ANALYSIS OF DIFFERENT BIOACTIVE COMPOUNDS IN WATER CRESS (*Enhydra fluctuans* Lour) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A Thesis

By

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Declaration

ANALYSIS OF DIFFERENT BIOACTIVE COMPOUNDS IN WATER CRESS (*Enhydra fluctuans* Lour) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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

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April, 2014

This work is dedicated to my Beloved PARENTS. Who sacrificed for me, inspired and blessed me in every ways

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ABSTRACT

Enhydra fluctuans Lour (Asteraceae) is an aquatic plant species common in South Asia. It is a medicinal plant used for many years as a remedy in the traditional medicine. The leaves of this floating marsh herb were used in folk medicine for their laxative activities and for herpetic treatment. The current study was conducted to evaluate the antioxidant and antimicrobial activities and to analysis different bioactive compounds as well as phytochemical constituents from the ethanol extract of *Enhydra fluctuans*. The yield of ethanol extract was found 3.3% w/w. Plant extract was subjected to preliminary phytochemical screening which revealed the presence of alkaloid, flavonoids, tannins, glycosides, steroids and saponin. In-vitro antibacterial activity was evaluated using disc diffusion method. The extract showed no antibacterial activity against three strains of *E. coli* in comparison of kanamycin. Following qualitative indication of the presence of antioxidant component(s), 4 ploy phenolic compounds were identified from crude extract by HPLC analysis while 5 and 2 polyphenolic compounds were identified from aqueous ethanol and n-Hexane fractions respectively. It is the first time when widely recognized bioactive polyphenolic compounds are detected in *E. fluctuans* extract. The findings of this study provide evidence that the extract of *E. fluctuans* possess significant antioxidative bioactive compounds, suggesting that this plant is a potential source of natural products with chemo-preventive and chemotherapeutic activities.

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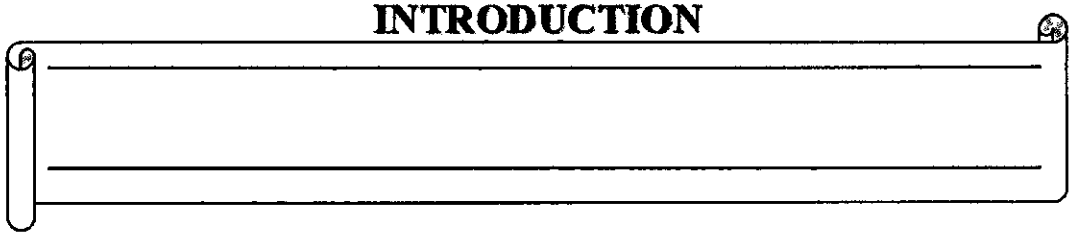
CHAPTER 1

INTRODUCTION



CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Living cells may generate free radicals and other reactive oxygen species by-products as a results of physiological and biochemical processes. Free radicals can cause oxidative damage to lipids, proteins and DNA, eventually leading to many chronic diseases, such as cancer, diabetes, aging, and other degenerative diseases in humans (Harman, 1998). Plants are endowed with free radical scavenging molecules, such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other metabolites, which are rich in antioxidant activity (Zheng and Wang 2001; Cai *et al.,* 2003).

Studies have shown that many of these antioxidant compounds possess anti-inflammatory, anti-atherosclerotic, antitumor, anti-mutagenic, anti-carcinogenic, antibacterial, and antiviral activities (Sala *et al.,* 2002; Rice-Evans *et al.,* 1995). The ingestion of natural antioxidants has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing, (Ashokkumar *et al.,* 2008; Veerapur *et al.,* 2009) and in recent years, there has been a worldwide trend towards the use of the natural phytochemicals present in berry crops, teas, herbs, oilseeds, beans, fruits and vegetables (Kitts *et al.,* 2000; Muselík *et al.,* 2007; Wang and Jiao, 2000).

Nature has been a supply of medicative agents for thousands of years and a formidable variety of recent medications were isolated from natural sources, several of that supported their use in ancient medication. In sight of this, attention of this study has been targeted significantly to *Enhydra fluctuans* Lour. (Family: Asteraceae), English name 'Water Cress' edible semi aquatic nonwoody vegetable plant with separate leaves. It is native to India, Bangladesh, Burma, Srilanka and a number of other places in South East Asia. This plant may be aprostate, spreading, and annual herb. (Ramjan *et al.,* 2013). It possesses biological value and its fuel extract has been reportable to own analgesic, cytotoxic, antimicrobial, hepatoprotective, hypotensive, CNS depressant, antidiarrheal activity

(Swain *et al.*, 2012; Amin *et al.*, 2012; Sannigrahi *et al.*, 2010; Roy *et al.*, 2011; Uddin *et al.*, 2011; Bhakta *et al.*, 2009).

Nowadays, the consumers' demand of new functional foods including those enriched with vitamins and biologically active compounds is clearly rising. The analysis of such bio active compounds by conventional methods usually involves tedious and time-consuming procedures, since it requires the use of numerous analytical and sample preparation protocols. Biologically active compounds and vitamins enriched food and vegetables are getting popular over the last five decades. Vitamins are a structurally heterogeneous group of essential food constituents that are required in small amounts for normal growth and maintenance of human health. They are generally divided in two main groups: lipid- and water-soluble vitamins (Klejdus *et al.*, 2004). Until recently, this classification has been used to determine the type of analytical method applied, being the most common approach the analysis of each vitamin individually by using HPLC or even a multiple water- or fat-soluble vitamins determination (Paixao *et al.*, 2003; Breithaupt and Kraut, 2006).

Even considering the enormous interest of vitamins as micronutrients in food, there exist other compounds with known biological activity that can, at the end, have a similar or even greater contribution in the functional activity of a food and/or vegetable (Kledjus *et al.*, 2004). 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. These food components have the common property of being labile and so degraded by light, heat or oxygen, also they are extremely complex, with dozens of very similar components or isomers which makes its separation extremely difficult. The basic feature of all polyphenols is the presence of one or more hydroxylated aromatic rings, which seemed to be, in fact, responsible for their properties as radical scavengers (Lule and Xia, 2005; Fukumoto and Mazza, 2000; Jaime *et al.*, 2005). Some analytical strategies have been reported (Escarpa and González, 2000) for the determination of polyphenols. Their presence may interfere in the analysis of vitamins, so usually a cleanup step is needed, namely solid-phase extraction, liquid extraction, etc. In spite of this difficulty, some analytical methods based on HPLC have already been developed to quantify some

phenolic compounds along with some vitamins (Tasioula-Margari and Okogeri, 2001; Blasco *et al.,* 2005). The goal of this work is to develop an HPLC method for the simultaneous detection and quantification of the bioactive compounds 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. 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 aquatic foods.

1.2 Objectives of the Study

Bangladesh has highly resourceful aquatic plants in fresh water body. These aquatic plants are a good source of antioxidant. Herbivore fishes may have taken antioxidant from aquatic plants. These antioxidants increase the immunity power of the fish body. The prime interest of the current study was screening the bioactive compounds responsible for antioxidant, phytochemical and antibacterial activities from *Enhydra fluctuans.* Although numerous studies in the literature exist with respect to plant-derived constituents but any report on bioactive compounds of water cress (*E. fluctuans)* has not been published yet. HPLC is a major tool for separation of individual bioactive compounds of the plant extract. Specific objectives of this study are:-

- Identification of different chemical groups present in this plant extract.
- To develop a HPLC method for the identification and analysis of bioactive compounds of *Enhydra fluctuans* Lour.
- Qualitative antioxidant assay and TLC analysis of different fractions of plant extract.
- Antimicrobial activity test.

1.3 Preview of the plant

Figure 1.1: *Enhydra fluctuans* whole plant with flower

Enhydra fluctuans Lour. (Family: Asteraceae) is a small genus of marsh herb, distributed in tropical and subtropical regions. The plant is a prostate, spreading, annual herb. This is also an edible semi aquatic herbaceous vegetable plant with serrate leaves. The leaves which are slightly bitter are used to treat inflammation, skin diseases, and small pox (Kirtikar and Basu, 2002). The stems are somewhat fleshy, 30 centimeters or more in length, branched, rooting at the lower nodes, and somewhat hairy. The leaves are stalk less, linear-oblong, 3 to 5 centimeters in length, pointed or blunt at the tip, usually truncate at the base, and somewhat toothed at the margins. The flowering heads are without stalks, are borne singly in the axils of the leaves, and excluding the bracts, are less than 1 centimeter in diameter. The outer pair of the involucres bracts is ovate and 1 to 1.2 centimeters long; the inner pair is somewhat smaller. The flowers are white or greenish-white. The acheness is enclosed by rigid receptacle-scales (Kirtikar and Basu, 1999).

Natural habitat

Enhydra fluctuans is a tropical herb, more sensitive to cold especially when very young. The species grows in and along ditches, water courses, margins of fish ponds and rice fields in the open, from sea-level up to 1,800 m. It is able to reproduce by fragmentation and may be so abundant that it clogs water courses (Ali *et al.,* 2013).

Characteristics

Climate: Tropical

Habitat: Hydrophytic

Habit: Herb

Flower colour: Beige, white (Dokosi, 1998).

Classification (Ali *et al.*, 2013)

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Asterales

Family: Asteraceae

Genus: Enhydra

Specific Epithet: fluctuans Lour

Species: *Enhydra fluctuans* Loureiro

Common names

Commonly known as Helencha (Bangla), Enhydra (English), Kankong-kalabau (Tagalog), Buffalo spinach (English), Marsh herb (English), Water cress (English), Zhao ju (Chinese). (Gupta, 2012).

Synonym/s

Enydra anagallis Gardner, *Meyera fluctuans* (Lour.) Spreng (Gupta, 2012).

Geographic distribution

This species is an old world species, possibly of Indochinese origin, which occurs in tropical Asia and Africa. It is common to all countries of Southeast Asia (Ali *et al.*, 2013).

Native

Bangladesh; Benin; Burkina Faso; Cambodia; Cameroon; Congo, The Democratic Republic of the; Côte d'Ivoire; Ghana; India; Indonesia; Kenya; Lao People's Democratic Republic; Malaysia; Mozambique; Myanmar; Nigeria; Philippines; Rwanda; Senegal; South Africa; Sri Lanka; Sudan; Tanzania, United Republic of; Thailand; Togo; Uganda; Viet Nam; Zambia; Zimbabwe (Gupta, 2012).

Traditional Uses

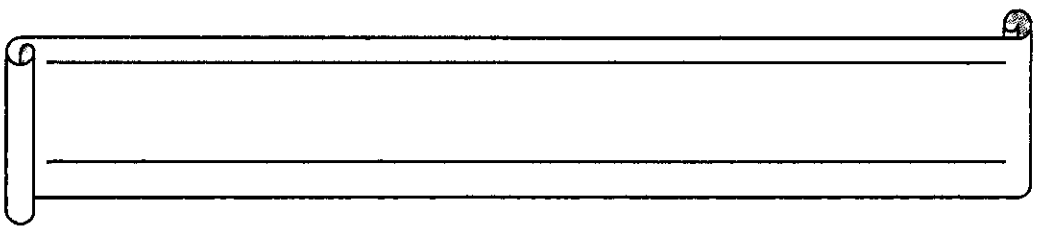
Plant is used in ascites, dropsy and anasarca. It is cooked with fish curry and taken to revive appetite after long weakness due to fever or typhoid. Leaves are laxative and antibilious; cure inflammation, leucoderma, bronchitis and biliousness; useful in skin and nervous affections; also useful in tropidity of the liver. Leaf paste is applied over head as a cooling agent and around the inflamed Brest to reduce inflammation (Yusuf *et al.*, 2009).

Chemical constituents

Plant is rich in protein and is a good source of β-carotene. It also contains saponins, myricyl alcohol, kaurol, cholesterol, sitosterol, glucoside, sesquiterpene lactones including germacranolide, enhydrin, fluctuanin and fluctuandin, a number of diterpenoid acids and their isovalerate and angelate derivatives, stigmasterol, cholesterol, sitosterol, glucoside, other steroids and gibberellins A9 and A13 have been isolated from this plant (Ghani, 2003).

CHAPTER 2

REVIEW OF LITERATURE



CHAPTER TWO

LITERATURE REVIEW

2.1 Based on chemical constituents

According to Ghosh *et al.* (2013), chemical, chromatographic, ESI-TOF-MS, and NMR analyses of the water extracted carbohydrate polymer (CP) of *E. fluctuans* leaves showed the presence of a 24 kDa arabinogalactan having a (1,3)-linked β-d-Gal*p* main chain, substituted at *O*-6 by (1,6)-linked β-d-Gal*p* side chains. The latter residues were substituted at *O*-3 by (1, 3)-, (1, 5)-, and (1, 3, 5)-linked α-l-Ara*f* chains, and non-reducing end-units of α-l-Ara*f* and β-d-Gal*p*. This polymer contained esterified phenolic acids. Biochemical analysis revealed similarity in antioxidative potential between the identified carbohydrate polymer and known standard antioxidants. The highly branched side chains and the phenolic acid residues of the arabinogalactan might be the functional sites. Fluorimetric and ultraviolet spectrometric analyses showed that the studied carbohydrate polymer can form complex with bovine serum albumin having binding constant $K = 2.42 \times 10^6$/M and changes its microenvironment. Thus, traditional aqueous extraction method provides a carbohydrate polymer, which stimulates a fair biological response: this could represent an interesting approach in phyto-therapeutic treatments.

Yadava and Singh (2007) identified a novel bioactive isoflavone glycoside 4',5,6,7-tetrahydroxy-8-methoxyisoflavone-7-O-beta-D-galactopyranosyl-(1 -- >3)-O-beta-D xylopyranosyl-(1 --> 4)-O-alpha-l-rhamnopyranoside from the methanolic extract of the leaves of *Enhydra fluctuans* Lour. Its structure was elucidated by various spectral analysis and chemical degradations. This compound exhibited anti-microbial activity against various bacteria and fungi.

Akter *et al.* (2012) studied on several medicinal plants like *Curcuma longa, Curcuma zedoaria, Streblus asper, Enhydra fluctunas* and *Scoparia dulcis* which are commonly available in Bangladesh. This study investigated the effect of ethanol extracts from these five medicinal plants on the viability of Brine Shrimp larvae. The lethal concentrations

(LC_{50} values) of the extracts were determined. LC_{50} values of *E. fluctunas* were greater than 250 µg/ml. Results indicate that *E. fluctunas* could be potential sources of bio-active compounds.

Krishnaswamy and Ramji (1995) reported that the extract of the leaves of *Enhydra fluctuans* gave, in addition to three known sesquiterpene lactones, two new chlorine containing melampolides. The structure and stereochemistry of the new compounds were elucidated by NMR techniques and chemical reactions.

Bohlmann *et al.* (1982) reported that the investigation of the aerial parts of a variety of *Enhydra fluctuans* afforded in addition to 4-hydroxyfarnesyl acetate and fluctuadin five new melampolides elucidated by high field H-NMR spectroscopy.

Muselli *et al.* (2000) studied on the chemical composition of *Enhydra fluctuans* essential oil from Vietnam and it was investigated by GC/retention indices and carbon-13 NMR spectroscopy. Myrcene, limonene, *trans*- and *cis*-dihydroperillaldehydes were the major components. This is the first report of the two isomers in nature.

According to Ali *et al.* (1972) the structures of enhydrin (2) and the co-occurring sesquiterpene lactones, viz. fluctuanin (3) and fluctuadin (4) isolated from *Enhydra fluctuans* Lour. have been established. The stereochemistry of enhydrin has been suggested from NMR data. Selective hydrolysis of the acetate and the glycidate group in 2 yielded the alcohols 11 and 16 indicating acyl migration during their formation. Acetylation of 11 led further to acetolysis of the glycidate ester function to afford the diacetate 17. The location of the two acyl groups in 2 (and also in 3 and 4) still remain to be settled.

Dewanji *et al.* (1993) investigated about the chemical composition of two semi-aquatic plants for food use. The seasonal variation in the nutrient composition of *Enhydra fluctuans* and *Marsilea quadrifolia*, two edible semi-aquatic plants, was studied in order to promote their consumption as green leafy vegetables. Both plants had high crude protein

content throughout all harvesting seasons. *Enhydra fluctuans* had low ash content and was a good source of beta-carotene (3.7 to 4.2 mg/100 g on a fresh weight basis). *Marsilea quadrifolia* exhibited wide fluctuations between seasons and was not very promising in nutrient composition when compared to other commonly used green leafy vegetables.

2.2 Based on biological activity

Sannigrahi *et al.* (2011) reported that the Flavonoids of *Enhydra fluctuans* exhibits analgesic and anti-inflammatory activity in different animal models. Total flavonoids of *E. fluctuans* (TFEF) were screened for analgesic and anti-inflammatory activity. Analgesic activity was studied in acetic acid induced writhing response and by hot plate method in Swiss albino mice. Anti-inflammatory activity was estimated by carrageenan and histamine induced acute inflammation and Freund's complete adjuvant (FCA) induced chronic inflammation in rats. Two flavonoids, baicalein 7-O-glucoside and baicalein 7-O-diglucoside, were isolated from the ethyl acetate fraction. Oral administration of TFEF at the doses of 200 and 400 mg/kg provide 27.05 and 55.49% protection respectively in acetic acid induced writhing method. It also increased the pain threshold in mice evidenced by hot plate method. TFEF showed more potent anti-inflammatory activity. The results of this study may be attributed to high free radical scavenging and antioxidant potential of the flavonoids present in ethyl acetate fraction of *Enhydra fluctuans*.

The Ethanol (EEF), Chloroform (CEF) and Pet-Ether (PEEF) extracts of *Enhydra fluctuans* were evaluated for reducing power, total phenolic content, the DPPH scavenging activity, NO scavenging activity and super oxide scavenging activity by Swain *et al.* (2012). In all the tests the ethanol extract was found to have higher antioxidant activity.

According to Uddin *et al.* (2005), the methanolic and aqueous extracts of *E. fluctuans* was evaluated in experimental diarrhoea, induced by castor oil in mice. Both methanolic and aqueous extracts, given orally at the dose of 250 mg kg^{-1} body weight, showed significant anti-diarrhoeal activity as compared to that of the control. Both the extracts at the above dose levels also significantly reduced the intestinal transit of charcoal meal in mice. The in vitro antimicrobial activity of the extracts showed variable result. The methanolic extract

moderately inhibited the growth of *Shigella dysenteriae, Shigella boydii* and *Shigella flexneri*, while the aqueous extract inhibited the growth of *Staphylococcus aureus, S. dysenteriae* and *S. boydii*. But both the methanolic and aqueous extracts did not show any significant effect on *Escherichia coli* and *Pseudomonas aerugenosa*. All the results indicated that the extract of *E. fluctuans* possesses anti-diarrhoeal activity.

Sannigrahi *et al.* (2010) evaluated the hepatoprotective potential of *Enhydra fluctuans* against carbon-tetrachloride-induced oxidative damage in rats. Results showed the flavonoid rich ethyl acetate fraction to have significant hepatoprotective activity, probably due to the extract's ability to inhibit lipid peroxidation and increase the anti-oxidant enzymatic activity.

Roy *et al.* (2011) studied Neuropharmacological effects of three fractions of aerial parts of *Enhydra fluctuans* using mice models. Results showed significant spontaneous motility depressant, sedative, anticonvulsant and anti-stress activity.

Rahman *et al.* (2002) evaluated the analgesic activity of *Enhydra fluctuans* that showed promising results in both acetic acidinduced writhing and the tail-flick methods.

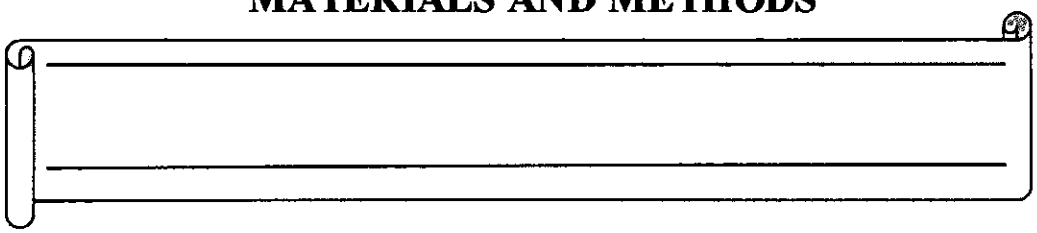
Bhakta *et al.* (2009) evaluated Antimicrobial efficacies of methanol extract of *Asteracantha longifolia, Ipomoea aquatica* and *Enhydra fluctuans* against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Micrococcus luteus*. Study results showed extract variations, but the methanol extract of *A. longifolia, I. aquatica* and *Enhydra fluctuans* showed antimicrobial activity against pathogenic bacteria - *S. aureus, P. aeruginosa, E coli* and *M. luteus*.

Amin *et al.* (2012) studied on Antimicrobial and Cytotoxic Activity of Three Bitter Plants - *Enhydra fluctuans, Andrographis Peniculata* and *Clerodendrum Viscosum*. In this study, three important medicinal plants (*Enhydra fluctuans* Lour, *Clerodendrum viscosum* Vent and *Andrographis peniculata* Wall) of Bangladesh were investigated to analyze their antimicrobial and cytotoxic activities against some pathogenic microorganisms and

Artemia salina (brine shrimp nauplii). The coarse powder material of leaves of each plant was extracted separately with methanol and acetone to yield methanol extracts of leaves of *Enhydra fluctuans* (MLE), *Clerodendrum viscosum* (MLC) and *Andrographis peniculata* (MLA), and acetone extracts of leaves of *Enhydra fluctuans* (ALE), *Clerodendrum viscosum* (ALC) and *Andrographis peniculata* (ALA). The disc diffusion method and the method described by Meyer were used to determine the antimicrobial and cytotoxic activities of each plant extract. Among the test samples, methanol extract of leaves of *Enhydra fluctuans* and acetone extract of leaves of *Andrographis peniculata* showed comparatively better antimicrobial activity against a number of bacteria and fungi with inhibition zones in the range of 06-15 mm and according to the intensity of activity, the efficacy against microorganisms were found in the order of *Enhydra fluctuans* > *Andrographis peniculata* > *Clerodendrum viscosum*. In cytotoxicity assay, all samples were found to be active against brine shrimp nauplii (*Artemia salina*) and ALA produced lowest LC_{50} value (7.03 µg/ml). So, according to the result, *Enhydra fluctuans* and *Andrographis peniculata* possesses significant antimicrobial and cytotoxic activities.

CHAPTER 3

MATERIALS AND METHODS



CHAPTER THREE

MATERIALS AND METHODS

3.1 Extraction of *Enhydra fluctuans* Lour

Extraction is the separation of medicinally active portions of plant (and animal) tissues using selective solvents through standard procedures. The products so obtained from plants are relatively complex mixtures of metabolites, in liquid or semisolid state or (after removing the solvent) in dry powder form and are intended for oral or external use (Prashant *et al.*, 2011). Extraction is first pre-purification step in the isolation and characterization of active compounds of a medicinal plant.

3.1.1 Collection of Plant Material/Part

For the present investigation the plant *Enhydra fluctuans* Lour was collected from Gollamari bazar, near Khulna University, Khulna, Bangladesh, in November 2013 at daytime. The fresh whole plants were used for the study.

3.1.2 Drying of Plant Material

The collected whole plants were separated from undesirable materials and then were washed with water. They were sun dried for 17 days to ensure the active constituents free from decomposition also to avoid any photochemical degradation.

3.1.3 Grinding of Plant Material

The dried whole plants were ground into a coarse 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.

3.1.4 Cold Extraction

About 300gm of powered material was taken in a clean, flat-bottomed glass container and soaked in 700 ml of 95% ethanol. The container with its contents was sealed and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture then

underwent a coarse filtration by a vacuum filter with a piece of clean, white cotton material and filter paper. This process was done for two times.

3.1.5 Evaporation of the Solvent

The filtrate was taken into beaker. Then the opening of beaker was wrapped by a sheet of aluminum foil to which perforation was done for evaporation of the ethanol and was kept in dry and cool place for several days. Then the filtrate (ethanol extract) obtained was evaporated under ceiling fan until dried. It rendered a gummy concentrate of greenish black color which was designated as crude ethanol extract of *Enhydra fluctuans*. Percentage yield in the extraction process was calculated by the following equation -

$$\text{Percent yield} = \frac{\text{Weight of concentrated extract}}{\text{Weight of powder macerated}} \times 100$$

3.1.6 Flow chart of extraction

Whole plant collection

↓

Drying

↓

Grining

↓

Powder of the dried whole plant *Enhydra fluctuans* Lour

↓

Macerated with ethanol for 15days (300g)

↓

Filtration by cotton plug

↓

Filtration by Whatman filter paper

↓

Crude ethanol extract

3.2 Chemicals

All chemicals were of highest purity. DPPH (1, 1-diphenyl 2-picryl hydrazyl), ferric chloride, HCl, and mercuric iodide were obtained from Sigma Chemical Co. Ltd, (St. Louis, MO, USA). Methanol, Ethanol, Dragendorff's reagent, were purchased from BDH, England, Ascorbic acid was obtained from SD Fine Chem. Ltd., India. TLC plates and Silica gel 60 were obtained from Merck (Darmstadt, Germany). All other chemicals and reagents were of analytical grade.

3.3 Solvent – Solvent Partitions

3.3.1 Materials used for solvent-solvent partition

Apparatus and Reagents

Separating funnel, Beaker, Ethanol, n-Hexane, Water

3.3.2 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
- aqueous ethanol

3.3.3 Separation Process

At first in separating funnel 5g of ethanol extract was added, which was obtained by cold extraction and evaporation of solvent, was dissolved into the mixture of 20 ml ethanol and 80 ml water. Then 100 ml of n-Hexane solvent were added to the 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. Then the separating funnel was set aside to allow 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. Those two fractions (n-Hexane and water fractions) were collected in separate two beakers.

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.

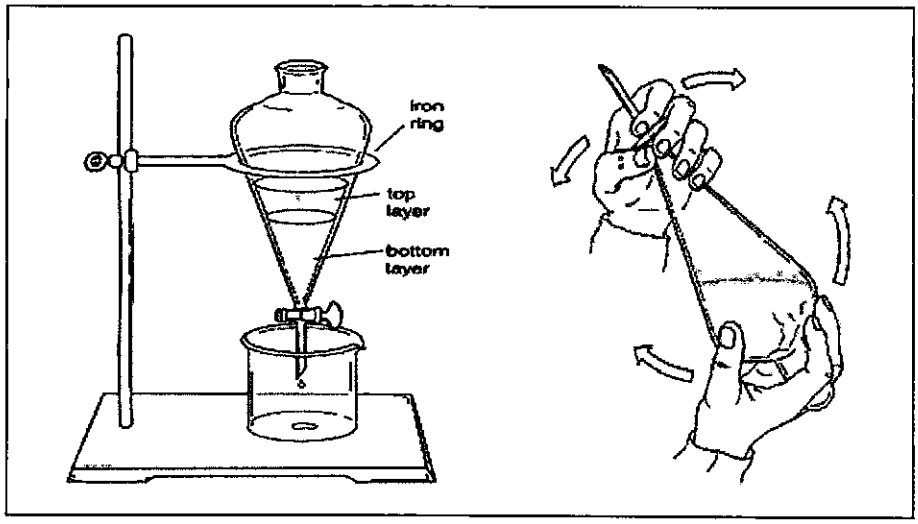


Figure 3.1: Illustration of partitioning

Then both the n-Hexane and aqueous ethanol fractions were evaporated under ceiling fan until dried. The dried n-Hexane part was designated as n-Hexane fraction of crude ethanol extract of the whole plant *Enhydra fluctuans*. The dried aqueous ethanol part was designated as aqueous ethanol fraction of crude ethanol extract of the whole plant *Enhydra fluctuans*.

3.4 Sub – Fractionation Using 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 or external pressure.

3.4.1 Materials used for column chromatography

Apparatus

Test tube, Sand, Silica gel 60, conical flask.

Reagents

Acetone, Ethanol, n-hexane.

3.4.2 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.

3.4.3 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 enough polar, 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 solvents are:-

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

3.4.4 Procedure of column chromatography

✓ 20g silica gel and .4g n-Hexane extract was mixed with ethanol. Then the slurry was quickly but carefully pours into the column immediately after mixing so that maximum amount of silica goes into the column instead of remaining behind in the beaker.

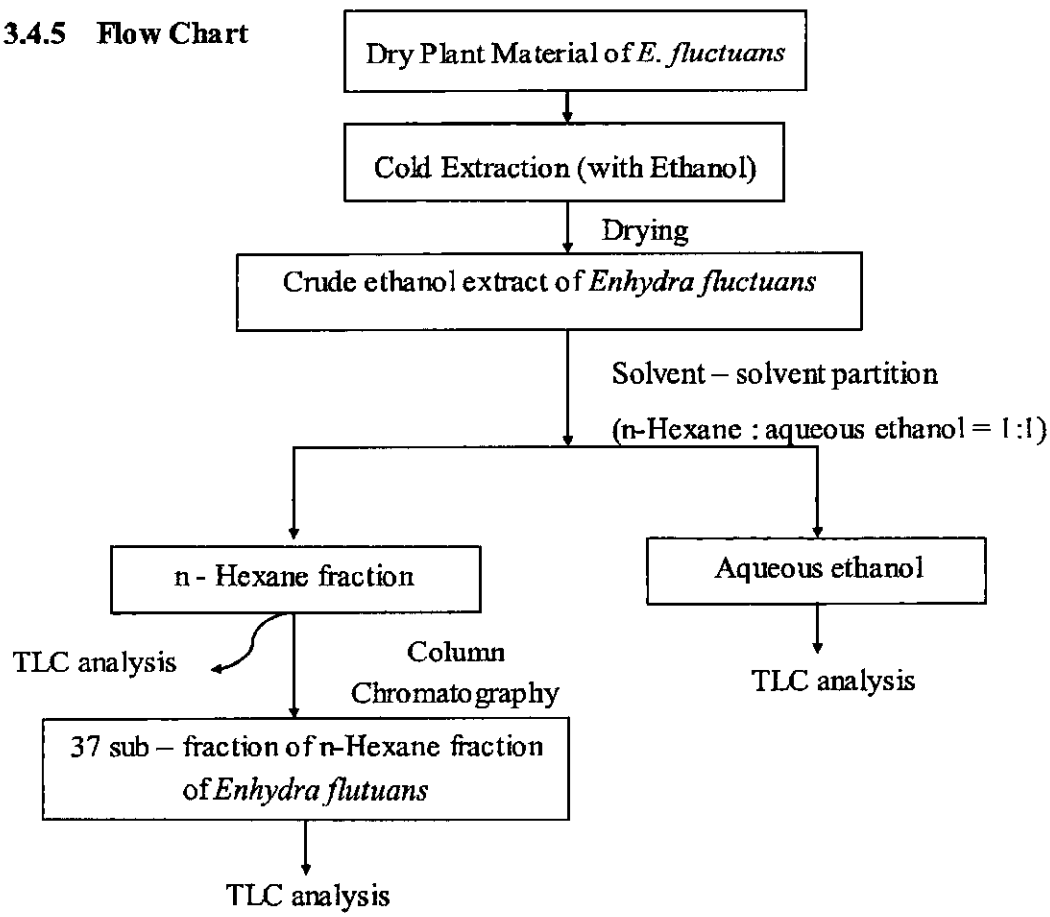
- ✓ The pinch clamp was removed to allow 60ml ethanol solvent to drip into a clean flask. Tap on the side of the column with a rubber stopper or tubing to help the silica settle uniformly.
- ✓ The same procedure was followed for acetone of 60 ml and n-Hexane : acetone solvent of 60 ml.

- ✓ A small amount of sand was added to surface of the silica gel. 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. 0.4 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 tubes of 2 ml respectively.

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

3.4.5 Flow Chart



3.5 TLC analysis

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.

3.5.1 Apparatus used

TLC plates containing fluorescent material, Glass jar with closure, Fine capillary tube, UV detector with short (254 nm) and long (366 nm) wave lengths, Spray gun, Filter paper, Dryer.

3.5.2 Reagents and Chemicals used

Ethanol, Methanol, Chloroform, Acetone, n-Hexane, Distilled water, H_2SO_4 , Ascorbic acid (as standard substance).

3.5.3 TLC plate preparation

TLC plate was cut into appropriate size of 10 cm long and 8 cm wide.

Table 3.1 Solvent system used for TLC analysis

Groups	Solvent system	Ratio
Non polar	n-Hexane: Acetone	3:1
Medium polar	$CHCl_3$: CH_3OH	5:1
Polar	$CHCl_3$: CH_3OH : H_2O	40:10:1

3.5.4 Sample application and Development of chromatogram

A. By polar solvent system

- The chromatogram was developed by ascending technique.
- A fine capillary tube was used as spotter for sample application in the TLC plates.
- A little amount of ethanol 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.
- 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 spatula.
- Two plates were spotted in such a manner. These plates were then kept in a jar containing a solvent system of chloroform and methanol and water (40:10:1).
- 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 manner 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 plates were sprayed with 10% H_2SO_4 by a spray gun.

B. By medium polar solvent

Again 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 plates were sprayed with 10% H_2SO_4 by a spray gun.

C. By non polar solvent

Again plates were spotted by the same process mentioned above and chromatogram was run in solvent system hexane and acetone (3:1). After developing the chromatogram plates were sprayed with 10% H_2SO_4 by a spray gun.

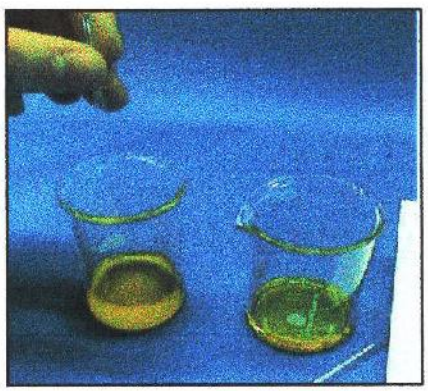


Figure 3.2: Capillary tube as sample spotter.

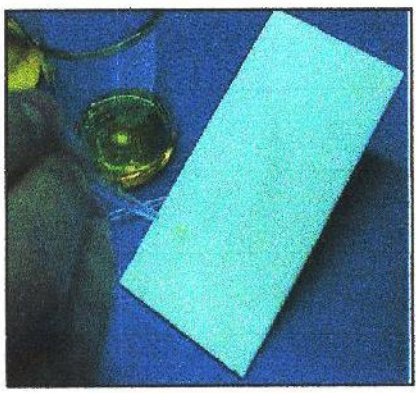


Figure 3.3: Spotting of sample.

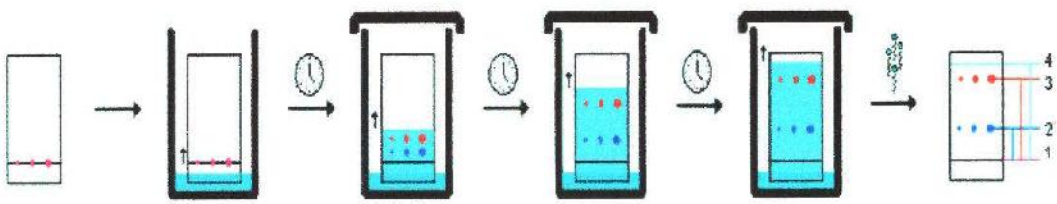


Figure 3.4: Development of a TLC Plate.

3.5.5 Solvent selection

The applied sample was run properly with different solvent systems and observed. The solvent system in which the sample separated well will be further used for fractionation of plant extract.

3.5.6 UV positive components detection

- After drying the plates, three plates from three solvent systems were observed visually under UV light and various regions were marked.

- Then these plates ware sprayed with 10% H_2SO_4 and dried on hot plate at 110-120°C for some time.

3.6 Phytochemical Group Test

Testing of different chemical groups present in extract represents the preliminary phytochemical studies. To identify the chemical constituents of plant extract standard procedures are followed (Ghani, 2003; Ghani, 2005; Trease and Evans, 1978; Trease and Evans, *1983*; Sofowara, 1982; Saxena and Saxena, 2012). To evaluate the pharmacological activity of the extract, identification of the nature of the compounds present in crude extract is essential. The crude extract was qualitatively tested for the presence of chemical constituents using the specific reagents and chemicals.

3.6.1 Materials used for phytochemical test

95% ethanol, a glass jar, Aluminum foil, Test tube.

3.6.2 Reagents of Chemical Group Tests

The following reagents were freshly prepared and used for the different chemical group test.

Molisch's Reagent

10% (w/v) alcoholic pure α-naphthol solution that means 10g pure α-naphthol was dissolved in sufficient amount of ethanol and the volume of solution was adjusted up to 100ml.

Benedict Reagent

17.3g sodium citrate and 10g anhydrous sodium carbonate were dissolved together in 50ml water and 1.73g cupric sulfate was dissolved in 30ml water. The latter solution was poured, with constant stirring, into the citrate-carbonate solution and the volume was made upto 100ml with water.

Fehling's Solution

Fehling's Solution A (copper sulfate solution)

6.928 gm copper sulfate was dissolved in sufficient waterand was diluted to 100 ml.

Fehling's Solution B (Alkaline tartrate solution)

34.6 gm of potassium sodium tartarate and 10 gm of sodium hydroxide were dissolved in sufficient water and diluted when cold to 100 ml.

Equal volume of the Fehling's solution A and Fehling's solution B were mixed at the time of use.

Mayer's Reagent

1.36g of mercuric iodide in 60ml of water was poured into a solution of 5g of KI in 10ml of water and sufficient water was added to make the solution upto100 ml.

Dragendroff's Reagent

0.17g bismuth nitrate was in a solution containing 2ml acetic acid and 8ml distilled water. This solution was mixed with a solution containing 4gm potassium iodide in 10ml acetic acid and 20ml water. The mixed solution was diluted upto 100ml with distilled water.

3.6.3 Test procedures

Tests for tannins

- **Ferric Chloride Test**

5 ml solution of the extract was taken in a test tube. Then 1 ml of 5%Ferric chloride solution was added. Formation of any one of the color of intense green or bluish green or black or purple or blue, that depends on the types of tannins present in the extract, indicates the presence of tannins such as green and blue color indicates hydrolysable and condensed tannins respectively. Here gallic acid /aqueous solution of catechu was used as standard.

➢ **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. Here gallic acid/ aqueous solution of catechu was used as standard.

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 and that was indicated as the presence of alkaloids. Here nicotine was used as standard.

➢ **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 and that was indicated as the presence of alkaloids. Here nicotine was used as standard.

Test for Steroids

Salkowski's Test: 1 ml solution of chloroform extract was taken and then added 1ml sulphuric acid. Red color indicates the presence of steroid.

Test for Flavonoids

A few drops of concentrated hydrochloric acid were added to a small amount of an alcoholic extract of the plant material. Immediate development of a red color indicates the presence of flavonoids. Here aqueous solution of rose petal /quercetin was used as standard.

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 saponnins.

Tests for Glycoside

Molisch's Test

An aliquot of 2ml of the aqueous extract solution was treated with Molisch's reagent in a test tube and carefully added 2ml of conc. sulfuric acid along the sides of the test tube. Formation of the bluish violet ring at the junction of two liquids indicates the presence of Glycosides. Here digoxin was used as standard.

3.7 HPLC analysis

3.7.1 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) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC), methanol (HPLC), acetic acid (HPLC), and ethanol was obtained from Merck (Darmstadt, Germany).

3.7.2 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 auto-samplier (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 (Dionix, USA) which was controlled at 30°C using a temperature controlled column compartment (TCC-3000). Data acquisition, peak integration, and calibrations were performed with Dionix Chromeleon software (Version 6.80 RS 10).

Figure 3.5: HPLC machine (connected to computer)

3.7.3 Chromatographic conditions

The phenolic composition of the ethanol extract samples of *Enhydra flutuans* was determined by HPLC, as described by Sarunya and 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 wave lengths 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.

3.7.4 Standard and sample preparation

A stock standard solution (100 µg/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 the mixed stock standard

solutions in methanol to give a concentration of 5 µg/ml for each polyphenols except (+)-catechin hydrate, caffeic acid, rutin hydrate (4 µg/ml) and quercetin (3 µg/ml). All standard solutions were stored in the 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 µg/ml for GA, CH, VA, EC, PCA, EA, MC, KF; 0.5 - 4.0 µg/ml for CH, CA, RH, and 0.25 - 3.0 µg/ml for QU. The calibration curves were constructed from chromatograms as peak area vs. concentration of standard.

A solution of ethanol extract samples of *Enhydra flutuans* at a concentration of 10 mg/ml was prepared in ethanol by vortex mixing (Branson, USA) for 30 min. The samples were stored in the 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.

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 ± standard deviations of triplicate independent analyses.

3.8 Anti Microbial Activity Test

Antibacterial activity of crude ethanol extract, n-Hexane and water extract and sub fractions of n-Hexane fraction of *Enhydra fluctuans* was determined by disc diffusion method (Bauer *et al.*, 1966; Ahmed *et al.*, 2003). Disc diffusion method is widely acceptable for the preliminary screening of antibacterial activity.

3.2 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

3.8.1 Microorganisms used for the test

Gram-negative bacterial strains of ***Escherichia coli*** were taken for the test. The 4 bacterial strains used for the investigation are listed in Table. These organisms were collected from the Microbiology Lab. of FMRT Discipline, Khulna University, Khulna.

Table 3.3: Bacterial strains used for investigation of antibacterial activity

1. *Escherichia coli* (EPEC)
2. *Escherichia coli* (ETEC)
3. *Escherichia coli* (ETEC)
4. *Escherichia coli*

Escherichia coli is a Gram-negative, rod-shaped bacterium ,commonly found in the lower intestine of warm-blooded organisms (endotherms). Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in humans, and are occasionally responsible for product recalls due to food contamination. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2, and by preventing the establishment of pathogenic bacteria within the intestine.

3.8.2 Culture media

A mixture of nutrients used in the laboratory to support growth and multiplication of a culture (a population of microorganisms) is called culture medium (Roland, 1982). The nutritional requirements of bacteria vary widely; there are great differences in the chemical compositions own in a medium containing only inorganic compounds, whereas others require a medium containing organic compounds (amino acids, sugars, purines, or pyrimidines, vitamins, or coenzymes).

In addition to specific nutrients, each kind of organism also requires specific physical conditions for growth. For example, some bacteria cannot grow below 40°c, some cannot grow above 20°c and some require a temperature close to that of the human body (i.e., 37°c). Light may be another important physical condition. The successful cultivation of bacteria requires an awareness of all of these factors (Pelczar *et al.*, 1993). Each kind of microorganism grows in a characteristic manner. On solid media, microbes grow as colonies-distinct, compact masses of cells that are macroscopically visible. Colonies are characterized by their size, shape, texture, consistency, color, and other notable features (Pelczar *et al.*, 1993).

The following media are used normally to demonstrate the antibacterial activity and to make subculture of the test organisms.

a) Nutrient agar media

b) Nutrient broth media

Composition of Nutrient agar and Nutrient broth media are as follows-

3.4 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)

3.5 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)

3.8.3 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.

3.8.4 Sterilization of different equipments and media

Agar 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. Loop and forceps were kept in a laminar hood and UV light was switched on before one hour working in a laminar hood to avoid accidental contamination.

3.8.5 Preparation of first culture

- The sterilized agar media was poured into petridishes and kept for few minutes to prepare agar slants.
- With the help of an inoculating loop, the attenuated test organisms kept in the refrigerator were transferred to the agar slants in an aseptic condition using laminar air hood.
- The inoculated slants were then incubated at 37°C for 16-18 hours to assure the growth of test organisms.

3.8.6 Preparation of second culture

- The sterilized agar media was poured into petridishes and kept for few minutes to prepare agar slants.
- With the help of an inoculating loop, the test organisms from the first culture were transferred to the agar slants in an aseptic condition using laminar air hood.
- The inoculated slants were then incubated at 37°C for 16-18 hours to assure the growth of test organisms of growth phase. This second culture could be used within 2 days.

3.8.7 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.

3.8.8 Preparation of seeded test plates

- Each of the test organisms of growth phase were transferred from the second culture to the vial containing 3 ml autoclaved nutrient broth media with the help of the sterilized inoculating loop.
- Then those vials were incubated for 2-3 hours at 37°C.
- After 2-3 hours when the turbidity was seen in vials then 10 µL solution from each vial containing test organisms was transferred from the vials to the test tube containing 15 ml autoclaved agar media with the help of the micropipette.
- 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.
- The petridishes were rotated several times, first clockwise and then anticlockwise, to assure homogeneous distribution of the test organisms.
- In this way 4 discrete media in petridishes were prepared for 4 bacterial strains used for screening and was properly marked.

3.8.9 Preparation of test sample

125 mg, 250mg and 500 mg of ethanol extract of *Enhydra fluctuans* were accurately measured by the electronic balance and taken in three small volumetric flasks. Then 3ml of ethanol was added and triturated in unidirectional manner using a vortex mixer, after proper mixing the volume was adjusted 5 ml by ethanol for each and the concentration of these solutions became 25µg/µl, 50µg/µl and 100µg/µl.

3.8.10 Application of discs

Three types of discs were used for antibacterial screening:

a) Sample discs

b) Standard discs and

c) Blank discs

Each agar plates were divided into five portions i.e. three for samples (25µg/µl, 50µg/µl and 100µg/µl), one for standard and one for blank.

3.8.11 Sample disc application

Three blank discs were placed into respected portions on agar plates 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 15mm to the edge of the plate and far enough apart to prevent overlapping the zones of inhibition. 10µl of the test sample from solutions were applied on the respective discs with the help of a micropipette in an aseptic condition under the laminar air flow to get per disc concentration of 250µg, 500µg and 1000 µg respectively. These discs were left for several hours (4-6h) in aseptic condition under the laminar air flow for complete removal of solvent as the solvent (ethanol) has some antimicrobial activity.

3.8.12 Blank disc application

4 blank discs were placed into respected portions on agar plates with the help of a sterile forceps to assure complete contact with medium surface. 10µl ethanol was applied on the blank discs as negative control. They ensure that the residual solvent's activity was not active themselves.

3.8.13 Standard disc application

Kanamycin (30 µg/ disc) antibiotic discs 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. Kanamycin (30 µg/ disc) was placed on the labeled portion of the petridish.

The plates were then inverted and kept in refrigeration for about half hours at 4°C to diffuse the plant materials into a considerable area of the medium. Finally the plates were incubated upside down at 37°C for 16-18 hours.

3.8.14 Determination of zone of inhibition

After proper incubation, the antibacterial activity of the test agent was determined by measuring the diameter of zone of inhibition in term of millimeter with calibrated digital slide calipers (Bauer *et al.* 1966; Ríos *et al.* 1988).

The antibacterial activity was assessed against a panel of 4 pathogenic bacterial strains (gram negative) at the dose of 250 µg/disc, 500 µg/disc and 1000 µg/disc and the results were compared with the activity of standard kanamycin (30 µg/disc)

3.8.15 Determination of Activity Index

From the result of zone of inhibition of sample and standard antibiotic kanamycin activity index was calculated (Gopalakrishnan *et al.*, 2012) by using the following formula

$$\text{Activity Index (AI)} = \frac{Inhibition\ \ zone\ \ of\ \ sample}{Inhibition\ \ zone\ \ of\ \ standard}$$

3.9 Anti-Oxidant Activity: Qualitative Analysis

DPPH based qualitative analysis was performed according to the method followed by (Sadhu *et al.*, 2003). Qualitative analysis was done by TLC technique.

3.9.1 Apparatus used

TLC plates containing fluorescent material, Glass jar with closure, Fine capillary tube, UV detector with short (254 nm) and long (366 nm) wave lengths, Spray gun, Filter paper , Dryer.

3.9.2 Reagents and Chemicals used

Ethanol, Methanol, Chloroform, Acetone, n-Hexane, Distilled water, DPPH (0.02 % w/v;0.02g/100ml) solution in ethanol, Ascorbic acid (as standard substance).

3.9.3 Procedure

TLC plate preparation

Commercially available TLC plate is cut into appropriate size of 10 cm long and 7 cm wide.

Table 3.6 Solvent system used for qualitative analysis

Groups	Solvent system	Ratio
Non polar	n-Hexane: Acetone	3:1
Medium polar	$CHCl_3$: CH_3OH	5:1
Polar	$CHCl_3$: CH_3OH : H_2O	40:10:1

Sample application

- A very little amount of plant extract and standard ascorbic acid was taken in two small vials separately and was diluted suitably with ethanol.
- A fine capillary tube was used as spotter.
- Very little amount of plant extract and ascorbic acid was applied in uniform size (about 0.3 cm) on the TLC plates.
- The plant extract and ascorbic acid 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 means of a spatula.

Development of chromatogram

The chromatogram was developed by ascending technique in where silica gel 60 F254 pre-coated plates (Merck, Germany) containing layer thickness 0.25mm was used as stationary phase and different solvent systems were used as mobile phase.

1. Non-polar solvent system in the ratio mentioned before was kept in a glass jar.
2. Then the spotted plate was placed in jar in such a manner that the sample spot was just above the solvent surface.
3. Filter paper was kept into jar by wetting with solvent system to keep the jar saturated, and then the jar was closed tightly.
4. The solvent was allowed to move up the plate by capillary action up to the scratched point.
5. The plate was then removed from the jar and dried with air suitably.

Figure 3.6: Development of Chromatogram

Following the similar manner again one plate was run in solvent system chloroform and methanol (5:1) and another plate in solvent system chloroform, methanol and water (40:10:1). And the plates were dried with air suitably.

Detection method

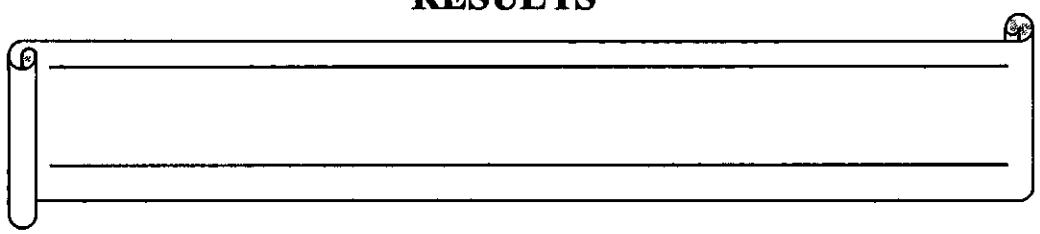
Antioxidant positive components detection

1. After drying the plates, they were viewed under UV detector both in short (254nm) and long (366nm) wave length. Components viewed in shorter wave length are marked by the sign ( ) and in longer wavelength are marked by < >.
2. One plate from each solvent system was sprayed with 0.02% ethanol solution of DPPH on it by spray gun.
3. The plates were dried with a current of air suitably.

After applying 0.02 % (w/v) DPPH solution on the TLC plate, the purple color of DPPH solution changed which indicates the presence of antioxidant compounds.

CHAPTER 4

RESULTS



CHAPTER FOUR
RESULTS

4.1 Yield from the extraction of *Enhydra fluctuans* Lour.

For cold extraction, 300g of macerated powder was taken and dissolved in ethanol and 10g of concentrated extract was obtained after filtration. So, yield in the extraction process was 3.3% w/w.

4.2 Solvent – Solvent Partition

Both the n-Hexane and aqueous ethanol fractions were evaporated under ceiling fan until dried.

Weight of the n – Hexane fraction = 2.97g (59.4%)

Weight of aqueous ethanol fraction = 2.03g (40.6%)

TLC analysis was performed on both fractions of crude ethanol extract of *E. fluctuans.*

4.3 Sub Fractionation Using Column Chromatography

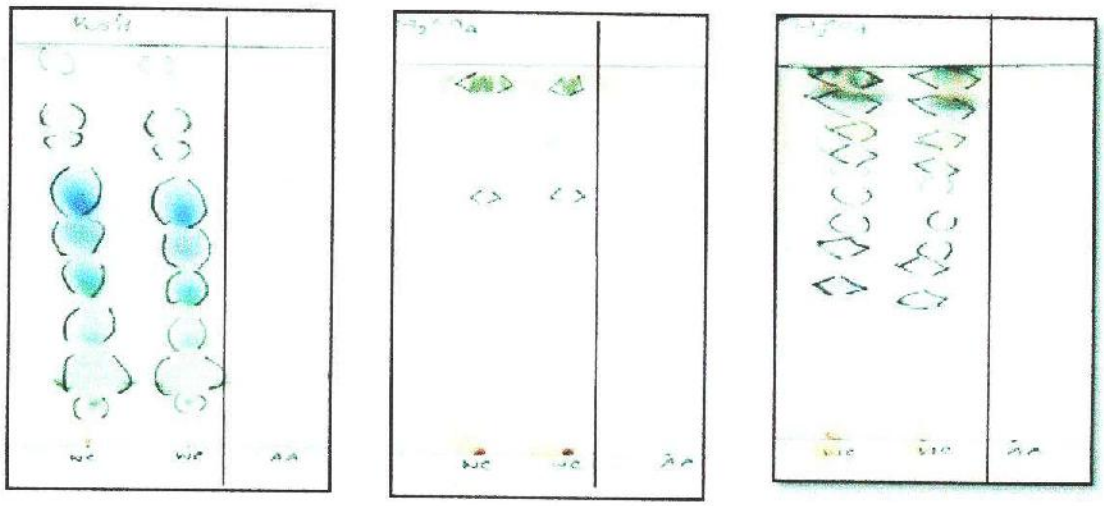
After solvent – solvent partition TLC analysis was performed where n-Hexane fraction exhibit better separation of compounds on TLC plate by providing more distinct spots at chromatogram than aqueous ethanol fraction. So, .4g n–Hexane extract was used in column chromatography containing 20g silica-60 gel for further separation. After column separation, 37 sub fractions were obtained and TLC analysis was performed on each sub – fraction.

4.4 TLC 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 spots were found in both wave lengths which indicated the presence of UV positive materials in the plant extract and they were marked. Spots viewed in shorter and longer wavelength are marked by the sign ( ) and < >

respectively. The chromatograms (on TLC plates) developed in different solvent systems are given below –

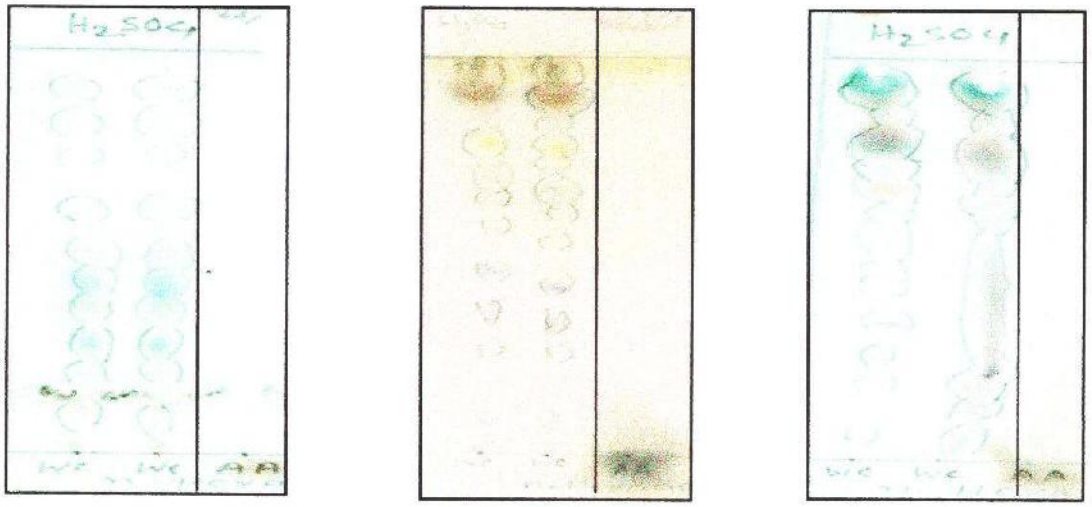
(Here in TLC plate, WC = *E. fluctuans*, AA = ascorbic acid).



E. fluctuans **Ascorbic acid** ***E. fluctuans*** **Ascorbic acid** ***E. fluctuans*** **Ascorbic acid**
(n-Hexane: Acetone = 3:1) ($CHCl_3$: CH_3OH =5:1) ($CHCl_3$: CH_3OH: H_2O =40:10:1)

Figure 4.1: Comparison among TLC Plates of Crude Ethanol Extract of *E. fluctuans* with Standard (Ascorbic acid) after Applying 10% H_2SO_4.

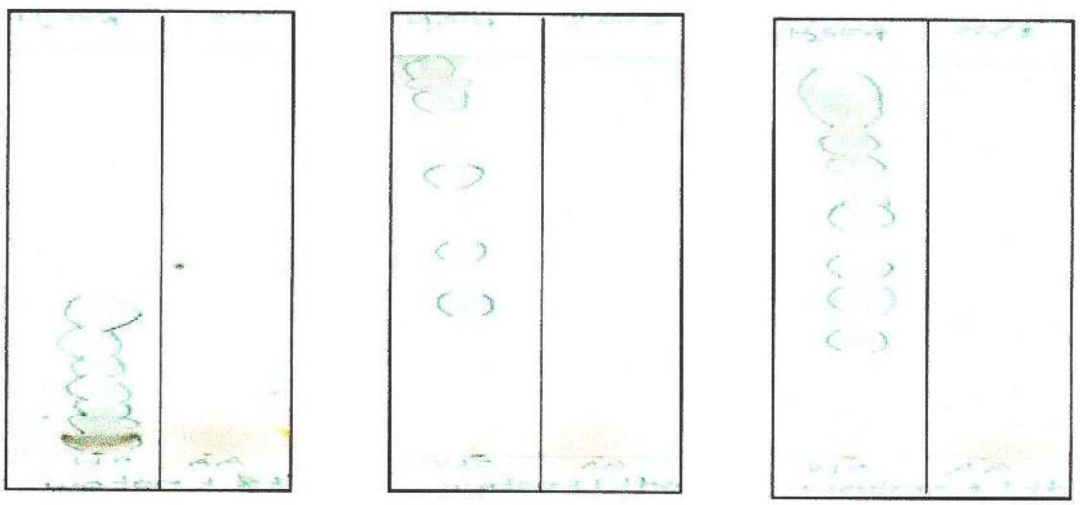
According to figure – 4.1, the TLC plates that was run in non polar (n-Hexane: Acetone = 3:1) and polar ($CHCl_3$: CH_3OH: H_2O =40:10:1) solvent systems exhibits more spots on chromatogram than the TLC plate run in medium polar ($CHCl_3$: CH_3OH =5:1) solvent. The TLC plate that run in medium polar ($CHCl_3$: CH_3OH =5:1) solvent system exhibit the lowest number of spots. This phenomenon indicated that compounds of *E. fluctuans* were not separated well in medium polar solvent. On the other hand, spots are too congested in case of polar solvent system while plant extract form comparatively separated spots in non polar solvent system. So, it indicated that compounds will be separated well by using non polar solvent. After solvent – solvent partition, crude ethanol extract has been divided in two parts – n-Hexane fraction (non polar part) and aqueous ethanol fraction (polar part). Chromatograms of these two fractions are given below -



E. fluctuans **Ascorbic acid** ***E. fluctuans*** **Ascorbic acid** ***E. fluctuans*** **Ascorbic acid**

(n-Hexane: Acetone = 3:1) ($CHCl_3$: CH_3OH =5:1) ($CHCl_3$: CH_3OH: H_2O =40:10:1)

Figure 4.2: Comparison among TLC Plates of n-Hexane fraction of *E. fluctuans* with Standard (Ascorbic acid) after Applying 10% H_2SO_4.



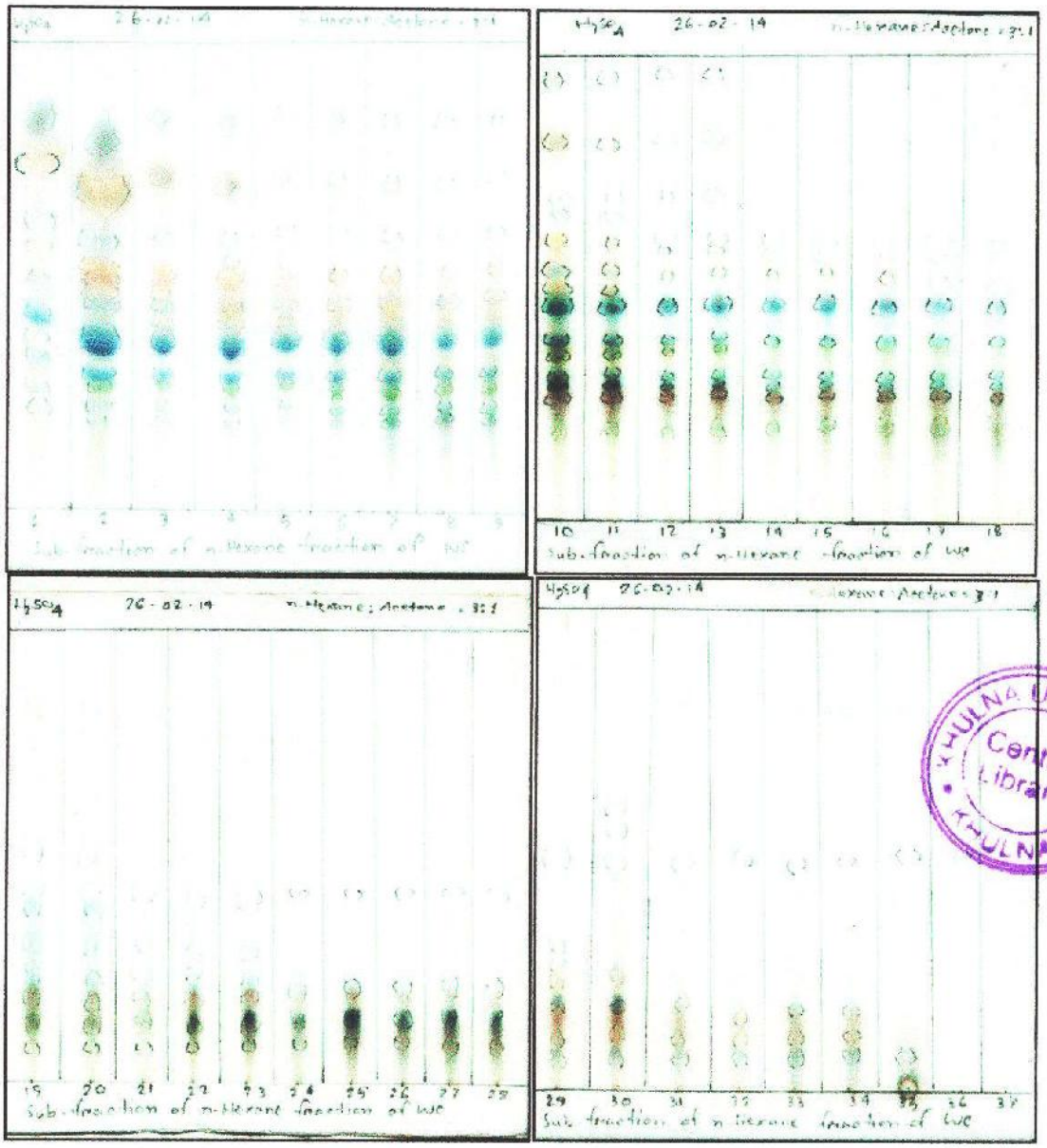
E. fluctuans **Ascorbic acid** ***E. fluctuans*** **Ascorbic acid** ***E. fluctuans*** **Ascorbic acid**

(n-Hexane: Acetone = 3:1) ($CHCl_3$: CH_3OH =5:1) ($CHCl_3$: CH_3OH: H_2O =40:10:1)

Figure 4.3: Comparison among TLC Plates of aqueous ethanol fraction of *Enhydra fluctuans* with Standard (Ascorbic acid) after Applying 10% H_2SO_4.

It appears that the chromatogram of n-Hexane fraction has given far more spots than aqueous ethanol fraction which means compounds from n-Hexane fraction could be identified and separated more effectively . So, n-Hexane fraction was used for further

fractionation by column chromatography using silica-60 (polar in nature). The chromatograms of 37 sub fractions of n-Hexane fraction are given below-



Solvent system – Non polar (n-Hexane: Acetone = 3:1)

Figure 4.4: Comparison among TLC Plates of Sub Fractions of n-Hexane Fraction of *Enhydra fluctuans* after Applying 10% H_2SO_4.

There are no spots at the lower region from sub fraction 1 to 18. But lower region spots become increased gradually from sub fraction 19 to 35 while upper region spots gradually disappear. It indicates that the compounds from later sub fractions are more polar than the compounds from earlier sub fractions.

Some fractions have similar chromatogram pattern. Such as – sub fractions 3 to 9 display same chromatogram pattern.

Sub fraction- 32, 33 and 34 has three distinct spots. The sub fractions which contain lesser spots (means lesser compounds) are easy to be used for further separation.

Sub fraction - 35 has 2 spots while sub fraction – 36 and 37 has no spot. It is possible that there is no compound in sub fraction 36 and 37. A particular spot has been found in all sub fractions except 37.

4.5 Phytochemical Screening

The crude ethanol extract of *Enhydra fluctuans* Lour was subjected for chemical group tests and results of different group tests are given below in Table 4.1.

Table 4.1: Results of Phytochemical Screening

Phytochemical Group	Test performed	Standard	Result		
			Crude ethanol extract	n-Hexane fraction	Aqueous ethanol fraction
Alkaloid	Mayer's test	Nicotine	+	+	-
	Dragendroff's test		+	+	-
Flavonoids	General test	Rose petal/ Quercetin	+	+	+
Tannins	Ferric chloride (5%w/v)	Catechu/ Gallic acid	+	+	+
	Potassium dichromate test		+	+	+
Glycoside	Molisch's test	Digoxin/ Tomato	+	+	+
Steroid	Salkowski's Test	Norgestrol	+	+	+
Saponin	Froth test	Detergent	+	-	-

(+) Indicates the presence of chemical constituents &

(-) Indicates the absence of chemical constituents

Phytochemical studies showed that alkaloids, glycoside, flavonoids, tannins, saponin and steroids are present in the ethanol extract of *Enhydra fluctuans* Lour which can extensively show different pharmacologic activities.

4.6 HPLC Analysis

The phenolic composition of three different samples of the ethanol extract of *Enhydra fluctuans* was determined by HPLC. Names of the three samples used in HPLC analysis are given below -

Table 4.2: Name and amount of samples used for HPLC analysis

Samples		Amount(g)
Sample – 1	Crude extract of ***Enydra fluctuans (CEEF)***	.20g
Sample – 2	n-Hexane fraction of ***Enydra fluctuans(NFEF)***	.20g
Sample – 3	Water : ethanol fraction of ***Enydra fluctuans (WEFEF)***	.20g

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 4.3).

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 singnal-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 µg/ml, respectively. In recovery assay, the samples were fortified with all standards at 2.5, and 5.0 µg/ml. The recovery tests of all compounds range of 95.0-104%.

Standard mixture solutions of two concentrations (low and high concentrations) were injected five times to determine the reproducibility of the peak areas and retention times under the optimum conditions. Intra-day and inter-day percentage relative standard deviations (%RSD) for retention time and peak areas are considerably low (Table 4.4)

indicating that the precision is good. Stability of crude ethanol extract was tested every 24 h for 7 days, and was found to be stable throughout the 7-day period (RSD < 2.0%).

Table 4.3: Parameters of calibration graphs for the eleven phenolic standards in this study.

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

[a] Data were expressed as mean of triplicate measurements.
[b] Recovery are expressed as mean ± standard deviation.

4.6.1 Analysis of Samples

Identification and quantification of individual phenolic compounds in the three different samples were analysed by HPLC. The chromatographic separations of polyphenols in ethanol extracts of *Enhydra fluctuans* – crude extract, n-Hexane fraction and water: ethanol fraction - are shown in Figures 4.6, 4.7 and 4.8 respectively. The content of each phenolic compound was calculated from the corresponding calibration curve and presented as the mean of determinations.

The experimental results indicated that the crude ethanol extract, n-Hexane fraction and the water: ethanol fraction of *Enhydra fluctuans* contained a high concentration of ellagic acid (251.24 mg per 100 g of dry weight). It was found that catachin, vanillic acid and p-coumaric acid were present in the extract, however concentrations were detected fairly low (16.32 mg, 3.37 mg, & 0.97 mg/100 g of dry weight, respectively).

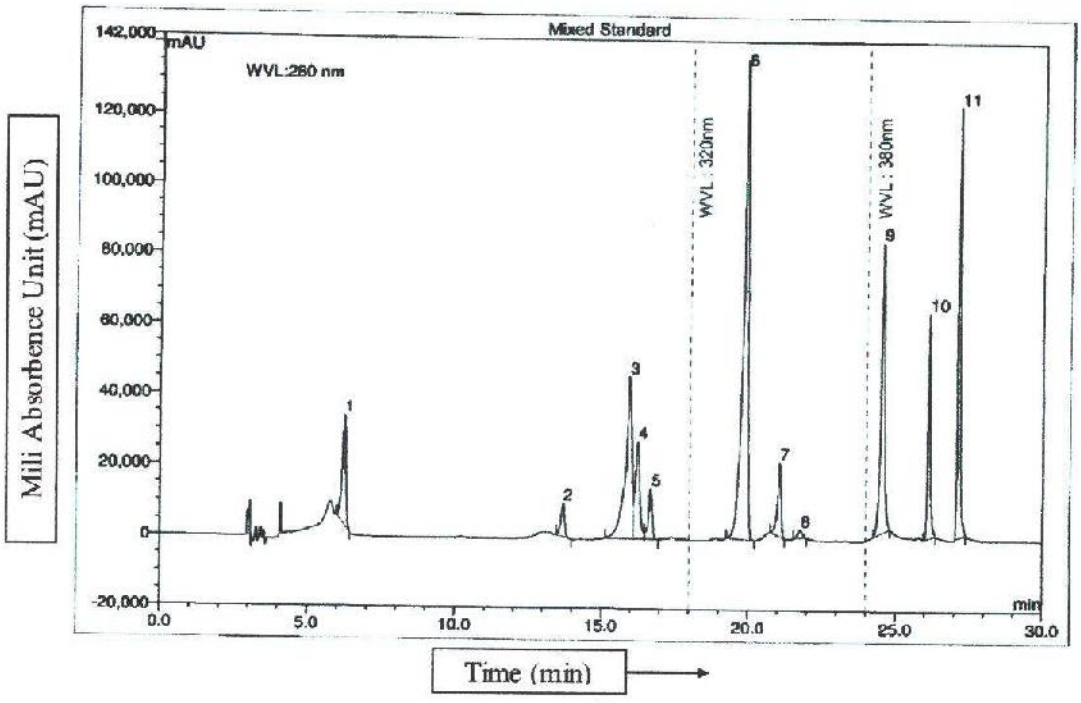


Figure 4.5: HPLC chromatogram of a standard mixture of polyphenolic compounds. Peaks: 1, gallic acid; 2, (+)-catechin; 3, vanillic acid; 4, caffeic acid; 5, (–)-epicatechin; 6, *p*-coumaric acid; 7, rutin hydrate; 8, ellagic acid; 9, myricetin; 10, quercetin; 11, kaempferol.

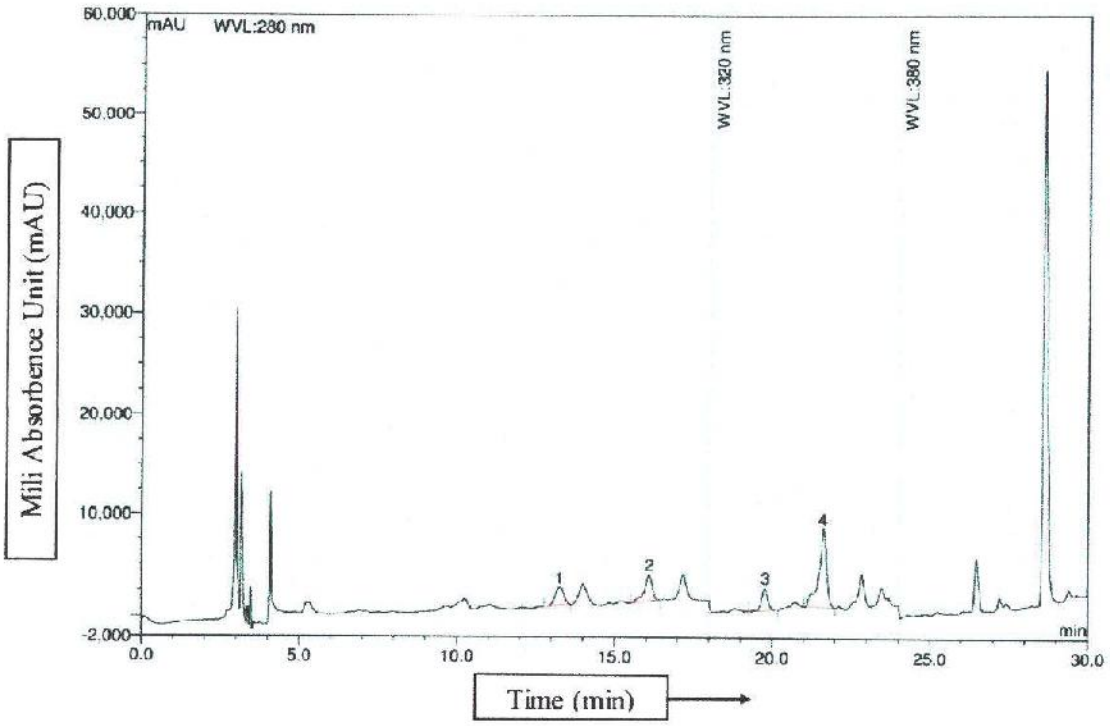


Figure 4.6: HPLC chromatogram of ethanol extract of **Sample** - Crude extract of ***Enydra fluctuans*** Peaks: 1, (+)-catechin; 2, vanillic acid; 3, *p*-coumaric acid; 4, ellagic acid.

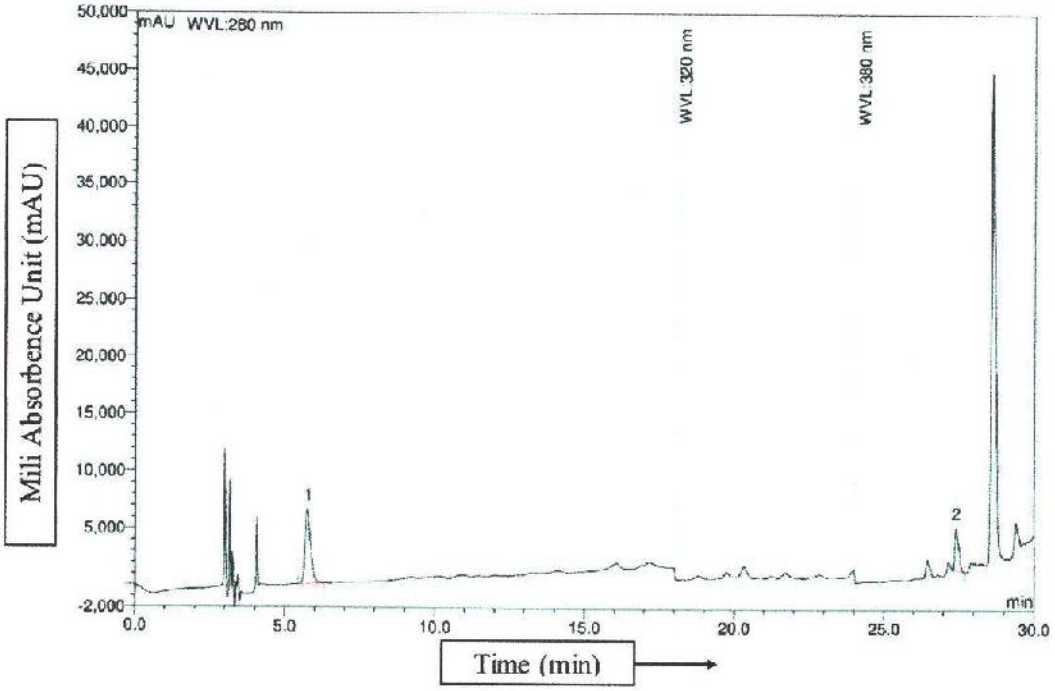


Figure 4.7: HPLC chromatogram of ethanol extract of **Sample** – n-Hexane fraction of ***Enydra fluctuans***. Peaks: 1, gallic acid; 2, kaempferol.

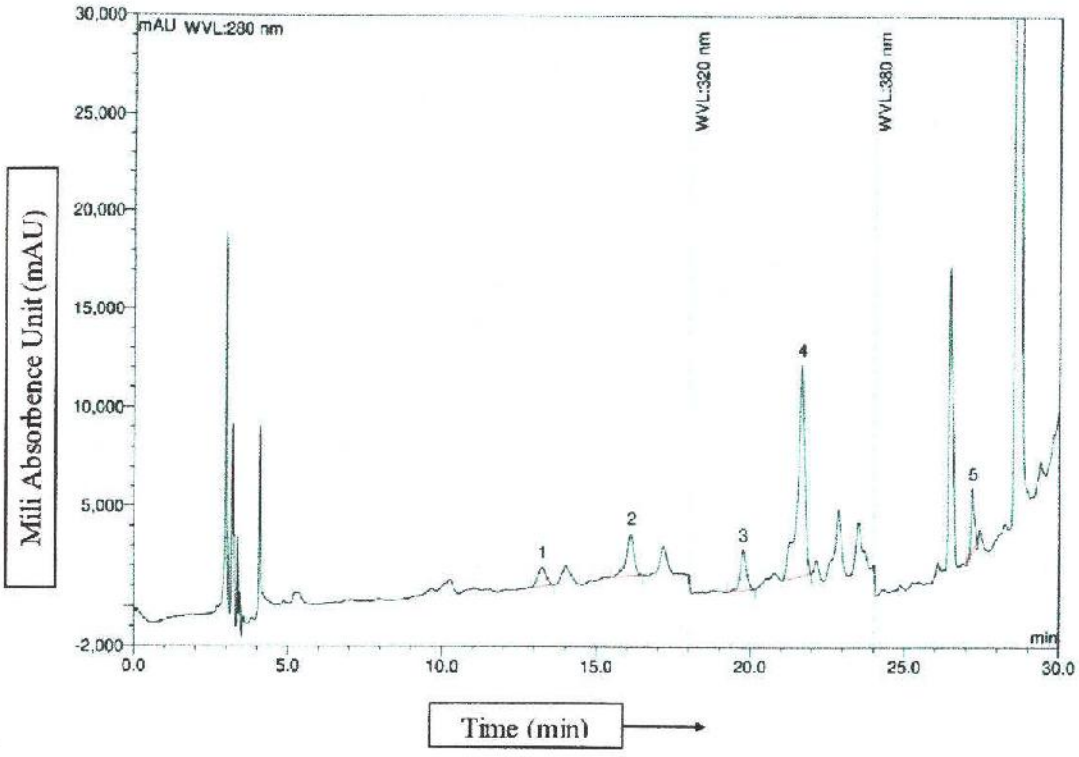


Figure 4.8: HPLC chromatogram of ethanol extract of sample - water: ethanol fraction of ***Enydra fluctuans***. Peaks: 1, (+)-catechin; 2, caffeic acid; 3, *p*-coumaric acid; 4, ellagic acid; 5, kaempferol.

4.6.2 Peak Characterization and Quantification

Percentage relative standard deviations (%RSD) for retention time and peak areas for three different samples are considerably low.

Table 4.4: Used concentration and obtained average area from mixed standards and three different samples (n=3).

Polyphenolic compound	Mixed standards		Crude extract		n-Hexane fraction		Aqueous ethanol fraction	
	Conc^n (µg/ml)	Avg. Area	Conc^n (µg/ml)	Avg. Area	Conc^n (µg/ml)	Avg. Area	Conc^n (µg/ml)	Avg. Area
GA	5.0	4538.196	10000	---	10000	1429.238	10000	---
CH	4.0	1390.189		573.010		---		296.314
VA	5.0	11479.90		775.716		---		---
CA	4.0	4910.547		---		---		615.770
EC	5.0	2152.048		---		---		---
PCA	5.0	26059.00		515.122		---		474.792
RH	4.0	3033.851		---		---		---
EA	5.0	424.195		2243.664		---		2092.138
MC	5.0	11393.0						---
QU	3.0	6330.564		---		---		---
KF	5.0	11342.70		---		553.490		381.958

4.6.2 Contents of Polyphenolic Compounds in Three Different Samples

Contents of polyphenolic compounds in three different samples are given below –

Table 4.5: Contents of polyphenolic compounds in three samples of *Enydra fluctuans* (n=3).

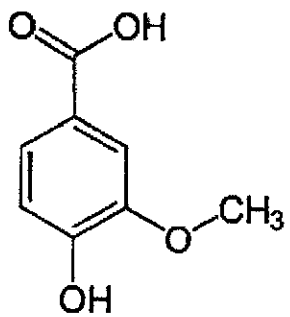
Polyphenolic Compound	Crude ethanol extract		n-Hexane fraction		Aqueous ethanol fraction	
	Content (mg/100 g of dry extract)	% RSD	Content (mg/100 g of dry extract)	% RSD	Content (mg/100 g of dry extract)	% RSD
CH	16.32	0.38	-	-	8.44	0.15
VA	3.34	0.05	-	-	-	-
PCA	0.97	0.01	-	-	0.90	0.02
EA	251.24	5.37	-	-	234.27	6.01
GA	-	-	15.59	0.73	-	-
KF	-	-	2.42	0.09	1.67	0.04
CA	-	-	-	-	4.97	0.08

According to Table 4.5, n-Hexane fraction has 1 and aqueous ethanol fraction has 3 different compounds that have not been detected in the crude ethanol extract (mother extract). It appears that the concentrations of those 4 compounds had increased after fractionation from mother extract.

The structures of 7 compounds identified by HPLC analysis has given below –

1. Vanillic acid:

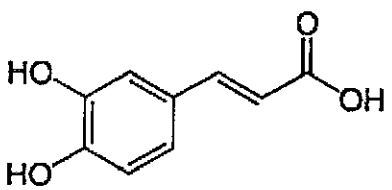
Structure:



IUPAC name: 4-Hydroxy-3-methoxybenzoic acid. The molecular formula is $C_8H_8O_4$.

2. Caffeic acid:

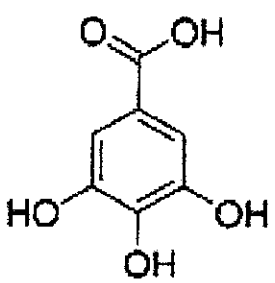
Structure:



IUPAC name: 3-(3,4-Dihydroxyphenyl)-2-propenoic acid. The molecular formula is $C_9H_8O_4$.

3. Gallic acid:

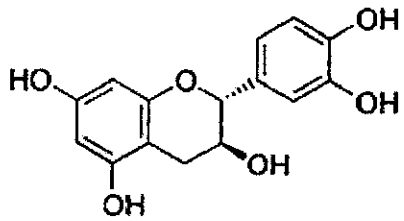
Structure:



IUPAC name: 3,4,5-trihydroxybenzoic acid. The chemical formula is $C_6H_2(OH)_3COOH$. The molecular formula is $C_7H_6O_5$.

4. Catechin:

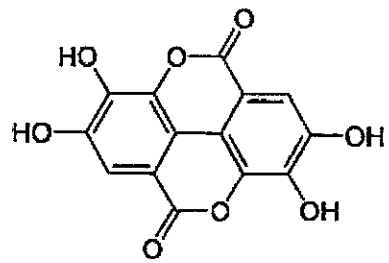
Structure:



IUPAC name: (2*R*, 3*S*)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2*H*-chromene-3,5,7-triol. The molecular formula is $C_{15}H_{14}O_6$.

5. Egallic acid:

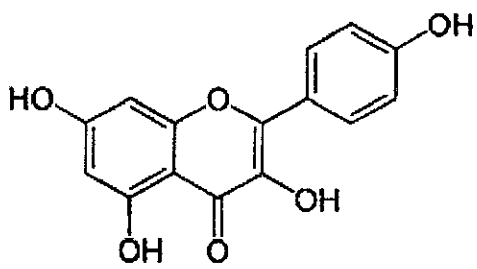
Structure:



IUPAC name: 2,3,7,8-Tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione. The molecular formula is $C_{14}H_6O_8$.

6. Kaempferol:

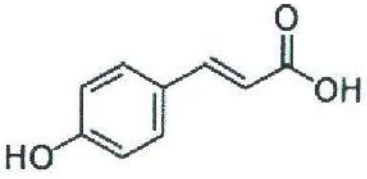
Structure:



IUPAC name: 3, 5, 7-Trihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one. The molecular formula is $C_{15}H_{10}O_6$.

7. *p*-Coumaric acid:

Structure:



IUPAC name: (E)-3-(4-hydroxyphenyl)-2-propenoic acid. The molecular formula is $C_9H_8O_3$.

4.7 Antibacterial Activity Test

Anti bacterial test was carried out for the following samples-

1. Crude ethanol extract
2. n-Hexane extract
3. aqueous ethanol extract
4. Sub fractions of n-Hexane fraction

The result of the investigation are given below in Table 4.6

Table 4.6: In vitro antibacterial activity of ethanol extract of *Enhydra fluctuans*

Bacterial Strains	Type of Bacterial Strains	Diameter of Zone of Inhibition in mm				
		Control	*Enhydra fluctuans* ***(Dose/disc)***			Kanamycin (30 µg/disc)
			250 µg	500 µg	1000 µg	
Escherichia coli EPEC	Gram(-ve)	0	0	0	0	20.30
Escherichia coli ETEC	Gram(-ve)	0	0	0	0	22.90
Escherichia coli ETEC	Gram(-ve)	0	0	0	0	21.50
Escherichia coli	Gram(-ve)	0	0	0	0	20.60

Gram (-ve): Gram Negative Bacteria

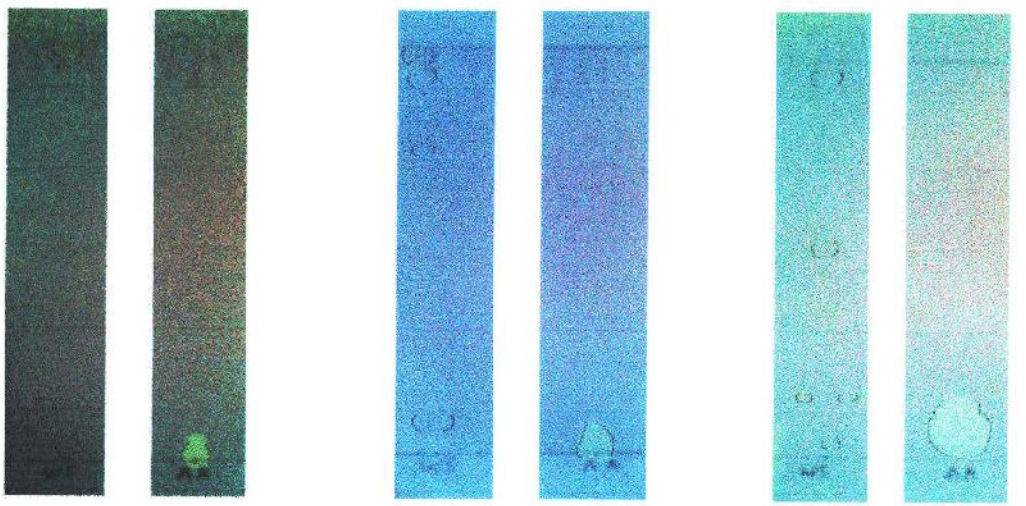
Although it is widely established that the plant *Enhydra fluctuans* has anti-bacterial activity against *Escherichia coli* but the results of antibacterial screening indicate that the plant has no antibacterial activity against these particular three *Escherichia coli* strains.

The obtained results may be due to personal errors, environmental factors and contamination. So, further study is needed to evaluate it.

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 researches are essential with other species of bacteria, viruses or other micro-organisms.

4.8 Anti-Oxidant Activity: Qualitative Analysis

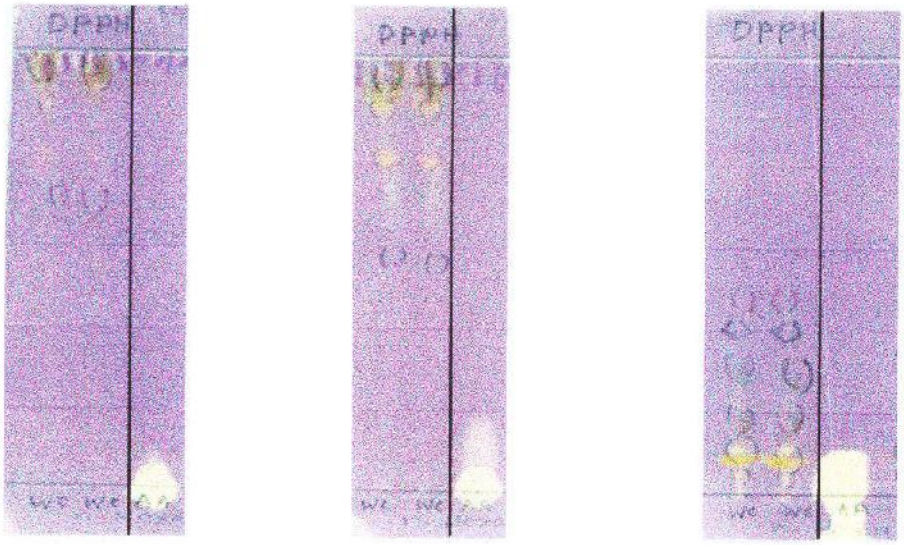
When the DPPH is exposed to antioxidant compounds the purple color of DPPH changed to yellow. The more yellowish color of DPPH observed the greater the antioxidant activity of the compounds tested. After applying 0.02% DPPH, extract show yellow spot on purple background but after few minutes later it turn into white which indicates the presence of antioxidant compounds.



E. fluctuans **Ascorbic acid** ***E. fluctuans*** **Ascorbic acid** ***E. fluctuans*** **Ascorbic acid**

($CHCl_3$: CH_3OH: H_2O =40:10:1) ($CHCl_3$: CH_3OH =5:1) (n-Hexane: Acetone = 3:1)

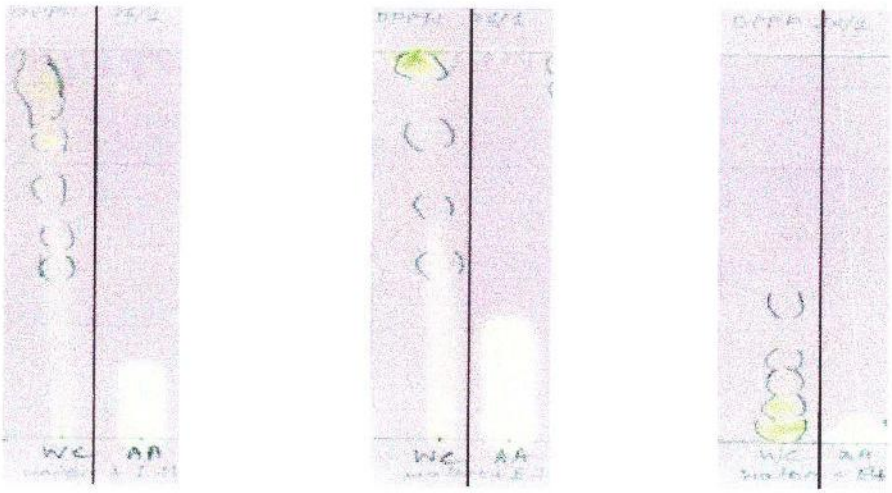
Figure 4.9: Comparison of TLC plates of crude ethanol extract of *E. fluctuans* with Standard (Ascorbic acid) after applying 0.02% DPPH.



E. fluctuans **Ascorbic acid** ***E. fluctuans*** **Ascorbic acid** ***E. fluctuans*** **Ascorbic acid**

($CHCl_3$: CH_3OH: H_2O =40:10:1) ($CHCl_3$: CH_3OH =5:1) (n-Hexane: Acetone = 3:1)

Figure 4.10: Comparison of TLC plates of n-Hexane fraction of ethanol extract of *Enhydra fluctuans* with Standard (Ascorbic acid) after applying 0.02% DPPH.



E. fluctuans **Ascorbic acid** ***E. fluctuans*** **Ascorbic acid** ***E. fluctuans*** **Ascorbic acid**

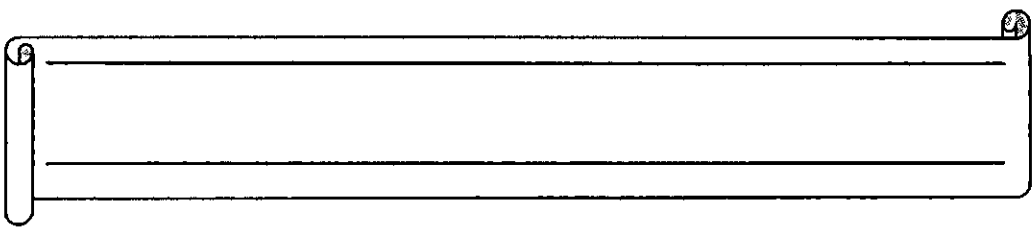
($CHCl_3$: CH_3OH: H_2O =40:10:1) ($CHCl_3$: CH_3OH =5:1) (n-Hexane: Acetone = 3:1)

Figure 4.11: Comparison of TLC plate of aqueous ethanol fraction of ethanol extract of *E. fluctuans* with Standard (Ascorbic acid) after applying 0.02% DPPH.

The result of DPPH scavenging activity assay in this study indicates that the plant is potently active and aqueous ethanol fraction provide greater antioxidant activity.

CHAPTER 5

DISCUSSION



CHAPTER FIVE
DISCUSSIONS

It is already reported that the polyphenolic compounds, as like phenolic acids, flavonoids and tannins, commonly found in different plants and exert multiple biological response, including antioxidant, antimicrobial, anthelmintic, antidiarrhoeal activity (Vinson *et al.*, 1995; Brown and Rice-Evans, 1998; Gil *et al.*, 1999; Kahkonen *et al.*, 1999; Prashant *et al.*, 2011; Halilu *et al.*, 2012).

The previous phytochemical screening of *Enhydra fluctuans* by Kuri *et al.* (2014) indicates the presence of carbohydrate, alkaloids, glycosides, flavonoids, tannin and saponin. Preliminary phytochemical screening of the present study revealed the presence of alkaloid, phenolic compounds, flavonoids, tannins, glycoside, steroids and saponin. The biological activities of this plant may be due to the presence of these various groups of chemical compounds (Vital and Rivera, 2011; Quideau *et al.*, 2011). So the result of the present study can correlate with the previous study.

This plant has proven its capability to scavenge the radical (DPPH) at low concentration, which may be due to the presence of phenol and alternative phyto-constituents in its crude extract and other fractions. The result of DPPH scavenging activity assay in this study indicates that the plant is potently active. This suggests that the plant extract contain compounds that are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radical's reactivity.

The ability of this plant extract to scavenge DPPH could also reflect its ability to inhibit the formation of ABTS+. This implies that the plant extract may be useful for treating radical related pathological damage (Wang *et al.*, 1998). Similar investigation was conducted with the Ethanol extract of *Enhydra fluctuans* and was evaluated for reducing power, total phenolic content, and the DPPH scavenging activity by Swain *et al.* (2012). In all the tests the ethanol extract was found to have higher antioxidant activity. Antioxidants are useful to cut back and forestall injury from radical reactions by donating electrons that neutralize the unconventional while not forming another. For example, vitamin C will lose associate degree negatron to a radical and stay stable itself by passing its unstable negatron round the inhibitor molecule (Seiler *et al.*, 2008).

The phytochemical analysis conducted on different fractions of *E. fluctuans* extract revealed the presence of alkaloid, tannins, flavonoids, steroids and saponins. Tannins are known to be useful in the treatment of inflamed or ulcerated tissues and they have remarkable activity in cancer prevention and anticancer (Ruch *et al.,* 1989; Motar *et al.,* 1985). Thus, *E. fluctuans* containing this compound may serve as a potential source of bioactive compounds in the treatment of cancer.

Flavonoids have been shown to exhibit their actions through effects on membrane permeability, and by inhibition of membrane-bound enzymes such as the ATPase and phospholipase A2 (Li *et al.,* 2003) and this property may explain the mechanisms of antioxidative action of *E. fluctuans*. Flavonoids serve as health promoting compound as a results of its anion radicals (Hausteen, 1983). These observations support the usefulness of this plant in folklore remedies in the treatment of stress-related ailments and as dressings for wounds normally encountered in circumcision rites, bruises, cuts and sores (Mathekga, 2001; Lourens *et al.,* 2004; Fergusion, 2001; Grierson and Afolayan, 1999).

Also, the plant extract was revealed to contain saponins, known to produce inhibitory effect on inflammation (Just *et al.,* 1998) and are major ingredients in traditional Chinese medicine and thus responsible for most of the observed biological effects (Liu and Henkel, 2002), and this tend to justify the use of *E. fluctuans* in traditional medicine. The plant extract was also positive for steroids which are very important compounds especially due to their relationship with compounds such as sex hormone (Okwu, 2001).

Alkaloid was also detected in this study plant. Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity (Nobori *et al.,* 1994), and their presence in this plant tend to increase the risk of poisoning by the plant.

According to the evaluation of Bhakta *et al.* (2009) and Amin *et al.* (2012), *Enhydra fluctuans* has antibacterial activity against *Escherichia coli*. But the present study was conducted on 4 strains of *Escherichia coli* and the results of antibacterial screening indicate that the plant has no antibacterial activity against these particular four *Escherichia*

coli strains. The obtained results may be due to personal errors, environmental factors and contamination. So, further study is needed to evaluate it.

In previously studies suggested that there was a direct relationship between antioxidant activity and total phenolic content found in the medicinal plants. Phenolic compounds had a major contribution to antioxidant activity (Lien *et al.*, 1997; Yang *et al.*, 2001). Phenolic compounds, secondary plant metabolites abundantly found in both edible and non edible plants possess biological properties of antioxidant, anti apoptosis, anti-aging, anti carcinogenic, anti-inflammatory, anti-atherosclerotic, cardiovascular protection, improvement of the endothelial function, as well as inhibition of oxidative damage of DNA (Ksouri *et al.*, 2009), angiogenesis and cell proliferation activity (Han *et al.*, 2007). Due to presence of hydroxyl groups (Mandal *et al.*, 2011), conjugated ring structures and carboxylic group (Hatano *et al*, 1989; Mandade *et al.*, 2011) phenolic compounds are capable to act as free radical scavenger (Ebrahimzadeh *et al.*, 2010), hydrogen donors, singlet oxygen quenchers (Hatano *et al.*, 1989; Rice-Evans *et al.*, 1995), metal chelator (Rice-Evans *et al.*, 1995), lipid peroxidator (Ksouri *et al.*, 2009) and other physiological properties. The most active dietary antioxidants belong to the family of phenolic and polyphenolic compounds so their possible usage in processed foods as a natural antioxidant (Zheng and Wang, 2001) have reached a new high in recent years (Mammadov *et al.*, 2011). The presence of the phenolic compounds in this plant contributed to their antioxidative properties and thus the usefulness of these plants in herbal medicament.

Among the many separation systems, the HPLC analysis include the use of a binary solvent system containing acidified water and a polar organic solvent were developed to specifically measure polyphenolic concentrations (Tsao and Yang, 2003; Sakakibara *et al.*, 2003). For method development, we had followed the method of Sarunya and Sukon (2006) with some modifications.

In this study we had used eleven different phenolic standards, C18 column with 250mm length, and rapid separation LC (RSLC) systems while the authors, Sarunya and Sukon used six standards, C18 column with 150mm length, and HP 1090, series II, liquid chromatography systems to determine the polyphenolic contents. 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. The wavelength between 210 and 380 nm was used for the detection of polyphenolic compounds (Cai *et al.,* 2004; Sakakibara *et al.,* 2003). Therefore, 280, 320 and 380 nm wavelength were selected for the appropriate wavelength for the detection of all standards in this study.

From the figure 4.5, it can be observed that a good separation can be achieved within 30 min using the above condition described. Symmetrical, sharp and well-resolved peaks were observed for the eleven polyphenolic standards. 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.

There are 7 ploy phenolic compounds that have been identified during HPLC analysis of three samples of *E. fluctuans.* These are – gallic acid, (+) catechin, egallic acid, vanillic acid, *p*- Coumaric acid, caffeic acid and kaempferol. The experimental results indicated that the crude ethanol extract, n-Hexane fraction and the aqueous ethanol fraction of *Enhydra fluctuans* contained a high concentration of ellagic acid (251.24 mg per 100 g of dry weight). It was found that catachin, vanillic acid and p-coumaric acid were present in the extract, however concentrations were detected fairly low (16.32 mg, 3.37 mg, & 0.97 mg/100 g of dry weight, respectively).

Catechin is a flavan-3-ol, a type of natural phenol and antioxidant. It is a plant secondary metabolite. It belongs to the group of flavan-3-ols (or simply flavanols), part of the chemical family of flavonoids (Zheng *et al.,* 2008). Catechin as well as their gallic acid conjugates is ubiquitous constituents of vascular plants, and frequent components of traditional herbal remedies (Nakai *et al.,* 2000).

Catechin was reported to induce longevity in the nematode worm *Caenorhabditis elegans* (Saul *et al.,* 2009). A Transcriptomic study shows that catechin reduces atherosclerotic lesion development in apo E-deficient mice (Auclair *et al.,* 2009). (+)-catechin seems to have stereospecific opposite effects on glycogen metabolism in isolated rat hepatocytes (Nyfeler *et al.,* 1983). (+)-Catechin inhibits intestinal tumor formation in mice (Weyant *et al.,* 2001). (+)-Catechin inhibits the oxidation of low densitylipoprotein (Mangiapane *et*

al., 1992). Incubation experiments with (+)-catechin show a prevention of human plasma oxidation (Lotito and Fraga, 1998).

Vanillic acid (4-hydroxy-3-methoxybenzoic acid) is a dihydroxybenzoic acid derivative used as a flavoring agent. It is an oxidized form of vanillin. It is also an intermediate in the production of vanillin from ferulic acid. Vanillic acid is one of the main catechins metabolites found in humans after consumption of green tea infusions (Lesage-Meessen *et al.,* 1996; Civolani *et al.,* 2000).

p-Coumaric acid is a hydroxycinnamic acid, an organic compound that is a hydroxy derivative of cinnamic acid. It is a crystalline solid that is slightly soluble in water, but well soluble in ethanol and diethyl ether. *p*-Coumaric acid has antioxidant properties and is believed to reduce the risk of stomach cancer (Ferguson *et al.,* 2005) by reducing the formation of carcinogenic nitrosamines (Kikugawa *et al.,* 1983).

Kaempferol is a natural flavonol, a type of flavonoid, previously that has been isolated from tea, broccoli, *Delphinium*, Witch-hazel, grapefruit, cabbage, kale, beans, endive, leek, tomato, strawberries, grapes, brussels sprouts, apples and other plant sources. Kaempferol is a yellow crystalline solid which is slightly soluble in water but soluble in hot ethanol and diethyl ether (Calderon-Montano *et al.,* 2011). Some epidemiological studies have found a positive association between the consumption of foods containing kaempferol and a reduced risk of developing several disorders such as cancer and cardiovascular diseases. Numerous preclinical studies have shown kaempferol and some glycosides of kaempferol have a wide range of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic, antiosteoporotic, estrogenic/antiestrogenic, anxiolytic, analgesic, and antiallergic activities (Calderon-Montano *et al.,* 2011).

Ellagic acid is a natural phenol antioxidant found in numerous fruits and vegetables. The antiproliferative and antioxidant properties of ellagic acid have spurred preliminary research into the potential health benefits of ellagic acid consumption. Ellagic acid is the dilactone of hexahydroxydiphenic acid (Ascacio-Valdés *et al.,* 2011). Ellagic acid has antiproliferative and antioxidant properties in a number of *in vitro* and small-animal models (Seeram *et al.,* 2005; Narayanan *et al.,* 1999). The antiproliferative properties of ellagic acid may be due to its ability to directly inhibit the DNA binding of certain carcinogens, including nitrosamines (Mandal *et al.,* 1988; Mandal and Stoner, 1990) and

polycyclic aromatic hydrocarbons. As with other polyphenol antioxidants, ellagic acid has a chemoprotective effect in cellular models by reducing oxidative stress (Vattem and Shetty, 2005). These properties have generated interest in potential human health benefits from the consumption of ellagic acid.

Gallic acid is a trihydroxybenzoic acid, a type of phenolic acid, a type of organic acid, also known as 3,4,5-trihydroxybenzoic acid, found in gallnuts, sumac, witch hazel, tea leaves, oak bark, and other plants (Reynolds and Wilson, 1991). Gallic acid is found both free and as part of hydrolyzable tannins (Fiuzaa *et al.,* 2004). Gallic acid seems to have anti-fungal and anti-viral properties. Gallic acid acts as an antioxidant and helps to protect human cells against oxidative damage. Gallic acid was found to show cytotoxicity against cancer cells, without harming healthy cells. Gallic acid is used as a remote astringent in cases of internal haemorrhage. Gallic acid is also used to treat albuminuria and diabetes. Some ointments to treat psoriasis and external haemorrhoids contain gallic acid (Nakai *et al.,* 2000).

Caffeic acid is an organic compound that is classified as hydroxycinnamic acid. This yellow solid consists of both phenolic and acrylic functional groups. It is found in all plants because it is a key intermediate in the biosynthesis of lignin, one of the principal components of plant biomass and its residues (Boerjan *et al.,* 2003).

Caffeic acid has a variety of potential pharmacological effects in *in vitro* studies and in animal models, and inhibitory effect of caffeic acid on cancer cell proliferation by oxidative mechanism in human HT-1080 fibrosarcoma cell line has recently been established (Rajendra Prasad *et al.,* 2011). Caffeic acid is an antioxidant *in vitro* and also *in vivo*. Caffeic acid also shows immunomodulatory and anti-inflammatory activity (Olthof *et al.,* 2001).

It is the first time when widely recognized bioactive polyphenolic compounds are detected in *E. fluctuans* extract. These biologically active polyphenols can be isolated by further separation and analysis of *E. fluctuans* extract. These compounds might be the reason behind the biological activities of *E. fluctuans* such as traditional remedies, antidiarrhoeal activities or anti inflammatory activities.

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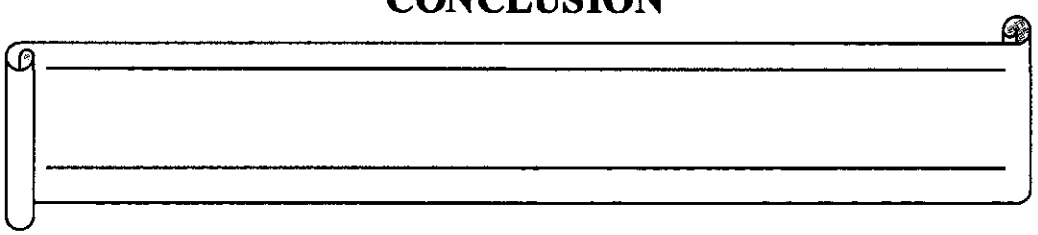
CHAPTER 6

CONCLUSION



CHAPTER SIX
CONCLUSION

In the present study, phytochemical test, antioxidant, antibacterial activity and TLC analysis have been carried out with ethanol extract of *Enhydra fluctuans.* Also, a HPLC method has been developed to identify the bioactive compounds in this plant extract. Phytochemical analysis showed the presence of alkaloid, tannin, flavonoid, saponin, glycoside and steroid which are the most valuable for therapeutic activity. The ethanol extract of *Enhydra fluctuans* Lour possess antioxidant activity and contains different polyphenolic components among them 7 compounds were identified. Further studies are required to isolate this bioactive compounds and should be directed to carry out *in vivo* investigation for the biologically active components in order to determine their exact mechanism of action and to improve nutritional profile and health benefits as well as to prepare natural pharmaceutical products of high value.

CHAPTER 7

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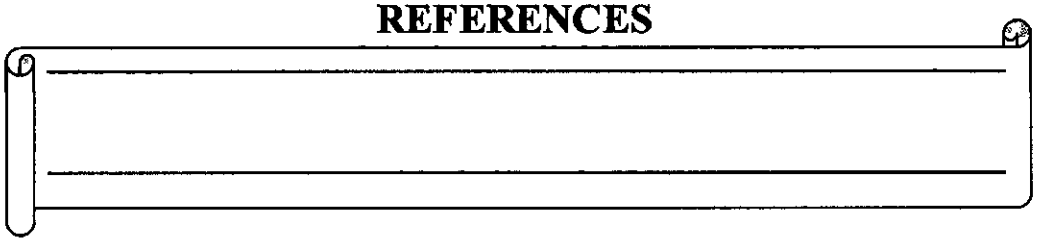

CHAPTER SEVEN

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