In-vitro determination of antioxidant capacity for methanolic extract of *Amaranthus gangeticus, Spinacia oleracea* L, and *Ipomoea aquatica* by DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay

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A thesis report submitted to the depart of pharmacy, East West University, in partial fulfillment of the requirements for the degree of bachelor of pharmacy

This paper is dedicated to my benevolent

CERTIFICATE

This research paper is submitted to the department of Pharmacy, East West University in conformity with the requirements for the degree of Bachelor of Pharmacy was carried out by Redwan Ahmed (2008-1-70-038) under my guidance and supervision and that no part of the thesis has been submitted for any other degree. I further certify that all the resources of the information in this research paper are duly acknowledged.

Sufia Islam, PhD

Associate professor& Chairperson

Department of Pharmacy

East West University, Dhaka.

CERTIFICATE

This is to certify that, the research work on “In-vitro determination of antioxidant capacity for methanolic extract of *Amaranthus gangeticus*, *Spinacia oleracea* L, and *Ipomoea aquatic* by DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay” submitted to the department of pharmacy, East West University, Mohakhali, Dhaka, in partial fulfillment of the requirement for the degree of bachelor of pharmacy (B.Pharm) was carried out by Redwan Ahmed, ID: 2008-1-70-038 under my guidance and supervision and that no part of the thesis has been submitted for any other degree. I further certify that all the resources of the information in this research paper are duly acknowledged.

Mr. Amran Howlader

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Mohakhali, Dhaka

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INDEX

Chapter – 01

INTRODUCTION

1.1 INTRODUCTION

One of the most pressing challenges for the next 50 years is to reduce the impact of chronic disease. Unhealthy eating is an increasing problem and underlies much of the increase in mortality from chronic diseases that is occurring worldwide. Diets rich in plant-based foods are strongly associated with reduced risks of major chronic diseases, but the constituents in plants that promote health have proved difficult to identify with certainty. This, in turn, has confounded the precision of dietary recommendations. Plant biochemistry can make significant contributions to human health through the identification and measurement of the many metabolites in plant-based foods, particularly those known to promote health (phytonutrients). Plant genetics and metabolic engineering can be used to make foods that differ only in their content of specific phytonutrients. Such foods offer research tools that can provide significant insight into which metabolites promote health and how they work. Plant science can reduce some of the complexity of the diet-health relationship, and through building multidisciplinary interactions with researchers in nutrition and the pathology of chronic diseases, plant scientists can contribute novel insight into which foods reduce the risk of chronic disease and how these foods work to impact human health.

A major challenge in human health over the next 50 years will be in the area of chronic, noncommunicable diseases, including heart disease, many cancers, type 2 diabetes, and obesity. In 2005, a chilling report from the World Health Organization highlighted the scale of the challenge: 80% of the mortality from chronic disease occurs in low and middle income countries, 60% of those with chronic conditions are aged between 18 and 64, the poor are most vulnerable to chronic disease because of their increased exposure to risks and lower access to

health care, and chronic disease causes poverty through lost capacity and income. Most significantly, the World Health Organization report projected that mortality from chronic disease would increase by 17% worldwide in the decade 2005 to 2015 due to longer average life span, tobacco use, decreasing physical activity, and perhaps most importantly, the increasing consumption of unhealthy foods. Because socio-behavioral risk factors contribute significantly to the incidence of and mortality from chronic disease, 36 million of the 388 million premature deaths predicted for 2005 to 2015 could be avoided if health, science, and public policies were reoriented toward prevention rather than cure. In 2007, the Oxford Health Alliance published a Grand Challenge document outlining how such reorientation of policies and priorities might occur. One of the five major objectives identified in this article was to modify the risk factors for chronic disease, and it is in this area, particularly in identifying and understanding the health-promoting components of food, that plant science could contribute significantly to addressing this Grand Challenge. Research on plants can lead to the identification of those metabolites that promote health and reduce the risk of chronic disease. Research on plants can also lead to the development of tools to assay the health-promoting effects of these metabolites. Greater understanding of the positive contributions that plant-based foods make to human health could contribute significantly to preventing the social and economic burdens of chronic disease globally.

The past 30 years have seen development of an enormous body of evidence on the importance of plant-based foods in preventing or reducing the risk of chronic disease. For cardiovascular disease, these claims began with the output of the Lyon-Diet study, which compared mortality from chronic heart disease on a national basis for European communities. Mortality showed a more or less linear relationship to dairy fat consumption, with countries consuming low dairy fat,

Mediterranean-style diets (Spain, Portugal, and Italy) having less than half the mortality from heart disease compared with countries with high dairy fat consumption, such as Germany, the United Kingdom, and Scandinavian countries.

Research on plant is also important to identify identification of nutritional factors in plant-based foods. While most foods are now labeled with nutritional information detailing their content of protein, carbohydrates (sugars), fats, and additives, many health promoting factors in foods are not measured or listed. Information on fiber content of processed foods is usually provided, although it would be useful for consumers also to know the fiber content of the fresh fruit and vegetables they consume. In addition, although viscous fiber (the sticky type of soluble fiber found in oats, barley, and beans, and certain vegetables, such as okra and aubergine) has been shown experimentally to lower the glycemic index of foods and to have a beneficial impact on obesity, type 2 diabetes, and risk factors for CVD, the relative content of viscous fiber compared with particulate cereal fiber in foods is usually not provided. This may be because the viscous fiber content of most foods in Western diets is very low. However, the content of cereal fiber (which in epidemiological studies has been correlated to reduced risk of CVD, type 2 diabetes, and obesity, but for which no metabolic experiments have shown impacts on glycemic index), particularlywhole grains, is advertised widely for foods together with claims for their health-promoting properties. With greater qualitative as well as quantitative information on the fiber content of different plant-based foods and information on the relative impact of that fiber on glycemic index, many processed foods in Western diets could be reformulated to include higher levels of plant fiber, especially viscous fiber and unprocessed grains, to improve glycemic control and body weight management.

Knowledge of vitamins and their presence in certain foods is long standing. Vitamins are groups of compounds not synthesized by humans but necessary for human life. They are therefore essential in the diet, and humans must consume vitamins periodically to avoid deficiency. So it is very clear to us how important is the research on plants.

Chapter – 2

PLANT REVIEW

PLANT REVIEW

2.1 Amaranthaceae (family of red amaranth)

The Amaranthaceae, the Amaranth family, represent the most species-rich lineage within the flowering plant order of Caryophyllales. Including the goosefoot family (Chenopodiaceae), the extended family contains approximately 180 genera and 2,500 species. Most of these species are annual or perennial herbs or subshrubs, some are shrubs; very few species are vines or trees. Some species are succulent. Many species have stems with thickened nodes. The wood of the perennial stem has a typical "anomalous" secondary growth, only in subfamily Polycnemoideae there is normal secondary growth.

The leaves are mostly alternate, sometimes opposite. They never possess stipules. The simple leaves are flat or trite; their shape is extremely variable, with entire or toothed margins. In some species, the leaves are reduced to minute scales. In most cases, neither basal nor terminal aggregations of leaves occur.

The flowers are solitary or aggregated in cymes, spikes, or panicles and typically perfect (bisexual) and actinomorphic. Some species have unisexual flowers. Bracts and bracteoles are either herbaceous or scarious. Flowers are regular with a herbaceous or carious perianth of (1 to) mostly 5 (rarely to 8) tepals, often joined.

The seeds of *Amaranthus* are edible and used like cereals.

Red amaranth (Lalshak) ***Amaranthus gangeticus***

2.2 Plant description

Amaranthus gangeticus is also known as elephant-head amaranth. It is an annual flowering plant with deep purple flowers. It can grow from 2-3 feet in height.

In Bangladesh, it has been used as a leafy vegetable. Scientific study suggests that it inhibits calcium retention. Another study suggested that due to high levels of antioxidants found in the plant, it could have a radioprotective role in mice.

Various types of amaranth seeds have been used since ancient times in Central and Latin America and in the countries of the Himalayas. Amaranth leaves are used across Asia.

The green-leaved varieties are popular in India and other places, the Chinese prefer their amaranth red-leaved and amaranth grain once was a staple in the diets of pre-Columbian Aztecs. Compared to other grains amaranth seeds have a much higher content of the minerals calcium, magnesium, and iron and of the amino acid Lysine.

Amaranth seeds are also high in potassium, zinc, Vitamin B and E and can contain over 20% protein (depending on the variety).

Like all fast growing leafy greens amaranth loves rich soil with steady moisture and a good supply of nutrients, especially nitrogen. But it isn't as fussed as spinach or silver beet would be. Amaranth is much harder. It can cope with heat and dry conditions a lot better than any other leafy green.

2.3 Taxonomical ladder

Domain Eukaryota

Kingdom Plantae

Subkingdom Viridaeplantae

Phylum Tracheophyta

Subphylum Euphyllophytina

Class Magnoliopsida

Subclass Caryophyllidae

Order Caryophyllales

Suborder Chenopodiineae

Family Amaranthaceae

Subfamily Amaranthoideae

Tribe Amarantheae

Genus Amaranthus

Specific epithet: gangeticus

Botanical name: *Amaranthus gangeticus*

Sanskrit synonym is Yavashaka.

Plant name in different languages

English : Country green, pig weeds

Hindi : Sag. Lalsag

Figure1: Amaranthus gangeticus plant.

2.4 Chenopodiaceae (family of spinach)

Chenopodiaceae were a family of flowering plants, also called the Goosefoot Family. They are now included within family Amaranthaceae. The vast majority of Chenopods are weeds, and many are salt and drought tolerant. A few food crops also belong to the family: spinach, beets, chard, quinoa, and sugar beets. Chenopod pollen is a common allergen, but most Chenopod crops do not produce pollen.

Current studies have classified the species of Chenopodiaceae to several distinct subfamilies (Betoideae, Camphorosmoideae, Chenopodioideae, Polycnemoideae, Salicornioideae, Salsoloideae and Suaedoideae).

Chenopod pollen is a common allergen. Most species bloom in summer months. Pollen at the microscopic level is mostly indistinguishable from species to species, but cross-reactivity has not been definitively established. Because flowering does not occur in the commercial planting cycle, localized pollen counts will not increase due to planting of Chenopod crops.

Spinach (Palong shak) ***Spinacia oleracea***

2.5 Plant description

Spinach (*Spinacia oleracea*) is an edible flowering plant in the family of Amaranthaceae. It is native to central and southwestern Asia. It is an annual plant (rarely biennial), which grows to a height of up to 30 cm. Spinach may survive over winter in temperate regions. The leaves are alternate, simple, and ovate to triangular-based, very variable in size from about 2–30 cm long and 1–15 cm broad, with larger leaves at the base of the plant and small leaves higher on the flowering stem. The flowers are inconspicuous, yellow-green, 3–4 mm diameter,

maturing into a small, hard, dry, lumpy fruit cluster 5–10 mm across containing several seeds.

Common spinach, *Spinacia oleracea*, was long considered to be in the Chenopodiaceae family, but in 2003 the Chenopodiaceae family was combined with the Amaranthaceae family under the family name 'Amaranthaceae' in the order Caryophyllales. Within the Amaranthaceae family there are now a subfamily Amaranthoideae and a subfamily Chenopodioideae, for the amaranths and the chenopods, respectively.

The Environmental Working Group reported spinach is one of the dozen most heavily pesticide-contaminated produce products. The most common pesticides found on spinach are permethrin, dimethoate, and DDT.

Types of spinach

A distinction can be made between older varieties of spinach and more modern ones. Older varieties tend to bolt too early in warm conditions. Newer varieties tend to grow more rapidly, but have less of an inclination to run up to seed. The older varieties have narrower leaves and tend to have a stronger and bitterer taste. Most new varieties have broader leaves and round seeds.

There are three basic types of spinach:

- **Savoy** has dark green, crinkly and curly leaves. It is the type sold in fresh bunches in most supermarkets heirloom variety of savoy is 'Bloomsdale', which is somewhat resistant to bolting. Other common heirloom varieties are 'Merlo Nero' (a mild variety from Italy) and 'Viroflay' (very large spinach with great yields).

- **Flat/smooth leaf spinach** has broad, smooth leaves that are easier to clean than 'Savoy'. This type is often grown for canned and frozen spinach, as well as soups, baby foods, and processed foods.
- **Semi-savoy** is a hybrid variety with slightly crinkled leaves. It has the same texture as 'Savoy', but it is not as difficult to clean. It is grown for both fresh market and processing. 'Five Star', a widely grown variety, has good resistance to running up to seed.

2.6 Taxonomical ladder

Kingdom *Plantae*

Subkingdom *Tracheobionta*

Superdivision *Spermatophyta*

Division *Magnoliophyta*

Class *Magnoliopsida*

Subclass *Caryophyllidae*

Order *Caryophyllales*

Family *Chenopodiaceae*

Genus *Spinacia* L.

Species *Spinacia oleracea* L.

Figure2: Spinacia oleracea L. plant.

2.7 Traditional uses of spinach

The plant is sweet, cooling, carminative, laxative, alexipharmic; useful in diseases of blood and brain, asthma, leprosy, biliousness; causes “kapha” (Ayurveda). It has been used in the treatment of urinary calculi. In experiments it has been shown to have hypoglycemic properties. The leaves are cooling, emollient, wholesome, antipyretic, diuretic, maturant, laxative, digestiblle, anthelmentic, useful in urinary concretion, inflammation of the lungs and the bowels, sore throat, pain in joints, thirst, lumbago, cold and sneezing, sore eye, ring worm scabies, leucoderma, soalding urine, arrest vomiting , biliousness, flatulence. The seeds are useful in fevers, leucorrhoea, urinary discharges, lumbago, and diseases of the brain and of the heart (Yunani). Seeds are laxative and cooling. They have been used in the treatment of difficulty in breathing, inflammation of the liver and jaundice. The green plant is given for the urinary calculi.

2.8 Convolvulaceae (family of water spinach)

Convolvulaceae, known commonly as the bindweed or morning glory family, are a group of about 60 genera and more than 1,650 species of mostly herbaceous vines, but also trees, shrubs and herbs.

Convolvulaceae can be recognized by their funnel-shaped, radially symmetrical corolla; the floral formula for the family has five sepals, five fused petals, five epipetalous stamens (stamens fused to the petals), and a two-part syncarpous and superior gynoecium. The stems of these plants are usually winding, hence their Latin name (from *convolvere*, "to wind"). The leaves are simple and alternate, without stipules. The fruit can be a capsule, berry, or nut, all containing only two seeds per one locule (one ovule/ovary).

The leaves and starchy, tuberous roots of some species are used as foodstuffs (e.g. sweet potato and water spinach), and the seeds are exploited for their medicinal value as purgatives. Some species contain ergoline alkaloids that are likely responsible for the use of these species as ingredients in psychedelic drugs (e.g. ololiuhqui).

Water spinach (Kolmi) *Ipomoea aquatica*

2.9 Plant description

Ipomoea aquatica is a semi aquatic, tropical plant grown as a leaf vegetable. It is found throughout the tropical and subtropical regions of the world, although it is not known where it originated. *I. aquatica* grows in water or on moist soil. Its stems are 2–3 metres (7–10 ft) or more long, rooting at the nodes, and they are hollow and can float. The leaves vary from typically sagittate (arrow head-shaped) to lanceolate, 5–15 centimetres (2–6 in) long and 2–8 centimetres (0.8–3 in) broad.

The flowers are trumpet-shaped, 3–5 centimetres (1–2 in) diameter, usually white in colour with a mauve centre. The flowers can form seed pods which can be used for planting.

Common names of *Ipomoea aquatica*

This plant is known in English as water spinach, river spinach, water morning glory, water convolvulus, or by the more ambiguous names "Chinese spinach", "Swamp cabbage" and "Kangkong" in Asia. Occasionally, it has also been mistakenly called "kale" in English, although kale is a different plant belonging to the *Brassica oleracea* Acephala Group and completely unrelated to water spinach. It is known as *phak bung* in Thai, *trokuon* in Khmer and *kangkung* in Malay and Indonesian. In the Philippines a variety of Kangkong is grown in canals dug by the Americans during the occupation after the Spanish American war. Another variety in the Philippines that grows on land is called "Chinese Kangkong" in the Philippines as opposed to the variety that is grown in water that is simply called Kangkong or "native" Kangkong.

Distribution

Ipomoea aquatica is most commonly grown in East and Southeast Asia. Because it flourishes naturally in waterways and requires little, if any, care, it is used extensively in Thai, Lao, Cambodian, Malay, Vietnamese and Chinese cuisine, especially in rural or *kampung* (village) areas.

The vegetable is also extremely popular in Taiwan, where it grows well. During the Japanese Occupation of Singapore in World War II, the vegetable grew remarkably easily in many areas, and became a popular wartime crop.

It has also been introduced to the United States, where its high growth rate has caused it to become an environmental problem, especially in Florida and Texas. It has been officially designated by the USDA as a "noxious weed" (the term "noxious" refers to its effect on the environment, not to any toxicity).

2.10 Uses

The plant when eaten raw may transmit *Fasciolopsis buski*, an intestinal fluke parasite of humans and pigs, causing fasciolopsiasis.

The vegetable is a common ingredient in Southeast Asian dishes. In Singapore, Indonesia and Malaysia, the leaves are usually stir-fried with chile pepper, garlic, ginger, dried shrimp paste (*belacan*/*terasi*) and other spices. In Penang and Ipoh, it is cooked with cuttlefish and a sweet and spicy sauce.

Chinese cuisine has numerous ways of preparation, but a simple and quick stir-fry, either plain or with minced garlic, is probably the most common.

In Thailand, where it is called *phak bung* (Thai: ผักบุ้ง), it is eaten raw, often along with green papaya salad or *nam phrik*, in stir-fries and in and curries such as *kaeng som*.

In the Philippines, *kangkóng* is usually sautéed in cooking oil, onions, garlic, vinegar, and soy sauce. This dish is called *adobong kangkong*.

In South India, the leaves are finely chopped and mixed with grated coconut to prepare *thoran* (തോരന്), a Keralan dish.

In Bangladesh and West Bengal, it is known as *kolmishak* (কলমীশাক) and stir-fried preparation of the leaves is a very popular dish.

2.11 Taxonomical ladder

Kingdom *Plantae* – Plants

Subkingdom *Tracheobionta* – Vascular plants

Superdivision *Spermatophyta* – Seed plants

Division *Magnoliophyta* – Flowering plants

Class *Magnoliopsida* – Dicotyledons

Order *Solanales*

Family *Convolvulaceae* – Morning-glory family

Genus *Ipomoea* L. – morning-glory

Species *Ipomoea aquatica* Forssk. – swamp morning-glory

Figure3: Ipomoea aquatica plant.

CHAPTER – 3

LITRRATURE REVIEW

REVIEW OF LITERATURE

3.1 Literature review on Red Amaranth

The edible amaranth (*Amaranthus tricolor* L or *Amaranthus viridis* L) is an annual leafy vegetables belonging to the amaranthaceae. *Amaranthus* is an herbaceous plant. It is a short lived annual plant, growing up to 1 meter in height. It is highly adapted under lowland condition. Grow well at day temperatures above 250C and night temperatures not lower than 150C. *Amaranthus* are quantitative short day plants. It consumes high amount of water and uses 6 mm/day.

Nutritional values

Amaranthus is especially high in Vitamin A and C and provide also a significant amount of B group Vitamins. Iron is important for red blood cell and a deficiency result into anaemia. Calcium is essential for skeletal development. Phosphorus together with calcium provides strong bone. Protein is needed for repair and maintenance of body tissue.

3.2 Pharmacological action

Several studies have shown that amaranth seed or oil may benefit those with hypertension and cardiovascular disease; regular consumption reduces blood pressure and cholesterol levels, while improving antioxidant status and some immune parameters. In traditional medicine amaranth is especially recommended for people with a low red blood cell count.

Amaranth seeds, like buck wheat and quinoa contain protein that is unusually complete for plant sources regular consumption reduces blood pressure and cholesterol levels.

Amaranth leaves are nutritionally similar to beets, swiss chard and spinach, but are much superior.

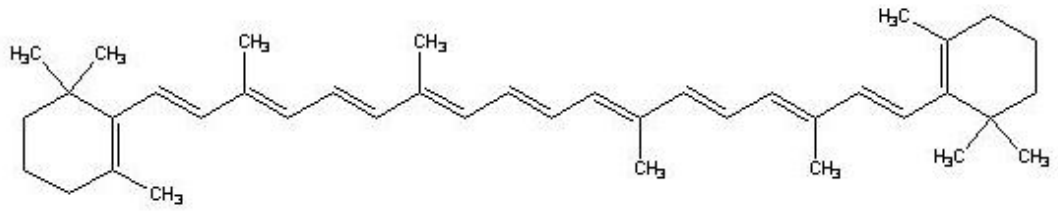
3.3 Phytochemical study

Phytochemical studies of *Amaranthus gangeticus* give finding of following chemicals:

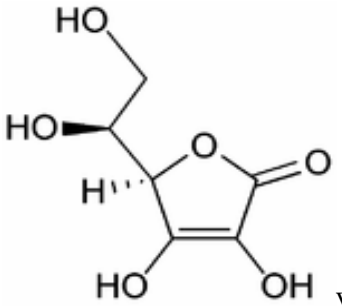
Table : chemicals found from *Amaranthus gangeticus*

Plant part	Chemical type
Leaves	Calcium, niacin, carotene, vitamin C, protein, and trace element
Seeds	protein

Some chemical structures found from *Amaranthus gangeticus* are shown here:



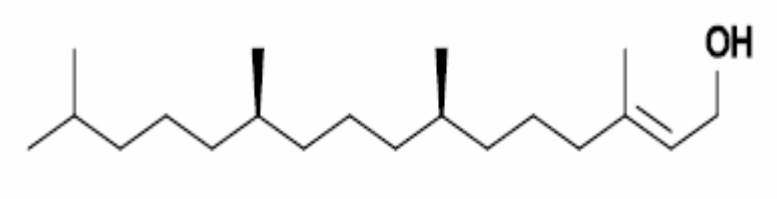
β-carotene



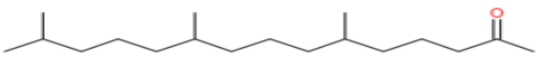
vitamin C

Table . List of detected organic compounds with chlorinated pesticides in red amaranth (*Amaranthus gangeticus*) sample.

Peak	Name of Compound	Mol. wt.	Mol. Fromula	Chlorinated Compound
01	2-Pentadecanone, 6,10,14-trimethyl	268	$C_{18}H_{36}O$	–
02	Trans- undec- 4 –enal	168	$C_{11}H_{20}O$	–
03	Hexadecanoic acid, ethyl caster	284	$C_{18}H_{36}0_2$	–
04	2-Hydroxy 1,1,10 – trimethyl-6,9- epidioxydecal	226	$C_{13}H_{22}O_3$	–
05	Cyclopentanol 2,4,4 –trimethyl	128	$C_8H_{16}O$	–
06	Phytol	296	$C_{20}H_{40}O$	–
07	Cyclopentanone, 2 (2-octenyl)	194	$C_{13}H_{22}O$	–
08	3, 7,11, 15 – tetramethyl -2 hexadecen -1-ol	296	$C_{20}H_{40}O$	–
09	Heptacosane, 1-chloro –SS	414	$C_{27}H_{55}Cl$	√
10	Tritetra contanne	604	$C_{43}H_{88}O$	–
11.	Phytol	296	$C_{20}H_{40}O$	–
12	11-oxa-dispiro(4.0.4. 1) udecan-1-ol	168	$C_{10}H_{16}O_2$	–
13.	3,7,11,15-tretramethyl-1-hexadecen-1-ol	296	$C_{20}H_{40}O$	–
14.	chdestan-3-ol,2-methylone (3.beta.5alpha)	400	$C_{28}H_{48}O$	–



Phytol



2-pentadecanone, 6, 10, 14-trimethyl

3.4 Literature review on spinach

3.5 Pharmacological action

Table : pharmacological activity of *Spinacea oleracea* L

Plant part	extracts	Pharmacological activities	references
	50% methanolic extract	Protective effect against radiation induced oxidative stress	Verma and Bhatia, 2003
Leaves	Water and 20,000g supernatant(acetone)	Antioxidant activity	Grossman, 2001
	Alcoholic extract	Hepatoprotective activity	Gupta and Singh, 2006
	Fresh juice and methanolic extract	Anthelmintic activity	Dave et al., 2009

This species has other pharmacological activities like Sulphite Oxidase Activity, inhibition of clastogenisity, anticancer activity, and CNS depressant activity.

Four kinds of assays (i) cell growth assay, (ii) colony forming assay, (iii) MTT colorimetric assay, and (iv) 3H-TdR incorporation assay, were used to study the effect of a fat-soluble extract of spinach powder (SPFE) on the proliferation of human gastric adenocarcinoma cell line (SGC-7901) in vitro.. The results indicated that SPFE inhibited the proliferation and colony forming ability of SGC-7901 cells. And in MTT assay, SPFE inhibited the viability of SGC-7901 cells, but no

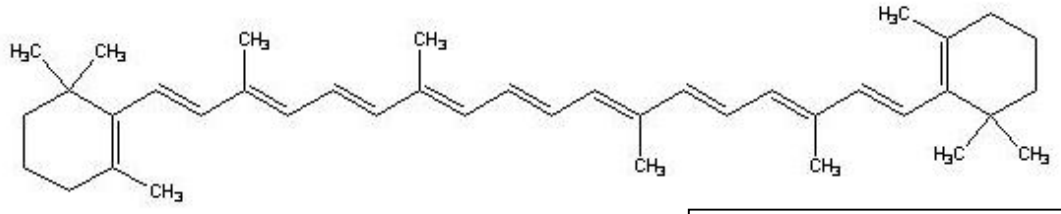
inhibitory effect of SPFE was observed on the viability of lymphocytes in peripheral blood of healthy people. Finally, in the 3H-TdR incorporation test, both SPFE and beta-carotene showed significant inhibitory effects on DNA synthesis in SGC-7901 cells, but SPFE was more effective than beta-carotene.

3.6 Phytochemical study

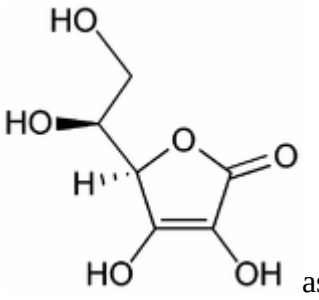
Phytochemical studies of *Spinacea oleracea* L give finding of following chemicals:

Table : chemicals found from *Spinacea oleracea* L.

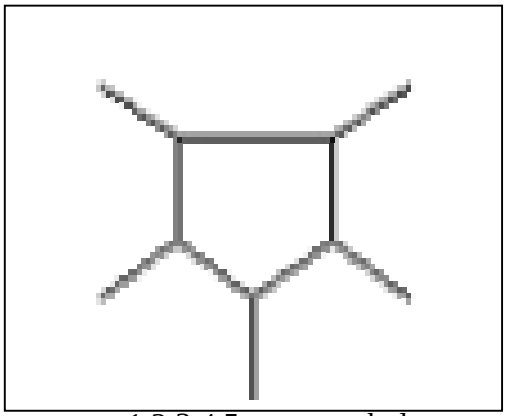
Plant part	chemicals
leaves	Carotene, lutein, ascorbic acid, flavonoids, p-coumaric acid



β-carotene



ascorbic acid

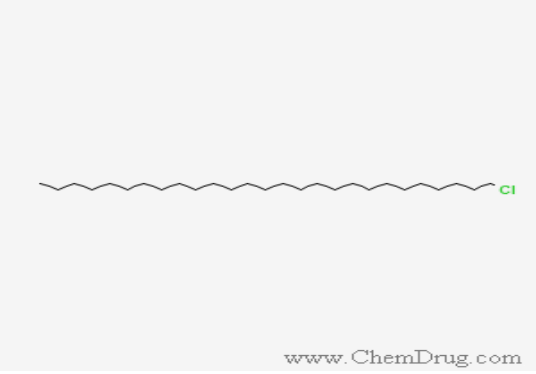


cyclopentane-1,2,3,4,5-pentamethyl

The organic compounds found in spinach sample are as follows:

Table : List of detected organic compounds with chlorinated pesticides in spinach (*Spinacea oleracea*) sample.

Peak	Name of Compound	Mol. Wt.	Mol. Fromula	Chlorinated Compound
01.	Cyclopentane-1,2,3,4,5- pentamethyl	140	$C_{10}H_{20}$	–
02.	1-chloro heptacosane	414	$C_{27}H_{55}Cl$	√
03.	Decane, 5- cyclohexyl	224	$C_{16}H_{32}$	–
04.	Tetratriacontane	478	$C_{34}H_{70}$	–
05.	Pentane,3-(2,2-dichloro-3-methylcyclopropyl	194	$C_9H_{16}Cl_2$	√
06.	1,3-cyclopentanediol, cis-SS	102	$C_5H_{10}O_2$	–
07.	2,3,5,6-Detetrahydrocyclohexanoe, 2,6-di-t-butyl-4-hydroxymethylene	234	$C_{15}H_{22}O_2$	–
08.	Decan, 1,1-oxybis –SS	298	$C_{20}H_{42}O$	–
09.	3,7,11,15-tetramethyl-2-hexadecen-1-ol	296	$C_{20}H_{42}O$	–
10.	1 – Octene 3,4 –dimethyl SS	140	$C_{10}H_{20}$	–
11.	Octadecane, 5-methyl	268	$C_{19}H_{40}O$	–
12.	9-Octadecenoic acid (z), hexyl ester	366	$C_{24}H_{46}O_2$	–
13.	Hexyl octyl ether –SS	214	$C_{14}H_{30}O$	–
14.	Nomanoyl chloride-SS	176	$C_9H_{17}ClO$	√



1-chloro heptacosane

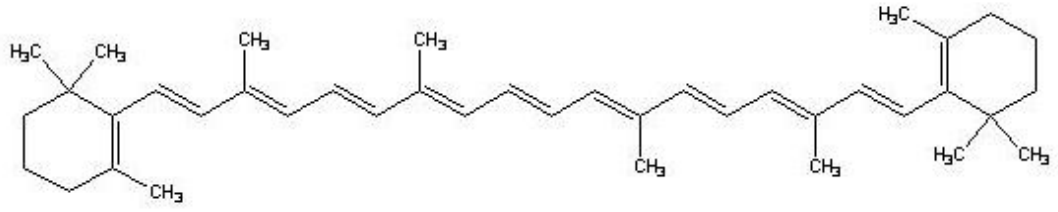
3.7 Literature review on water spinach

3.8 Pharmacological activities

In a study published on October 28th 2004 (ejournal sinica edu) "Antioxidant and antiproliferative activities of water spinach (*Ipomoea aquatica* Forsk) constituents," Huang et al conclude that the flavonoids and phenolic compounds in the plant "may have a significant effect on antioxidant and anticancer activities." Extracts from the stems of the plant were more potent than the leaves it was found. In other studies extracts from the plant decrease cholesterol and triglycoside in rats and are thought to have possible uses in the treatment of diabetes mellitus. However further studies need to be done on humans and more needs to be discovered about how the flavonoids and phenolic compounds work.

3.9 Phytochemical studies

The plant is rich in the minerals calcium and iron, as well as containing sodium, magnesium, phosphorous, manganese, copper and zinc. It also contains some of the B-complex vitamins, notably B2 (riboflavin) along with vitamins C and K. It contains flavonoids such as catechin and phenolic compounds. 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.



β-carotene

Chapter – 4

Statement of purpose

4. Statement of purpose (SOP)

The Present Study was designed to observe the antioxidant properties of the pure compounds isolated from the crude extracts of the plant *Amaranthus gangeticus*, *Spinacia oleracea L*, and *Ipomoea aquatica*. These three have traditional uses as diets and healers as well as scientifically discovered other activities.

Here determination of Antioxidant activity of crude extracts of *Amaranthus gangeticus, Spinacia oleracea L,* and *Ipomoea aquatic* is done using DPPH.

Chapter – 5

Equipments and method of testing

5.1 Equipments and reagents

Equipments

Pipette (5ml)

Analytical balance

UV- visible spectrophotometer

Beaker (100 & 200ml)

Test tube

Aluminum foil

Spatula

Micro-pipette

Reagents

1, 1-diphenyl-2-picrylhydrazyl

Distilled water

Distilled methanol

5.2 Antioxidant test

5.2.1 Antioxidants

Antioxidant compounds in food play an important role as a healthprotecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Plant sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. Some compounds, such as gallates, have strong antioxidant activity, while others, such as the mono-phenols are weak antioxidants.

The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease.

Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases.

5.2.2 In-Vitro Determination of Antioxidant Capacity

Antioxidant capacity can be measured by both in-Vivo and in- Vitro method. Here only the in-Vitro methods are discussed. The various conventional and latest methods comes under invitro are listed in table below. It is very difficult to select a suitable antioxidant assay method. Antioxidants act by several mechanisms and no

one assay can capture the different modes of action of antioxidant. (Badarinath, et al. 2010)

Here are the in-Vitro methods by which the antioxidant tests can be done.

a. Hydrogen Atom Transfer methods (HAT)

i. Oxygen radical absorbance capacity (ORAC) method
ii. Lipid peroxidation inhibition capacity (LPIC) assay
iii. Total radical trapping antioxidant parameter (TRAP)
iv. Inhibited oxygen uptake (IOC)
v. Crocin bleaching Nitric oxide radical inhibition activity
vi. Hydroxyl radical scavenging activity by p-NDA (p-butrisidunethyl aniline)
vii. Scavenging of H2O2 radicals
viii. ABTS radical scavenging method
ix. Scavenging of super oxide radical formation by alkaline (SASA)

b. Electron Transfer methods (ET)

i. Trolox equivalent antioxidant capacity (TEAC) decolourization
ii. Ferric reducing antioxidant power (FRAP)
iii. DPPH free radical scavenging assay
iv. Copper (II) reduction capacity
v. Total phenols by Folin-Ciocalteu
vi. N,N-dimethyl-p-Phenylenediamine (DMPD) assay

c. Other Assays

i. Total oxidant scavenging capacity (TOSC)
ii. Inhibition of Briggs – Rauscher oscillation reaction
iii. Chemiluminescence

iv. Electrochemiluminescence
v. Fluorometric Analysis
vi. Enhanced chemiluminescence (ECL)
vii. TLC bioautography
viii. Cellular antioxidant activity (CAA) assay
ix. Dye-substrate oxidation method (Badarinath, et al. 2010)

Among these methods the most most widely methods are described below.

5.2.3 Ferric-Raducing Antioxidant Power (FRAP) assay

In order to assess the modifying effect of tea flavonoids on plasma antioxidant status, a variety of methods has been employed. Commonly used is the FRAP assay. This is a colorimetric assay that measures the ability of plasma to reduce the intense blue ferric tripyridyltriazine complex to its ferrous form, thereby changing its absorbance. (Benzie & Strain, 1999)

5.2.4 Total Radical Trapping Antioxidant Parameter (TRAP)

Another assay which has been applied in human plasma is the total radical trapping antioxidant parameter (TRAP). In this assay, the rate of peroxidation induced byAAPH(2'-azobis(2-amidinopropane) hydrochloride) is monitored through the loss of fluorescence of the protein R-phycoerythrin (R-PE). In the TRAP assay the lag-phase induced by plasma is compared with that induced by Trolox in the same plasma sample. (Huang, Ou & Prior,2005)

5.2.5 Trolox Equivalent Antioxidant Capacity (TEAC)

This assay is based on the ability of molecules to scavenge the stable free radical of 2,2'- azinobis (3-ethylbenzothiozoline-6-sulfonic acid) in comparison with Trolox, a water soluble analogue of vitamin E. The activity of a compound is

therefore expressed as TEAC. Of these assay, the ECL seems the least suitable to determine plasma antioxidant capacity because it relies on enzymatic activity. This technique has not been widely applied, which limits the possibility to compare results from different studies. All the other assays have been applied in plasma reproducibility.

5.2.6 ABTS {2,2' – azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid)}

Miller et al (1993) described another technique for TAC measurement based on colorimetry. This assay is based on the principle that when 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) {ABTS} is incubated with a peroxidase (such as metmyoglobin and H2O2, a relatively stable radical cation, ABTS+ , is formed (see equation below).

The formation of ABTS+ on Interaction with Ferryl Myoglobin produces a relatively stable blue-green color, Measured at 600nm. Antioxidants in the fluid sample suppress this color production to a degree that is proportional to their concentrations. In this equation, HX-Fe III= metmyoglobin, X – [Fe IV = 0] = ferrylmyoglobin, ABTS = 2,2'– azino-di-[3-ethyl-bensthiazoline sulphonate]. (Miller & Rice-Evans, 1993)

5.2.7 Oxygen Radical Absorbing Capacity (ORAC) Assay

Basically the same principle is applied as in the TRAP assay. The ORAC assay is another commonly applied antioxidant assay based on the ability of a test substance to inhibit the oxidation of B-phycoerythrin by reactive oxygen species, relative to Trolox. Proteins interfere with the analysis, partially protecting R-PE when all plasma antioxidants are exhausted. Determination of the lag-phase TRAP and ORAC assays can be performed with different radicals and thus different results will be obtained depending on the radical. For these reasons, results

obtained with the TRAP or the ORAC assay in plasma has to be interpreted with care. (Caldwell, 2001) The advantage of the AUC approach is that it implies equally well for both antioxidants that exhibit distinct lag phase and those that have no lag phases. ORAC assay has been broadly applied in academy and in the food and dietary supplement industries as a method of choice to quantify AOC. (Caldwell, 2001) ORAC is limited to measurement of hydrophilic chain but ignores lipophilic antioxidants. It requires fluorometers, which may not be routinely available in analytical laboratories. Temperature control decreases reproducibility.

5.2.8 DPPH Free Radical Scavenging Assay

A simple method that has been developed to determine the antioxidant activity of foods utilizes the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The structure of DPPH and its reduction by an antioxidant are shown above. The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. The color turns from purple to yellow as the molar absorptivity of the DPPH radical at 517 nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolorization is stoichiometric with respect to number of electrons captured.

Antioxidant compounds may be water-soluble lipid-soluble, insoluble, or bound to cell walls. Hence, extraction effciency is an important factor in quantifcation of antioxidant activity of foods. Trolox (as the reference standard) and the sample are reacted with DPPH solution in methanol/water for four hours at 35°C in a vessel mounted on a rotary shaker and the absorbance changes are measured at 517 nm. The quantity of sample necessary to react with one half of the DPPH is expressed

in terms of the relative amount of Trolox reacted. Antioxidant activity of a sample is expressed in terms of micromole equivalents of Trolox (TE) per 100 grams of sample, or simply Trolox units per 100 gm or TE/100g. (Prakash, Rigelhof & Miller)

5.2.9 Photochemiluminescence Method (PCL)

In the PCL assay (photochemiluminescence) the photo-chemical generation of free radicals is combined with the sensitive detection by using chemiluminescence. The PCL is based on the photo-induced autoxidation inhibition of luminol by antioxidants, mediated from the radical anion superoxide (Of) and is suitable to measure the radical scavenging properties of single antioxidants as well as more complex systems in the nanomolare range. Luminol works as photosensitiser as well as oxygen radical detection reagent. The antioxidant potential is measured by means of the lag phase at diiïerent concentrations, calculated by a TroloxY calibration curve and expressed as mmol equivalents in antioxidant activity of a reference compound (i.e. Trolox). The PCL rnethod was carried out with the procedure described by Popov and Lewin (Popov& Lewin, 1999) and can be conducted by two difîerent protocols ACW and ACL that consent to measure the antioxidant capacity of the water- and lipid-soluble components respectively. In the water soluble fraction antioxidants such are ñavonoids, ascorbic acid, aminoacids, etc. are detected, while in the lipid soluble fraction tocopherols, tocotrienols, carotenoids, etc. are measured. The most widely used methods for measuring antioxidant activity involve the generation of radical species and the presence of antioxidants determining the dis-appearance of these radicals. Most of the assays determine the antioxidant activity in the micromolar range needing minutes or hours. The PCL assay, which is easy and rapid to perform, and although less reliable with lipohilic substrates, presents numerous advantages: it does not

requires high temperatures to generate radicals and it is more sensitive to measure, in few minutes, the scavenging activity of antioxidants against the superoxide radical which is one of the most dangerous reactive oxygen species (ROS) also occurring in human body (Sch1esier, Harwat, Bohm, Bitsch, 2002).

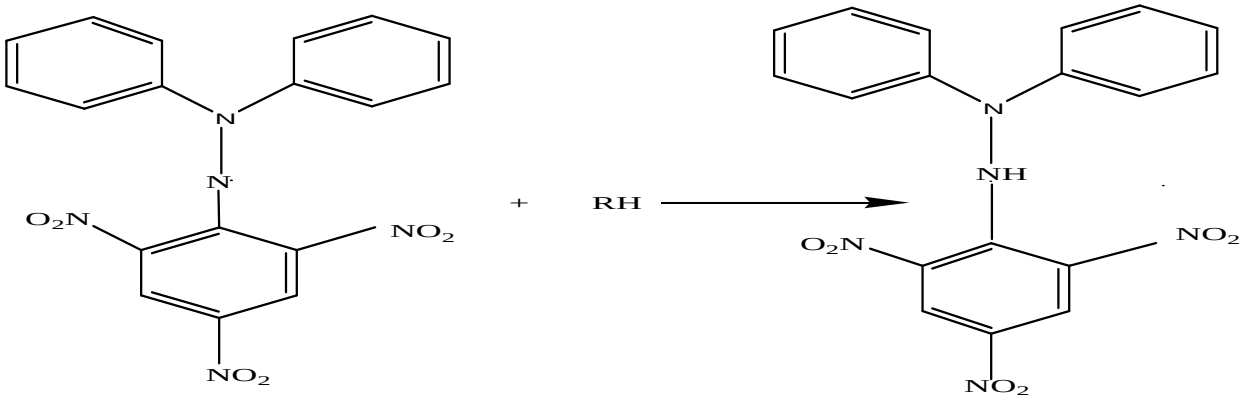
The methods presented can be divided into two groups depending on the oxidising reagent. Five methods use organic radical producers (TEAC I-III, TRAP, DPPH, DMPD, PCL) and one method works with metal ions for oxidation (FRAP). Another difference between these tests is the reaction procedure.

5.3 Methodology

Determination of DPPH radical scavenging assay (Quantitative analysis)

5.3.1 Principle

A rapid, simple and convenient method to measure free radical scavenging capacity of antioxidants involves the use of the free radical, 1, 1-Diphenyl-2-picrylhydrazyl (DPPH). DPPH is a stable nitrogen centered free radical with purple color and the odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm. When the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H, then the color turns from purple to yellow as the molar absorptivity of the DPPH radical reduces from 9660 to 1640 at 517 nm. Scavenging of DPPH free radicals by antioxidants decreases the absorbance. The lower the absorbance at 517 nm, the greater the free radical scavenging capacity of the crude extracts.



*DPPH (Oxidized form)

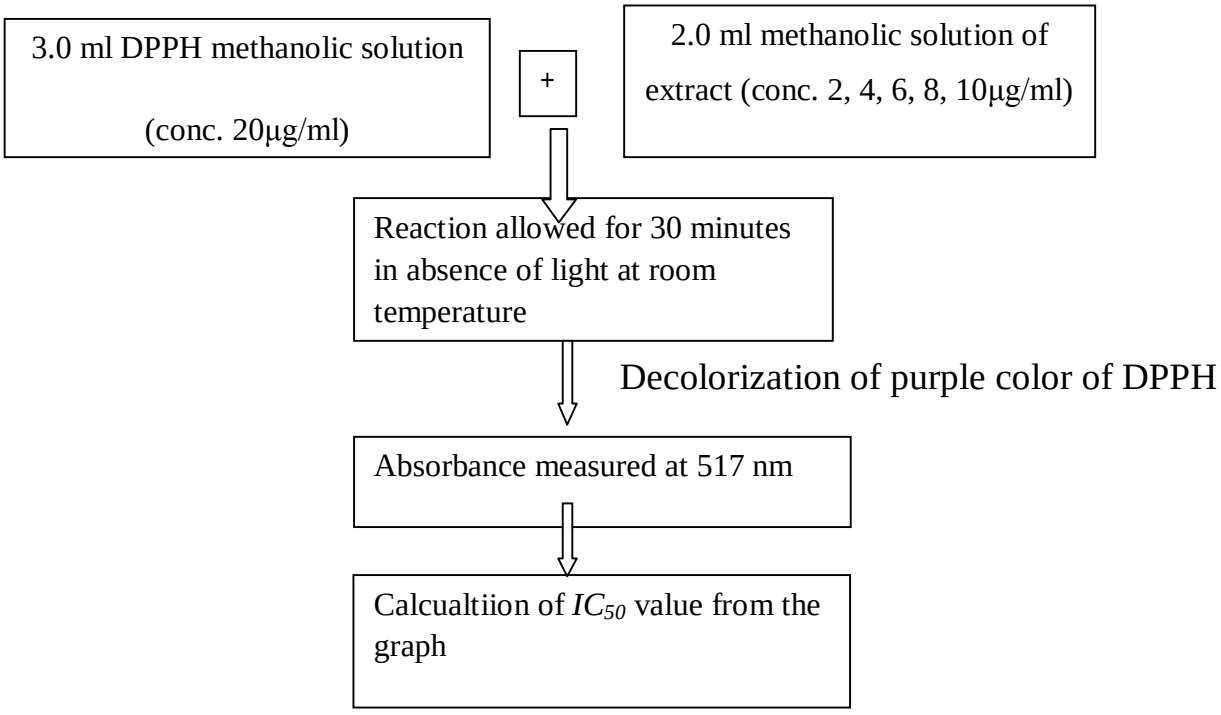
DPPH (Reduced form)

5.3.2 Procedure

- 2.0 ml of a methanol solution of the extract at different concentration (400, 200, 100, 50, 25, etc. μg/ml) were mixed with 3.0 ml of a DPPH methanol solution (20 μg/ml).
- After 30 min reaction period at room temperature in dark place the absorbance was measured against at 517 nm against methanol as blank by using a UV- visible spetrophotometer.
- Inhibition free radical DPPH in percent (I%) was calculated as follows:

$$(I\%) = (1 - A_{sample}/A_{blank}) \text{ X } 100$$

Here A_{blank} is the absorbance of the control reaction (containing all reagents except the test material). Schematic representation of the method of assaying free radical scavenging activity is shown here:



Chapter – 6

Calculation

6.1 DPPH radical scavenging assay (quantitative analysis)

Concentration (µg/mL)	Aborbance		
	Amaranthus gangeticus	*Spinacia oleracea* L	*Ipomoea aquatica*
400	0.585	0.219	0.378
200	0.273	0.140	0.218
100	0.149	0.150	0.103
50	0.132	0.195	0.067
25	0.094	0.194	0.077
12.5	0.197	0.196	0.142
6.25	0.282	0.190	0.191
3.12	0.319	0.168	0.223
1.56	0.356	0.178	0.231
0.78	0.358	0.188	0.233

Absorbance of the blank was 0.419.

6.2 IC50 calculation for *Amaranthus gangeticus*

Serial No.	A_{Blank}	Concentration (µg/ml)	A_{Sample}	% inhibition of free radical DPPH = (1 – A_{Sample}/A_{Blank}) X 100	IC_{50} µg/ml
1		0.78	0.358	54.15	
2		1.56	0.356	77.25	
3		3.12	0.319	89.84	
4		6.25	0.282	95.50	
5	0.419	12.5	0.197	98.42	
6		25	0.094	99.62	
7		50	0.132	99.74	
8		100	0.149	99.85	
9		200	0.273	99.86	
10		400	0.585	99.86	

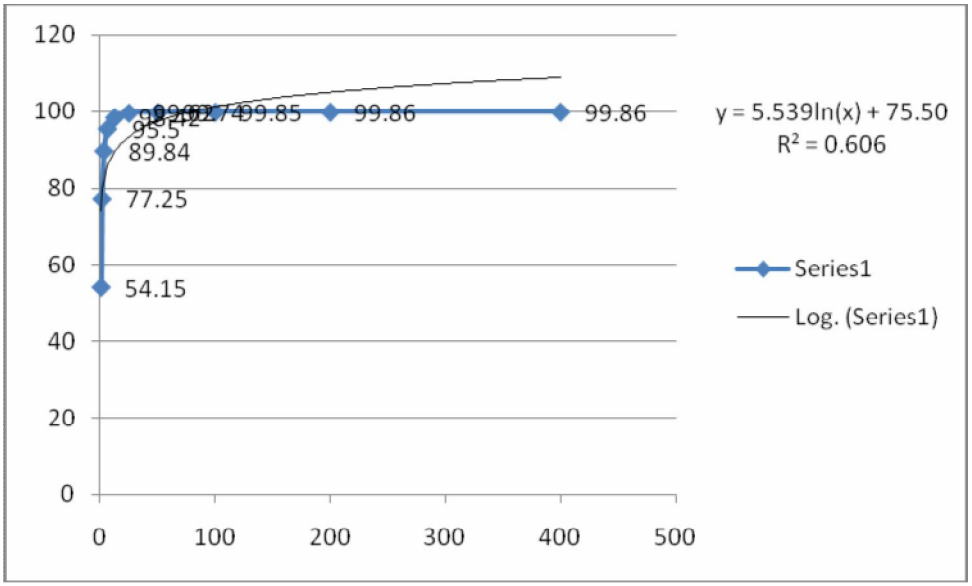


Figure : DPPH scavenging potential and IC50 value of methanolic extract of *Amaranthus gangeticus*

6.3 IC50 calculation for *Spinacia oleracea* L

Serial No.	A_{Blank}	Concentration (µg/ml)	A_{Sample}	% inhibition of free radical DPPH = (1 – A_{Sample}/A_{Blank}) X 100	IC_{50} µg/ml
1		0.78	0.188	75.90	
2		1.56	0.178	88.60	
3		3.12	0.168	94.62	
4		6.25	0.190	96.96	
5	0.419	12.5	0.196	98.43	

6		25	0.194	99.23	
7		50	0.195	99.60	
8		100	0.150	99.85	
9		200	0.140	99.93	
10		400	0.219	99.96	

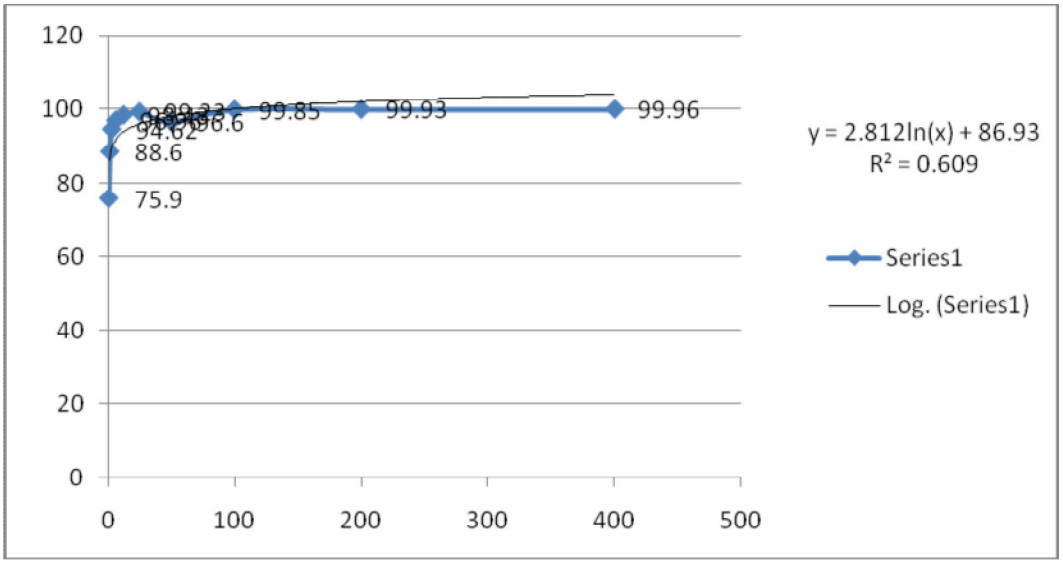


Figure : DPPH scavenging potential and IC50 value of methanolic extract of *Spinacia oleracea* L

6.4 IC50 calculation for *Ipomoea aquatica*

Serial No.	A_{Blank}	Concentration (µg/ml)	A_{Sample}	% inhibition of free radical DPPH = (1 – A_{Sample}/A_{Blank}) X 100	IC_{50} µg/ml

1		1.56	0.416	0.743	
2		3.12	0.381	9.127	
3		6.25	0.337	21.576	
4	0.419	12.5	0.278	33.746	50.149
5		25	0.146	65.179	
6		50	0.103	75.327	
7		100	0.120	71.369	
8		200	0.133	68.365	
9		400	0.041	90.124	

The graph plotted by using these values of concentration versus % inhibition of free radical is given below:

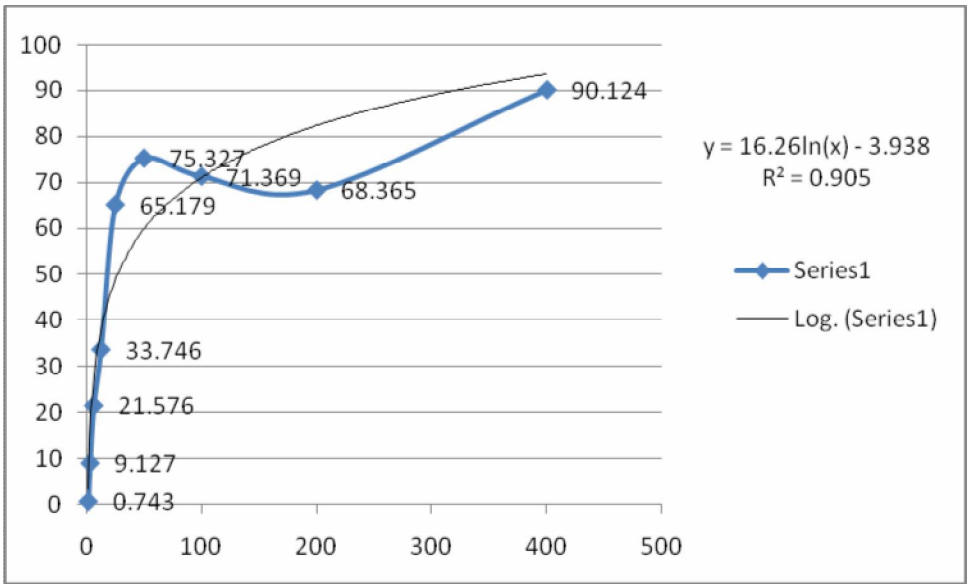


Figure : DPPH scavenging potential and IC50 value of methanolic extract of *Ipomoea aquatic*

Chapter – 7

Result and discussion

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