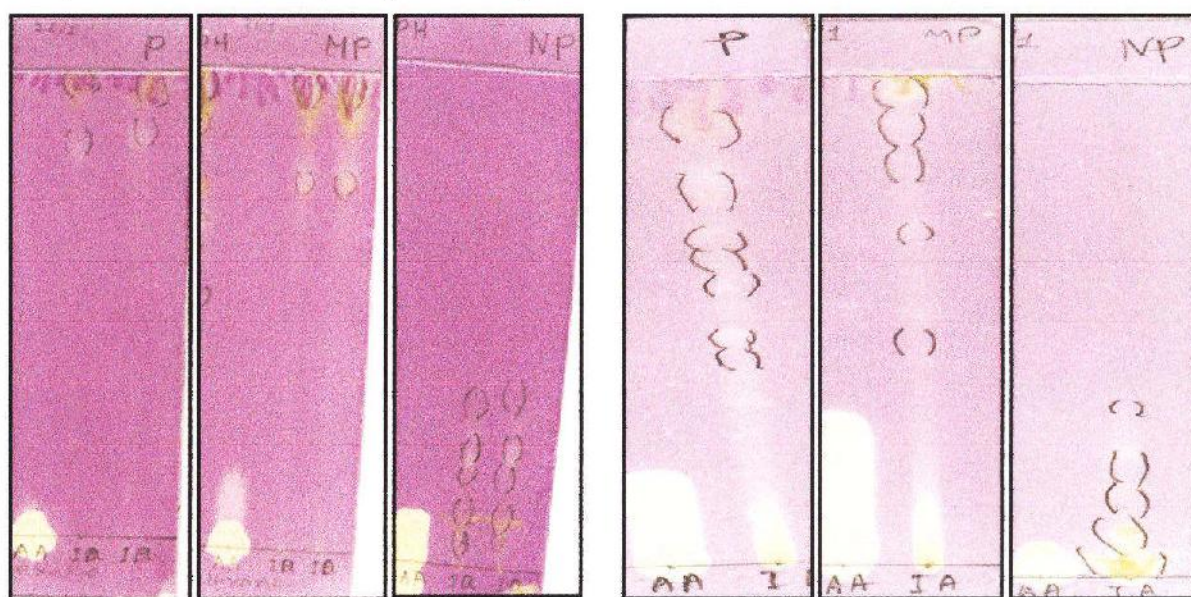
4.8 In-vitro qualitative anti-oxidant test

As shown in Fig.18 and Fig.19, qualitative anti-oxidant test was performed by TLC method. When the DPPH is exposed to antioxidant compounds the purple color of DPPH changed to yellow. The more yellowish color of DPPH was observed the greater the antioxidant activity of the compounds tested in the crude ethanolic extract, n-Hexane fraction and aqueous ethanol fraction of water spinach (*I. aquatica*) in polar, medium polar and non-polar solution.



(CHCl₃: CH₃OH: H₂O =40:10:1) (CHCl₃: CH₃OH =5:1) (n-Hexane: Acetone = 3:1)

Fig. 18 TLC plates for *I. aquatica* of crude ethanolic extract after applying 0.02% DPPH. Here, AA=Ascorbic acid, Ia=*Ipomoea aquatica*.



n-Hexane fraction

Aqueous ethanol fraction

Fig. 19 TLC plates for *I. aquatica* of n-Hexane fraction and aqueous ethanol fraction after applying 0.02% DPPH. These TLC plates were marked after solvent-solvent partition.

Chapter Five

Discussion

Chapter 5

5.0 Discussion

Plant is a source of antioxidant compounds. The number of chemical compounds is called phytochemical constituents which found in the plant kingdom is truly vast and their range of bioactive compounds is equally as great. Some of the phytochemicals which found in these constituents (phenolic compounds, steroid, tannins and alkaloids) are reported to demonstrate pain and inflammation reducing properties. So identification of antioxidant compounds present in extracts is essential to evaluate the biological activity of the plant extract.

A previous study reported that *Ipomoea batatas* leaves can be considered a high source of phytochemical constituents (e.g., flavonoids, phenolic acids, tannins, alkaloids, steroids, saponins and tocopherols) with a potential medicinal usage and a viable alternative source of antioxidants. The antimicrobial test showed the negative results (Pochapski *et al.*, 2011). The present study showed the phytochemicals constituents (e.g., alkaloid, tannins, glycoside and steroid), seven bioactive compounds and no antimicrobial activities from the plant extract of water spinach (*I. aquatica*). So, it can be said that result of the present study can correlate with the finding of Pochapski *et al.* (2011)

In the previous study, TLC analysis show one spot for fractions 2, 4 and 6, two distinct spots for fraction 3 and two joined spots for fraction 5 (Amarowicz *et al.*, 2003). In the present study, TLC analysis of the silica column showed one spot for sub-fractions 31, 32,33,34, 35, 36 and two joined spots for sub-fractions 23, 24, 25, 30 and two distinct spots for sub-fractions 7, 8, 11 and three distinct spots for sub-fractions 9, 10, 13 to18. So, the result of the present study can correlate with the previous study.

The phytochemicals of flavonoids, glycosides, tannins, phenols, carbohydrates, terpenoids, gums, mucilage and alkaloids show antimicrobial activity against microorganisms (*Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Proteus vulgaris*) using the agar disc diffusion method (Sivaraman *et al.*, 2010). In the present study extract didn't show dose dependent antibacterial activity against gram negative bacteria of *E. coli*. So, the result of the present study can't correlate with the previous study. So, further investigation is required to find out the chemical constituents responsible for these effects.

The finding of previous study, bioactive compounds namely ascorbic acid, carotenoids and flavonoids are identified by using HPLC in baby spinach (Bergquist, 2006). The present study showed chromatographic separations of polyphenolic compounds including catechin, vanillic acid, *p*-coumaric acid, rutin hydrate, caffeic acid, ellagic acid and gallic acid in water spinach. So, it is newly investigated bioactive compounds of water spinach than the previous finding.

Research finding of previous study, flavonols are detected at 370 nm and the flavonols myricetin, quercetin, kaempferol and isorhamnetin has retention times of 23.8, 26.7, 31.2, 32.6 min respectively. The carotenoids are detected at 450 nm and lycopene, α -carotene and β -carotene has retention times of 9.5, 18.5 and 19.8 min respectively (Lako *et al.*, 2005). The research showed that four and three chromatographic separations namely gallic acid, (+)-catechin, vanillic acid, caffeic acid, *p*-coumaric acid, rutin hydrate and ellagic acid peaks in plant extract of water spinach (*I. aquatica*) were observed at 280nm and 320nm wavelength for 6.25, 13.69, 15.95, 16.24, 19.89, 21.07 and 21.79 min respectively. So, new bioactive compounds are found in the present study of wave length vs time than the previous studies.

In the experimental result of the previous study, recovery rates are in the range 90.21~110.19%, and their RSD values are less than 4%. The good linearity (correlation coefficient values $R^2 > 0.998$) was achieved in relatively wide concentration ranging from 1.67 to 167 $\mu\text{g/ml}$ for all the compounds (Choi *et al.*, 2010). The findings of the present study showed that recovery tests of all compounds ranged 95.0-104% and their RSD values were less than 2.0%. The good linearity (correlation coefficient values $R^2 > 0.999$) was achieved in relatively wide concentration ranging from 1.0 to 5.0 $\mu\text{g/ml}$ for all the compounds. So, the finding of the present study is agreed with Choi *et al.* (2010).

Research finding of previous study revealed that the plant extract of *Viscum album* contained a high concentration of gallic acid (39.93 $\mu\text{g/g}$ dry). It is found that caffeic acid, *p*-coumaric acid, kaempferol and quercetin are present in the extract with very low concentration (13.61 μg , 1.82 μg , 7.30 μg & 6.05 $\mu\text{g/g}$ of dry weight respectively) (Vicas *et al.*, 2011). The experimental results of the present study indicated that the crude ethanolic extract of water spinach (*I. aquatica*) contained a high concentration of ellagic acid (337.26 mg per 100 g of dry extract). Catechin, vanillic acid, *p*-coumaric acid and rutin hydrate were present in the extract with low amount (19.35 mg, 2.84 mg, 1.16 mg, & 031.31 mg/100 g of dry weight

respectively. The n-Hexane fraction of water spinach (*I. aquatica*) contained a moderate concentration of gallic acid (13.91 mg per 100 g of dry extract). The aqueous ethanol fraction of water spinach (*I. aquatica*) contained a high concentration of ellagic acid (280.06 mg per 100 g of dry extract). Catachin, caffeic acid, p-coumaric acid and rutin hydrate were present in the extract with low concentration (30.88 mg, 2.98 mg, 1.70 mg & 26.67 mg/100 g of dry weight, respectively). So, it is the new finding and not append in previous studies.

The previously studies suggested that there are a direct relationship between antioxidant activity and total phenolic content found in the medicinal plants. Phenolic compounds has a major contribution to antioxidant activity (Lien *et al.*, 1997). Among the many separation systems of the HPLC analysis is included to use a binary solvent system containing acidified water and a polar organic solvent has been developed to specifically measure polyphenolic concentrations (Tsao & Yang, 2003; Sakakibara *et al.*, 2003). The authors, Sarunya & Sukon used six standards, C18 column with 150 mm length, and HP 1090, series II, liquid chromatography systems to determine the polyphenolic contents. The wavelength between 210 and 380 nm is used for the detection of polyphenolic compounds (Cai *et al.*, 2004; Sakakibara *et al.*, 2003).

The research showed that eleven different phenolic standards, C18 column with 250mm length, and rapid separation LC (RSLC) systems were used. Therefore, 280, 320 and 380 nm wave length were selected for the appropriate wavelength for the detection of all standards. The HPLC system was developed and run the mobile phase with the following gradient elution program: 0 min, 5%A/95%B; 10 min, 10%A/80%B/10%C; 20 min, 20%A/60%B/20%C and 30min, 100%A for perfect separation of polyphenolic compounds. So, the result of the present study are developed by using HPLC system to detect antioxidant compounds than the finding of the previous authors.

The present finding showed that bioactive compounds namely vanillic acid, caffeic acid, gallic acid, catechin, ellagic acid and *p*-Coumaric acid were activated as flavoring agent, biosynthesis of lignin, prevention cancer, traditional herbal, health benefits and prevention stomach cancer. So, the result of the present study are the new investigation of antioxidant compounds than the finding of the previous authors.

Chapter Six

Conclusion

Chapter 6

6.0 Conclusion

Aquatic plants produce a variety of bioactive compounds of known human nutrition and can be utilized as food and feed. The profiling of bioactive components can play an important role on guidance for good agriculture practice of water spinach (*I. aquatica*). Water spinach leaves demonstrated the presence of phytochemical constituents (e.g., of alkaloid, tannins, glycoside and steroid) with potential biological activities. No antimicrobial activity was observed in plant extract. But it contains effect of antioxidant components. The antioxidant activity and health effects vary among the individual compounds in each of the groups of bioactive compounds studied. A high concentration of bioactive compounds may be positive not only for human health but also for produce storability. The crude ethanolic extract and aqueous ethanol fraction of water spinach (*I. aquatica*) are contained high concentration of ellagic acid such as 337.26 mg and 280.06 mg per 100 g of dry extract. So, it is important that the levels of bioactive compounds in fruit can be maintained from harvest to consumption.



Chapter Seven

References

Chapter 7

7.0 References

- Ali, H., Houghton, P.J. and Soumyanath, A., 1998. Amylase inhibitory activity of some Malaysian plants used to treat diabetes with particular reference to *Phyllanthus amarus*. *Journal of Ethnopharmacol.* 107: 449-55.
- Alkiyumi, S.S., Abdullah, M.A., Alrashdi, A.S., Salama, S.M., Abdelwahab, S.I. and Hadi, A.H., 2012. *Ipomoea aquatica* Extract Shows Protective Action Against Thioacetamide-Induced Hepatotoxicity. ppt. 6147
- Amarowicz, R., Shahidi, F. and Wiczkowski, W., 2003. Separation of individual catechins from green tea using silica gel column and HPLC. *Food and nutrition press*.pp.166
- Arts, I.C.W. and Hollman, P.C.H., 2005. Polyphenols and disease risk in epidemiologic studies. *American journal of clinical nutrition.* 81: 317-325.
- Austin, D.F., 2007. Water Spinach (*Ipomoea aquatica*, Convolvulaceae) A food gone wild. *Ethnobotany Research & Applications.* 5:123-146
- Badarinath, A.V., Mallikarjuna, R.K., Madhu, S.C.C., Ramkanth, S., Rajan, T.V.S., Gnanaprakash, K., 2010. A Review on in-vitroAntioxidant methods: comparisions, correlations and considerations. *International Journal of Pharm Technology Research.* 2(2): 56-60
- Baysa, M.C., Anuncio, R.R.S., Chiombon, M.L.G., Cruz, J.P.R.D. and Ramelb, J.R.O., 2006. Lead and Cadmium Contents in *Ipomoea aquatica* Forsk. Grown in Laguna de Bay. *Philippine Journal of Science.* 135 (2): 139-143.
- Bergquist, S., 2006. Bioactive compounds in baby spinach (*Spinacia oleracea* L.). Effects of Pre and Post-harvest Factors. *Horticulture and Agricultural Science.* pp.21.
- Buttriss, J., 1999. Omega-3 fatty acids and antioxidants in edible wild plants. *Biol Res.* pp.1

- Cai, Y., Luo, Q., Sun, M. and Corke, H., 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Science*. 74: 2157-2184.
- Castillo, F.S., 1982. Oraphyl-a saline mouthwash powder from Kangkong (*Ipomoea reptans*) leaves. pp.128.
- Chang, S.K., Nagendra Prasad, K. and Amin, I., 2013. Carotenoids retention in leafy vegetables based on cooking methods. *International Food Research Journal*. 20(1): 457-465.
- Chen, B.H. and Chen, Y.Y., 1992. Determination of carotenoids and chlorophylls in water convolvulus (*Ipomoea aquatica*) by liquid chromatography. *Food Chemistry*. 45: 129-134.
- Choi, J.S., Kim, S.W., Kim, H.W., Woo, M.H., Lee, J.H. and Min, B.S., 2010. Quantitative Determination of Bioactive Compounds in Some Artemisia capillaries by High Performance Liquid Chromatography. *Natural Product Sciences*. 16(4) : 233-238.
- Christen, W.G., Gaziano, J.M. and Hennekens, C.H., 2000. Design of Physicians' Health Study II a randomized trial of beta-carotene, vitamins E and C, and multivitamins, in prevention of cancer, cardiovascular disease, and eye disease, and review of results of completed trials. *Annals of epidemiology*. 10: 125-134.
- Chu, Y. F., Jie, S., Xianzhong, W., Hai, L. R., 2002. Antioxidant and antiproliferative activities of common vegetables. *J. Agric. Food Chem*. 50:6910-6916.
- Edie, H. H. and Ho, B. W. C., 1969. *Ipomoea aquatica* as a vegetable crop in Hong Kong. *Economical Botany*. 23: 32-36.
- Fasiheh, L., Mahlagha, G. and Arian, S., 2013. Changes in antioxidant activity and content of phenolic compounds during the ripening process of elm-leaved blackberry fruit. *International Journal of Agronomy Plant Product*. 4 (1): 88-93.
- Fukumoto, L.R. and Mazza, G., 2000. Assessing antioxidant and prooxidant activities and phenolic compounds. *Journal of Agriculture and Food Chemistry*. 48: 3597-3604.

- Gabay, O., Sanchez, C., Salvat, C., Chevy, F., Breton, M., Nourissat, G., Wolf, C., Jacques, C., Berenbaum, F., 2010. Stigmasterol: a phytosterol with potential anti-osteoarthritic properties. *American Journal of Clinical Nutrient*. 18 (1): 106–116.
- Ghani, A., 2003. Medicinal Plants of Bangladesh. 2nd edition, 415. The Asiatic Society of Bangladesh.
- Hamid, K., Ullah, M.O., Sultana, S., Howlader, M.A., Basak, D., Nasrin, F. and Rahman, M.A., 2011. evaluation of the Leaves of *Ipomoea aquatica* for its Hypoglycemic and Antioxidant Activity. *Journal of Pharmacy Science & Research*. Vol.3(7): 1330-1333.
- Ho, B.W.C. and Edie, H.H., 1969. White rust (*Albugo ipomoeae- aquaticae*) of *Ipomoea aquatic* in Hong Kong. *Plant Disease Reporter*. 53: 959-962.
- Huang, D.J., Chen, H.J., Lin, C.D. and Lin, Y.H., 2005. Antioxidant and antiproliferative activities of water spinach (*Ipomoea aquatica* Forsk) constituents. *Natural Product Sciences*. 46: 99-106.
- ICH Q2A, International conference on harmonization: guidance for industry: text on the validation of analytical procedures availability, Fed. Regist., 1995; 60(40): 11260-11262.
- Jaime, L., Mendiola, J.A., Herrero, M., Soler Rivas, C., Santoyo, S., Señorans, F.J., Khan, M.J., Saini, V., Bhatil, V.S., Karchuli, M.S. and Kasture, S.B., 2005. Anxiolytic activity of *Ipomoea aquatica* leaves. *European Journal of Experimental Biology*. 1 (1): 63-70
- Khan, M.J., Saini, V., Bhatil, V.S., Karchuli, M.S., Kasture, S.B., 2011. Anxiolytic activity of *Ipomoea aquatica* leaves. *European Journal of Experimental Biology*. 1 (1): 63-70
- Khaw, K.T., Bingham, S., Welch, A., Luben, R., Wareham, N., Oakes, S. and Day, N., 2001. Relation between plasma ascorbic acid and mortality in men and women in EPIC-Norfolk prospective study: a prospective population study. *Food and nutrition press*. 357: 657-663.

- Klejduš, B., Mikelová, R., Adam, V., Zehnálek, J., Vacek, J., Kizek, R. and Kubán, V., (2004). Isolation and characterization of bioactive compounds from plant resources: The role of analysis in the ethnopharmacological approach. *Anal. Chim. Acta.* 517.
- Knekt, P., Jarvinen, R., Reunanen, A. and Maatela, J., 1996. Flavonoid intake and coronary mortality in Finland: a cohort study. *British medical journal.* 312: 478-481.
- Kritchevsky, S.B., 1999. Beta-carotene, carotenoids and the prevention of coronary heart disease. *Journal of nutrition.* 129: 5-8.
- Kulkarni, M.S., Bhati, V.S., Saini, V., Khan, M.S. and Kasture, S.B., 2011. Anxiolytic activity of *Ipomoea aquatica* leaves. *European Journal of Experimental Biology.* 1 (1): 63-70.
- Lako, J., Trenerry, V.C., Wahlqvist, M., Wattanapenpaiboon, N., Sotheeswaran, S. and Premier, R., 2005. phytochemical flavonols, carotenoids and the antioxidant properties of a wide selection of Fijian fruit, vegetables and other readily available foods. *Horticultural Science.* 35(4): 588-592.
- Lakshmi, B. and Vimala, V., 2000. Nutritive value of dehydrated green leafy vegetables. *Journal of Food Science Technology.* 37: 465-471.
- Lien, E.J., Ren, S., Bui, H.H. and Wang, R., 1997. Quantitative structure activity analysis of phenolic antioxidants, Free Radical. *Biological Medicine.* 26: 285-294.
- Ly, J., Samkol, P. and Preston, T.R., 2002. Nutritional evaluation of aquatic plants for pigs: pepsin/pancreatin digestibility of six plant species. *Livestock Research for Rural Development.* 14(1).
- Manach, C., Mazur, A. and Scalbert, A., 2005. Polyphenols and prevention of cardiovascular diseases. *Current opinion in lipidology.* 16: 77-84.
- Mendiola, J.A., Marin, F.R., Senorans, F.J., Reglero, G., Martín, P.J., Cifuentes, A. and Ibanez, E., 2007. Profiling of Different Bioactive Compounds in Functional Drinks by HPLC. Instituto de Fermentaciones Industriales (CSIC). pp.75-80.

- Milner, A., 1999. Functional foods and health promotion. *Journal of Nutrition*. 129: 1395–1397.
- Monic, S., Yerram, R.B. and Jagadeesh, B., 2012. Phytochemical screening and in- vitro antioxidant activity of aqueous and hydroalcoholic extract of *Bacopa monnieri* Linn. *International Journal of Pharm Science Report*. 3(9): 3418-3424.
- Montero, O., Macías-Sánchez, M.D., Lama, C.M., Lubián, L.M., Mantell, C., Rodríguez, M. and De La Ossa, E.M., 2005. Quantitative Determination of Bioactive Compounds in Some *Artemisia* capillaries by High-Performance Liquid Chromatography *Journal of Agriculture and Food Chemistry*. 53: 9701.
- Pelczar, M.J., Chan, J.R., Noel, E.C.S. and Kreig, R., 1993. Microbiology. Tata McGraw-Hill Publishing Company Ltd. New Delhi. pp. 280-291
- Phokrai, A., Akaracharanya, A., Chareonpornwattana, S., Leepipatpiboon, S., Nakamura, T., Sivaraman, D. and Muralidaran, P., 2010. Nootropic Effect of *Ipomoea aquatic* Forsk in Rat Hippocampus. *International Journal of Pharm Technology Research*. 2(1).pp.477.
- Pochapski, M.T., Fosquiera, E.C., Esmerino, L.A., Santos, E.B.D., Farago, P.V., Santos, F.A. and Groppo, F.C., 2011. Phytochemical screening, antioxidant, and antimicrobial activities of the crude leaves' extract from *Ipomoea batatas* (L.) Lam. *Pharmacogn Magazine*. 7(26): 165–170.
- Prasad, K.N., Prasad, M.S., Shivamurthy, G.R. and Arashya, S.M., 2006. Callus induction from *Ipomoea aquatica* Forsk. Leaf and its antioxidant activity. *Indian journal of Biotechnology*. Vol.5. pp.107-111.
- Prior, R. and Cao, L. G., 2000. Antioxidant photochemical in fruits and vegetables. Diet and health implications. *Horticulture Science*. 35: 588-592.
- Ramarathnam, N., Osawa, T., Ochi, H. and Kawakishi, S., 1995. The contribution of plant food antioxidants to human health. *Trends in Food Science and Technology*. 6: 75-82.

- Rangan, D., Rao, K.S., Singh, K., Kaluwin, C., Donals, E., Rivett, G. and Jones, P., 1990. Lipid, fatty acid, amino acid and mineral composition of five edible plant leaves. *Journal of Agriculture and Food Chemistry*. 38: 2137–2139.
- Rao, T.V.R.K. and Tuhina, V., 2002. Iron, calcium, β -carotene, ascorbic acid and oxalic acid contents of some less common leafy vegetables consumed by the tribals of Purnia district of Bihar. *Journal of Food Science Technology*. 39: 560–562.
- Roland, R., 1982. Antibiotics-An introduction. Hoffman La-Roche & Co, Basle, Switzerland. pp. 70-71.
- Sakakibara, H., Honda, Y., Nakagawa, S., Ashida, H. and Kanazawa, K., 2003. Simultaneous determination of all polyphenols in vegetables, fruits, and teas. *Journal of Agriculture and Food Chemistry*. 51(3): 571.581.
- Sakata, K., Yamamoto, K., and Watanabe, N., 1994. Antioxidative compounds from marine organisms. In: Food Phytochemicals for Cancer Prevention II. Teas, Spices and Herbs, C.T. Ho, T. Osawa, M.T. Huang and R.T. Rosen (Eds.), American Chemical Society, Washington DC. pp 164-182.
- Santwani, K., Thakar, A.B., Shukla, V.J. and Harisha, C.R., 2011. A preliminary Phytochemical assay of ashwagandha granules a pilot study. *International Journal of Pharm Technology Research*. 2(4): 1026-1032
- Sarower, M.G., Kumar, S., Sayeed, M.A.B., Sabbir, W., Hasanuzzaman, A.F.M., Alam, M.I. and Rahaman, S.M.B., 2008. Ecological diversity and distribution of aquatic and semi-aquatic weeds in Khulna. *South Asian Journal of Agriculture*. 3: 163-168.
- Sarunya, C. and Sukon, P., 2006 . Method development and determination of phenolic compounds in Broccoli Seeds Samples. *Journal of Science*. 33(1): 103-107.
- Schneider, O., Seret, V., Edin, E. H. and Verreth, J. A. J., 2005. Analysis of nutrient flows in integrated intensive aquaculture systems. *Aquaculture Engineering*. 32: 379–401.
- Schoefs, B., 2005. Developing high-speed HPLC separations for the analysis of bioactive compounds. *In advanced food nutrition research*. p. 41.

- Shah, W., Rane, N., Kekare, M.B. and Vaidya, V., 2010. Estimation of two Bioactive Compounds from *Azadiracta India* A.Juss. Leaves using. *International Journal of Pharma and Bio-sciences*. V1(2): 1-2
- Shahid, M., 2011. Antioxidants and Nutrition. *Aquaculture*. pp.65-70
- Sharma, V.K., Tiwari, M., Chauhan, N.S. and Nema, R.K., 2102. Phytochemical investigation on the Ethanolic Extract on the Leaves of *Zizyphusxylopyrus* (Retz.) Willd. *International Journal of Agronomy Plant Product*. 3 (1): 26-37.
- Sivaraman, D., Muralidaran, P. and Kumar, S., 2010.evaluation of Anti-microbial and anti-inflammatory activity of methanol leaf extract of *Ipomoea aquatica* forsk. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*. vol.1(2): 261
- Sivasankari, K.V., Janaky, S. and Sekar, T., 2011. Antioxidant status of leaves of *Caesalpinia bonduc*. *International Journal of Pharmacology Application*. 2(4): 262-266.
- Snyder, G.H., Morton, J.F. and Gentung, W.G., 1981.Trials of *Ipomoea aquatica*, nutritious vegetable with high protein-and nitrate-extraction potential. *Proceedings of the Florida State Horticultural Society*. 94: 230-231.
- Sundar Rao, K., Dominic, R., Singh, K. and Kaluwin, C., 1990. Lipid, fatty acid, amino acid, and mineral composition of five edible plant leaves. *Journal of Agriculture and Food chemistry*. 38: 2137-2139.
- Tofern, B., Mann, P. and Kaloga, M., 1999. Aliphatic pyrrolidine amides from two tropical convolvulaceous species. *Phytochemistry*. 52: 1437-1441.
- Trease, G.E., Evans, W.C., 1983. Pharmacognosy. Bailliere Tindale, London. 12th Edition: 256-257
- Tsao, R. and Yang R., 2003. Optimization of a new mobile phase to know the complex and real polyphenolic composition: towards a total phenolic index using high performance liquid chromatography. *Journal of Chromatogrm Academy*.1018: 29-40.

- Van Rijn, J., 1996. The potential for integrated biological treatment systems in recirculating fish culture a review. *Aquaculture*.139: 181-201.
- Van Valkenburg, J. L. C. H. and Bunyaphatsara, N., 2001. Plant Resources in South-East Asia. Number 12. Backhuys Publishers, Leiden, The Netherlands.
- Vicas, S.I., Rugina, D., Leopold, L., Pintea, A. and Socachu, C., 2011. HPLC Fingerprint of Bioactive Compounds and Antioxidant Activities of *Viscum album* from Different Host Trees. *Notulae Botanical Horticulture and Agrobotanica Cluj-Napoca*. 39(1): 48-57.
- Villano, D., Fernandez-Pachón, M.S., Moyá, M.L., Troncoso, A.M. and Garcia-Parrilla, M.C., 2007. Chromatography in bioactivity analysis of compounds. *Talanta*. 71: 230.
- Watt, G., 1972. A Dictionary of the Economic Products of India. *International Journal of PharmTech Research*. 6(3): 1889-1892.
- Westphal, E., 1993. Ipomoea aquatica forsk in Plant Resources in South-East Asia. Number Vegetables. Pudoc Scientific Publishers, Wageningen. pp.181-184.
1973. Energy and protein requirements. *WHO Technical Report Survey*. 55: 522
- and Ranga, A., 1996. Determination of carotenoids in Chinese vegetables. *Chemistry*. 56: 451-455.
- wale, L.A. and Nade, V.S., 2008. Effect of home processing on ascorbic betacarotene content of spinach (*Spinacia oleracea*) and amaranth (*tricolor*) leaves. *Indian Journal of Pharmacology*. 40: 32-36.
- myo, S., and Sano, H., 2006. Sulfate Phytoremediation through *Ipomoea aquatica* Forsk. Harboring Rice (*Oryza sativa* L.) Cysteine *Journal of Science Research*. Vol. 31: pp.69.

LIST OF ABBREVIATIONS

μg = Microgram

Concⁿ. = Concentration

DPPH = 2, 2-diphenyl-1 - picryl hydrazyl

et al= et alia

hr = Hour

Min = Minute

R^2 = correlation coefficients

SD = Standard Deviation

TLC= Thin Layer Chromatography

HPLC= High Performance Liquid Chromatography

RPC= Reversed phase chromatography

UV = Ultra-violet

WHO = World Health Organization

Wt = Weight

MEIAF= Methanol extract *Ipomoea aquatica* Forsk

IAF= *Ipomoea aquatica* Forsk

FTC= Total flavonoid content

RSD= Relative Standard Deviation

mAU = milli absorbance units

