EFFECTS OF POLYPHENOLIC CONSTITUENTS OF *OCIMUM SANCTUM*, *OCIMUM BASILICUM* AND *AZADIRACHTA INDICA* LEAVES ON FOODBORNE PATHOGENS AND ANTIBIOTIC RESISTANT BACTERIA



By

SHAFQAT ULLAH

Dissertation Submitted to the University of Peshawar in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY IN

CHEMISTRY

INSTITUTE OF CHEMICAL SCIENCES

UNIVERSITY OF PESHAWAR, PESHAWAR

PAKISTAN

(December, 2015)

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DECEMBER, 2015

ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious and the Most Merciful

Alhamdulillah, all praises to Allah for the strengths and His blessing in completing this thesis. Special appreciation goes to my supervisor, ***Dr. Rasool khan,*** *For his invaluable help of constructive comments and suggestions throughout the experimental and thesis works have contributed to the success of this research.*

I would like to express my appreciation to the Voice Chancellor University of Peshawar, Dr Rasool Jan, Director Admission University of Peshawar Dr. Hizbullah, Chairman of Pharmacy Department, Dr. Zafar Iqbal, Director Institute of Chemical Science Dr Yousaf Iqbal, Prof. Dr. Imtiaz Ahmad, Dr. Asad Ullah, Dr. Waseem Hassan, Dr.Muhammad Imran and also for their support and help towards my postgraduate affairs. My acknowledgement also goes to all the technicians and office staffs of Mycotoxin laboratory PCSIR Peshawar as well Institute of Chemical Science for their co-operations, especially Dr. Arshad Hussain (SSO) PCSIR labs complex Peshawar and Izhar Khan.

Sincere expressions of deep regards are due to all my teachers, friends and staff members of this institute, for support during my study.

My deepest gratitude goes to my beloved parents; Mr. Muhammad Zafran (late) and *Mrs. Muhammad Zafran, my wife and two little angles Ammama Shafqat and Ammana Shafqat, also to my sisters and my cousins for their endless love and prayers. Last but not least my cousin Dr. Asad Ullah for his encouragement. To those who indirectly contributed in this research, your kindness means a lot to me. Thank you very much.*

Shafqat Ullah

Dte 19-5-17 Cont of Exam COP

DECLARATIONS

This is to certify that this dissertation prepared by Shafqat Ullah entitled "Effects of Polyphenolic Constituents of *Ocimum sanctum*, *Ocimum basilicum* and *Azadirachta indica* Leaves on Foodborne Pathogens and Antibiotic Resistant Bacteria" is accepted in the present form by the Institute of Chemical Sciences, University of Peshawar as fulfilling this part of the requirements for the degree

of

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1 INTRODUCTION

1.1 BRIEF DESCRIPTION OF PHENOLIC COMPOUNDS

Phenolic compounds are a diverse and abundant group of naturally occurring plant substances [1]. The term "phenolic" can be defined chemically as a substance possessing an aromatic ring bearing at least one hydroxyl substituent and functional derivatives like esters, glycosides, methyl ethers *etc*. These compounds have the ability to chelat metals, quench singlet oxygen, scavenge free radicals and break radical chain reactions. [2, 3]. Phenolic compounds exhibit biological activities like anti-bacterial, anti-fungal, anti-viral, anti-ulcer, anti-allergenic and anti-inflammatory [4-7]. These compounds also appear to be effective in certain kind of cancerous process and some cardiovascular [7, 8]. Phenolic compounds are also used as natural antioxidant or as food additive to preserve food for organoleptic and nutritional quantities [9, 10]. Phenolic compounds are the secondary metabolites of plant and are derived from phenylalanine, which is widely reported for their physiological and pharmacological role and their health benefits [11].

There are two types of plant metabolites primary and secondary. Primary metabolites play a vital role in the nutrition and essential metabolic process such as growth, development and reproduction. Carbohydrates, proteins and lipids are the example of primary metabolites. While, secondary metabolites plays important ecological roles between environment and plant, such as defensive compounds against pathogens and herbivores or as being pigments in plant leaves and flowers. There are more than thousand known and many unknown compounds. Phenolics, terpenoids and Alkaloids are the examples of secondary metabolites, and are also called non-nutritive

plant chemicals [12, 13]. These plant chemicals protect plant's cells from environmental hazards such as UV exposure, stress, drought, pollution and pathogenic attacks. Plant reduces these chemicals to protect themselves, humans and animals against diseases [14, 15].

From the previous research, it is cleared that these secondary metabolites have a great impact in human and animal health, although these compounds are not essential nutrients or not required by the human body to sustain life, but having a great role to fight against many common diseases. Because of these properties many researchers have been performed to reveal the beneficial health effect of these compounds [16, 17].

1.2 CLASSIFICATION OF PHENOLIC COMPOUNDS

It is estimated that more than 8000 structures are currently known belongs to phenolic having a common structure features, an aromatic ring with at least one -OH hydroxyl substituent [18]. Phenolic compounds have more than dozen of classes and can be divided into different classes based on their chemical structures, but mainly classified into simple phenols and polyphenols. The simple phenols are phenolic acid possessing one carboxylic acid either in the form of hydrobenzoic C_6-C_1 or hydrocinnamic acid C_6-C_3, while the polyphenol are flavonoids consisting of 15 carbon atoms arranged in three rings C_6-C_3-C_6 [19].

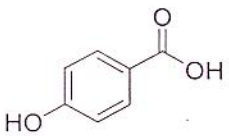
1.2.1 PHENOLIC ACIDS

Simple phenols are categorized into hydrobenzoic acid derivatives and hydrocinnamic acid derivatives. Hydrobenzoic and hydrocinnamic acid have similar

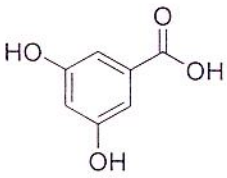
backbones of an aromatic ring with variation in the hydroxylation and methoxylation pattern in structure.

1.2.1.1 Hydroxybenzoic acids

The hydrobenzoic acids C_6-C_1 are simple phenols that possess carboxylic acid –COOH functionality along with hydroxycinnamic acid, constitute the phenolic acid. Variation in the hydroxybenzoic acids occurs due to differing patterns of methoxylation and hydroxylation of their aromatic rings [20]. Hydroxybenzoic acids are present nearly in all plants. Common hydroxybenzoic acids are p-hydroxybenzoic, 3, 5-dihydroxybenzoic acid, salicylic, gallic, vanillic and syringic acids.



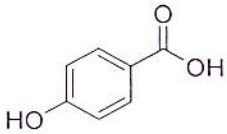
p-Hydroxybenzoic Acid



3, 5 Dihyrobenzoic acid



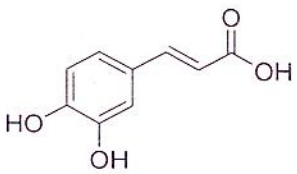
Gallic Acid



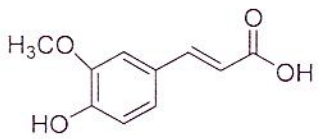
p-Hydroxybenzoic Acid

1.2.1.2 Hydroxycinnamic acids

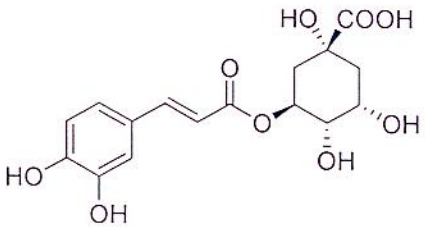
The hydroxycinnamic acids (C6-C3) largely consist of cinnamic acid derivatives and coumarins, collectively known as phenylpropanoids. Hydroxycinnamic acids are widely distributed acids in plants. Common cinnamic acids are p-coumaric, caffeic, ferulic, sinapic and chlorogenic acids [21].



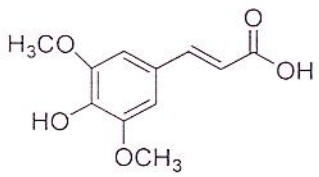
Caffeic Acid



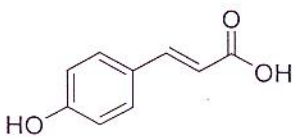
Ferulic Acid



Chlorogenic Acid



Sinapic Acid



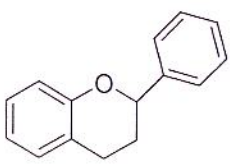
p-Coumaric Acid

These compounds are found in many species of plants and have many pharmacological properties in vitro. Several authors demonstrated the antimicrobial and antioxidant activities of phenolic acid. Some of them are also active against Human Immunodeficiency Virus (HIV), colon cancer and prostate cancer. According to previous study phenolic acid also have analgesic, diuretic, anti-inflammatory and

anti-ulcer activities [22-24]. Phenolic acids were found to be active against antioxidant, antimicrobial, Cancers, Human Immunodeficiency Virus (HIV), Endothelial Dysfunction, Analgesic activity, High-Density Lipoprotein (HDL), Atherosclerosis, Colon cancer, Diuretic Activity, Anti-inflammatory, Prostate cancer, Ulcers [11, 25-27]. Now a day's these phenolic acids are available commercially in the form of dietary supplement such as green tea extracts and grape seed extracts which contain in sufficient amount. These products are marketed as antioxidants, but the scientist and researcher suggests that eating fresh fruits, vegetables and grains is more beneficial than these antioxidant supplements.

1.2.2 FLAVONOIDS

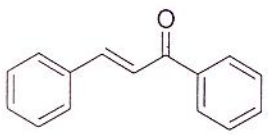
Flavonoids is an important and largest class of phenolic compounds, generally known to be present in plants and plant base products, associated with a broad spectrum of health-promoting effects. Flavonoids chemically may be defined as a group of phenolic compounds consisting of substances having two substituted benzene rings connected by the chain of three carbon atoms and an oxygen bridge as shown in figure. On the basis of skeleton flavonoids can be classified into six major subgroups.



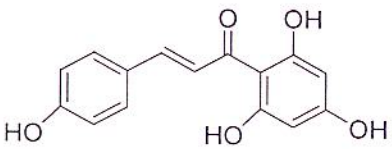
Basic Flavonoid skeleton

1.2.2.1 Chalcones

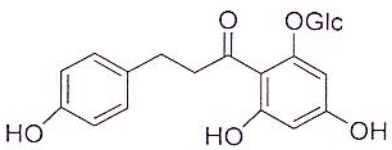
Chalcones is a subclass of flavonoids they are characterized by the absence of Carbon-ring of the basic flavonoids skeleton and can also be referred as open chain flavonoids. Chalcones mainly include Arbutin, Phloridzin, Chalconaringenin and Phloretin. These compounds occur in significant amount in pears, bearberries, strawberries, tomatoes and certain wheat products. These compounds have garnered considerable attention because of numerous biological and nutritional benefits [28, 29].



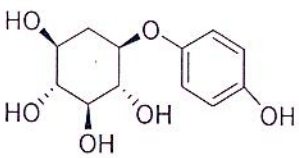
Chalcone skeleton



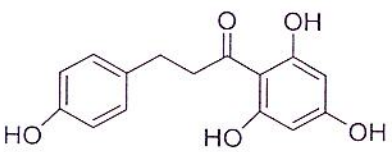
Chalconanringenin



Phloridzin



Arbutin

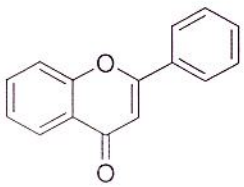


Phloretin

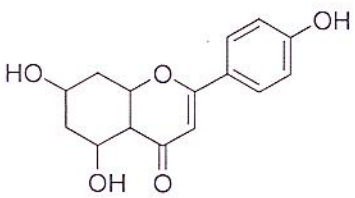
1.2.2.2 Flavones

Flavones are one of the important subgroups of flavonoids, widely present in plants leaves, flowers, and fruits as glucosides. luteoline, apigenin and tangeritin belong to this sub class of flavonoids.

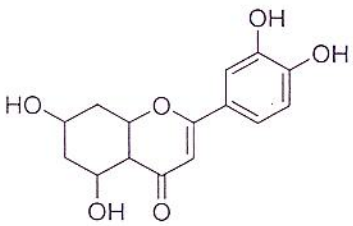
Apigenin, Tangeritin and Luteolin belong to this sub-class of Flavonoids. Chamomile, ginkgo biloba, celery, parsley, mint and red peppers are the major sources of flavones which have various biological functions [30, 31].



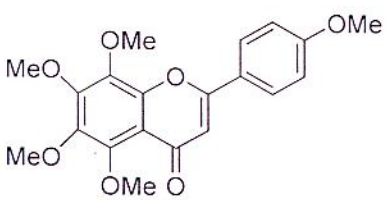
Flavanone



Apigenin



Luteolin



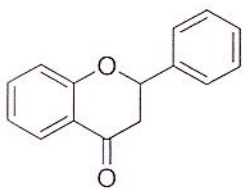
Tangeretin



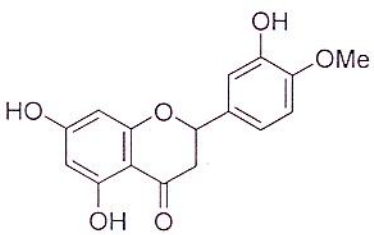
Baicalein

1.2.2.3 Flavanones

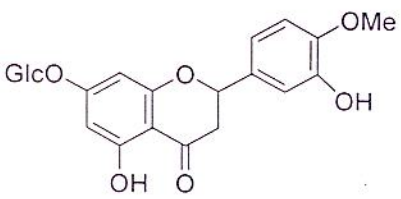
Flavanones are another important subclass of flavonoids associated with a number of health benefits because of their cholesterol lowering, anti oxidative and anti-inflammatory properties. Naringenin, Eriodictyol and Hesperidin are the examples of this class, which is generally present in all the citrus fruits. These compounds are responsible for the bitter taste of citrus fruits [32, 33]. Structures of some commonly studied Flavanones are as under.



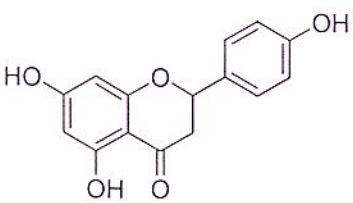
Flavavone



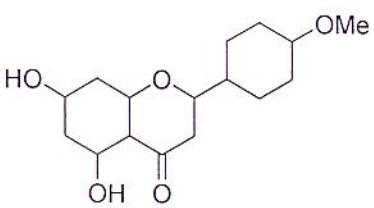
Hesperetin



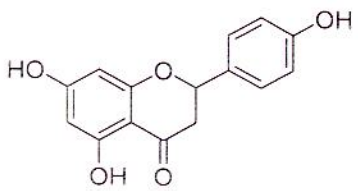
Hesperidin



Naringenin



Naringin



Eriodictyrol

1.2.2.4 Flavanols

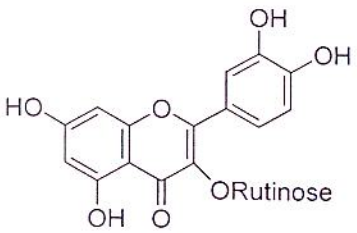
Flavanols are flavonoids contain ketone group associated with wide range of health benefits including anti-inflammatory, anti-oxidative potential and reduced risk of vascular diseases. The most studied flavanols are quercetin, Fisetin, Myricetin and Kaempferol. Apple, grape, kale, tomato, onions and barriers are the good sources of flavanols [34, 35].



Flavonol



Quercetin



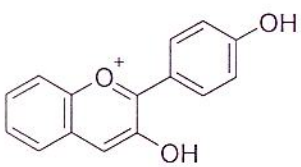
Rutin



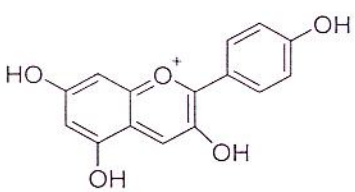
Myricetin

1.2.2.5 Anthocyanins

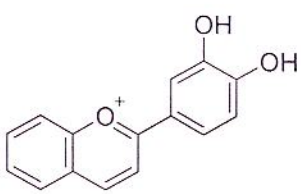
Anthocyanins are the sub group of flavonoids. Peonidin, Malvidin, Pelargonidin, Delphinidin and Cyanidin are the most studied compounds in the sub group of Anthocyanins. These compounds are reported anti oxidant, anti bacterial, anti inflammatory and anti carcinogenic activities [36]. They also exhibit significant effects on blood platelets and blood vessels and reduce the risk of coronary heart diseases. Black berry, black current, raspberry, strawberry, blue beery, bilberry, cranberry and red grape are the good sources of Anthocyanins [37].



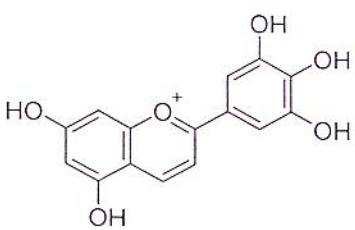
Anthocyanins



Pelagonidin



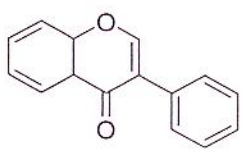
Cyanidin



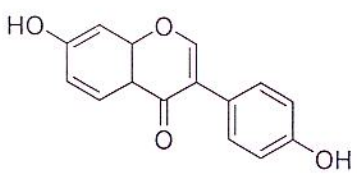
Delphinidin

1.2.2.6 Isoflavonoids

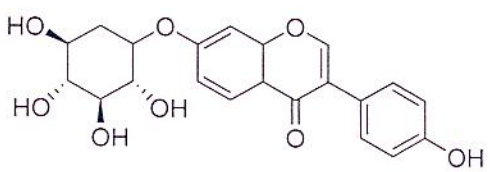
Isoflavonoids are the large and distinctive subgroup of flavonoids exhibit tremendous potential to fight a number of diseases. Genistein, Genistin, Daidzein, Diadain and Glycitein are the most studied compound of this sub group posses numerous biological properties. For example they play an important role in protecting of breast cancer, prostate cancer and help in preventing hinder cell growth. In addition consumption of these compounds reduce the buildup of arterial plaques, which further help in the reduce risk of cardiovascular complaints and coronary heart diseases; they may also relieve menopausal symptoms [38, 39]. Genistein and Daidzein are examples of key Isoflavonoids.



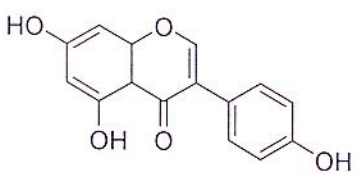
Isoflavoniods



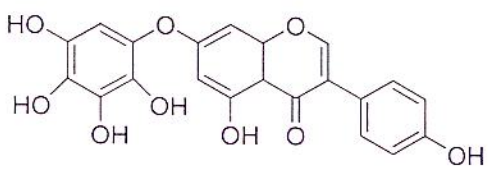
Daidzein



Daidzin



Genistein



Genistin

1.3 BRIEF DESCRIPTION OF PLANTS USED IN THE PRESENT STUDY

In recent years, the nutraceutical and phytochemical constituents of the plants have gained much scientific interest due to their biological and antioxidant activities. In the last two decades, the scientists recognized the value of the plants, vegetables and fruits, not only as a food sources but also due to their potential medicinal benefits. Phenolic are broadly dispersed in the plant kingdom however, the presence and concentration of these of these compounds varies even in same plant at different ripening stages and organs of the same plant. Some plants are found to be an excellent source of phenolic compounds and suggested to use for preservation of food as well as to get health benefits. [40]. These compounds such as syringic, sinapic, ferulic, p-coumaric acids and flavonoids are considered to be powerful antioxidants [41].

1.3.1 *OCIMUM SANCTUM*:



Figure 1: *O. sanctum* Plant and Leaves

Botanical Name	:	*Ocimum sanctum*
Family	:	Lamiaceae
Species	:	Ocimum tenuiflorum
Genius	:	Ocimum
Part used	:	Leaves

Ocimum sanctum (*O. Sanctum*) also known as *Ocimum tenuiflorum*, 'Holy basil' in English and 'Tulsi' in Hindi is an aromatic plant belongs to the family Lamiaceae. *O. sanctum* is one of the rich nutritive members of the family as discovered by various researcher, even the same genus do not possess the same chemical constituents.

It is native throughout the Eastern world and is wide spread as cultivated plant. *O. sanctum* grows up to 60cm high with red and purple sub quadrangular branches. Leaves of the plant are simple, hairy and serrate, flowers are purple in color and the fruits are smooth and very mucilaginous when wetted. It is propagated by means of seeds. The seeds directly planted in the ground. Young plants can be transplanted when they attain up to 10cm height [42, 43]. Different parts root, stem, leaves, flower and even whole plant of *O. sanctum* have been recommended for the treatment of

infectious diseases like bronchitis, bronchial asthma, diarrhea, malaria, dysentery, arthritis, skin diseases and chronic fever in traditional system of medicine [44, 45]. It has been also suggested to posse's antioxidant, antimicrobial, anticancer activities and effective in a cardio protective, hepatoprotective, adaptogenic and analgesic properties.

The leaves of *O. Sanctum* are very effective in lungs, intestinal and cardiovascular diseases. The extracts of leaves are useful in skin disorders, pain, swelling and headache. The seeds are effective in reducing blood sugar and cholesterol [46, 47].

1.3.2 *OCIMUM BASILICUM:*



Figure 2: *O. basilicum* Plant and Leaves

Botanical Name	:	*Ocimum basilicum*
Family	:	Lamiaceae
Species	:	Ocimum *basilicum*
Genius	:	Ocimum
Part used	:	Leaves

Ocimum basilicum (*O. basilicum*) in Urdu Basil belongs to family Lamiaceae family, used as an ornamental and culinary. The genus *Ocimum* contains more than

hundred species of shrubs and herbs in the tropical regions of Asia, south/central America and Africa. *O. basilicum* or sweet basil grows to a size of 1-2 feet in height. It prolifically produces large green leave around 2 inches in length and greenish throughout the summer.

Some of the *basilicum* cultivars such as Dark Opal have purple color leaves. The purple color is due to the presence of anthocyanins and small amount of peonidin compound. These cultivars are considered a potential source of red pigments for the food industry. Other basil cultivars include lemon, Italian, curly and lettuce-leaf basil, the names of these cultivars give way to their variance [48]. The *O. basilicum* has been used as a traditional medicinal plant in the treatment of diarrhea, coughs, headaches, worms, warts, constipation and kidney malfunctions [49, 50]. The plant is also used in food and oral care products. *O. basilicum* is a good source of magnesium, which promotes muscles and blood vessels and also helps in cardiovascular health, thus improve blood flow and reduce the risk of irregular heart rhythms or spasming of heart muscle or blood vessel. Natural components from the aqueous extract of *O. basilicum* have long been extensively used to flavor food products, oral products, dental products and in the treatment of constipation, diuretic, headaches, diarrhea, coughs, galactagogue, intestine ache, worms, kidney, and warts [51, 52].

1.3.3 *AZADIRACHTA INDICA*:



Figure 3: *A. indica* Plant and Leaves

Botanical Name	:	*Azadirachta indica*
Family	:	Meliaceae
Species	:	*A. indica*
Genius	:	*Azadirachta*
Part used	:	Leaves

Azadirachta indica (*A. indica*) is known by Urdu name Neem, is a member of mahogany family Meliaceae. *A. indica* trees are fast growers and estimated that grow to 20 feets within three years from seed planting. It mostly grows in the areas where rainfall is only 18 inches per year and has productive lifespan of 150-200 years. It is mainly cultivated in Asia, Africa, central and south America and especially in the Indian subcontinent, while the small scale plantations of *A. indica* are also reported in Europe and United States of America. The world's largest plantations of *A. indica* on 10sq kilometer in the plains of Arafat, Saudi Arabia. 50,000 Neem trees planted by Saudi philanthropist to shade and comforts to the two million pilgrims [53, 54].

The tree is considered incredible and has been declared by the United Nation as "Tree of the 21st century". The National Academy of Science US published a

report entitled "Neem: A tree for solving global problems" in 1992. In the beginning of history, *A. indica* has been extensively used to treat various ailments by mankind [55, 56]. Different parts (roots, stem, bark, leaves, flower, fruits, and seeds) of the *A. indica* have been used traditionally for the treatment of infections, inflammation, fever, dental disorders and skin diseases [57-59]. More than 140 active compounds have been isolated from *A. indica*, including phenolic compounds, alkaloids, triterpenoids, flavonoids, carotenoids, ketones and steroids. These chemical constituents possess antioxidant, antimicrobial, antiviral, antimutagenic, antihyperglycaemic, antiulcer and anticarcinogenic and anti-inflammatory properties [60-63].

1.4 EXTRACTIONS OF PHENOLIC COMPOUNDS

The extraction of phenolic compounds from a wide range of the sample mostly depends on the nature of sample and chemical properties of phenolic group. The yield of phenolic may influence on several parameters like temperature, extraction time, solvent-to-sample ratio, solvent type as well as the number of repeat extraction of the sample. Different extraction solvents and procedures are reported in literature for extraction of phenolic compounds [64, 65]. A number of reports in the previous literatures shown that ethanol and methanol are more efficient for extraction of a variety of phenolic compounds. Some literatures depicted the efficacy of aqueous methanol and acetone to extract phenolic compounds from plant material [66, 67]. However a single solvent may not be able to extract all types of phenolic compounds from plant material. The Plant sources contained several thousand secondary

metabolites, needs to develop standard procedures for the extraction of these compounds in order to increase the yield and commercial appeal. Previous researchers have employed a variety of extraction procedures influences the yield of phenolic extracted. Some of the commonly used methods include Microwave Assisted Extraction (MAE), Ultra sonic Assisted Extraction (UAE), Supercritical Fluid Extraction (SFE), Liquid Liquid Extraction (LLE), Accelerated Solvent Extraction (ASE) and Solid Phase Extraction (SPE) [68]. For instant the most promising extraction of phenolic compounds is being carried out using traditional method ultrasonic and orbital shaker.

1.4.1 ORBITAL SHAKER EXTRACTION (OSE) PROCEDURE

In addition to selecting the optimal extraction procedure, there are two main parameters time and temperature which affect the yield of phenolic compounds. Although high temperature and extended time extraction also not favorable because plant phenolic are generally degraded and undergo undesirable reaction [69].

Over the years the liquid extraction has been adopted by a number of researchers for a wide variety of phenolic compounds from numerous sources. Researchers have shown that phenolic can be extracted with methanol, ethanol, water and combination of these [70, 71]. There are conflicting reports in the literature in selection of best extraction solvent for phenolic compounds to use in this procedure.

1.4.2 ULTRASONIC EXTRACTION (USE) PROCEDURE

Ultrasonic Extraction (UAE) involves passing waves of ultrasonic energy through a liquid solvent containing the solid particles. Ultrasound has been used for emulsification, cleaning of equipment, accelerated drying and freezing, preservation and most recently for extraction of various compounds [72]. Extraction is carried out

using an ultrasonic bath or ultrasonic probe system when compared with other extraction method it is relatively cheap and has low instrumental requirements. Herrera, M, et al used USE to extract phenolic compounds form wheat [73]. They used a response surface design to determine the optimum condition for extraction based on total phenolic content. The optimized conditions were 60°C for 25minutes with 64%. Additionally Rostagno et al., (2002) Extract phenolic from soybean by USE method. They found the maximum recoveries when using 60°C for 20min extraction with 50% ethanol. It was reported that time of extraction was the most significant factor influencing extraction efficiency and the maximum recoveries was 406mg/l using USE method for 60minutes [74].

1.5 IDENTIFICATION OF PHENOLIC COMPOUNDS BY GC-MS

1.5.1 GAS CHROMATOGRAPHY

GC is an analytical technique in which gas is used as a mobile phase. This technique is mostly used for the compounds, which is volatile and stable at the temperature employed by system. But there are several other non volatile compounds, which can also be separated by GC but need to be derivatized before GC separation [75, 76]. The main purpose of the mobile phase (Gas Carrier) in GC system is to transport volatile substances through column. Helium, Nitrogen and Hydrogen are the most common carrier gases. These all are inert and cannot react with sample or the stationary phase. The samples analyzed by GC are mostly liquid form which are injected with syringe of 1μl or less or may by auto sampler.

1.5.2 DETECTORS

There are several detector which can be employed, some of most common detectors were Flame Ionization Detector (FID) and Thermal Conductivity Detector (TCD), but form the last two decades Mass Spectrometry (MS) has increased its impact and is nowadays the most commonly used detect in combination with gas chromatography [77].

1.5.3 MASS SPECTROMETRY

Mass spectrometry consist of an ion source, a mass analyze and detector. Electron ionization (EI) is one of the most frequently used ion source. When the separated compound enter the EI ion source the bombardment of electron hits the molecule resulting in formation of charged molecules, that's enter the mass analyzer by the force of electrically charged lenses. Inside the ion source both positive and negative ions are formed, however it is not common to detect negative ions due to increase sensitivity for positive ionization mode. Fragmentation is the result of the excess energy in molecules.

$$M + e^- \rightarrow M_o^+ + 2e^-$$

$$M + e^- \rightarrow [M - X]^+ + X_o + 2e^-$$

The purpose of MS is to separate the ions based on their mass to charge ratio (m/z) and to detect the quantitatively [78].

1.5.4 Analysis of phenolic compounds

To make the phenolic compounds compatible with chromatographic analysis, derivatization is often needed. Derivatization is performed prior to GC, to convert temperature labile compounds suited for GC analysis [79].

There are three common type of derivatization for GC, Silylation, Alkylation and Acylation. Silylation is one the most commonly used for phenolic compound on GC. This method is also used in this thesis. By the Silylation, these compounds become more volatile and thermal stable then original compound. The trimethylsilyl (TMS) group replaces the active hydrogen on the compound.

However a great verity of other Silylation reagent trimethylchlorosilane (TMCS), N,O-bis (Trimethylsilyl) acetamide (BSA), N-trimethylsilyimidazole (TMSI), hexamethyldisilazane (HMDS) and N,O-bis (trimethylsilyl) trifluroacetamide (BSTFA) etc are also available.

1.6 QUANTIFICATION OF PHENOLIC COMPOUNDS BY HPLC

1.6.1 HPLC THEORY

In liquid chromatographic separation, the analyte separate on the bases of their differential partitioning between two non miscible phases, i.e. the mobile phase and the stationary phase. The stationary phase is either a solid, porous or surface active material. The mobile phase is a liquid that carries the analyte to be separated.

1.6.2 HPLC SEPARATION MODES

Different HPLC modes have been developed for the separation of a wide range of analytes. The common modes include: Reverse Phase Liquid Chromatography (RPLC), Normal Phase Liquid Chromatography (NPLC), Non Aqueous Reverse

Liquid Chromatography (NARP), Ion Exchange Chromatography (IEX), Size Exclusive Chromatography (SEC), Hydrophilic Interaction Chromatography (HILIC) and Ion Pair Chromatography (IPC). In current study only RPLC will be addressed in following section.

1.6.2.1 Reverse Phase Liquid Chromatogrophy (RPLC)

RPLC is the dominant mode of HPLC used for the separation of mixtures of organic compounds. In RPLC the column acts as nonpolar retaining stationary phase, packed with derivatized silica and the binary mobile phase consists of an aqueous phase and an organic phase. Methanol and Acetonitrile are the preferred organic solvents, whilst Tetrahydrofuran and Isopropanol may also be used, while aqueous phase may be slightly acidified.

1.6.3 HPLC INSTRUMENTATION

The general components of an HPLC system are discussed in this section in detail. The mobile phase in a reservoir is drawn via a pump, which generates sufficient pressure to drive the mobile phase through column. The auto sampler is used for injecting the specific amount of sample in to the column. The separation column is typically placed within the column oven. The oven regulates and controls the temperature of column. The detectors are used to responds the changes in column effluent composition and the attached data system monitors the output of the detector and processed the data.

1.6.3.1 Solvent Reservoir, Filtration And Degassing

In isocratic elution system a single reservoir is required using a premixed mobile phase. But for gradient application or online blended mobile phase, more than one reservoir is used. The mobile phase must be filtered through polyamide membrane to free from particulate matters prior to filling to reservoir. The mobile phase is also degassed prior to use because the air bubbles may creates problems in the operation of HPLC system. Degassing may be done via ultrasonication.

Beside selectivity the selection of mobile phase is based on criteria such as purity, boiling point, viscosity, UV transparency, toxicity, price, and inertness with respect to sample analytes [79].

1.6.3.2 Gradient Systems in HPLC

The HPLC pump unifies two features: it must be capable of generating high pressure and it must deliver flow accuracy and precision at the selected flow rate. In isocratic elution system the composition of mobile phase kept constant during chromatographic separation process. However the complex samples cannot be accomplished at isocratic system so gradient elution is usually preferred. In gradient elution system, the concentration of mobile phase altered. The increase of strong mobile phase leading to an in the elution strength of mobile phase and a reduction in analyte retention. Which reduces peak tailing effect and prevent peak broadening [80].

1.6.3.3 Autosamplers and injection systems

The latest HPLC mostly equipped with auto samplers. Auto samplers introduce the sample in to the column. The sample injection to the column requires a measured quantity of the sample to the flowing and pressurized mobile phase which is

not accompanied by manual injection of sample to HPLC. The sample injection must be accurate, precise and automatic so auto sampler should be required.

1.6.3.4 Stationary phase or column

The column in HPLC may be considered as the 'Heart' of HPLC system. The separation of analytes takes place in column. For analytical purpose columns are mostly made of stainless steel with different column length of 50-250mm and diameter of 2.0 to 4.6mm. The most common support for the packing of HPLC column is silica, either in the form of particles or monoliths. Silica based columns provide higher efficiency as compare to other inorganic support materials such as alumina, zirconia, titinia and graphitized carbon.

1.6.3.5 Column ovens

The HPLC column situated in column oven, which plays an important role in HPLC efficiency, selectivity and retention. The use of column oven is recommended for maintained of constant column temperature. It is also plays an important role in preheat of mobile phase to the temperature of column. The preheating of mobile phase is a particular concern during High Temperature Liquid Chromatography (HTLC), if the mobile phase does not preheat; a simple preheated may be connected to the column inlet.

1.6.3.6 Detectors

Detector may be defined as transducer which converts a chemical property of an eluted analyte into electrical signals. HPLC detector is positioned directly after column. Some important characteristics of an ideal HPLC detector: high sensitivity

and predictable response, predictable specificity, respond to all solutes, be reliable and convenient to use, be unaffected by changes in temperature and carrier composition, be robust and cheap, be non destructive, provide linear response and qualitative information on the detected peak. Some common detectors used in HPLC: Ultra Violet Visible Detector (UVD), Florescence Detector (FLD), Electrochemical Detector (ECD), Diode Array Detector (DAD) and Mass Spectrometric Detector (MSD). The bulk detector or universal detector which measures the properties that is common to all compounds are Light Scattering Detector (LSD), Refractive Index Detector (RID) and Corona Discharge Detector (CDD). The sample specific detectors which can measure a unique sample characteristic include FLD, UVD and ECD as well as conductivity, radioactivity and chiral detector. The most common sample specific detector is UV. Thus special attention will devoted to UV-VIS detector and its application in the characterization of phenolic compounds in section 1.6.3.7.

1.6.3.7 UV-Visible detector

UV-Visible is one the most commonly used detector in HPLC for the detection of wide range of compounds and based on the absorption of light in the UV region and Visible region (190-700nm). The light source is a deuterium lamp, provides acceptable light intensity form 190-400nm and when measurement at visible wavelength (400-700nm) are required a higher energy tungsten halide lamp is often used. UV detectors are ideal for use with gradient and isocratic elution. It capable of high sensitivity and relatively insensitive to changes in mobile phase flow and temperature. The lamp light passes through UV transmitting flow cell connected to column and is transmitted on to a diode that measures the light intensity (I).

1.7 HPLC METHOD DEVELOPMENT FOR QUANTIFICATION

The review in this section will give overview of the basic principles regarding HPLC coupled with UV-Vis detector. Special attention will be devoted to the development of HPLC method for the separation of phenolic compounds. Furthermore, the method validation parameter and their importance will also be discussed.

1.7.1 METHOD DEVEOLPMENT

The key components for HPLC method development are; sample preparation (pH, %organic, sample age, sample size and shaking/sonication), analysis condition of HPLC; (pH, %organic, temperature, flow rate and wavelength), and standardization (standard concentration, wavelength, integration and response factor correction).
For method development, these parameters must be investigated, which is challenge for scientists to evaluate each parameter critically and streamline the final method optimization. In this approach, these critical components for a HPLC method development will first be investigated individually.

In reverse phase liquid chromatography mobile phase composition play an important role in separation, Methanol (MeOH), Acetonitrile (ACN) and tetrahydrofuan (THF) are commonly used solvents having low UV cut-off of 190, 205, and 212nm respectively. These solvents are easily miscible with water. During method development, mixture of water and ACN is the best initial choice for optimization of mobile phase. Selection of column is one of the parameter for method development. In RP-HPLC separation the C-18 column packed with C8 C-bonded silica are used for separation of a wide range of organic compounds.

Selection of detector is one the important parameter for method development. Various detectors included UV/visible, PAD, FLD, MSD *etc.*

HPLC method development and validation are key elements to identify and quantifying compounds of interest.

1.7.2 METHOD VALIDATION

Validation parameters are well defined by the international organization for standardization (ISO). The results of a developed method does not touch the highest and lowest level and will be in the range of IUPAC [81]. HPLC method can be validated for qualitative, semi quantitative (1-5ppm) and quantitive (>5ppm). The various validation parameters are linearity, LOD, LOQ, accuracy and precision.

1.7.2.1 Linearity

The linearity is one of the important parameter for method validation. Linearity of an analytical procedure is its ability to obtain test results that are directly proportional to the concentration of analyte. Generally, a value of correlation coeffient R^2 >0.998 is an evidence of an acceptable fit of the data to the regression line [82]. Linearity is determined by the series of three to six injection of standard. The peak areas of the calibration standard are plotted against the standard concentration and the value of co-relation coefficient determined from the linearity of the plotted curve. For linear range the positive and negative deviations should be equally distributed.

1.7.2.2 Accuracy

Accuracy for a development of new method is defined by ISO as "closeness of agreement between a measured quantity and a true value of a measurement" it is

qualitative characteristic that can be expressed as numerical value. Accuracy has inverse relation to errors, higher accuracy means lower errors.

Accuracy of an analytical procedure is evaluated by analyzing different concentration of drug. For accuracy a known amount of relative substances and drugs are spiked with standard and determined in triplicate. The accuracy was determined by comparing the found concentration with the added concentration [82].

1.7.2.3 Precision

Precision define as the closeness of results between a series of measurements from multiple analysis of the same sample under the prescribed condition. Precision of analysis measures by the same operator in one laboratory on same equipment. Precision may be considered as reproducibility and repeatability. Reproducibility expresses precision of analysis by different analysts in different laboratories and environmental condition but is still within the specified parameter of the method. The objective of precision is to verified the method despite differences in humidity, room temperature, operators, different characteristic of equipments, variation in material and instrumental conditions (e.g. in HPLC, Mobile phase, flow rate of mobile phase, pH, column), reagents, consumables, solvents and other material of different ages and different quality. ICH recommends a minimum of nine determinations covering the specific range for the procedure for evaluation of repeatability which should be reported as standard deviation or relative standard deviation.

1.7.2.4 Selectivity and Specifcity

Selectivity and specificity of an analytical method is defined by the ISO as "property of a measuring system, used with a specified measurement procedure. Specificity and selectivity are used interchangeably to describe the same concept in

method validation. The specificity of an analytical procedure is determined by comparing results obtained of samples containing impurities with those obtained from analysis of samples without impurities. Specificity can be demonstrated by resolution between the analyte peak and the other closely eluting peak.

1.7.2.5 Limit of Detection and Quantification

Limit of detection (LOD) of an analytical procedure is the lowest concentration of an analyte which can be detected but cannot be quantified as an exact value. Whereas limit of quantification (LOD) can be define as the lowest amount of analyte in a sample which can exactly quantified with precision and accuracy.

ICH guidelines recommend different method for determination of LOD and LOQ including visual evaluation method, which may be used for both instrumental and non instrumental method. By this method LOD and LOQ is determined by diluting the known concentration of analyte to minimum level at which the analyte can be detected and quantified with acceptable accuracy and precision [83].

A method which is extensively used for LOD and LOQ using HPLC is S/N ratio. This method can only be applied to analytical procedure which exhibit base line. A S/N ratio of 3:1 is acceptable for estimation of LOD (RSD≤10%) whereas for LOQ the S/N ratio of 10:1 is appropriate with RSD≤3%.

1.1 FOOD BORN PATHOGENS:

Majority of bacteria are harmless even beneficial, few types of them are pathogenic which causes different diseases, such as pneumonia, tetanus, diphtheria, typhoid fever, leprosy, syphilis and other foodborne illnesses. There are more than

hundreds of naturally occurring biological pathogens and 80,000 chemicals, heavy metals, parasites and toxins that cause serious illnesses, although these diseases can be control through antibiotics, fungicide and vaccination [84]. Foodborne diseases are an increasingly serious public health problem all over the world [84]. Aspergillus flaws and Aspergillus parasiticus is one of them cause's serious infections are briefly described in this section.

Food and food product are vulnerable to attack by fungi that produces toxin metabolites. *Aspergillus flavus* and *Aspergillus parasiticus* produces toxic metabolites of Aflatoxin. Aflatoxins are a group of highly oxygenated heterocyclic compounds with closely related structure. Aflatoxin can be produces on crops in the field and during storage, especially under warm and moist condition. These are highly toxic and suspected carcinogen [84]. Aflatoxins were discovered in 1960s in Great Britain, when thousand of turkey poults died with severs lesions of the liver. The disease was traced in contaminated Brazilian peanut meal used in poultry feed. These toxic substances can persist at high temperature for long time even the fungi have been killed and contaminate foods. These compounds are stable that are not destroyed during food processing and cooking [85].

1.2 ANTIBIOTICS RESISTANCES

The word "antibiotic" is derived from Greek words 'anti' means against and 'bios' means life and define that a substance which kills or inhibits any living organism. However according to medical definition antibiotic is a substance which is produce by a living organism which kills bacteria [86].

For example penicillin: among the 1st antibiotic to be investigated to use as medicine was named and characterized by Alexander Flaming in 1928 [87]. Penicillin is produce naturally by the *Pencillium chrysogenum* and *Pencillium notatum*. It's also called antimicrobial. The word antimicrobial comes from Greek words anti mean against, micro means small and bios mean life, define any substance which inhibits or kill bacteria. Antimicrobial include antibiotic that's produce by other organism that produced by other organism, chemically modified antibiotic as well as chemically synthesize antibiotic. Pencillium and other discovered antibiotics became widely used to treat different infections. These antibiotics are often referred as miracle drugs and considered to be one of the greatest achievements in public health [87].

The discovery of antibiotics was a turning point in human history. Antibiotics are the revolutionized medicine and countless lives have been saved. These are used against bacterial, fungal, viral and parasitic infections. Regrettably the use of these antibiotics has been accompanied by the rapid appearance of resistant strains. A number of biochemical and physiological mechanism may be responsible for resistance. From of the introduction of these drugs, millions of metric tons of antibiotics have been employed for a variety of purposes. Improvement in production and action to cure different infectious diseases encourage non prescription and off label uses [87].

2 LITRATURE REVIEW

Study on natural products of traditional medicinal plant and herbs; fruits, vegetable etc is always an interesting target for scientists over decades. A critical review of Kähkönen, *et al.*, (1999) reported that plants are good source of wide varieties of compounds including phenolic compounds, nitrogen compounds, terpenoids, vitamins and some other secondary metabolites [41]. These compounds are found to be rich in valuable bioactivities that's are anti oxidant, anti inflammatory, anti carcinogenic, anti bacterial, anti viral, anti mutagenic and anti tumor [88, 89]. Mukhtar et al., (2000) demonstrated that phenolic compounds also appeared to be effective in some cardiovascular disorders and certain kind of cancerous process [90]. Visioli *et al.*, (2002) demonstrated that these phenolic compounds are derived from phenylalanine, which is widely reported for their physiological and pharmacological role and their health benefits. [7]

Rai, *et al.*, (2011) studied the functions of phenolic compounds in plants they reported that these chemicals protect plants cells from environmental hazards such as UV exposure, stress, drought, pollution and pathogenic attacks. Plant produces these chemicals to protect themselves, humans and animals against diseases [15]. Shallenberger et al., (1971) estimated that more than 8000 structure are currently known belongs to phenolic [18]. (Zhou, *et al.*, (2004) reported that Hydroxybenzoic acids (p- hydrobenzoic, 3,5-dihydrobenzoic acid, salicylic, gallic, vanillic and syringic acids) are present nearly in all plants [20].

Hijova, (2006) reported that flavonoids have associated with a number of health benefits because of their cholesterol lowering, anti oxidative and anti inflammatory properties. They also demonstrated the antimicrobial and antioxidant

activities of flavonoids [29]. Joshi *et al.*, (2011) studied 10 different medicinal plant leaves for screening of chemical composition including *O. sanctum* and *A. indica*. The results indicated the concentration of crude fat, crude fiber and crude protein of *O. sanctum* and *A. indica* were moderated among other plant leaves [91]. Maisuthisakul *et al.*, (2008) studied the relationship between antioxidant properties and chemical composition of Thai plants, the fat content value were 3.1, 6.7 and 3.2g/100g for *B. alba*, *O. basilicum* and *O. sanctum* respectively, which were recorded lowest as compared with other plant leaves [92]. The ash and fiber content of *O. sanctum* and *O. basilicum* were relatively high as compared with other plant leaves. *O. sanctum* and *A. indica* leaves were found good source of protein and fiber which reduces stress, lowers cholesterol, improve digestion and regulate blood pressure and blood sugar. Edeoga *et al.*, (2010) studied the proximate composition of *Ocimum* (moisture, ash, Fat, protein, crude fat and crude fiber) and determined the phyto constituent (alkaloids, phenolic, tannin, flavonoids, saponins) [93].

Several of the current human health problems related to deficiency of minerals. Allen, (2000) reported that iron deficiency cause enema which affected one third of population. Cu helps in production of red and white blood cells and triggers the release of iron to form hemoglobin [94]. Gupta *et al.*, (2014) reported that the deficiency of Cu also causes hypertension, hyperglycemia, insomnia, manic disorders, allergies, osteoporosis and antibiotic sensitivity [95]. Ling *et al.*, (2010) studied Minerals composition of different plants [96]. The concentration of Ca and Mg were found 1.48 and 1.26ppm in *A. indica*, while 1.0 and 1.05ppm for *O. sanctum* leaves. The concentration of Cu and Zn were highest in *O. sanctum* leaves that are 12.31

and 81.66ppm. From previous literature and research the mineral results reveals that the aerial parts of plants are rich source of minerals elements.

Selvam *et al.*, (2013) studied the secondary metabolites and antioxidant properties of *O. sanctum*, they found some important bioactive phytochemical constituent include phenolic, flavonoids, alkaloids, saponins, tannin and essential oils. [97]. Joseph et al., (2013) reported the phytochemical, antibacterial and immunomodulatory properties of *O. Sanctum* [98]. Fathiazad *et al.*, (2012) studied pharmacongnostical and phytochemical screening of various Ocimum species, available in south eastern Odisha state of India [99]. Rabeta et al., (2013) have determined antioxidant capacity of vacuum drying, freeze drying, fermented and unfermented leaves of *O. sanctum*. The vacuum drying method produce higher quality product of antioxidant property than freeze drying [100]. Rout *et al.*, (2012) studied the antimicrobial activity of different leave extract of *O. sanctum* by well diffusion method against *Staphylococcus aureus, Escerichia Coli* and *Pseudomonas aeruginosa*. The results showed the extract inhibits the growth of *Staphylococcus aureus* and *Escerichia Coli* [101]. Ramteke *et al.*, (2012) have observed antibacterial activity of 10 different medicinal plant leaves extract including *O. sanctum* against bacterial strains e.g. *Escerichia Coli, Staphylococcus aureus, Salmonella typhimurium, Bacillus subtilis, Klebsilla pneumnial, Pseudomonas aeruginosa* and *Shigella* [102]. Dahiya et al., (2012) studied antibacterial activity of chloroform extract of leaves of Tulsi against the pathogenic bacteria i.e. *Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhimurium* that cause diarrhea [103]. Review on therapeutic potential of *O. sanctum* in prevention and treatment of cancer was investigated by Singh *et al.*, (2012) [104]. Shirazi *et al.*, (2014) reported that extract

of *ocimum* was potent in radio protective, antioxidant, antihermetic, antimicrobial, antiviral, anticancer, anti distress, cardio protective and wound healing activity [105]. Joseph et al., (2013) presented a comprehensive study on effect of *Ocimum* in numerous cancers such as skin, lungs, oral gastric, breast, cervical and prostate [106]. Al-Jadidi *et al.*, (2015) determined the antimicrobial activity of different crude extract of *A. indica* leaves by disc diffusion method against food born pathogen *Staphylococcus aurous* [107].

TPC in plant are mostly determined using Folin-Ciocalteu reagent method, commonly known as total phenols or phenolic assay and the results were expressed as gallic acid equivalent (GAE)/g of dry leaves extract [108]. (Wang *et al.*, (2006) studied the TPC and TFC by different extraction procedure, results revealed that ultrasonic assisted extraction is more effective for good yield of phenolic compounds [109]. The ultrasonic assisted extraction causes less degradation of phenolic compounds and is relatively effective and faster process for extraction of phenolic compounds as compared with other extraction method including microwave assisted, solid liquid, and subcritical water method [73]. Hrastar *et al.*, (2013) reported that in some particular cases the antioxidant effectiveness and phenolic content appreciably increases by thermal processing as a result of conversion of ester linked phenolic compounds into free phenolic that exhibit more antioxidant activity [110]. Gull *et al.*, (2012) reported that the variation of TPC and TFC depend on several factors, such as genetic differences, maturity at harvest and environmental condition etc [111].

Gas chromatography has been applied for the characterization of wide verities of compound in plants, vegetables, fruits and other food products. This technique is mostly used for the compounds, which is volatile and stable at the temperature

employed by system. But there are several other non volatile compounds, which can also be separated by GC but need to be derivatized before GC separation. Therefore it has limited use in characterization of phenolic compounds. For the characterization of phenolic compounds these are to derivatize to their trimethylsilyl esters.

In the characterization of phenolic compounds from propolis using GC-MS, [112] identified Twenty four compounds and eight of them were new. Choudhury *et al.*, (2014) applied LC-MS method for quantitative analysis of phenolic acid, by this method seven phenolic acid including ferulic, coumaric, syringic, gallic, chlorogenic and caffeic acids were found in *O. Sanctum* [113]. Zuo *et al.*, (2002) reported a GC-MS method for characterization of phenolic acids as their trimethylsilyl derivatives in cranberry. Fifteen phenolic acids (sinapic, ferulic, phthalic, vanillic, cinnamic, benzoic, p-coumaric, p-hydroxybenzoic, p-hydroxyphenyl acetic, o-hydrobenzoic, m-hydrobenzoic, o-hydroxycinnamic, 2,3-dihydroxybenzoic, 2,4-dihydrixybenzoic acid) were identified by proposed method [114]. Barbieri *et al.*, (2004) studied the volatile components of O. sanctum leaves, they identified more than 50 aroma constituents [115]. Proestos *et al.*, (2006) characterized phenolic compounds by GC after Silylation with BSTFA [116]. Bankova *et al.*, (1987) studied the phenolic compounds by GC-MS after Silylation. Fifteen phenolic acids, eight ester of phenolic acid, six flavanones and two flavonoids were tentatively detected [117]. Li *et al.*, (2001) developed a fast silyl derivatization technique for simultaneous determination of phenolic by GC-MS. He also studied the impact of different silyl reagent on phenolic composition. The extracts were silylated with TMCS, HMDS, and BSTFA [118].

The phenolic compounds can be quantified by different chromatographic techniques like TLC, HPLC, UHPLC, and GC. But during the last two decades RP-

HPLC column technology has been extensively used for the identification and quantification of phenolic compounds. Jobert was the 1st to developed an analytical HPLC method for quantification of arpalathin and nathofagn in rooibos tea Joubert, (1996) [119]. In addition a standard mixture of phenolic acid (caffeic, ferulic, p-coumaric, vanillic, p-hydroxybenzoic, protocatechinic) and flavonoids (rutin, isoqueritrin, orientin, vitexin, isoorientin) were also analyzed. Separations were achieved under RP gradient elution system on C-18 column (5um, 250×4.0mm) at 38°C in total run time of 125 minutes. Phenolic compounds were separated by HPLC from medicinal plants leaves [14, 88] wine and bear [120], seeds and seed oil [121]. [122] separated three flavanols namely Kaempferol, Myricetin and quercetin in tomato based product using reverse phase HPLC with UV detection. Siddhuraju *et al.*, (2003) identified and quantified quercetin and Kaempferol content in *Moringa oleifera* leaves using HPLC with UV detection [123]. Bramati *et al.*, (2002) reported a new HPLC method for quantification of phenolic compounds in aqueous and methanol extract of tea-bags. The separations were carried out using acidified water and Acetonitrile in gradient elution system at room temperature on 5um C-18 column [124]. Lee *et al.*, (2009) reported HPLC method for quantification of phenolic compounds in *Ocimum* using Diode Array detector. They separated 11 phenolic acids in *O. basilicum* leaves. The major phenolic acids were caffeic, chicoric and cafatric acid, these have been found as dominant phenolic acids in *Ocimum* by numerous other researchers [125]. Zheng *et al.*, (2001) studied 12 medicinal herbs for characterization of phenolic compounds by HPLC using PDAD. The separation were carried out using gradient elution of acidified water and Acetonitrile at flow rate of 1.0mL/min. 12 different phenolic compounds are identified by retention time and reference authentic

standard [126]. To date, RPLC coupled with MS or UV detector is the dominant technique for identification and quantification of phenolic compounds. [127].

Antimicrobial agents are those chemical which kill or inhibit the growth of micro organism and used against the microbial infections. These antimicrobial agents produced naturally by microbes or synthetically in laboratory. Antibiotics, antifungal, antiviral and other plant bioactive compounds such as phenolic used as antimicrobial agents.

Foodborne diseases are an increasingly serious public health problem all over the world. *Aspergillus flavus* and *Aspergillus parasiticus* are one of them cause's serious infections are briefly described in this section. Oka *et al.*, (1972) studied the effects of phenolic compounds against *Aspergillus flavus*. They found quercetin more effective against *Aspergillus flavus* [128]. Batovska *et al.*, (2007) found that flavanones possessed activity against the opportunistic pathogen *Candida albicans* [129]. Sher, (2004) isolated the flavanols from *Terminalia belerica* fruits has also been shown to posses activity against *Candida albicans* [130]. Chikezie *et al.*, (2015) isolated two new flavanones from *Artemisia giraldi* have been reported to exhibit activity against *Aspergillus flavus*. *Aspergillus flavus* and *Aspergillus parasiticus* produces toxic metabolites of Aflatoxin [131]. Bouddine *et al.*, (2012) evaluated the anti *Aspergillus niger* effect of eugenol, carvacol and thymol. The results revealed that thymol and carvacol proved to have better anti *Aspergillus* effect than eugenol [132]. Omidbeygi *et al.*, (2007) evaluated the inhibitory effect of three phenolic compounds of *Aspergillus parasiticus*. The MIC value of individual phenolic compounds showed that isoeugenol, carvacol and thymol were the most active against *Aspergillus* growth [133].

Many research groups isolated the flavonoids from plants sources that possessed antibacterial activity, Such as Basile *et al.*, (1999) isolated apigenin and evaluated the effect against different pathogenic strains [134] galangin by Cushnie et al., (2005) [135], Naringenin by (Rauha *et al.*, (2000) [136], Luteolin by Sato *et al.*, (2000) [137], quercetin by Salazar, *et al.*, (2015) [138] rutin by Hendra *et al.*, (2011) [139] and Kaempferol by Sadiq, *et al.*, (2015) [140] etc. These activities suggested that phenolic compounds can be used as disinfectant. Cai, *et al.*, (2004) identified some individual phenolic compounds having antimicrobial activity. They found that caffeic acid and Chlorogenic acid have demonstrated active against pathogenic bacteria [88]. Gülçin, (2006) determined the antimicrobial activity of phenolic compounds and found that caffeic, ursolic and chlorogenic acid were active against pathogenic bacterial strains [141]. Nascimento *et al.*, (2000) found that the caffeic and Chlorogenic acid did not demonstrate activity against *MRSA* [142]. Xu et al., (2001) reported that kaempferol did not inhibit *Staphylococcus epidermidis* however it demonstrated activity against *MRSA* strains. Rutin did not demonstrate any activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* [143]. The results of Skočibušić et al., (2004) were contradictory to these, where neither kaempferol nor quercetin demonstrated activity against *Staphylococcus aureus* [144].

Borges *et al.*, (2013) studied the activity of ferulic and Gallic acid against pathogenic bacteria. *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus* they found that gallic and ferulic acid activity against all tested pathogenic bacteria the MIC was 500µg/mL for *Pseudomonas aeruginosa* 1500µg/mL for *Escherichia coli* and 1250 for *Staphylococcus aureus* [145]. Ali et al., (2012) evaluated the antibacterial activity of two flavonoids orientin and vicenin obtained from *O. sanctum* leaves. The results

of both flavonoids showed positive studied against *Escherichia coli* and *Staphylococcus aureus* [146]. Dhayanithi *et al.*, (2012) studied the leaves extract of *A. marina* against isolated multi drug resistance *Staphylococcus aureus*. The results showed activity against different *MRSA* strains, which was resistant against Methicillin, vancomycin and ciprofloxacin [147]. Balakumar *et al.*, (2011) assessed antifungal activity of crude extracts of *O. sanctum* against isolated dermatophytic fungi including *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Microspore gypseum*, *Microsporum nanum* and *Epidermophyton flocossum* [148]. Liu *et al.*, (2009) studied the effect of kaempferol on antibacterial resistant bacteria *MRSA* they found that kaempferol showed strong activity against *MRSA* they also found that combination of kaempferol greatly reduced the MIC of fluoroquinolones in *MRSA* [149].

Irfan *et al.*, (2008) evaluated resistant rated in isolated *MRSA* against Clindamycin tetracycline, cotrimoxazole, Rifampicin, chloramphenicol, and fusidic acid. They found high resistance rates to tetracycline 82% and Clindamycin 79%. While the resistance to chloramphenicol and fusidic acid was 10 and 9% [150]. Soon *et al.*, (2011) studied the antibacterial activity of *A. indica* against clinically isolated strains of pathogenic bacteria and fungi. They found the most susceptible strain was *Staphylococcus aureus* (MIC=16µg/mL), while most resistive was *K. Pneumonia* (MIC=128 µg/mL). The MIC value recorded for *Aspergillus niger* was 32µg/mL [151].

3 EXPERIMENTAL

The research works presented in this dissertation were conducted in the Pakistan Council of Scientific and Industrial Research (PCSIR) labs complex Peshawar and Institute of chemical Sciences university of Peshawar.

3.1 COLLECTION OF PLANTS SAMPLES

The *O.Sanctum* and *O.basillicum* plant samples were collected from botanical garden of Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories Complex Peshawar, KPK, Pakistan, while *A.indica* leaves from Islamia college university of Peshawar. The fresh fully green leaves were selected at the initial stage of maturity during April- July 2012.

All the sample leaves (5kg after removing the foreign material) were washed with plenty of water, dried in vacuum oven at 40°C for 48hours. The dry leaves were crashed with commercial grinder and 80-mesh sieved. The powder sample 3.2, 3.4 and 3.7kg leaves of *O.santctum*, *O.basillicum* and *A.indica* were stored in poly ethylene bags in desiccating cabinet for further analysis.

3.2 COLLECTION OF BACTERIAL CULTURES

MRSA (n=80) were collected from the microbiology laboratory of tertiary care hospitals Khyber Teaching Hospital, Lady Reading Hospital and Hyathabad Medical complex Peshawar KPK Pakistan. The collected *MRSA* strains were carried out to microbiological laboratory (ISO-17025) PCSIR labs complex Peshawar and cultured on the same day within 2 hours of collection.

3.3 REAGENTS AND STANDARDS

3.3.1 CHEMICALS

Copper Sulphate ($CuSO_4$), Fluka chemical, Switzerland; Sodium Hydroxide (NaOH) and Potassium Sulphate (K_2SO_4), Riedel-de Haen; Germany, Boric Acid (H_3BO_3), Ferric Chloride ($FeCl_3$) , Ferric Sulphate ($FeSO_4$) and Citric Acid ($C_6H_8O_7$), Scharlau Barcelona Spain; Gallic Acid ($C_7H_6O_5$), Caffeic Acid ($C_9H_8O_4$), Ferulic Acid ($C_{10}H_{10}O_4$), Sinapic Acid ($C_{11}H_{12}O_5$), Chlorogenic Acid ($C_{16}H_{18}O_9$), Syringic Acid ($C_9H_{10}O_5$), Benzoic Acid ($C_7H_6O_2$), Vanillic Acid ($C_8H_8O_4$), Quercetin ($C_{15}H_{10}O_7$), Luteolin ($C_{15}H_{10}O_6$), Rutin ($C_{27}H_{30}O_{16}$), Apigenin ($C_{15}H_{10}O_5$), Kaempferol ($C_{15}H_{10}O_6$), Hesperitin ($C_{16}H_{14}O_6$), Catechins ($C_{15}H_{14}O_6$), from Sigma Aldrich, Germany; 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Alfa Aesar, UK., Phenolphthalein Indicator ($C_{20}H_{14}O_4$), Methyl Red Indicator ($C_{15}H_{15}N_3O_2$) Fisher Scientific, UK.

3.3.2 REAGENTS:

Folin-Ciocalteu phenol reagent and Mayer's reagent were purchased from Sigma Aldrich while *N,O* Bis(trimethylsilyl) trifluoroacetamide (BSTFA) and TMCS from thermo scientific.

3.3.3 SOLVENTS:

Chloroform ($CHCl_3$), Sulphuric acid (H_2SO_4) and Hydrochloric Acid (HCl) from lab-Scan analytical sciences; Ethanol (C_2H_5OH), Methanol (CH_3OH) , and Acetone (C_3H_6O) from Scharlau, Barcelona, Spain; Acetonitrile (C_2H_3N), Ethyl Acetate ($C_4H_8O_2$) and Dimethyl Sulfoxide(C_2H_6OS) from sigma Alderich Germany. HPLC grade solvents Acetonitrile (C_2H_3N), Methanol (CH_3OH) and Chloroform ($CHCl_3$) were purchased from Fisher Scientific, UK and Sigma Aldrich Germany.

3.3.4 INSTRUMENTS

3.3.4.1 Low Technology Instruments

Analytical balance: CPA Sartorius, (Model) CPA26P, Germany;

Oven: Ontherm Scientific Ltd, (Model-8150) New Zealand;

Desiccating cabinet: Today' scientific (Model-2360) Japan;

Water bath: Gemmy, (Model-YCW-01), Taiwan;

Ph meter: Hanna Instrument (model HI5221), USA;

Orbital shaker: Heidolph Unimax, (Model-2010), Germany;

Digital refractometer: Rudolph Research analytical (Model J-47, USA;

Ultrasonic bath: Ceia (Model CP-104), Italy;

Filtration assembly: (Model Rocker-300), Taiwan;

Centrifuge: Hittich (Model D-78532) Germany;

Luminar flow: Pak Scientific Lahore Pakistan;

Eclectrical Autoclave: (Model KT-30L) Tokyo, Japan;

Fuming hood: Esco (Model L-25) Singapore;

UV Spectrophotometer: Optima (Model-SP3000) Tokyo, Japan;

Rotory Avaporator: Buchi Rotavapor (Model R-200) Switzerland.

3.3.4.2 High Technology Instruments

Atomic Absorption Spectrophotometer: (Model Zeeman Z-8000 Japan) equipped with hollow cathode lamp as a radiation source.

GC-MS: Shimadzu (Model GC 17-A) supported by Joel JMS-600H and Joel JMS HX110 quadruple Mass Spectrometer, Capillary Column, DB-5 (Optima)

HPLC: HPLC (Hitachi-2000) equipped with dual pumps (L-2130) auto injector (L-2200) with analytical column Intersil ODS-3 C18 (GL Sciences Inc.

Tokyo Japan 5um, 250+ 4.6mm) in column oven (L-2300) and UV-Vis Detector (L-2400)

3.4 PROXIMATE ANALYSIS

3.4.1 MOISTURE CONTENT

The moisture content of each sample was determined gravimetrically using vacuum oven according to official method Association of Official Analytical Chemist (AOAC, 2000) procedure reported by Ishida *et al.*, (2000) [152]. China dishes were pre-dried in oven at 105°C for six hours and then cooled at ambient temperature at 25°C in desecrator. Briefly, each sample was accurately weighed 2 to 5g into a pre-weighted dish using analytical balance, dried in a vacuum oven at 70°C for 16- 20 hours until constant weight achieved. After drying the sample was cooled in a glass desecator and weighted for moisture content. Each sample was analyzed in triplicate, and determined by given formula.

$$Moisture\ (\%) = \frac{\mathrm{WS} - \mathrm{DS}}{WS - D} \times 100$$

Where:

WS = Weight of sample before drying with dish

DS = Weight of dry sample with dish

D = Weight of dry dish

3.4.2 ASH CONTENT

Ash content of each sample was determined by AOAC method reported by Agrahar et al., (2005) [153] using a muffle furnace. Briefly each sample was accurately weighted 2g into a pre-weighted crucible and carbonized by heating on a

gas flame. The samples were then transferred to muffle furnace and ashed at 550°C until constant mass was achieved.

Ash content of each sample were determined using following formula.

$$Ash\ (\%) = \frac{\text{Weight of Ash (g)}}{Weight\ of\ sample\ (\text{g})} \times 100$$

3.4.3 CRUDE FAT CONTENT

The crude fat was estimated according to the method of Indrayan *et al.*, (2005) [154]. 5g of each sample was taken in a thimble and placed in soxhlet extractor. A dried and pre weighted round bottom flask 250mL was connected to the soxhlet assembly containing 150mL n-hexane and heated for 8 hours in heating mental. After the complete evaporation of n-hexane the flask containing crude fat was weighted, and the crude fat contents were determined using the following formula.

$$\text{Crude Fats (\%)} = \frac{W1 - W2}{W3} \times 100$$

Where W1 = weight of round bottom flask along with crude extract

W2 = weight of empty round bottom flask

W3 = weight of sample taken in thimble

3.4.4 CRUDE FIBER CONTENT

The crude fiber content was determined by reported method of Noblet *et al.*, (1994) [155]. 5g of each dried powder sample was taken in a thimble and defatted using soxhlet extractor for 8 hours with n-hexane solvent as mentioned in section 2.3.3. 2g of defatted sample was boiled for 30 min in 200mL of 0.255N solution of H_2SO_4. The mixture was then filtered through linen cloth and the residues were washed with distilled water till obtained the acid free sample.

After H_2SO_4 treatment the residue was boiled for 30min in 200mL NaOH solution (0.313N). The mixture was filtered through pre weighted Gooch crucible with asbestos mat and dried in an oven. The dry sample was weighted and ignited in a muffle furnace at 600°C for 4 hours. After ignition the sample was again weighted and the % crude fiber content was determined using following formula.

Weight of loss on ignition = Weight of residue – Weight of ash

$$\text{Crude Fiber (\%)} = \frac{\text{Weight of loss on ignition (g)}}{\text{Weight of sample (g)}} \times 100$$

3.4.5 PROTEIN CONTENT

The protein content of each sample was determined as total nitrogen by official method AOAC, 2000 reported by Sze et al., (2000) [156] method. Each sample 1g in duplicate sets was added to 2g digestion mixture of (copper sulphate : ferric sulphate : Potassium sulphate in the ratio of (1:9:90), after that concentrated H_2SO_4 20mL was added. The solution was heated until to clear the solution and then make the volume 100mL with distilled water.

The final solution was poured into flask containing 10mL of 2% boric acid, added few drops of methyl red indicator turn to pink colour. Distillate was titrating with 0.1N sulphuric acid H_2SO_4 till the color changed from yellow to pink during neutralization. 5mL of digest was added to 10mL of 40% sodium hydroxide solution and the distillation was completed in ten minutes, with the change of color of boric acid to yellow due the formation of ammonium borate. Titrate against 0.1N H_2SO_4, until the color changed again to pink. The volume of acid used wad noted and the calculation

was made by using formula given below. The total protein was calculated at conversion factor of 6.25.

The crude protein was calculated by using below formulas

$$Nitrogen\ (\%) = \frac{\text{Vol. of 0.1 N } H_2SO_4 \text{ used } \times \text{Vol. of dilution } \times 0.0014}{Weight\ of\ sample\ (\text{g}) \times \text{Vol. of dilution used}} \times 100$$

$$Crude\ protein = \%\ Nitrogen \times 6.25$$

3.4.6 TOTAL ACIDITY

The total acidity in each sample was determined by official method AOAC, 2000 reported by Sze et al., (2000) [156] method. 10 g of each sample was added in 100mL distilled water and boiled for 30min. The mixture was filtered through wattman filter paper to remove the residue. 10mL of extract was poured in a titration flask, added a few drops of phenolphthalein indicator and titrated against 0.1N sodium hydroxide solution. The appearance of light pink color showed the end point. The %total acidity of the sample was determined using following formula.

$$Total\ Acidity\ (\%) = \frac{\text{Factor} \times \text{ N } \times \text{titer } \times \text{ dilution}}{S_w \times S_v} \times 100$$

Where S_W = weight of the sample taken (g)

S_V = volume of the sample extract

3.4.7 PH AND TOTAL SOLUBLE SOLIDS

Each sample 5g was boiled for 30min in 100mL of distilled water cooled at room temperature and filtered through linen cloth. The pH of each sample was determined using calibrated digital pH meter and the total soluble solid content by digital refractometer.

3.5 PRELIMINARY PHYTOCHEMICAL ANALYSIS OF DRY LEAVES

The preliminary phytochemical analysis of plant leaves were carried out by reported method Ayoola *et al.*, (2008) and Koleva *et al.*, (2002) [157, 158]

3.5.1 TEST FOR ALKALOIDS

1 g of each dry powder sample was taken in a test tube and added 3mL of ammonia solution. The samples were allowed to stand for few minutes. Then added 10mL chloroform to the sample test tubes, shacked well and filtered to remove the powder samples. The chloroform was evaporated using water bath and added 2mL Mayer's reagent. A cream colored ppt was produced which indicates the presence of alkaloids.

3.5.2 TEST FOR FLAVONOIDS

2g of each dry sample was extracted with 10mL water and filtered. A few drops of extract were added to 10mL diluted ferric chloride solution. The solution was mixed thoroughly and observed for color change. The black/green color indicated the presence of flavonoids.

3.5.3 TEST FOR CYANOGENIC GLYCOSIDES

2g of each powdered sample was extracted with enough water in 250mL conical flask. A thread was used to suspend pictate paper in flask. The flask was heated in a water bath at 40°C for an hour. The colour changed from yellow to brick red of the Picrate paper was the indication of presence of glycosides.

3.5.4 TEST FOR REDUCING SUGAR

2g of each powder sample was added to 7.5mL of a mixture of Fehling solution I and II in a test tube. The mixture was heated in water bath for 5min at 40°C and observed for color change to brick red ppt.

3.5.5 TEST FOR TANNINS

1g of each sample was boiled with 20mL distilled water in test tube and filtered, 5mL of filtrate was then mixed with 0.1% ferric chloride solution and observed the color. Green or blue color indicated the presence of tannins.

3.5.6 TEST FOR SAPONINS

The aqueous crude extract of each sample (1mL) was diluted with 10mL distilled water and then the test tube was shaken for 15 min. the formation of foam layer on the surface of the top of the test tube showed the presence of saponins.

3.5.7 TEST FOR TRITERPENOIDS

5g of each crude extract was dissolved in 2mL chloroform and then 1mL acetic anhydride. One mL of conc. H_2SO_4 was also added to the solution and observed the color. The formation of reddish violet color shows the presence of triterpenoids.

3.5.8 TEST FOR STEROIDS

1gm of each dry powder sample was dissolved in 10mL chloroform and added 1mL conc. H_2SO_4 into the test tube by wall sides. The upper layer color turned to red

and the H_2SO_4 layer yellow with green fluorescence, which indicated the presence of steroids.

3.6 MINERAL ANALYSIS

The mineral analysis were carried out by atomic absorption spectrophotometer [159]. The concentration of micro and macro minerals such as cobalt, copper, iron, nickel, zinc, sodium, potassium, calcium and magnesium in *O. sanctum*, *O. basilicum* and *A. indica* were determined by Hitachi atomic absorption spectrophotometer (Model Zeeman Z-8000 Japan) equipped with hollow cathode lamp as a radiation source. The standard operating condition for each element was mentioned in table.

Table 1: Standard Operation Condition for Atomic Spectrophotometer

Parameter	Co	Cu	Zn	Fe	Ni	Mg	Ca	Na	K
Lamp (mA)	12.5	7.5	5.0	12.5	10	7.5	7.5	10	10
Wave Length (nm)	240.7	324.8	213.9	248.3	232.0	285.2	422.7	589.0	766.5
Slit (nm)	0.2	1.3	1.3	0.2	0.2	1.3	1.3	1.3	1.3
Burner Head	Standard	Standard	Standard	Standard	Standard	Standard	Standard	Standard	Standard
Burner Height (mm)	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Flame	Acetylene	Acetylene	Acetylene	Acetylene	Acetylene	Acetylene	Acetylene	Acetylene	Acetylene
Gas pressure (kpa)	160	160	160	160	160	160	160	160	160
Fuel Gas flow rate(Mn)	2.2	2.2	2.0	2.0	2.2	2.2	2.4	2.2	2.4

3.6.1 Extraction for Mineral analysis:

2g dried powdered of each plant leave sample was taken in a digestion tube, added 10mL concentration HNO_3 and allowed to stand overnight. After 24 hours 4mL perchloric acid was to each sample and allowed for 25 minutes, the sample were then heated till it evaporated to a small volume. The samples were then cooled and diluted with distilled water. 100mL solution was obtained and filtered. All the solutions were marked with names and coded numbers and were analyzed through atomic absorption spectrophotometer for quantitative detection of the following elements. [160] . The elemental analyses were done for *O. sanctum*, *O. basilicum* and *A. indica*.

3.7 ANTIMICROBIAL ANALYSIS

3.7.1 PREPARATION OF EXTRACT FOR ANTIMICROBIAL ACTIVITY

For the determination of antimicrobial activity the sample were extracted according to reported method of Tepe *et al.*, (2005) [161]. 50g of powder leaves of *O. sanctum*, *O. basilicum* and *A. indica* were socked in 250mL methanol for 72 hours at 28° C on orbital shaker, thereafter the extract filter through whatman filter paper to remove the residue. Each extract was then evaporated in rotary evaporated at 40°C to concentrate the extract and stored at 4°C prior to use. The concentrated extracts were then dissolved in dimethylsulphoxide (DMSO) at the concentration of 1mg/mL for antibacterial and antifungal activities.

3.7.2 ANTIBACTERIAL ACTIVITY

The antibacterial study of the crude extracts was carried out using agar well diffusion method [162] and modified by Hanif *et al.*, (2011) [163]. Antibacterial assay was studied against eight different bacterial strains including *Staphylococcus aureus*

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(ATCC 27853), *Salmonella typhi*, *Escherichia coli* (ATCC 25922), *Bacillus cereus*, *Pseudomonas aeruginosa* (ATCC 27853), *Klebsilla pneumonia* (ATCC 700603), *Proteus mirabilis* (ATCC 13315), and *Enterococcus fecalis* (ATCC 29212). Eight concentration of each extract (0.2µg/mL to 1.0µg/mL) were prepared by serial dilution. Plates were prepared by pouring 50mL of sterilized nutrient agar medium in 14cm Petri plates. On the next day fresh bacterial culture was poured on the plate containing nutrient agar. The plate was rotated to make equal distribution of the culture and allowed to solidify. Wells were made in plates by using 8mm sterile borer. The leaves extracts of *O. sanctum*, *O. basilicum* and *A. indica* were screened for antimicrobial activity by this method. All extract were first dried in vacuum evaporator and then suspended in sterile DMSO to get stock solution.

50µl of sample was added using micropipette in well according to bacterial culture and equal quantity of DMSO was added serving as negitive control. These plates were then inoculated at 37°C for 24 hours and zone of inhibition (mm) were recorded on next day.

3.7.3 ANTIFUNGAL ACTIVITY

The antifungal study of the crude extracts was also carried out using agar well diffusion method [121]. The antifungal activity was evaluated against three fungal strains including *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus parasiticus*. Eight concentration of each extract (0.2µg/mL to 1.0µg/mL) were prepared by serial dilution. Plates were prepared by pouring 50mL of sterilized potato dextrose agar medium in 14cm Petri plates. On the next day fresh fungal culture spore suspension was poured on the plate containing potato dextrose agar. The plate were rotated to make equal distribution of the culture spores and allowed to solidify. Wells were made in plates by using 8mm sterile borer.

50µl of sample was added using micropipette in well and equal quantity of DMSO was added serving as negitive control. These plates were then incubated at 30°C for 3 to 5 days and zone of inhibition (mm) were recorded.

3.7.4 MINIMUM INHIBITORY CONCENTRATION (MIC)

MIC concentration was determined by Ansari et al., (2007) [164] method. The extract which revealed high antibacterial activity was further diluted by serial dilution method to get MIC.

3.8 EFFECTS OF EXTRACTION MEDIUM/TECHNIQUE ON THE ANTIOXIDANT ACTIVITY

The efficacy of the extraction solvent and process towards obtaining optimum extract yield with efficient of antioxidants were evaluated; two different techniques

(orbital shaker and ultrasonic extraction) and three different solvent (ethanol, methanol and acetone) were employed.

3.8.1 Extraction by orbital shaker

The samples were extracted with ethanol, methanol and acetone according to the method of Khattak *et al.*, (2007) [165]. The collected leaves were air dried at room temperature. 100g powder sample were extracted with 500mL 98.8% methanol on orbital shaker for 3 hours at room temperature 30°C. The extract were than filtered through Whatman No. 1 filter paper and the excess solvent were removed at 45°C under reduce pressure using rotary evaporator and stored in refrigerator for further analysis.

3.8.2 Extraction by Ultra Sonicator

Each 100g dried leaves were extracted with 100mL each extraction solvents by ultra sonicator bath at 30°C for 1 hour. After the sonication treatment the samples were filtering through Whatman No.1 filter paper to remove the residues. The residue were re-extracted twice with fresh each extracting solvent and then concentrated under reduce pressure at 45°C, using rotary evaporator. The crude concentrated extract were then stored in a refrigerator, until used for analysis.

3.9 EVALUATION OF ANTIOXIDANT ACTIVITY OF EXTRACTS

3.9.1 DETERMINATION OF ANTIOXIDANT ACTIVITY

The Antioxidant potential of the different extract of *O. sanctum*, *O. basilicum* and *A. indica* leaves was determined spectrophotomatrically following the DPPH free radical scavenging method reported by Sokmen *et al.*, (2004) [166]. For stock

solution 1 mL of each extract was diluted with 9 mL methanol, centrifuged at 6000 rpm and the supernatant was decanted. Five different concentration of each extract (50-250ppm) were prepared from stock solutions. 1 mL of each concentration was added with 2 mL of freshly prepared DPPH solution (0.004% w/v in Methanol) and incubated for 30 min. For control 1mL methanol was added to 2mL DPPH solution. The absorbance was measured at 517 nm using UV-VIS spectrophotometer and the scavenging activities were determined using following equation.

$$\% \text{ Scavenging Activity} = \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of the control}} \times 100$$

EC-50 value define as the concentration of antioxidant sample leading to 50% reduction of of intial DPPH concentration. EC-50 value was calculated from linear regression of different concentrtion of the test extracts against the %inhibition of DPPH.

3.9.2 DETERMINATION OF TOTAL PHENOLIC CONTENT (TPC)

The TPC of each sample was determined by Lachman *et al.*, (2010) [167]. 2 mL of Folin-Ciocalteu reagent was added to each extract and gallic acid standards. Five concentration of gallic acid 100 to 500µg/mL were prepared for calibration curve. After 10 min sodium carbonate saturated solution was added to each and allow for 30 minutes at room temperature. After incubation the mixture was measured at 760 nm against blank on a UV spectrophotometer and the calibration curve of absorbance vs concentration was plotted. The TPC was expressed as

gallic acid equivalent mgGAE/l, calculated from calibration curve of gallic acid: $Y = 0.143x + 0.221, R_2 = 0.996$.

3.9.3 DETERMINATION OF TOTAL FLAVONOIDS CONTENT (TFC)

The TFC of each sample was determined by Kumar et al., (2013) [168] method. For the calibration curve five different concentration solutions of catechin (50, 100, 150, 200 and 250 µg/mL) were prepared 1:2 with water from standard solution (500 mg/100mL methanol). Standard solutions (0.5 mL) and sample was mixed with 2mL of deioized water and 150µl of 5% sodium nitrate. After 5 min at room temperature 150µl of 10 % aluminum chloride was added and allowed for 6 min. Then added 1 mL of 1M sodium hydroxide and adjust the total volume to 5mL. The absorbance was read at 510nm. The total flavonoids content were determined from the calibration curve of Catechins: $Y = 0.062x + 0.059, R_2 = 0.999$ and expressed as catechin equivalents (mg/l)

3.10 IDENTIFICATION OF PHENOLIC COMPOUNDS BY GC-MS

3.10.1 SAMPLE PREPARATION FOR GC-MS

The extractions and derivatization of phenolic compounds were carried out using reported method Mahmood, (2011) [169]. Briefly, the plant extracts were further extracted with ethyl acetate. The ethyl acetate fraction was then treated with $NaHCO_3$ (0.5 M). The solvent were evaporated to dryness under the nitrogen gas stream. 400µl of the dry phenolic solution was transferred to vial containing 100µl BSTFA and TMCS. The vial was then heated at 70°C for 15minutes. 1µl sample was

applied to GC for characterization of phenolic compounds. The GC operating conditions presented in table.

Table 2: GC-MS Conditions for the Identification of Phenolic Compounds

Column	Fused silica capillary column HP-5MS (30 m × 0.25 mm, 0.25µm)
Injector	Auto injector, split mode (split ratil : 100), temperature 280°C injection volume µl
Column oven programmed	Initial temperature 80°C, holdup 2 minutes, 80-120°C (ramp rate 5°C/minute), 120 t0 275°C (ramp rate 8oC), holdup 10 minutes
Mobile phase	Helium gas at flow rate 1.5 mL/minute
GC-MS Detection	MS transfer line temp. 290°C. Electron impact ionization, Scanning range, 50-550 m/z @ 1.5 scan/sec

3.11 HPLC ANALYSIS OF PHENOLIC COMPOUNDS

3.11.1 EXTRACTION OF PHENOLIC ACID

0.5g of dry residues was re dissolved in 10mL ACN and centrifuge at 5000rpm for 10 minutes. The clear supernatant was then filtered through 0.45µm cellose (Millipore) to inject HPLC.

3.11.2 CHROMATOGRAPHIC CONDITION

The mobile phase of HPLC was optimized to obtain the desired phenolic acids. The ACN as a (solvent A) and 0.1% aqueous acidic acid solution at 3.0 pH as a (solvent B), proven to be a best optimal mobile phase system in the following gradient elution program for the purpose of chromatographic separation. 0 minute, 10%A-

90%B; 5minutes 20%A-80%B: 10minutes 40%A-60%B; 15min 60%A-40%B; and 20minutes 80%A-20%B at flow rate of 1.0 mL/minute. The UV wavelength was set 210nm and column temperature was kept at 25°C for optimal separation. The Identification of each phenolic acid based on retention time and on spectral data in comparison with authentic standards and quantified by establishing calibration curve of each compound standard.

3.11.3 VALIDATION OF QUANTITATIVE ANALYSIS

Standard stock solution of Gallic, Chloroganic, Syringic, Benzoic and Vanillic acids were prepared by dissolving 0.01g in 10mL Acetonitrile (1mg/mL) and further diluted to 5, 10, 15, 20, 25 and 30µg/mL for standard curve. Precision was determined by injecting 10 µl of each standard solution (n=6). For linearity the calibration curves were drawn by plotting the peak areas against concentrations. The peak areas were automatically measured by an integrator of HPLC instrument. The LOD and LOQ under the present chromatographic conditions were evaluated at S/N of 3 and 10, respectively. The accuracy of the method was determined for each component by analyzing the recovery in percent of *O. sanctum* leaves extracts spiked with six different concentrations and the robustness was investigated by changing the flow rate, the chromatographic temperature and the detection wavelength, and found to be a fine robustness of the proposed method.

3.11.4 EXTRACTION OF FLAVONOIDS

The extraction was achieved by using Khan *et al.*, (2014) [170] with some modification. The dry residue of ethyl acetate were re-dissolved in 10mL ACN and centrifuge at 5000rpm for 10min. the supernatant was then mixed with chloroform in

separately funnel. The chloroform layer was collected and then washed with water to remove sugars and other polar compounds. The final solution was then dried under nitrogen stream at 40°C. The residue was then dissolved in mobile phase and filtered through polyamide membrane prior to inject HPLC.

3.11.5 CHROMATOGRAPHIC CONDITION FOR SEPRATION OF FLAVONOIDS

The optimum chromatographic separation was achieved with binary mobile phase A and B. organic phase Acetonitrile as a mobile phase A and acidified aqueous solution with acidic acid at pH 3 as a mobile phase B. the gradient elution program was as follows. 0-5min, 10%A; 5-8min, 10-40%A; 8-15min, 40-80%A; finally returning to the initial condition in 15 min with a flow rate set of 1mL/min. A wavelength ( ) of 280nm was set for UV-visible detector. The column temperature was kept at 35°C and the injection volume was 10ul. Chromatographic peaks were identified by comparing the retention time of sample with those of standard phenolic compound and quantification was achieved by internal standardization.

3.11.6 VALIDATION OF QUANTITATIVE ANALYSIS

In previous work for the quantification of phenolic compounds several mobile phases based on citric acid, citric acid/water, acetic acid/water, formic acid, formic acid/water and aqueous phosphoric acid as a mobile phase A and Acetonitrile and methanol as mobile phase B were used as isocratic and gradient elution system [126, 171-173]. In this work to optimized the mobile phase for characterization of phenolic compounds all the possible combination of two mobile phase at different flow rate

(0.2 to 1.2mL/min) at different pH (5-3) were tested for better chromatographic separation. The decrease in pH of mobile phase dramatically reduce the retention time of phenolic but more acidic mobile phase is not ideal for column life so that why pH=3.0 was choose as the optimum pH of mobile phase for UV as well as florescent detection. The wavelength between 210 and 320 is commonly used for the detection of phenolic compounds by UV-Vis absorbance [174]. The standard compounds were determined by 210, 250, 280, 320nm for the detection at appropriate wavelength. In which strong absorption were found on 280nm, which was set according to published data [175], The optimized method reduces analysis time from 60 to 35 minutes with respect to other published data.

3.12 BACTERIAL CULTURES TRANPORT AND PROCESSING

The clinical isolated *MRSA* strains were sub-cultured on sterile nutrient agar plates and incubated at 37°C for 24 hours. After incubation the plates showing growth were subjected to Mannitol salt agar for differentiation. The MSA plates showing yellow colonies of organism were considered S. aurous. The S. aureus from MSA plates were isolated, inoculated in sterile nutrient broth and incubated overnight at 37°C. Thereafter the turbidity of inoculums was adjusted to 0.5 Mc Farland using 0.9% (w/v) sterile normal saline and then used to bacterial lawns on sterile MHA plates. Methicillin disc were applied on plate and incubated over night at 37°C. After incubation plates with zone of inhibition <10mm diameter or zero zone of inhibition were considered to be *MRSA*.

3.12.1 ANTIBIOTIC SENSITIVITY ASSAY

Susceptibility of clinical isolated *S. aurous* and ATCC 43300 against selected antibiotics, phenolic compounds and combination of antibiotic + phenolic were studied by disk diffusion method. The susceptibility tests were carried out using sterile MHA plates after 12 hours incubation at 37°C. The zone of inhibition >10mm diameter was consider active, while the zone of inhibition <10mm diameter were regarded inactive.

Statistical Analysis

Statistical analyses of presented data were performed using Statistica 6.1 and the difference between fresh and stored extracts were evaluated by the Duncan test at 5 % level of significance.

4 RESULTS AND DISCUSSION

4.1 PROXIMATE COMPOSITION OF PLANT LEAVES

This part of study comprises the data for the determination of moisture, ash, crude fat, crude fiber, crude protein, total acidity *etc* in *O. sanctum*, *O. basilicum* and *A. indica*.

The results in (Table-3) revealed the proximate composition in %. The moisture contents were found 5.30, 4.80 and 7.31% in dry powder of *O. sanctum*, *O. basilicum* and *A. indica* leaves. Rai *et al.*, (2004) reported high value of moisture content in *Ocimum*. The previous literature demonstrated that moisture contents not only varied among the species but also between the different phonological states of plant. [176]. Oboh, (2005) also stated that moisture content varies in different species [177]. The moisture contents in the present case are much less than that of cited result which indicated that the sample were sufficiently dried for analysis. The overall ash content ranged from 1.87 to 2.81%. When organic maters are decomposed at high temperature the remaining residues is called ash and the total ash determined in % indicated the presence of minerals. The overall highest ash content (2.81%) was recorded in *A. indica* followed by (2.50%) in *O. sanctum* and (1.87%) in *O. basilicum*. These results are in contradiction with those of Al-Khalifa et al., (1999) [178] but are in line with those of Edeoga *et al.*, (2006) [179] and Hosseini *et al.*, (2010) [180]. The percent ash in leaves samples indicated that these leaves are rich in minerals and hence it was important to carry out mineral analysis.

Table 3: Proximate Analysis of *O. Sanctum*, *O. Basilicum* and *A. Indica*

S.No	Parameter	Proximate analysis		
		O. sanctum	*O. basilicum*	*A. indica*
1	Moisture (%)	5.30 ± 0.4	4.80 ±0.81	7.31 ±0.87
2	Ash (%)	2.50 ±0.3	1.87 ±0.07	2.81 ±0.10
3	Crude Fat (%)	0.90 ±0.06	1.01 ±0.01	0.88 ±0.01
4	Crude Fiber (%)	9.80 ±0.3	10.2 ±0.76	7.75 ±0.45
5	Crude Protein (%)	0.80 ±0.01	1.02 ±0.01	0.76 ±0.01
6	Total Acidity (10% Sol.)	0.06 ±0.01	0.08 ±0.005	0.17 ±0.02
7	TSS (10% Solution)	1.01 ±0.04	0.91 ±0.1	1.05 ±0.04

Crude fat is a part of complex organic material that is soluble in Hexane and ether and extremely rich source of energy. The crude fat varied from 0.88 to 1.01%. The minimum percent of crude fat in leaves is a closed agreement with other researcher. They found high level of crude fat in seeds and fruits of medicinal plants while minimum in leaves. Ramadan *et al.*, (2006) reported the crude fat content in stem was greater than leaves [181]. Surveswaran *et al.*, (2007) also found very low fat content in leaves as compare with stem and seeds [182]. Our results are also in line with other researchers.

Among the proximate analysis the fiber content were found in maximum amount in leaves samples. The overall crude fiber content ranged from 7.75 to 10.20%. The maximum fiber content 10.20% found in *O.basilicum* followed by 9.80% in *O.sanctum* and 7.75% in *A. indica*. Hussain et al (2009) estimated fiber content in

various medicinal plant leaves which varied from 9.5% to 12.20% [183], this range is similar to the result in present case. The use of plants with efficient fiber content reduce tracolonic pressure which is beneficial in diverticular diseases. These are also adequate for rumination and digestion in ruminant animals. Fiber is an important constituent of balance diet that decreases blood cholesterol level, heart risks, diabetes and colon cancer.

The crude protein content varied from 0.76% to 1.02%. These results revealed that the amount of crude protein is very low in plants leaves, which is also a closed agreement with other researchers. Crude protein refers to all the nitrogenous compounds present in plant except alkaloids [184]. The total acidity of 10% solution were found in ranged of 0.06 to 0.17% while the total soluble solid in rang of 0.91 to 1.05%.

4.2 MINERAL ANALYSIS

The minerals composition studied in the leaves of *O. sanctum*, *O. basilicum* and *A. indica* and presented in (Table-4). The Zn and Fe are essential micronutrient for human health, and the deficiency of these two nutrients remains a global problem especially among women and children's in developing countries. The concentration of Zn and Fe determined in *O. sanctum*, *O. basilicum* and *A. indica* leaves were in the range of 43.4 to 71.30ppm and 33.80 to 41.20ppm. The health benefits of Zn include control of diabetes, digestion, hair care, and improvement in stress level. Zn also plays a vital role in synthesis of protein and helps in regulation of the cell production in the immune system of human body.

Ni and Co are associated trace elements and considered essential to human health. The concentration of Ni and Co determined in *O. sanctum*, *O. basilicum* and *A. indica* were in the range of 9.76 to 11.30ppm and 3.70 to 6.30ppm.

The content of K and Na found in leaves of *O sanctum*, *O. basilicum* and *A. indica* as a macro nutrients. The highest concentration of K and Na were found (187, 161mg/100) in *O. sanctum* followed by (181, 154ppm) in *O. basilicum* and (164, 111ppm) in *A. indica*. Both K and Na are reported to play an important role in maintaining blood pressure. Hypertension is one of the major public concerns of the developing countries and controlled amount of K and Na in the diet could lead controlled blood pressure. The increase intake of K and reduce intake of Na have been linked to prevent hypertension.

The Ca and Mg in the investigated leaves were found in the range of 45-73ppm and 19-27ppm. The overall concentration of Ca was recorded highest 73ppm in *A. indica* followed by 54ppm *O. basilicum* and 45ppm in *O. sanctum*, while the highest concentration of Mg was recorded 27ppm in *O. basilicum* followed by 21ppm in *O. sanctum* and 19ppm in *A. indica*. Ca is the major component of bone and assist in teeth development. The mineral content also fluctuated at different reproductive stages. Rogiers *et al.*, (2015) studied the minerals content in different reproductive stages and found varied concentration of minerals [185]. Both micro and macro minerals analysis showed that these leaves may provide the needed minerals for undernourished people especially childrens and womens. Our finding confirmed the use of these leaves as a source of mineral for our diet as commercial product if used in calculated formula.

Table 4: Minerals Composition of *O. Sanctum*, *O. Basilicum* and *A. Indica* Leaves

Element	Micro Mineral analysis (ppm)		
	O. Sanctum	*O. Basilicum*	*A. Indica*
Cobalt (Co)	3.7	4.9	6.3
Cupper (Cu)	3.1	4.1	2.1
Iron (Fe)	35.4	33.8	41.2
Nickel (Ni)	10.4	11.3	9.76
Zinc (Zn)	71.3	65.9	43.4
	Macro Mineral Analysis (ppm)		
Potassium (K)	181	187	164
Sodium (Na)	154	161	111
Calcium (Ca)	45	54	73
Magnesium (Mg)	21	27	19

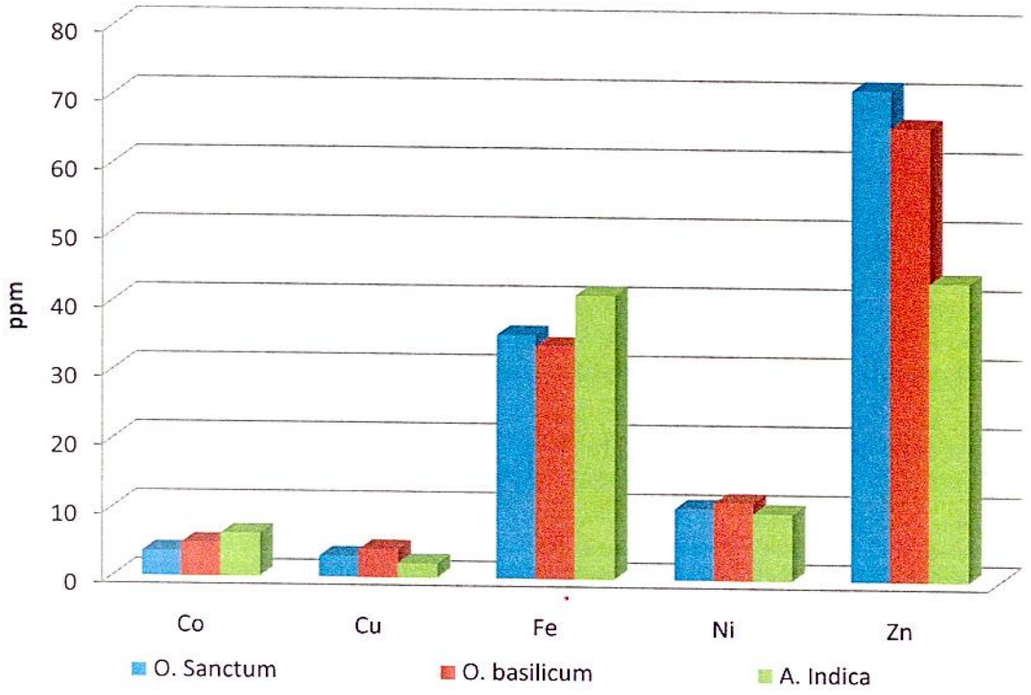


Figure 4: Micro Mineral Content of *O. sanctum*, *O. basilicum* and *A. indica*

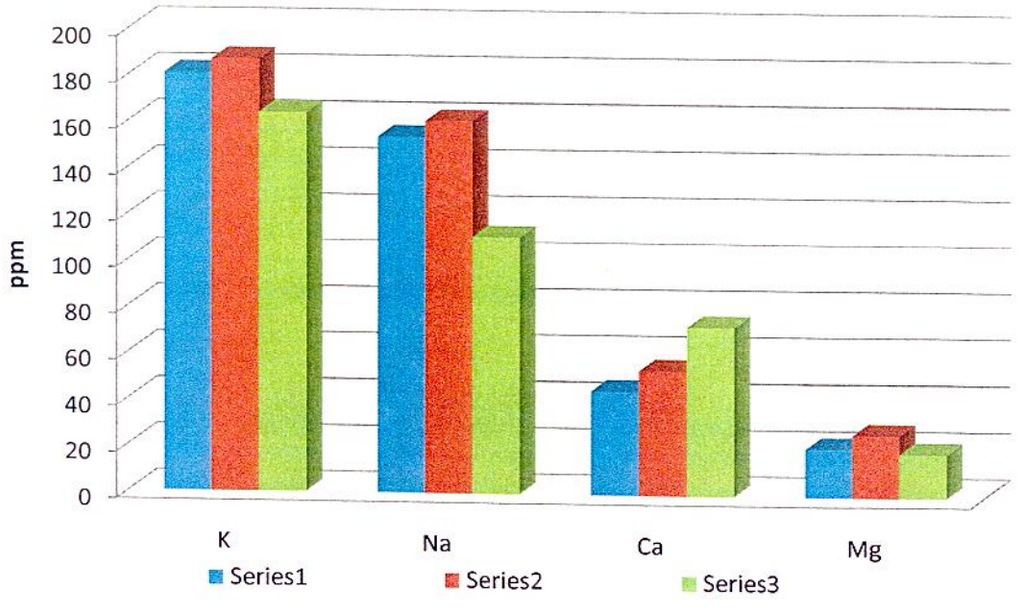


Figure 5: Macro Mineral Content of *O. sanctum*, *O. basilicum* and *A. indica*

4.3 PHYTOCHEMICAL ANALYSIS

The results of the preliminary phytochemical analysis of *O. sanctum*, *O. basilicum* and *A. indica* are shown in the following (Table-5). Which showed that all leaves samples contained alkaloids, glycosides, carbohydrates, triterpenoids, saponins, tannins and flavonoids. These phytochemical are non nutritive plant chemicals that having disease prevention properties. Saponins were detected in *O. sanctum*, *O. basilicum* and *A. indica* posses both beneficial and deleterious properties. It is because of some saponins which is highly toxic under experimental condition, although it have beneficial effects such as lowering of cholesterol level, increasing bone health and stimulation of immune system [186]. Tannins were also found in all selected leaves. The presence of tannin in herbs made them beneficial when applied on mucosal coating in month and therefore these herbs are widely used in mouth washes, snuff, eye washes and also treat rectal disorders [187]. However long term and excess use of herbs containing high concentration of tannins is not recommended. Flavonoids is also one of the important phyto-nutrient which is widely distributed in plants having antioxidant properties and responsible for medical qualities. These compounds having strong ability to scavenge free radicals quench singlet oxygen, chelat metals and break radical chain reaction. The presence of these phytochemical in leaves have medicinal properties in various disease condition such as diabetes, nausea, arthritis, arthrosclerosis, asthma, diarrhea, colitis, dysentery and cancer [188-190].

Table 5: Qualitative Screening of *O. Sanctum*, *O. Basilicum* and *A. Indica* for the Presence of Phytochemical

S.No	Name of Test	Observation	inference
1	**Alkaloids**	Formation of Creamy precipitate	Alkaloids present in *O. sanctum* and *O. basilicum*
2	**Flavonoids**	Black color appeared	Flavonoids present in all plant leaves
3	**Cyanogenic glycosides**	Yellow Picrate Paper turned to bricks red color	Cyanogenic glycosides present in all plant leaves
4	**Reducing sugar**	Bricks red color appeared	Reducing sugar present in all plant leaves
5	**Tannins**	Formation of green precipitate	Tannins present in all plant leaves
6	**Saponins**	Foam layer formed on top surface	Saponins present in all plant leaves
7	**triterpenoids**	Reddish violet color appeared	Triterpenoids present in all plant leaves

4.4 ANTIBACTERAIL ACTIVITY:

During the study of medicinal plant prior to characterization of individual phenolic compounds. The antibacterial assay of *O. sanctum*, *O. basilicum* and *A. indica* was study against eight bacterial strains including *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus*, *Klebsilla pneumonia*, *Proteus mirabilis* and *Enterococcus fecalis*. The activity had shown the potential of each crude extract as antibacterial and antifungal effects.

The results in (Table-6) showed that crude methanol extract of *O. sanctum* showed antibacterial activity against all selected bacterial strains accept *Salmonella typhi*. The highest activity of *O. sanctum* extract was observed against *Klebsilla Pneumonia* at all concentration. The *Bacillus cereus* showed maximum resistance in range of 0.6mg/mL. the MIC value of *O. sanctum* extract was 0.2mg/mL against *Staphylococcus aurous, Klebsilla pneumonia, Enterococcus fecalis* and 0.4mg/mL against *Escherichia coli, Proteus mirabilis* and *Pseudomonas aeruginosa.*

The antimicrobial activities of *O. sanctum* extract were also studied by Poonam et al., (2011) and the crude extract were found to be active against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Escherichia coli*. [191] Nisa *et al.*, (2013) assessed antifungal activity of crude extract of *O. sanctum* against isolated dermatophytic fungi including *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Microspore gypseum*, *Microsporum nanum* and *Epidermophyton flocossum* [192].

Table 6: Antibacterial Activity of O. Sanctum Against Different Pathoganic Strains

S/No	Antibacterial activity of *O. sanctum* (Zone of Inhibition in mm)						
	Bacterial strains	0.2mg/mL	0.4mg/mL	0.6mg/mL	0.8mg/mL	1.0mg/mL	MIC mg/mL
1	*Salmonella typhi*	R	R	R	R	R	R
2	*Escherichia coli*	R	11	14	17	21	0.4
3	*Staphylococcus aureus*	9	14	17	21	24	0.2
4	*Bacillus cereus*	R	R	R	11	13	0.8
5	*Pseudomonas aeruginosa*	R	08	15	20	25	0.4
6	*Klebsilla pneumoniae*	11	17	22	26	30	0.2
7	*Proteus mirabilis*	R	11	14	17	19	0.4
8	*Enterococcus fecalis*	10	16	21	25	29	0.2
9	Water(negative control)	0	0	0	0	0	R

R=Resistant

Table 7: Antibacterial Activity of *O. Basilicum* Against Different Pathoganic Strians

S/No	Anti bacterial activity of *O. basilicum* (Zone of Inhibition in mm)						
	Bacterial strains	0.2mg/mL	0.4mg/mL	0.6mg/mL	0.8mg/mL	1.0mg/mL	MIC mg/mL
1	*Salmonella typhi*	R	R	R	R	R	R
2	*Escherichia coil*	R	09	14	17	21	0.4
3	*Staphylococcus aureus*	R	08	11	13	18	0.4
4	*Bacillus cereus*	R	R	R	11	14	0.6
5	*Pseudomonas aeruginosa*	R	09	12	15	21	0.4
6	*Klebsilla pneumoniae*	11	15	19	23	28	0.2
7	*Proteus mirabilis*	R	R	09	11	13	0.6
8	*Enterococcus fecalis*	09	11	15	17	19	0.2
9	Water(negative control)	0	0	0	0	0	R

R=Resistant

Table 8:Anti Bacterial Activity of *A. Indica* Against Different Pathoganic Strians

S/No	Anti bacterial activity of *A. indica* (Zone of Inhibition in mm)						
	Bacterial strains	0.2mg/mL	0.4mg/mL	0.6mg/mL	0.8mg/mL	1.0mg/mL	MIC mg/mL
1	*Salmonella typhi*	R	R	R	09	12	0.8
2	*Escherichia coil*	11	16	21	27	32	0.2
3	*Staphylococcus aureus*	10	14	17	21	25	0.2
4	*Bacillus cereus*	R	R	R	10	14	0.8
5	*Pseudomonas aeruginosa*	12	16	21	26	31	0.2
6	*Klebsilla pneumoniae*	11	15	19	21	24	0.2
7	*Proteus mirabilis*	11	14	19	23	26	0.2
8	*Enterococcus fecalis*	R	09	13	14	16	0.4
9	Water (negative control)	0	0	0	0	0	0

R=Resistant

The crude methanol extract of *O. basilicum* also showed antibacterial activity against all selected bacterial strains accept *Salmonella typhi* (Table-7). The highest activity of *O. basilicum* extract was observed against *Klebsilla pneumonia* and *Enterococcus fecalis* at all concentration, while the MIC of extract was 0.4mg/mL against *Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli.*

The *Bacillus cereus* and *Proteus mirabilis* showed maximum resistance in range of 0.6mg/mL. The MIC value of *O. basilicum* extract was 0.2mg/mL against *Klebsilla pneumonia* and *Enterococcus fecalis.*

The antibacterial activity of crude methanol extract of *A. indica* is given in (Table-8). The extract was found active against all selected strains, while the *Salmonella typhi* was found resistant against the crude extract of *O. sanctum* and *O. basilicum.* The highest activity of *A. indica* (MIC value 0.2mg/mL) was found against Escherichia *coli, Staphylococcus aurous, Pseudomonas aeruginosa, Klebsilla pneumoniae* and *Proteus mirabilis.* The MIC value was 0.4mg/mL against *Enterococcus fecalis,* and 0.8mg/mL against *Bacillus cereus* and *Salmonella typhi.* Dhayanithi, *et al.,* (2012) studied the leaves extract of *A. marina* against isolated multi drug resistance *Staphylococcus aureus*. The results showed activity against different MRSA strains, which was resistant against Methicillin, vancomycin and ciprofloxacin [147]. Khan *et al.*, (2011) studied the antibacterial activity of *A. indica* against clinically isolated strains of pathogenic bacterial and fungal strains. They found the most susceptible strain was *Staphylococcus aurous* (MIC=16μg/mL), while most resistive was *Klebsilla pneumoniae* (MIC=128 μg/mL). The MIC value recorded for *Aspergillus niger* was 32μg/mL. [193]

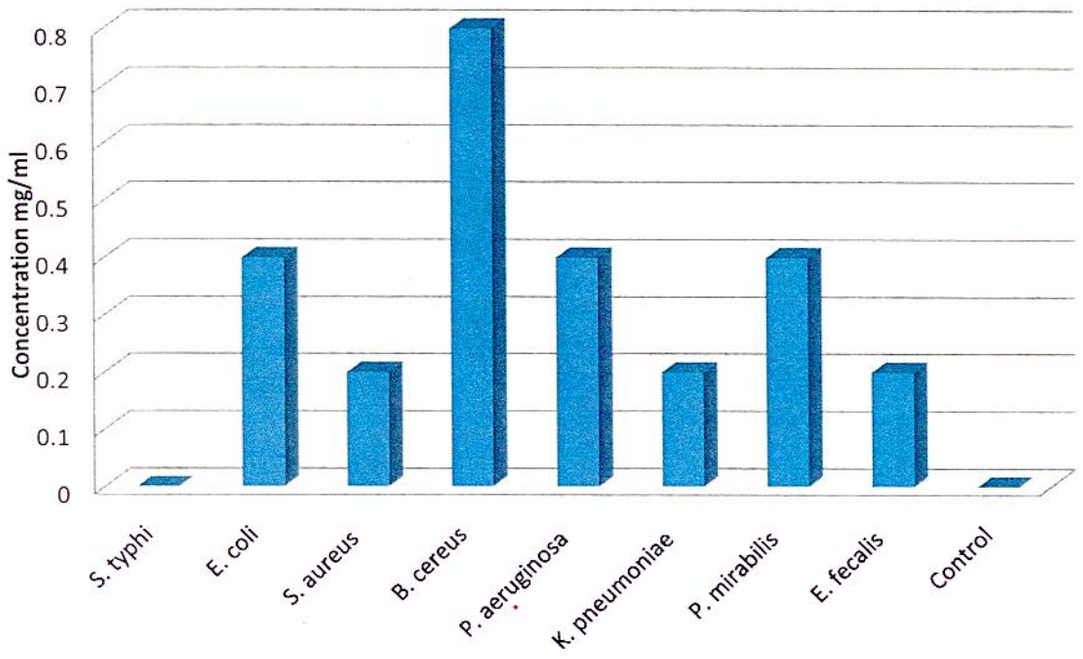


Figure 6: MIC of *O. Sanctum* Extract Agsinst Different Pathoganic Bacteria

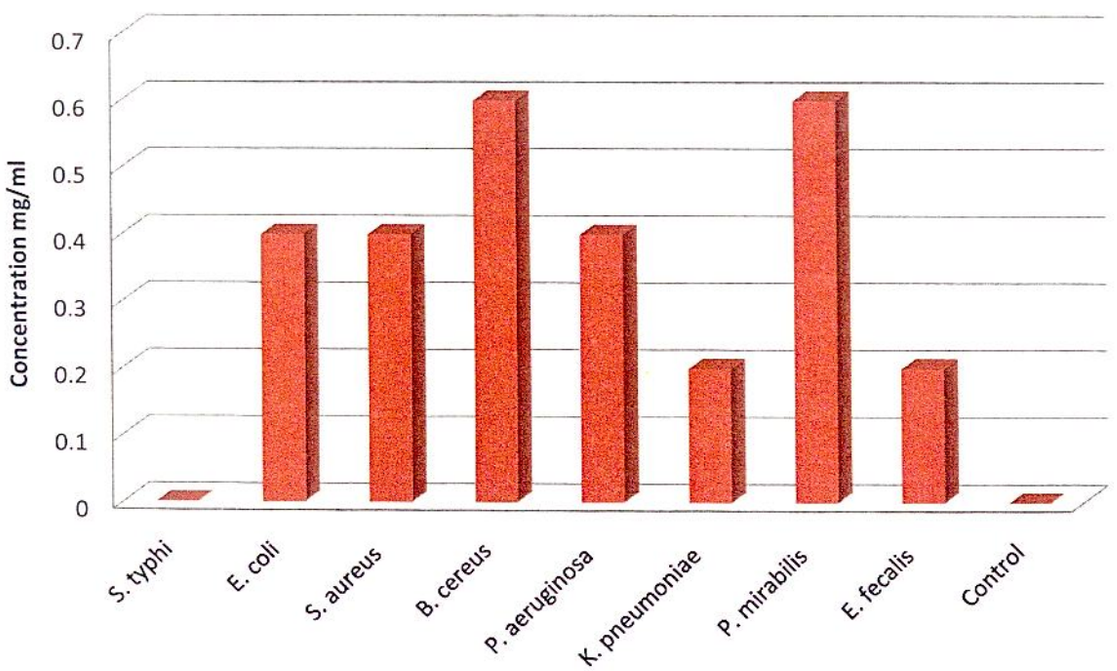


Figure 7: MIC of *O.Basilicum* Extract Agsinst Different Pathoganic Bacteria

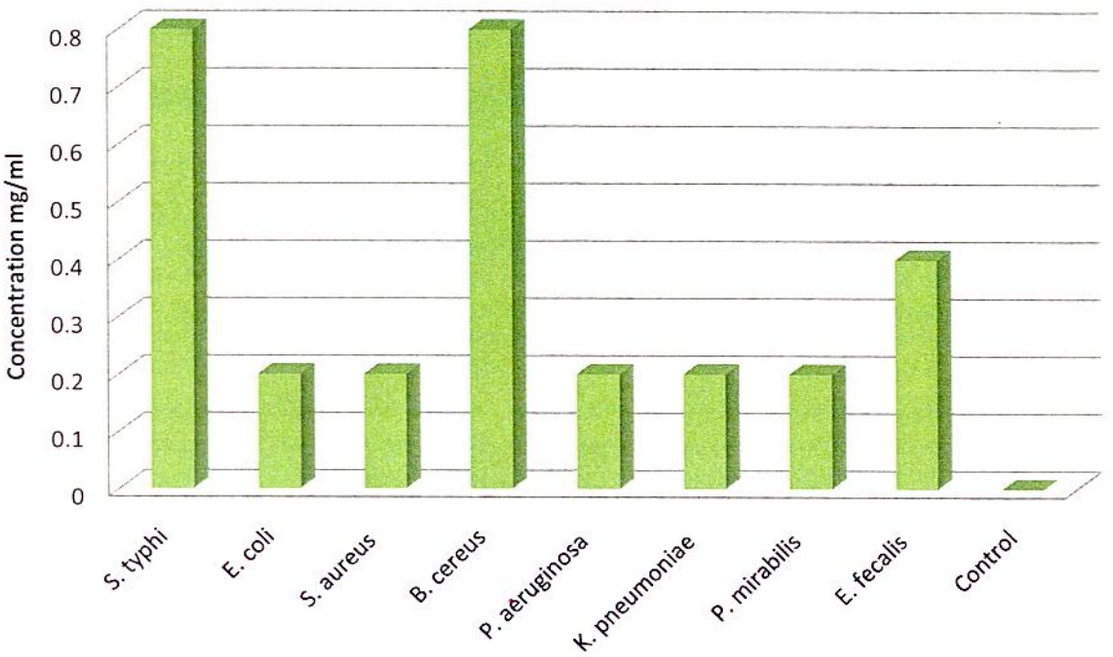


Figure 8: MIC of *A. Indica* Extract Agsinst Different Pathoganic Bacteria

4.5 ANTIFUNGAL ACTIVITY

The antifungal assays of *O. sanctum*, *O. basilicum* and *A. indica* were studied against three fungal strains including *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus parasiticus*. The crude methanol extract of *O. sanctum*, *O. basilicum* and *A. indica* showed antifungal activity against all selected strains (Table-9). The highest activity of *O. sanctum*, *O. basilicum* and *A. indica* extract was observed against *Aspergillus niger* at all concentration. The *Aspergillus parasiticus* showed maximum resistance in range of 0.4mg/mL and 0.2mg/mL against *O. sanctum* and *O. basilicum*. The MIC value of *A. indica* extract was 0.2mg/mL against all tested fungal strains.

Cushnie *et al.*, (2005) found that flavanones in plants leaves possessed activity against the opportunistic pathogen *Candida albicans* [135]. Ahmad et al., (2001) isolated the flavanols from *Terminalia belerica* fruits has also been shown to posses activity against *Candida albicans* [194].

Table 9: Antifungal Activity of *O. Sanctum*, *O. Basilicum* and *A. Indica* Against Different Fungi

Extracts	Microorganism	Antifungal activity (Zone of Inhibition in mm)					
		0.2mg/mL	0.4mg/mL	0.6mg/mL	0.8mg/mL	1.0mg/mL	MIC mg/mL
	Aspergillus niger	10	13	16	19	21	0.2
O. sanctum	*Aspergillus flavus*	R	09	13	15	16	0.4
	Aspergillus parasiticus	R	R	08	09	11	0.6
	Aspergillus niger	07	09	15	19	23	0.2
O. basilicum	*Aspergillus flavus*	09	11	16	20	24	0.2
	Aspergillus parasiticus	R	09	13	16	20	0.4
	Aspergillus niger	14	19	23	27	31	0.2
A. indica	*Aspergillus flavus*	11	15	18	22	25	0.2
	Aspergillus parasiticus	09	12	16	19	22	0.2
Control	Water	R	R	R	R	R	R

R=Resistant

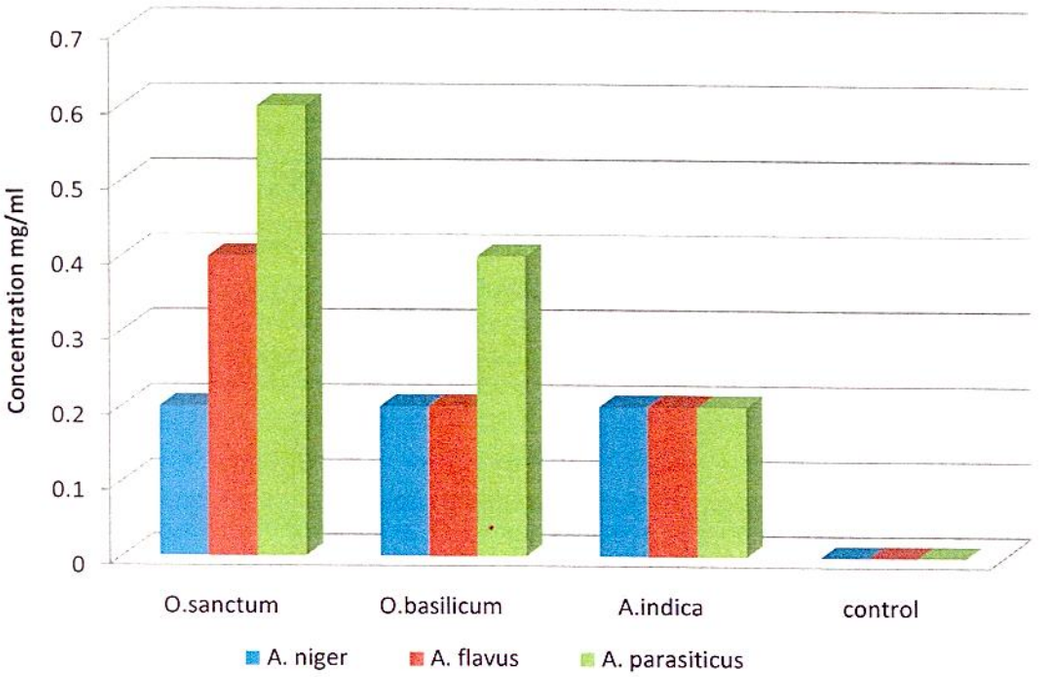


Figure 9: MIC Value of *O. Sanctum*, *O. Basilicum* and *A. Indica* Against Fungi

4.6 EFFECTS OF SOLVENTS AND EXTRACTION TECHNIQUES ON ANTIOXIDANT POTENTIAL

The antioxidant potential of *O. sanctum*, *O. basilicum* and *A. indica* were evaluated by DPPH method. DPPH solution exhibits a deep purple color with maximum absorption at 517nm. The presence of antioxidant in sample disappear the purple color because of quenching the free radicals of DPPH and convert them to colorless solution resulting decrease in absorbance [195]. DPPH free radical scavenging in % was directly proportional to the antioxidant concentration. The antioxidant activities were calculated from the % inhibition of DPPH free radicals.

Taking into consideration the industrial requirements for extraction, both yield and economic parameters were primarily considered during evaluation of antioxidant potential of selected leaves in this study. (Tables 10-12) depict the effects of three different organic solvent (ethanol, methanol and acetone) at two different extraction procedure Orbital Shaker Extraction (OSE) and Ultra Sonic Extraction (USE) on antioxidant potential of five different concentration (50, 100,150,200 and 250ppm) of each extract of *O. sanctum*, *O. basilicum* and *A. indica*.

Table 10: Scavenging Activity of Different Extracts of *O. Sanctum* by OSE and USE Procedures

S. No	Conc. PPM	Orbital Shaker Extraction (OSE)			Ultra Sonic Extraction (USE)			Vitamin-C
		Ethanol	Methanol	Acetone	Ethanol	Methanol	Acetone	
1	50	11.2*	15.0	09.4	18.1	21.3	15.6	38.1
2	100	27.4	33.0	23.1	36.4	37.6	32.4	53.2
3	150	39.5	42.8	31.7	45.4	47.8	40.9	65.7
4	200	49.4	53.0	38.5	56.6	61.4	51.7	73.3
5	250	58.6	61.9	47.3	64.3	71.3	57.7	83.6

*= Percent Scavenging of DPPH free radicals

Table 11: Scavenging Activity of Different Extracts of *O. Basilicum* by OSE and USE Procedures

S. No	Conc. PPM	Orbital Shaker Extraction (OSE)			Ultra Sonic Extraction (USE)			Vitamin-C
		Ethanol	Methanol	Acetone	Ethanol	Methanol	Acetone	
1	50	10.4*	23.1	11.4	12.1	28.4	11.2	38.1
2	100	23.4	32.3	19.3	25.2	37.7	19.5	53.2
3	150	34.2	41.6	27.6	36.8	46.1	31.3	65.7
4	200	42.7	50.8	38.1	45.4	55.1	41.7	73.3
5	250	53.1	58.8	51.3	57.7	61.4	53.4	83.6

*= Percent Scavenging of DPPH free radicals

Table 12: Scavenging Activity of Different Extracts of *A. Indica* by OSE and USE Procedures

S. No	Conc. PPM	Orbital Shaker Extraction (OSE)			Ultra Sonic Extraction (USE)			Vitamin-C
		Ethanol	Methanol	Acetone	Ethanol	Methanol	Acetone	
1	50	21.3*	37.4	19.1	26.1	41.3	23.1	38.1
2	100	29.2	46.6	28.4	33.9	49.1	31.1	53.2
3	150	41.6	54.3	37.5	46.7	58.6	40.2	65.7
4	200	52.2	63.5	45.2	55.6	67.8	48.6	73.3
5	250	62.2	74.1	53.4	67.8	78.1	57.1	83.6

*= Percent Scavenging of DPPH free radicals

The results in (Table-10) showed the highest scavenging activity of *O. sanctum* by USE procedure was in ranged of 21.3 to 71.3% in methanol extract followed by 18.1 to 64.3% in ethanol extract and 15.6 to 57.7% in acetone extract while by using OSE procedure these values were in the ranged of 15.0 to 61.9% , 17.2-58.6% and 09.4 to 47.3%. The same trend had observed in *O. basilicum* and *A. indica*. The highest scavenging activity of *O. basilicum* by using USE procedure was in ranged of 28.4 to 61.4% in methanol extract followed by 12.1 to 57.7% in ethanol extract and 11.2 to 53.4% in acetone extract while by using OSE procedure these values were in the ranged of 23.1 to 58.8, 10.4 to 53.1% and 11.4 to 51.3% respectively.

As compared to other leaves the maximum yield of antioxidant found in *A. indica*. The highest scavenging activity of *A. indica* by using USE procedure was in ranged of 41.3 to 78.1% in methanol extract followed by 26.1 to 67.8% in ethanol extract and 23.4 to 57.1% in acetone extract while by using OSE procedure these values were in the ranged of 37.4 to 74.1%, 21.3 to 62.2% and 19.1 to 53.4% respectively.

Considerable variations were observed in the antioxidant potential using different solvent system and extraction techniques. The results revealed that higher extraction of antioxidant yield was achieved using ultra sonic assisted extraction procedure followed by orbital shaker regardless of the solvent used. In different solvent system, methanol was found most effective for extraction of antioxidant compounds. The highest yield achieved using ultrasonic assisted stirring technique and methanol solvent in *O. sanctum*, *O. basilicum* and *A. indica*.

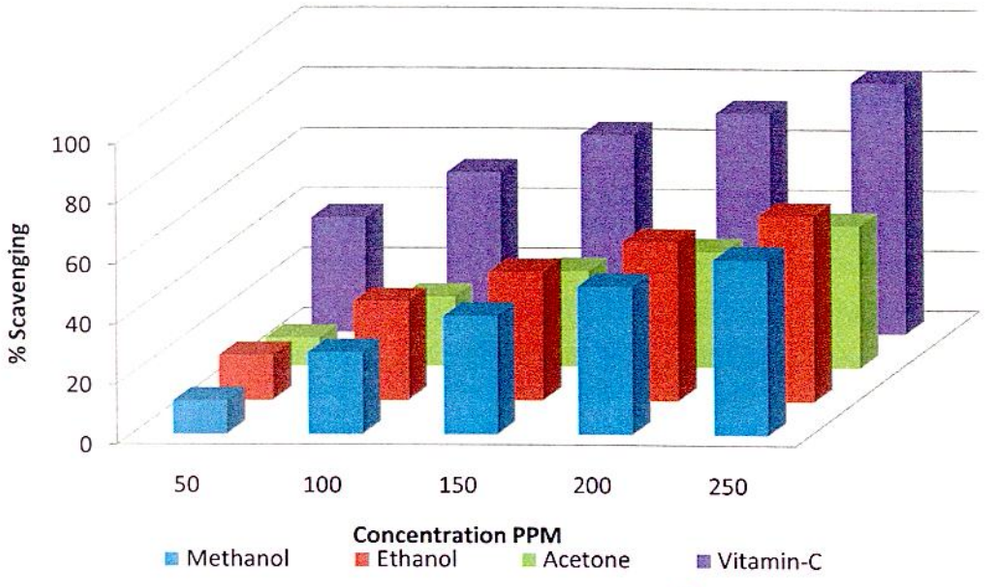


Figure 10: Scavenging Activity of *O. Sanctum* by OSE Procedure

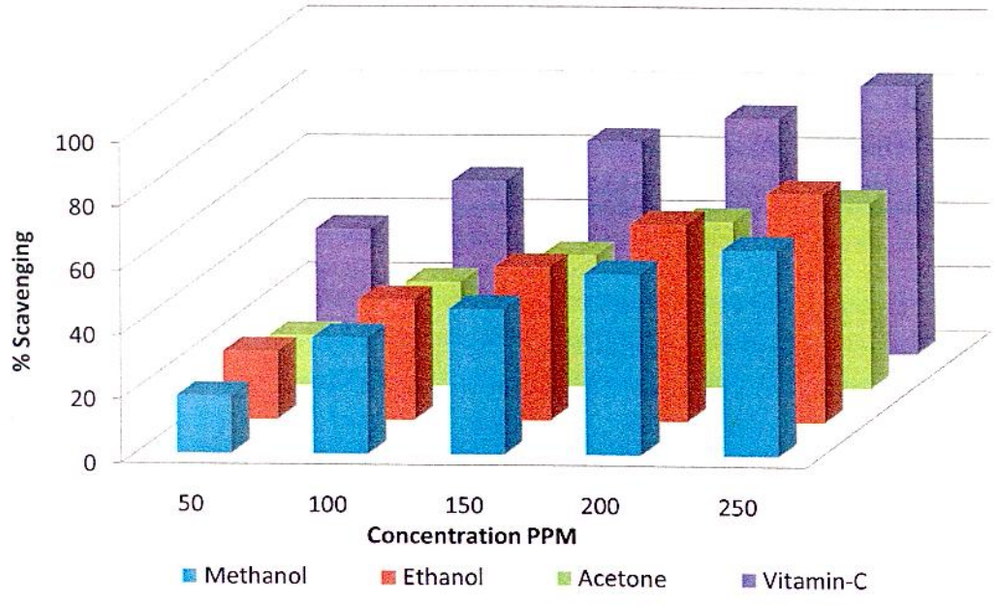


Figure 11: Scavenging Activity of *O. sanctum* by USE Procedure

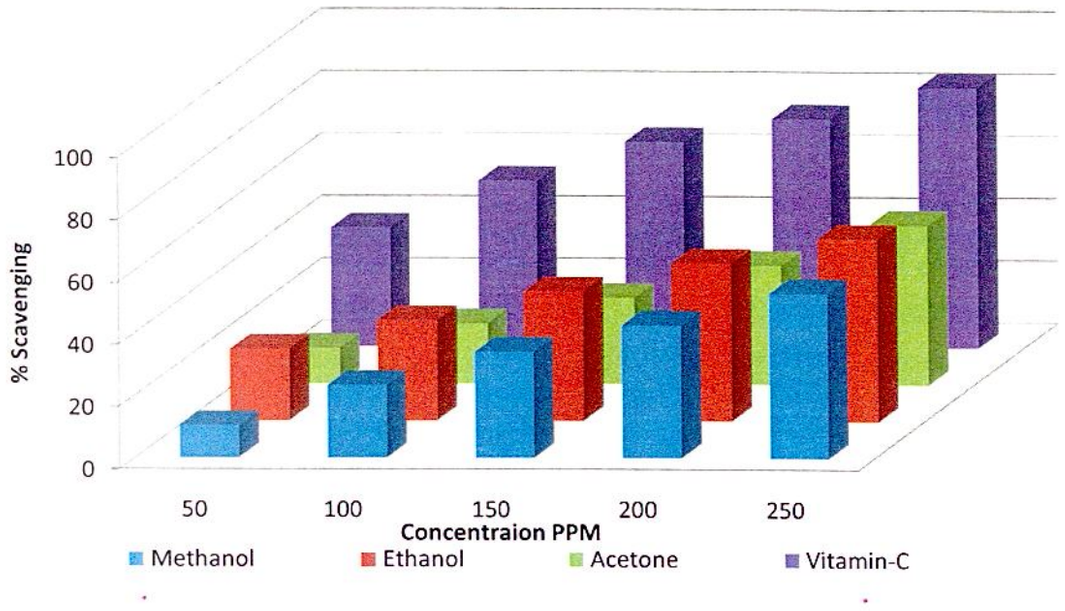


Figure 1: scavenging activity of *O. basilicum* by orbital shaker method

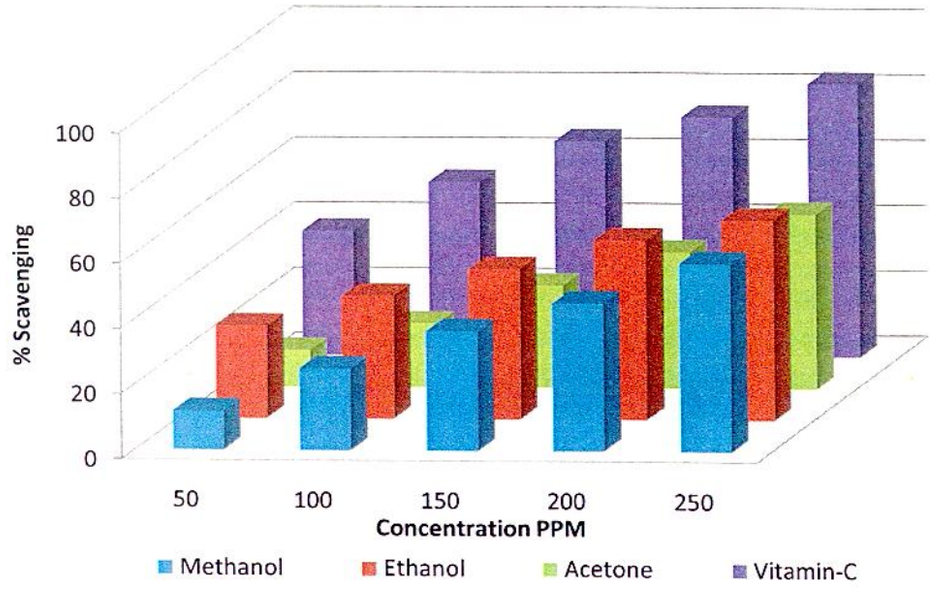


Figure 13: Scavenging Activity Of *O. Basilicum* by USE Procedure

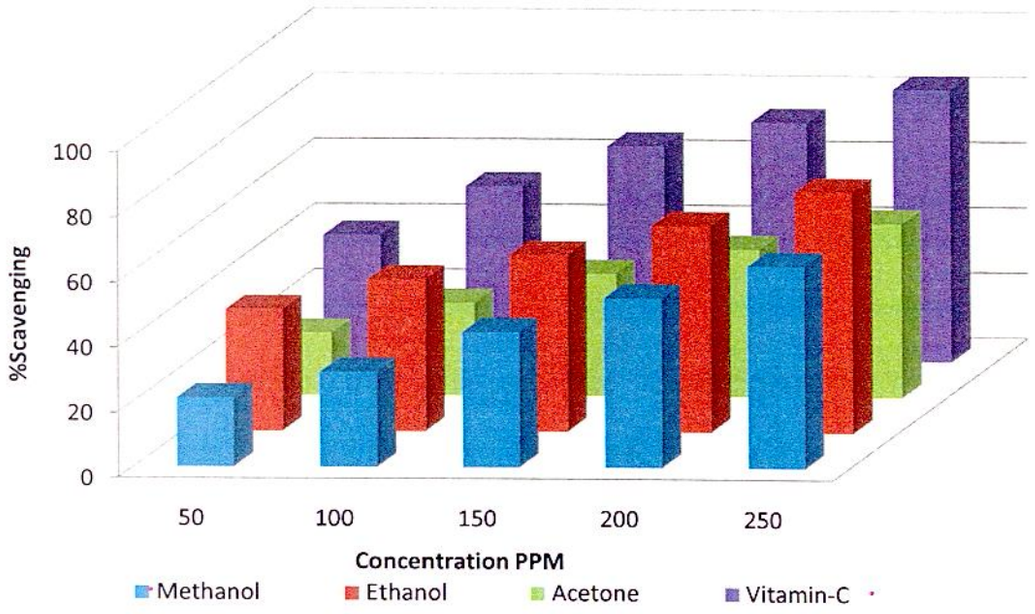


Figure 14: Scavenging Activity of *A. Indica* by OSE Procedure

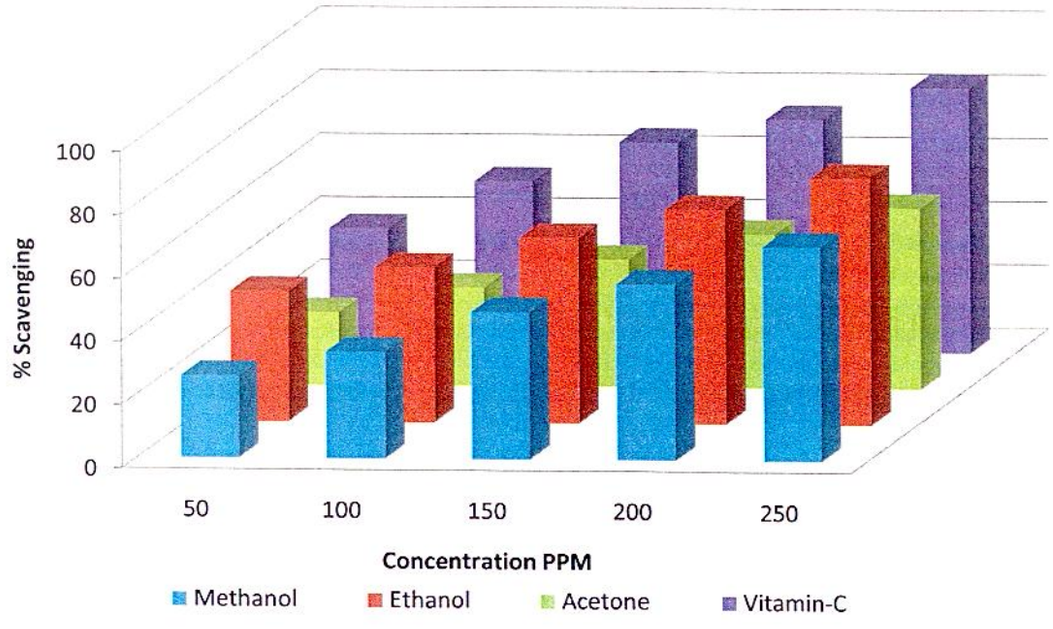


Figure 15: Scavenging Activity of A. Indica by USE Precedure

The EC-50 values were calculated from the calibration curve of different concentration of each sample extracts by using different techniques from Figure 16-20)

(Table-13) depicts the EC-50 value of different extract *O. sanctum*, *O. basilicum* and *A. indica* by using OSE and USE procedures. The concentration of antioxidant sample at which the inhibition reaches to 50% is its EC-50 value. This value is negatively related to the antioxidant activity. EC-50 is the amount of antioxidant needed to scavenging 50% free radicals.

The results revealed that USE procedure is more efficient for extraction of phenolic compounds than OSE procedure. In the previous research ethanol and methanol have been extensively used to extract antioxidant compounds for various plants [196]. Naseer *et al.*, (2014) found in the extraction of *Phellinus baumii* that more efficient extraction solvent was aqueous methanol. In agreement with our present finding, [197] reported highest extraction yield of antioxidant potential was achieved by using methanol [198].

In the literature, fewer reports were available regarding to the yield of antioxidant potential from plant leaves used in the present study. The present results showed that best yield of phenolic composition can be achieved by using USE procedure, regardless of the solvent system used. Ultra sonic waves are also reported for hydration and swelling of material to cause enlargement of pores, by which mass transfer improves and hence improved extraction yield of antioxidant compounds [199].

In the solvent system methanol proved to be the most effective for extraction of antioxidant compounds from selected plant leaves. The highest yield achieved by using USE procedure and methanol solvent in *O. sanctum*, *O. basilicum* and *A. indica*.

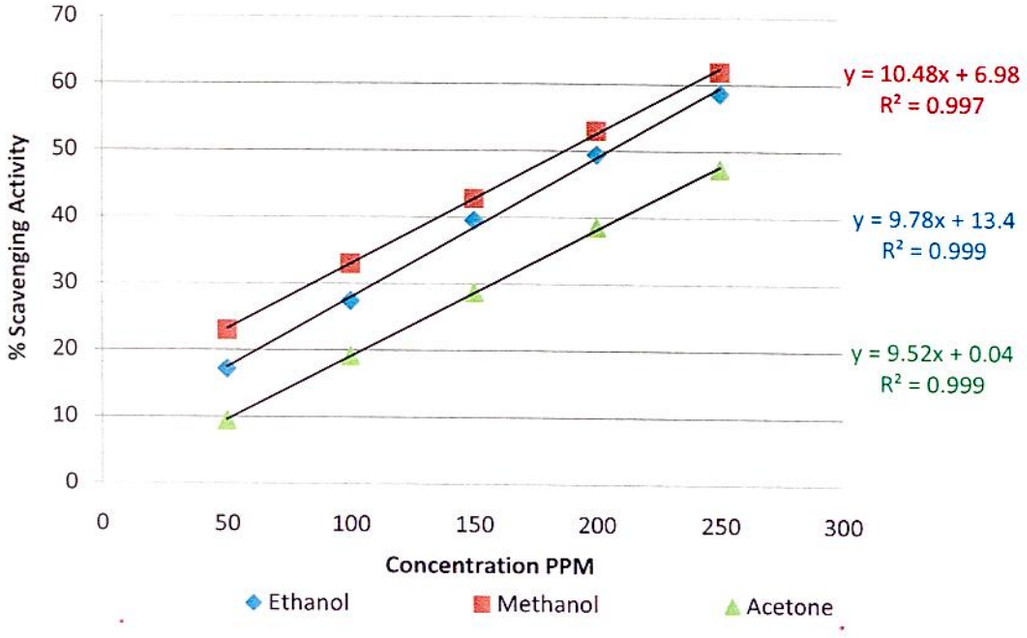


Figure 16: Linear Curve of *O. sanctum* Extracts by OSE Procedure

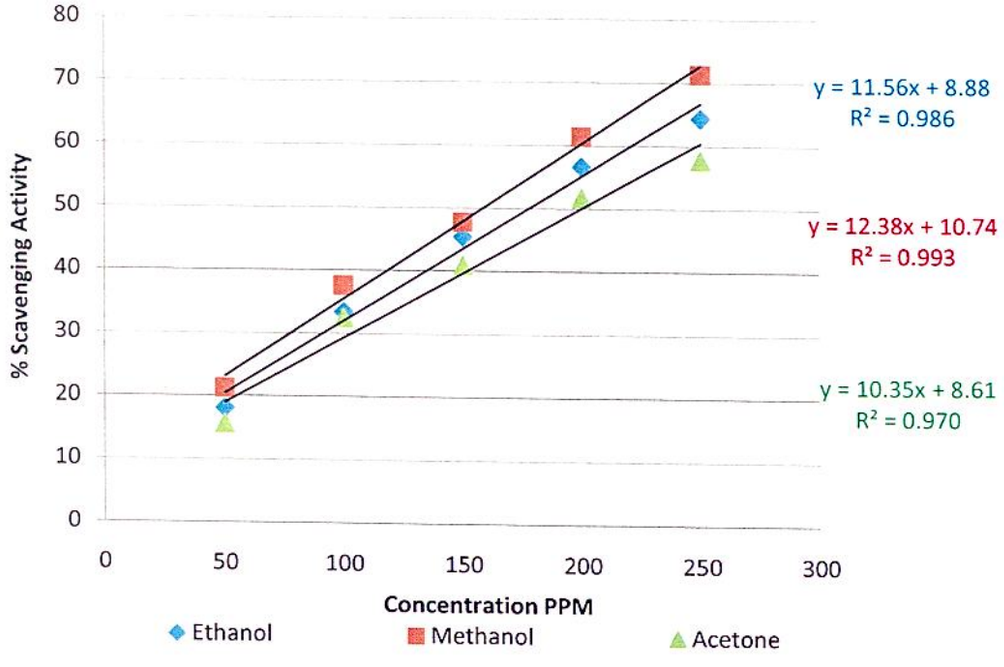


Figure 17: Linear Curve of *O. sanctum* Extracts by USE Procedure

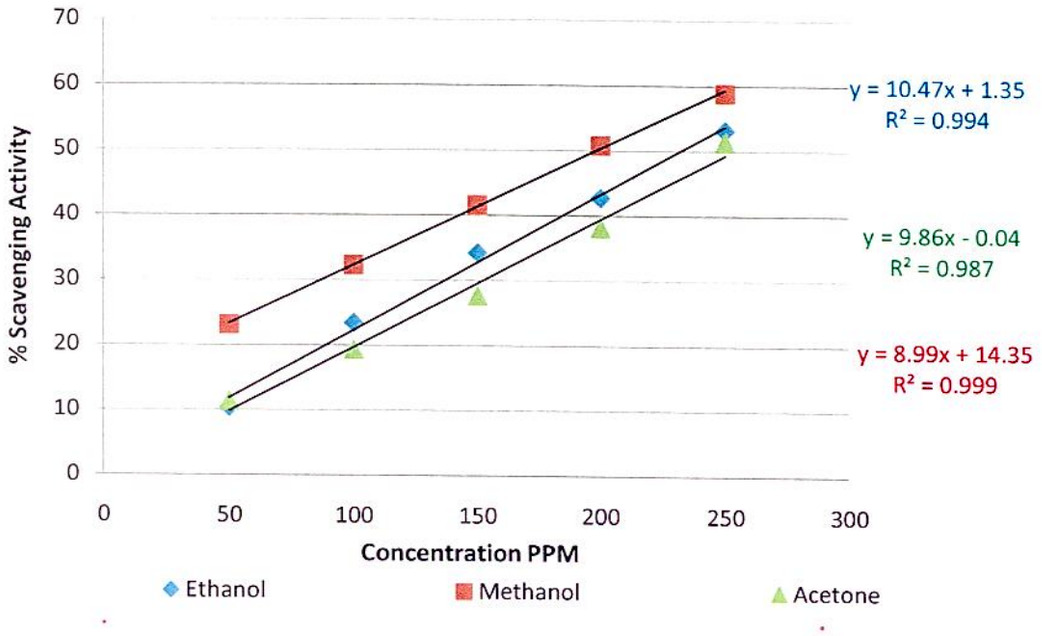


Figure 18: Linear Curves of *O. basilicum* Extracts by OSE Procedure

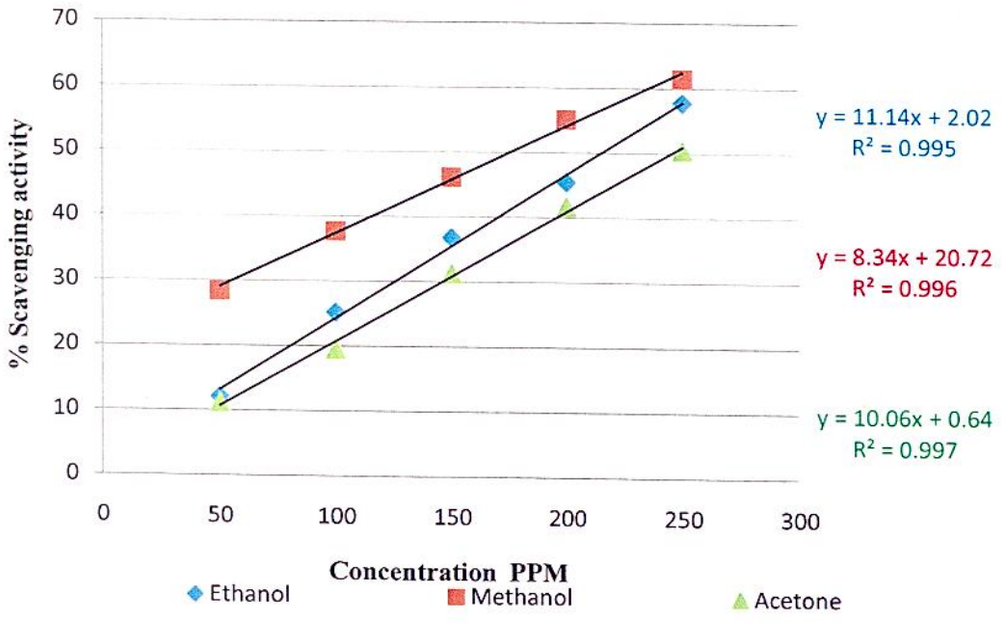


Figure 19: Linear Curves of *O. basilicum* Extracts by USE Procedure

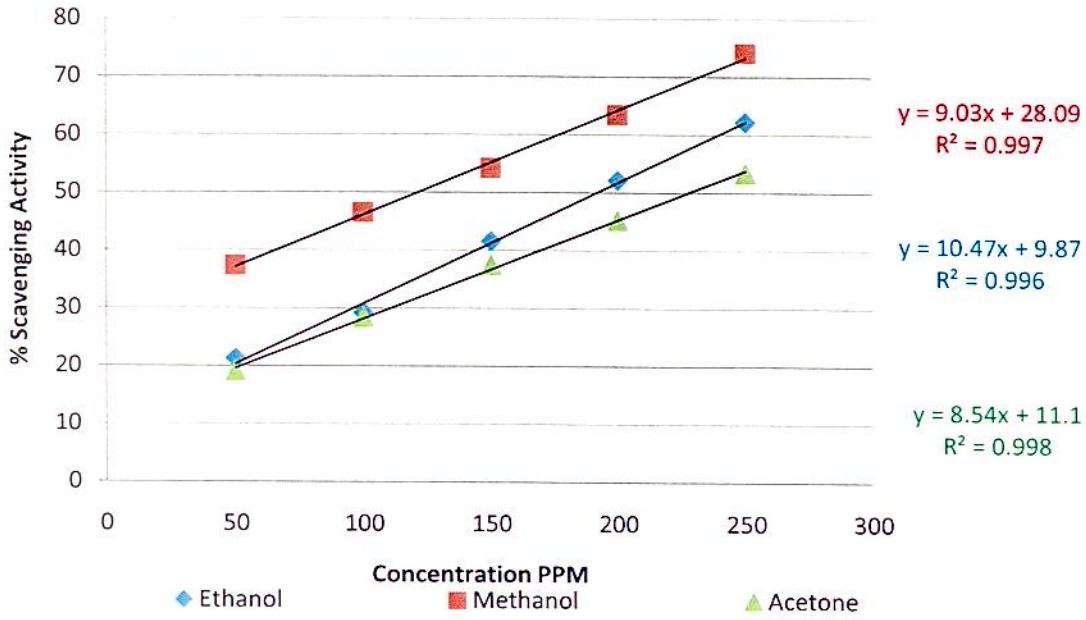


Figure 20: Linear Curves of *A. indica* Extracts by OSE Procedure

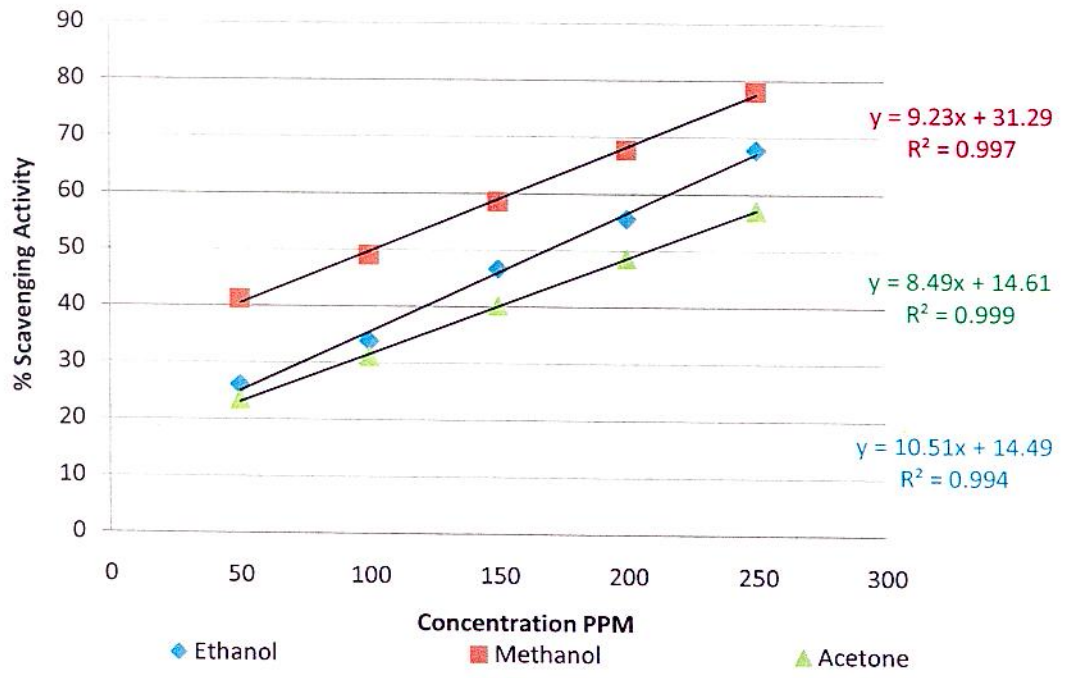


Figure 21: Linear Curves of *A. indica* Extracts by USE Procedure

Table 13: IC-50 Value Calculated from % Scavenging in PPM

S.No	Plant leaves	Extracts	EC-50	
			Orbital shaker method	Ultra Sonic method
		Ethanol	205.5	177.4
1	*O. sanctum*	Methanol	187.4	158.2
		Acetone	262.0	199.6
		Ethanol	232.2	215.3
2	*O. basilicum*	Methanol	198.6	175.7
		Acetone	253.1	245.6
		Ethanol	191.8	168.5
3	*A. indica*	Methanol	121.6	101.1
		Acetone	227.3	208.0

4.7 EFFECTS OF SOLVENTS AND EXTRACTION TECHNIQUES ON TOTAL PHENOLIC CONTENT (TPC)

TPC in selected plant leaves in the present work were determined using Folin-Ciocalteu reagent method, commonly known as the total phenols or phenolic assay and the results were expressed as gallic acid equivalent (GAE)/g of dry leaves extract [108]. TPC of *O. sanctum*, *O. basilicum* and *A. indica* leaves by different solvent system (ethanol, methanol and acetone) and using two different extraction procedure OSE and

USE are presented in (Table-14). The overall highest TPC were found 313µg/g in methanol extract of *A. indica* using ultrasonic method.

TPC in *O. sanctum* ranged from 46.0 to 164 µg/g of dry leave extract by using OSE procedure and 106 to 186µg/g by using USE procedure. The TPC in *O. basilicum* ranged from 115 to 296µg/g by using OSE procedure while by using USE it were found in the range of 139 to 400µg/g. The TPC found in A. Indica was in the range of 76 to 216µg/g by using OSE procedure while 186 to 313µg/g by using USE procedure. The figures 1 and 2 depicted the concentration of TPC in different extract of *O. sanctum*, *O. basilicum* and *A. indica* using two different extraction procedures.

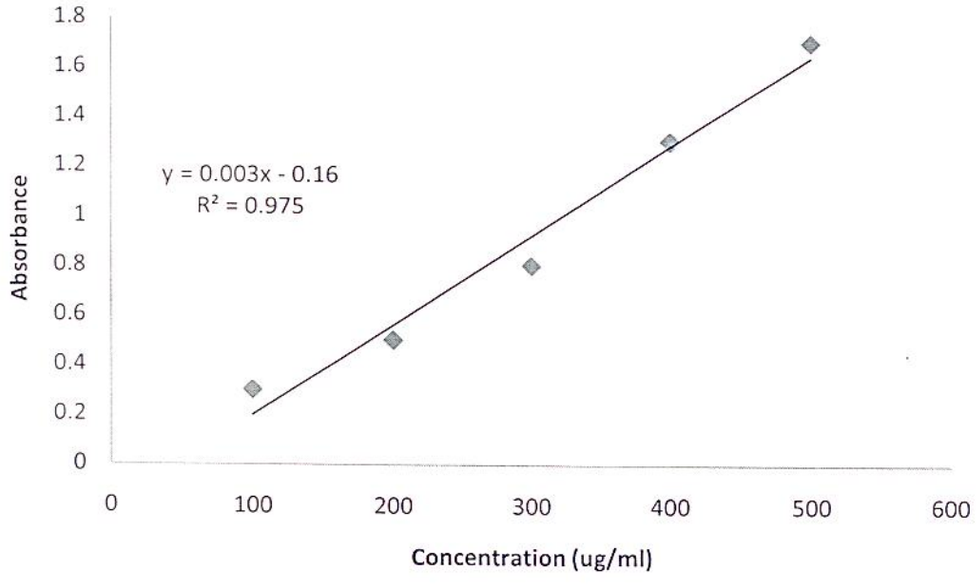


Figure 22: Linear Curve of Gallic acid

Table 14: TPC in Different Extract of Leaves by Different Procedure

Plant leaves	Extracts	Extraction Method	
		OSE	USE
O. sanctum	Ethanol	98.0	218.5
	Methanol	164.5	186.0
	Acetone	46.0	106.5
O. basilicum	Ethanol	208.5	276.0
	Methanol	296.0	300.0
	Acetone	115.5	139.5
A. indica	Ethanol	180.5	286.0
	Methanol	216.5	313.5
	Acetone	76.5	186.5

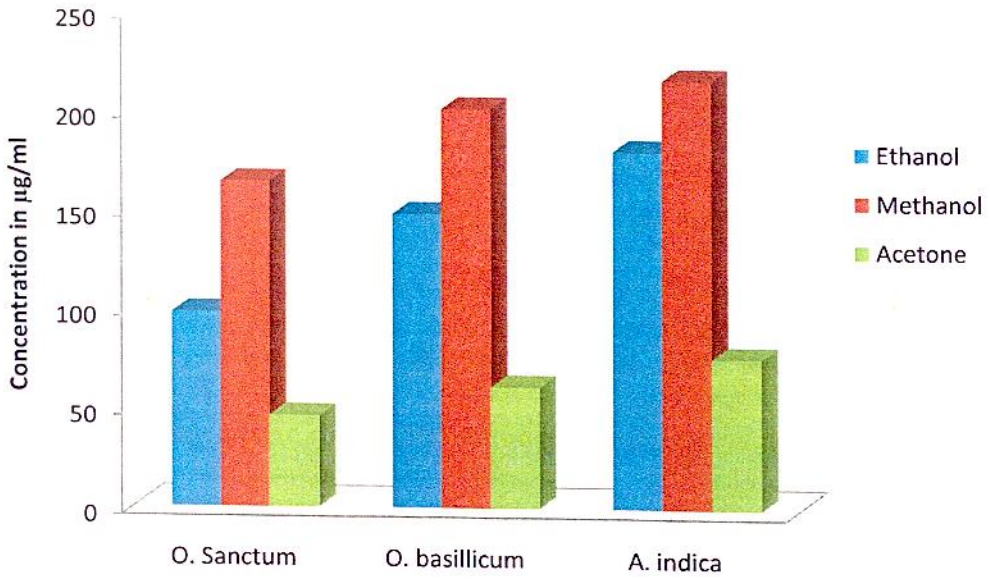


Figure 23: TPC of *O. sanctum*, *O. basilicum* and *A. indica* by OSE Procedure

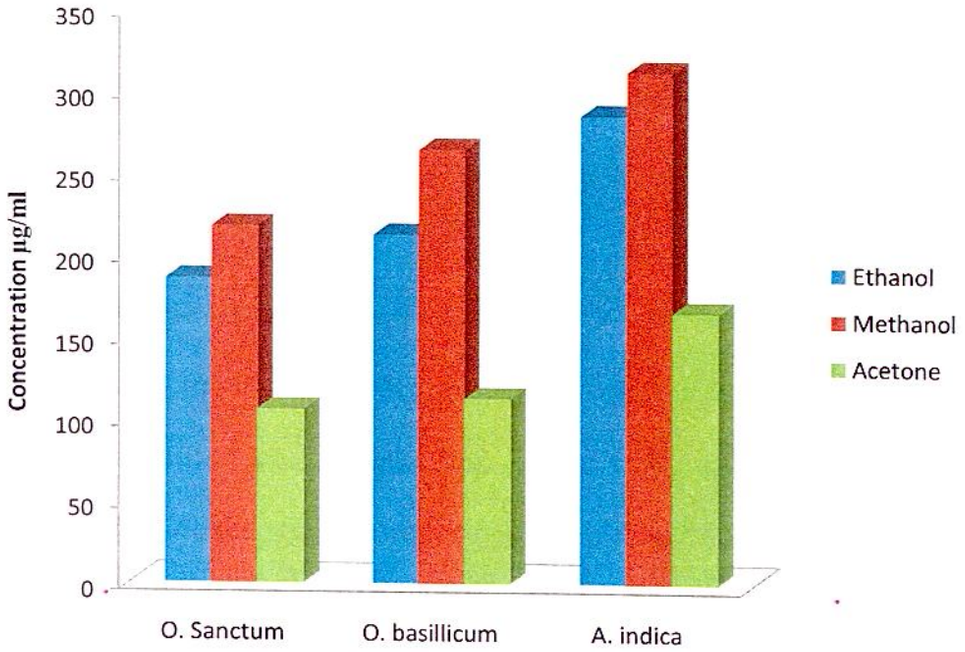


Figure 24: TPC of *O. Sanctum*, *O. Basilicum* and *A. Indica* by USE Procedure

The previous literature reveals that USE causes less degradation of phenolic compounds and is relatively effective and faster process for extraction of phenolic compounds as compared with other extraction method including microwave assisted, solid liquid, subcritical water method [109].

The extraction of phenolic compounds may also be influenced by other factors such as particle size of sample and solvent to solid ratio. Increases in solvent to solid ration have been found to be effective for enhancing phenolic yields and on the other hand lowering the particle size of sample material also enhances the yield of phenolic. [200, 201]. In some particular cases the antioxidant effectiveness and phenolic content appreciably increases by thermal processing as a result of conversion of ester linked phenolic compounds into free phenolic that exhibit more antioxidant activity [202].

4.8 EFFECTS OF SOLVENTS AND EXTRACTION TECHNIQUES ON TOTAL FLAVONOIDS CONTENTS (TFC)

The TFC in selected plant leaves in the present work were determined using three different solvent ethanol, methanol and acetone by two different extraction techniques OSE and USE. The results were expressed as quercetin equivalent (QE) and shown in (Table-15).

The TFC in *Ocimum* ranged from 69.0 to 314µg/mL by using orbital shaker and 125 to 401.5µg/mL using ultrasonic assisted stirring while the *O. basilicum* ranged from 61.5 to 246.5µg/mL by using orbital shaker and 113.0 to 365.0µg/mL by ultrasonic assisted stirring and in *A. indica* ranged from 256.0 to 491.5µg/mL by using orbital shaker and 346.0 to 529.0µg/mL using ultrasonic assisted stirring. The result depict that *A. indica* has higher TFC than other plant leaves. By studying different extraction solvent the effect of solvents on the recovery of TFC were methanol > ethanol > acetone.

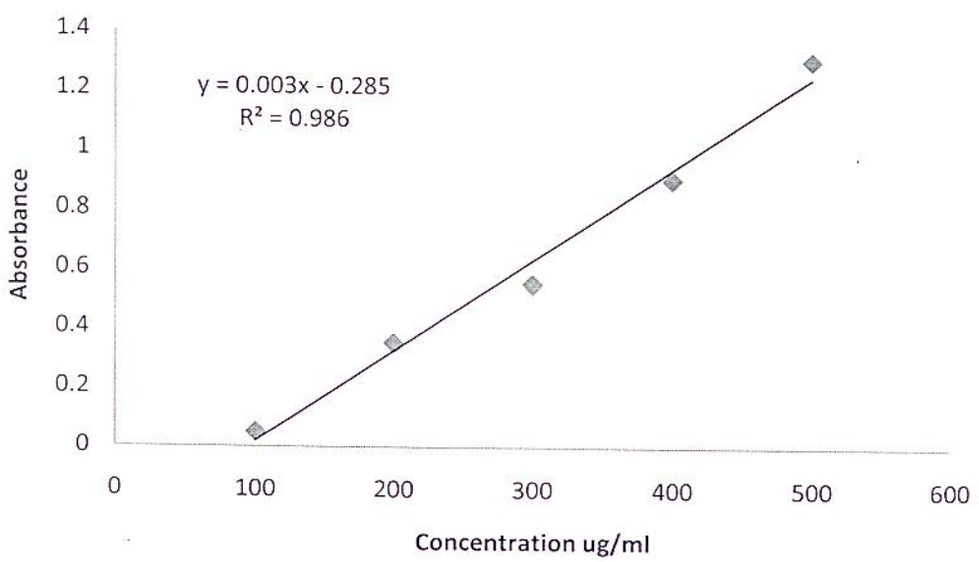


Figure 25: Linear Curve of Quercetin

Table 15: TFC in Different Extract of Leaves by Different Procedure

Plant leaves	Extracts	Extraction Method	
		OSE	USE
O. sanctum	Ethanol	246.5	293.0
	Methanol	314.0	401.5
	Acetone	69.0	125.0
O. basilicum	Ethanol	246.5	293.0
	Methanol	301.5	365.0
	Acetone	61.5	113.0
A. indica	Ethanol	400.5	463.0
	Methanol	491.5	529.0
	Acetone	256.0	346.0

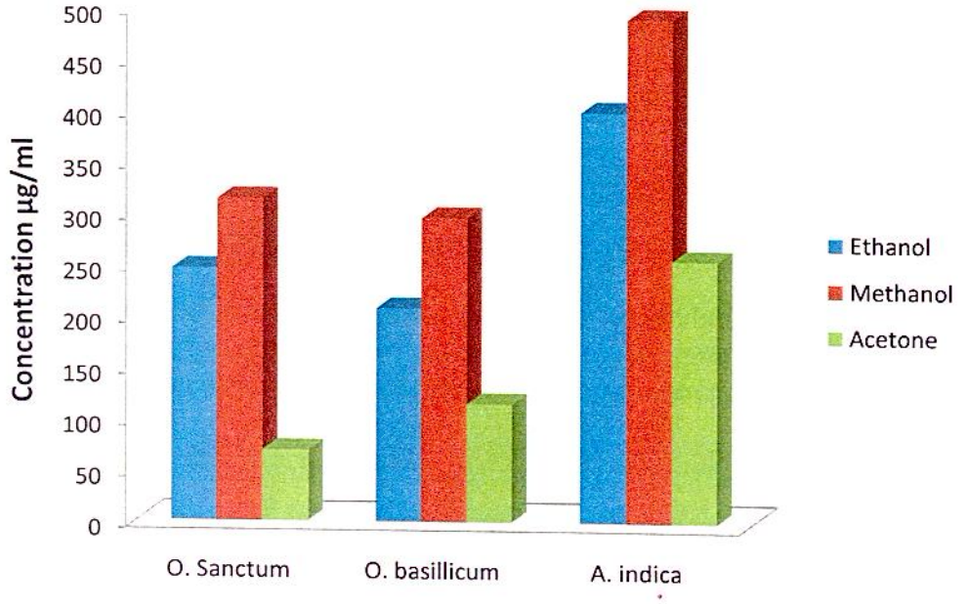


Figure 26: TPC of *O. Sanctum*, *O. Basilicum* and *A. Indica* by OSE Procedure

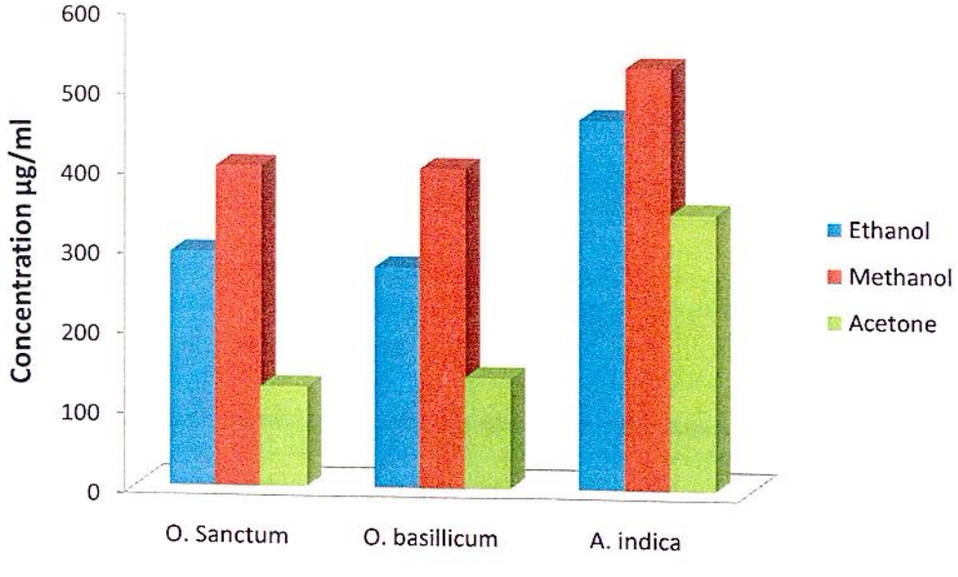


Figure 27: TPC of *O. Sanctum*, *O. Basilicum* and *A. Indica* by USE Procedure

4.9 GC-MS ANALYSIS

Gass Chromatographic technique is mostly used for the separation of gases and volatile substances, therefore it has limted use for the characterization of phenolic compounds. The non volatile phenolic compounds can be seprated by GC after deriviatization. The deriviatization increses the volatility, detectability and stability of these compounds. There are three common types of derivitization prior to GC analysis silylation, alkylation and acylation. Derivitization by silylation is an ideal procedure for the derivitization of non volatile and thermolabile compounds prior to GC analysis. The TMS derivatives are more volatile, thermotolerant and less polar. Silylation is a nucleophilic subsitution reaction in which the active hydrogen in –NH, -NH2, -OH and –COOH is replaced by a TMS group.

Zhang et al., (2004) developed a simple GC method for phenolic acids using TMS derivitization. They seprate nine phenolic acids within 16 minute run time [203]. Proestos, *et al.*, (2006) also reported the GC/MS method for identification of silyle derivatives of phenolic compounds in different medicinal plants. They also studied the optimization of derivitizing reagents, temprature and reaction time. They found BSTFA was the best derivitizing reagent [120].

The deveolped procedure used in our study [204] allowed the indentification of 10 phenolic compounds in *O. sanctum*, 11 in O. bsilicum and 10 in *A. indica*. The TIC chromatogram of phenolic compounds of *O. sanctum*, O.basilicum and *A. indica* is presented in (Figure 28).

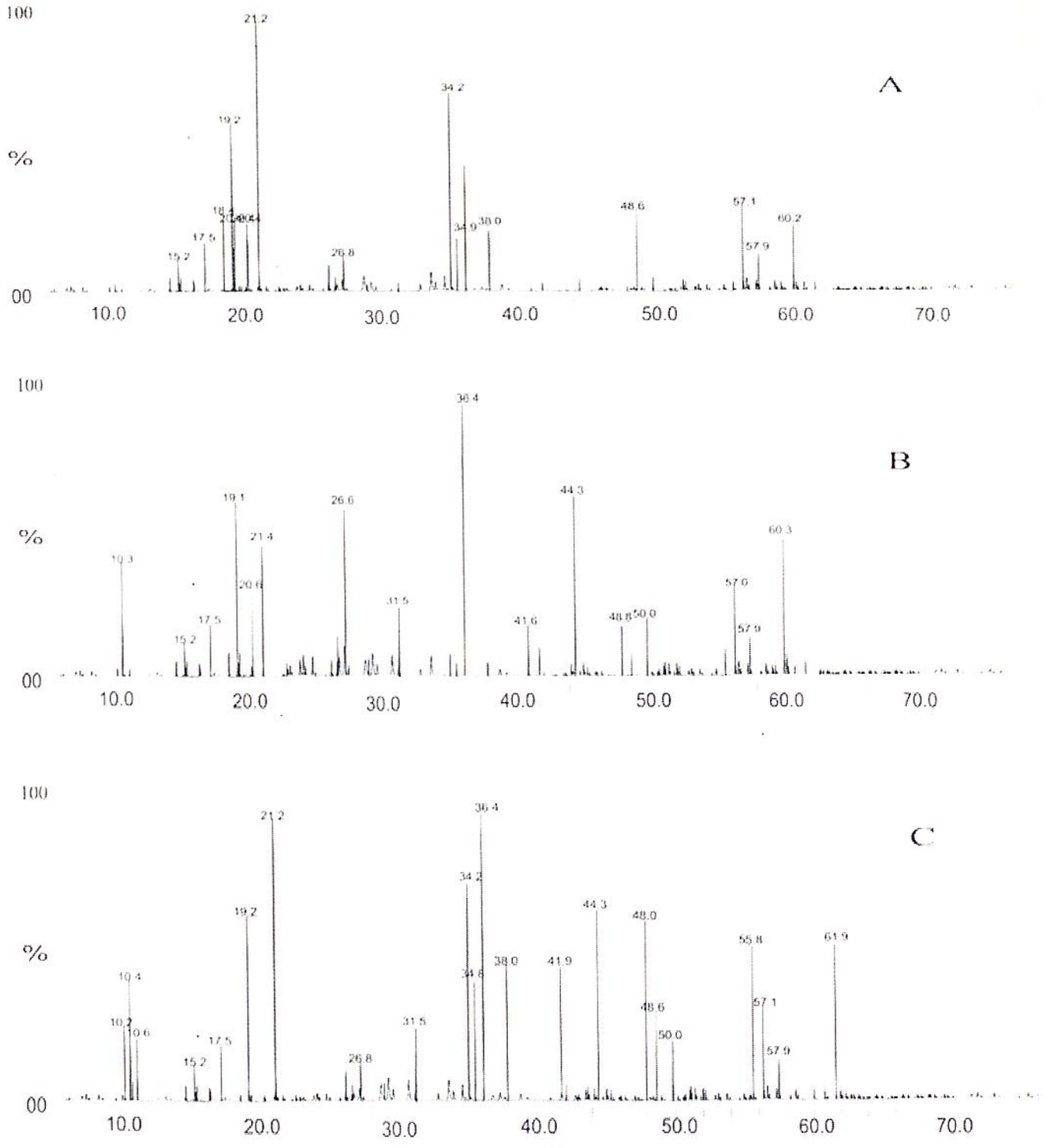


Figure 37: TIC Chromatogram of Silylated Phenolic Compounds A, B and C of *O. sanctum*, *O. basilicum* and *A. indica* Leaves

The retention time of these silylated phenolic compounds are tabulated in (Table-16).

Table 16: The Retention Time of Silylated Phenolic Compounds in Leaves Extracts of *O. Sanctum*, *O. Basilicum*a and *A. Indica*

S.No	Phenolic Compound	Retention Time (RT)		
		O. sanctum	*O. basilicum*	*A. indica*
1	p-Coumaric acid	ND*	10.3	10.4
2	Vanillic acid	15.2	15.2	15.2
3	p-Hydroxybenzoic acid	19.2	19.1	19.2
4	Gallic acid	20.4	20.6	ND
5	Quercetin	26.8	26.6	26.8
6	Ferulic acid	31.5	31.5	31.5
7	(+)-catechin	34.9	ND	34.8
8	(−)-Epicatechin	41.2	41.6	ND
9	Caffeic acid	48.6	ND	48.6
10	Syringic acid	ND	50.0	50.0
11	Kaempferol	57.1	57.0	57.1
12	Sinapic acid	57.9	57.9	57.9
13	Hydroxytyrosol	60.2	60.3	ND

*ND= Not Detected

The TIC chromatogram of *O. sanctum* showed the presence of five phenolic acid including vanillic acid (RT= 15.2), p-hydrobenzoic acid (RT= 19.2), gallic acid (RT=

20.4), caffeic acid (RT= 48.6) sinapic acid (RT= 57.9) and five flavonoids including quercetin (RT= 26.8), catechin (RT= 34.9), epicatechin (RT= 41.2) kaempferol (RT= 57.1), and hydroxytyrosol (RT= 60.2).

In the TIC chromatogram of *O. basilicum* 7 phenolic acid including p-coumaric acid (RT= 10.3), vanillic acid (RT= 15.2), p-hydrobenzoic acid (RT= 19.1), gallic acid (RT= 20.6), ferulic acid (RT= 31.5), syringic acid (RT= 50.0) and sinapic acid (RT= 57.9) while four flavonoids including quercetin (RT= 26.6), epicatechin (RT= 41.6) kaempferol (RT= 57.0), and hydroxytyrosol (RT= 60.3) were identified.

In the TIC chromatogram of *A. indica* 7 phenolic acid including p-coumaric acid (RT= 10.4), vanillic acid (RT= 15.2), p-hydrobenzoic acid (RT= 19.2), ferulic acid (RT= 31.5), caffeic acid (RT= 48.6), syringic acid (RT= 50.0) and sinapic acid (RT= 57.9) while three flavonoids including quercetin (RT= 26.8), catechin (RT= 34.8) and kaempferol (RT= 57.1) were identified.

The MS spectrogram of each phenolic compound TIC peaks are presented in (Figures 29-33). These phenolic compounds were identified by the present method as TMS derivatives, based upon the NIST and Wiley libraries.

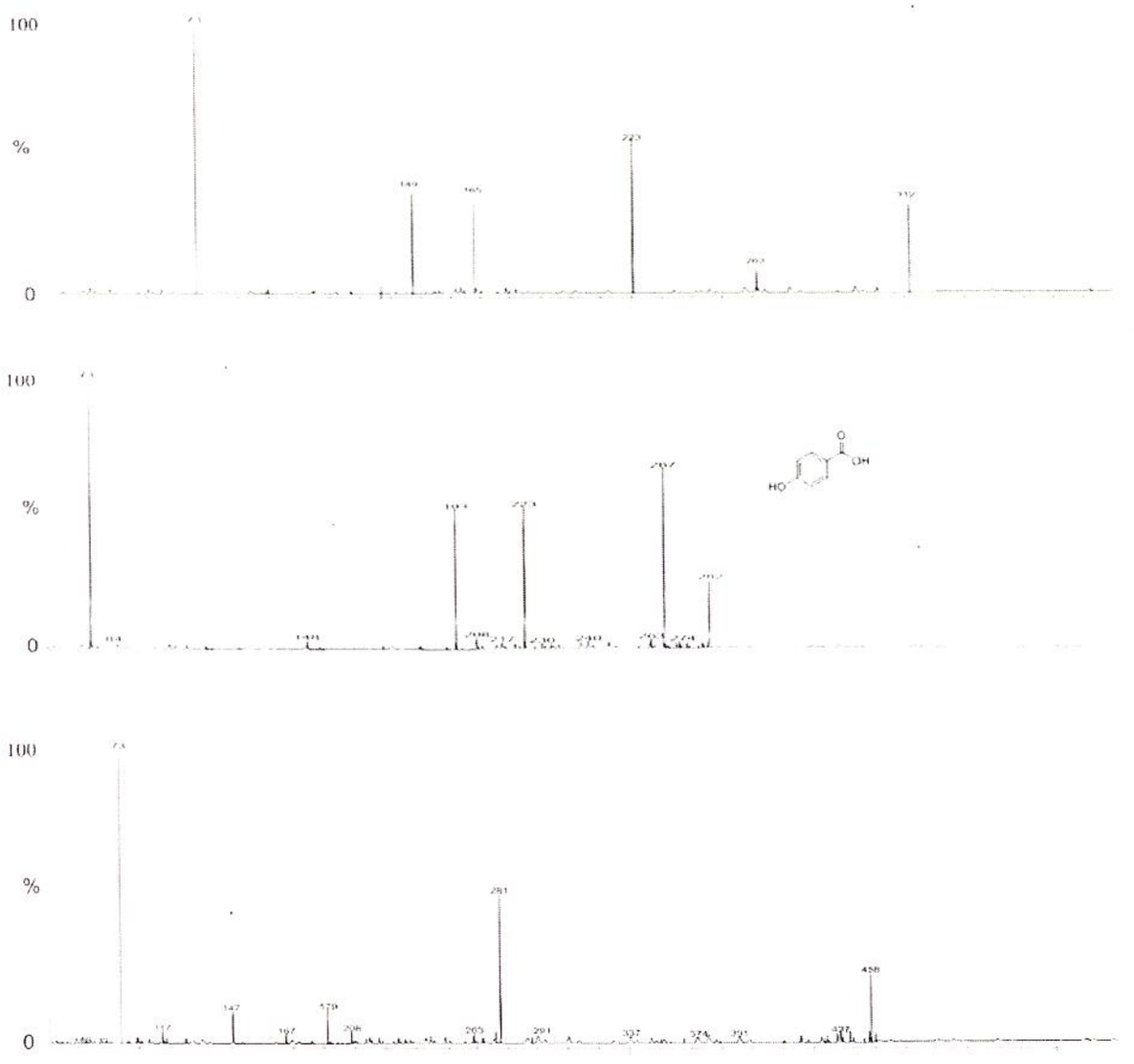


Figure 38: MS Spectrograms of TIC Peaks Vanillic, hydroxybenzoic and Gallic Acid

The moleculer ion of silylated compounds appears at 312 m/z for vanillic acid, 282 m/z for p-Hydroxybenzoic acid and 458 m/z for gallic acid, while the main peak appears at 223m/z, 267m/z and 281m/z respectively.

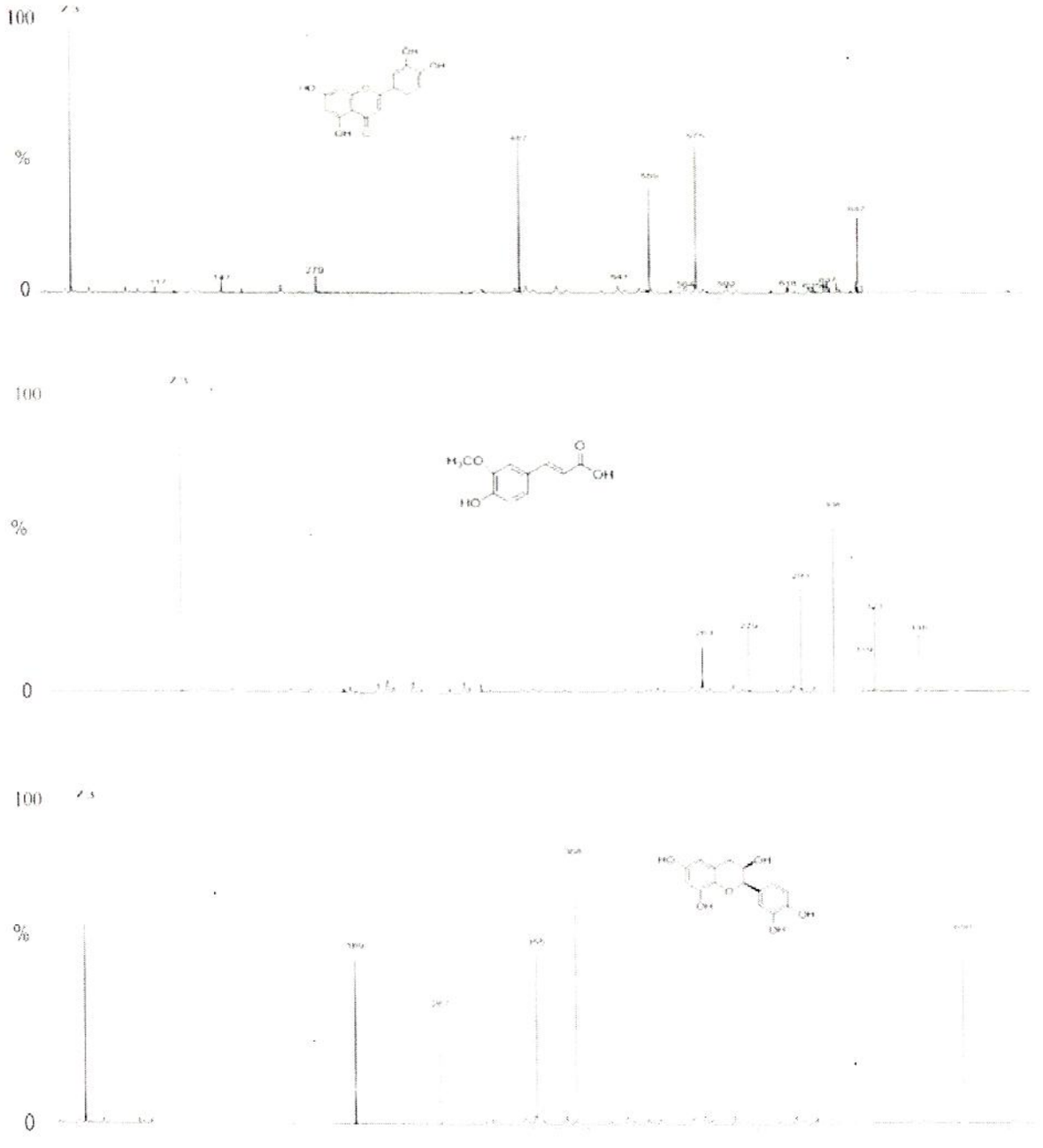


Figure 39: MS Spectrograms of TIC Peaks Quercetin and Ferulic Acid and Catechins

The moleculer ion of silylated compounds appears at 647 m/z for quercetin, 338 m/z for ferulic acid, 650 m/z for catechins, while the main peak appears at 575 m/z, 308 m/z and 368m/z respectively.

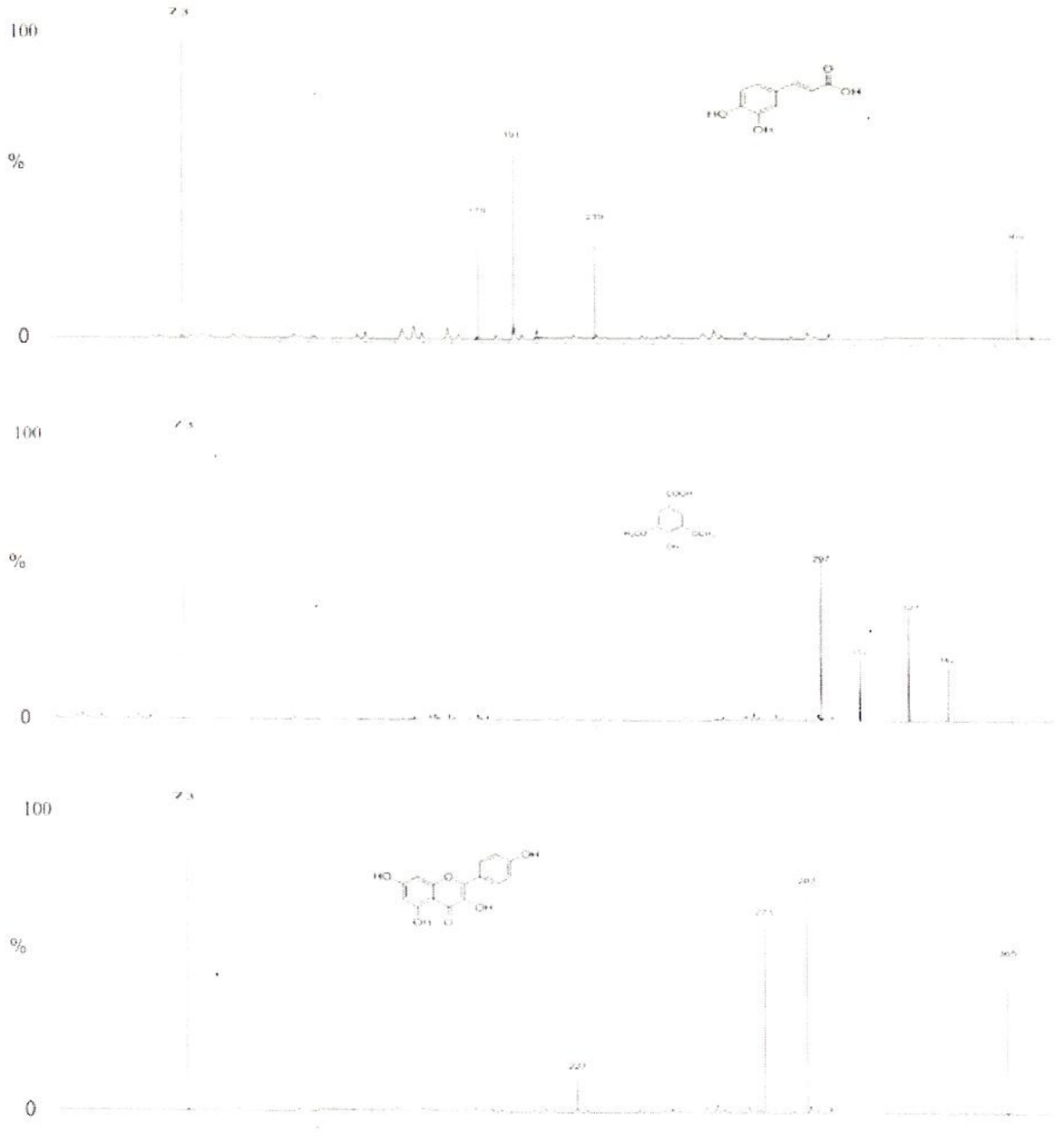


Figure 40: MS Spectrograms Of TIC Peaks caffiec acid, syringic acid and Kaempferol

The moleculer ion of silylated compounds appears at 396 m/z for caffeic acid, 342 m/z for syringic acid and 365 m/z for kaempferol, while the main peak appears at 219 m/z, 327 m/z and 287 m/z respectively.

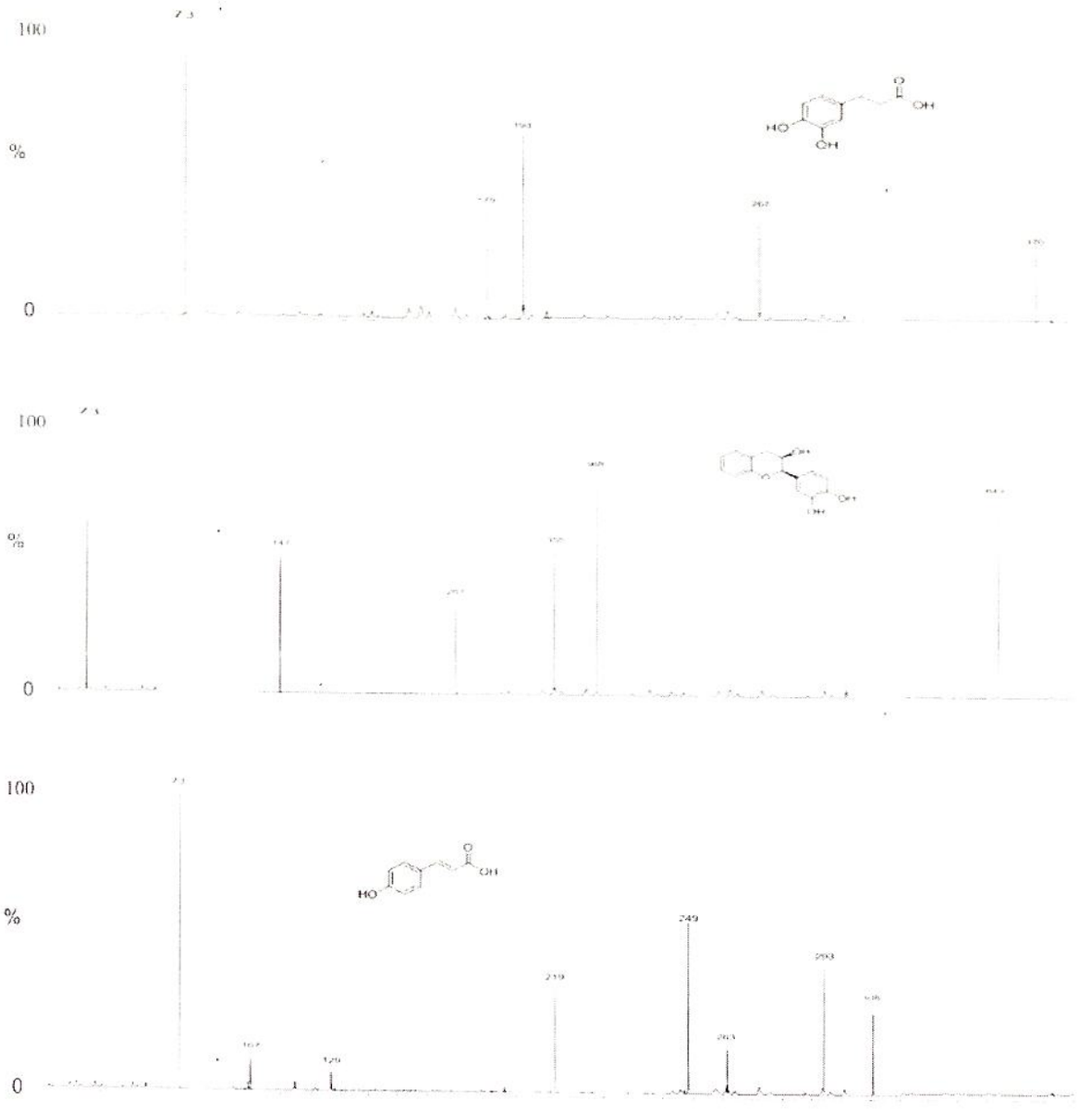


Figura 41: MS Spectrograms Of TIC Peaks Sinapic acid, -Epicatchins, Coumaric acid

The moleculer ion of silylated compounds appears at 368 m/z for sinapic acd, 649 m/z for (-)-epicatechin and 308 m/z for coumaric acid, while the main peak appears at 267 m/z, 368 m/z and 293 m/z respectively.

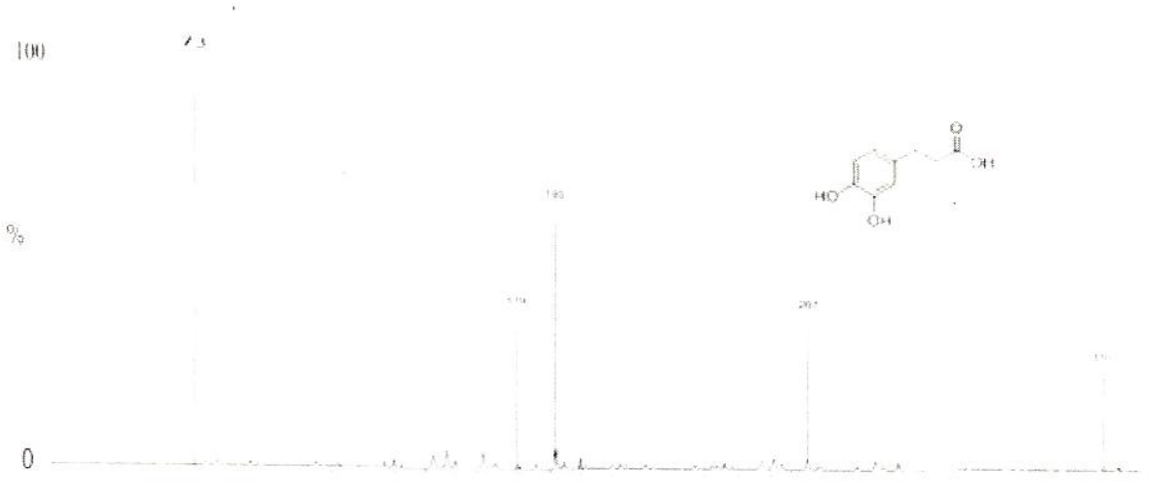


Figure 42: MS Spectrograms of TIC Peaks of Hydroxytyrosol

The moleculer ion of silylated compounds appears at 370 m/z for hydroxytyrosol, while the main peak appears at 267m/z.

The interpretation of GC-MS mass spectrum was evaluated using the database of National Institute Standard and Technology (NIST). The unknown spectrum was compared with known spectrum stored in the NIST library. Their name, molecular weight and structure of each components of the test material were ascertained.

The moleculer weight and characteristec fragments of these silylated phenolic in leaves extracts are presented in (Table-17). The fragmentation mechanism of phenolic compounds has been already studied by other researcher [116, 205]. For example Gallic acid shows a molecular ion (M+) at m/z 458 and a main peak at m/z 281(M+ - 177).

Table 17: Moleculer Weight and Fragments of Identified Phenolic Compounds

S.No	Phenolic Compounds	MW	Identified Ions (m/z)
1	p-Coumaric acid	308	219, 249, 293, 308
2	Vanillic acid	312	149, 165, 223, 312
3	p-Hydroxybenzoic acid	282	193, 223, 267, 282
4	Gallic acid	458	147, 179, 281, 458
5	Quercetin	647	487, 559, 575, 647
6	Ferulic acid	338	279, 308, 323, 338
7	(+)-catechins	650	267, 355, 368, 650
8	(−)-Epicatechin	650	267, 355, 368, 649
9	Caffeic acid	396	179, 191, 219, 396
10	Syringic acid	342	297, 312, 327, 342
11	Kaempferol	365	227, 271, 287, 365
12	Sinapic acid	368	323, 338, 353, 368
13	Hydroxytyrosol	370	179, 193, 267, 370

Hossain *et al.*, (2013) carried out the GC-MS analysis of various crude extract of the fruit of *Datura metel L.* without any derivatization. The results showed the presence of some polyphenol and their derivatives, hydrocarbons, flavonoids, and terpenoids (Hossain *et al.*, 2013). Karthikeyan et al., (2014) studied the active compounds in *Cynanchum callialatum* through GC-MS and LC-MS. The GC-MS analysis showed 52 volatile compounds while LC-MS used for nonvolatile constituents. These phenolic

ingredients in plants leaves extract are considered to be a part of plant defense system [206].

A GC-MS-SIM procedure was adopted by Chiou *et al.*, (2007) for analysis of low molecular weight phenol. 13 common phenols were identified after their conversion to volatile TMS derivatives including hydroxytyrosol, catechins, epicatechin, ferulic acid, gallic acid, coumaric acid, syringic acid, protocatechic acid, vanillic acid and p-hydrobenzoic acid in total retention time of 40 minutes [207]. Plessi *et al.*, (2006) developed a method for identification of phenolic acids after derivatization with (BTSFA). By the developed method none phenolic acids identified in balsamic vinegar including hydrobenzoic, vanillic, protocatecuic, syringic, isofeurolic, p-coumaric, gallic, ferulic, caffeic acid in total retention time of 10 minutes [208]. GC-MS analysis was carried out in order to get inside into the content of individual phenolic compounds.

Shareef *et al.*, (2004) studied the phenolic compounds by GC-MS after derivatization with two different reagents TMCS and BSTFA. They also studied the role of different variable in the derivatization procedure such as temperature of reaction, reaction time and amount of siltated reagents [209].

In the characterization of phenolic compounds from propolis using GC-MS, Bankova *et al.*, (2000) identified Twenty four compounds and eight of them were new [210]. Kalili et al., (2011) applied LC-MS method for quantitative analysis of phenolic acid, by this method seven phenolic acid including gallic, cholorogenic, caffeic, vanillic, syringic, coumaric and ferulic acid were found in Ocimum sanctum [211]. Proestos, *et al.*, (2006) reported a GC-MS method for characterization of phenolic acids as their trimethylsilyl derivatives in cranberry. Fifteen phenolic acids (sinapic, ferulic, phthalic,

vanillic, cinnamic, benzoic, p-coumaric, p-hydroxybenzoic, p-hydroxyphenyl acetic, o-hydrobenzoic, m-hydrobenzoic, o-hydroxycinnamic, 2,3-dihydroxybenzoic, 2,4-dihydrixybenzoic acid) were identified in cranberry [120]. Makri et al., (2008) studied the volatile components of basil and thyme leave he identified more than 50 aroma constituents in basil [212]. Popova *et al.*, (2005) studied the phenolic compounds by GC-Ms after silyation fifteen phenolic acids, eight ester of phenolic acid, six flavanones and two flavonoids were tentatively detected [213]. Li, *et al.*, (2001) developed a fast silyl derivatization technique for simultaneous determination of phenolic by GC-MS [118]. He also studied the impact of different silte reagent on phenolic composition. The extracts were silylated with TMCS, HMDS, and BSTFA.

Shareef *et al.*, (2006) reported the GC-MS method for characterization of silyl derivatives of phenolic from different aromatic compounds. They optimized silyation reagent, reaction time and temperature against derivatization process and found the best derivatizing reagent was BSTFA to convert analyte into volatile trimethylsilyl derivatives [214].

4.10 HPLC METHOD DEVELOPMENT FOR QUANTIFICATION OF PHENOLIC ACIDS

There are different Chromatographic techniques to analyze phenolic acid in plant extracts, such as TLC, HPLC, UHPLC and GC [215-217]. These separation techniques were commonly used for the separation and determination. These techniques were found to be sensitive sensitive, reproducible and reliable but its time consumable and cannot be applied for routine characterization in plant extract.

To date these analytical method have been reported for the characterization and quantification of phenolic compounds, but HPLC-MS method is considered to be more effective and useful than other analytical method, because HPLC-MS method provided information about the molecular masses and structures of unknown components [120]. However these instruments are most costly to purchase and maintain so therefore many researcher prefer to use HPLC-UV method for quantification of phenolic compounds for routine analysis, although it is tedious job to identify and quantify each phenolic compounds in plant extract.

The purpose of the current study was to develop a simple analytical procedure for the determination of phenolic acid by HPLC in plant extracts. HPLC is most popular technique as compare with other analytical techniques used for detection and quantification of phenolic acids. Such a separation is simple rapid and provides high resolution and sensitivity. It has many advantages like fast analysis, isolation of compounds, a small amount of sample is required for qualitative and quantitative analysis and highly sensitive and accurate [218]. The newly reported method was utilized to analyze the targeted phenolic acids including Gallic acid, Chloroganic acid, Syringic acid, Benzoic acid and vanillic acid in *O. sanctum*, *O. basilicum* and *A. indica* leaves extracts. These phenolic acid were separated by analytical column Intersil ODS-3 C18 (GL Sciences Inc. Tokyo Japan 5um, 250+ 4.6mm) using mobile phase ACN and acetified water in a total run time of 30 minutes, by using UV-Vis Detector. These entire compounds have already been described by different detector.

Mettila, *et al.*, (2002) developed a method for simultaneous determination of 15 phenolic compounds using RP-HPLC with UV-Vis detection. The optimized mobile

phases consisting 1% acetic acid in ACN as A and B was 1% acetic acid in water using gradient elution system. By the proposed method flavonoids were detected at 270nm while phenolic acid including caffeic, ferulic, sinapic and chlorogenic acid were detected at 320nm [219]. These entire compounds have already described by different detector. The most common detector chosen for separation of phenolic acid was Diode Array Detector DAD [219], but here it is reported by the UV-VIS detection.

In method development the selection of mobile phase and column become crucial for achieving good separation. In the optimization of solvent system for the separation of phenolic acids different ratio and different mobile system were used containing acidified water and a polar organic solvent. The increasing of acidity improves the separation by suppressing the ionization of acidic groups [220]. In trail assay elution program of acetic acid, phosphoric acid solution at different pH (3-8) and ACN and methanol were tested. However ACN and acetic acid in gradient elution program was found good for phenolic acids separation.

The peak area of different concentration of each phenolic acid standard was automatically measured by an integrator of HPLC instrument. Linearity was determined by drawing calibration curves. For the construction of calibration curve, six calibration standard solutions (5 to 30µg/mL) was injected in triplicate. Their peak area is given in (Table-18).

A linear equation was drawn between the concentration and peak area of each compound (Figures 35-39) as Y= mx +c here y is peak area; x is concentration while m and c are constant. A good linearity was found in the range 0.995-0.999 for all the compounds (Table 19).

Table 18: Peak Area of Each Phenolic Acid at Different Concentration

S.NO	Standard	Peak Area of Concentration µg/mL					
		5	10	15	20	25	30
1	Gallic Acid	17682	25455	34567	42965	51345	59432
2	Chlorogenic Acid	31456	38765	45643	53235	61543	71565
3	Syringic Acid	15243	22345	31425	39265	48536	56543
4	Benzoic Acid	24356	31876	39465	45876	53806	60238
5	Vanallic Acid	29656	36765	43236	49879	58376	66567

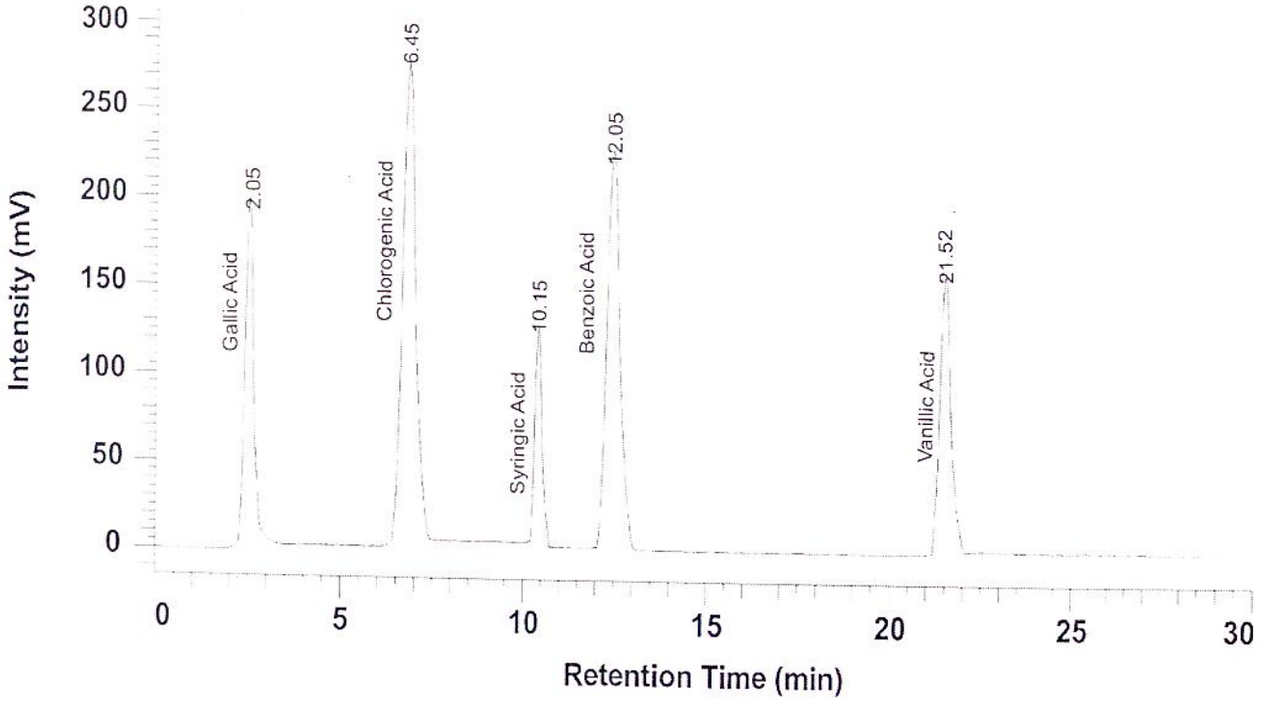


Figure 34: HPLC Chromatogram of Standard Phenolic Acids

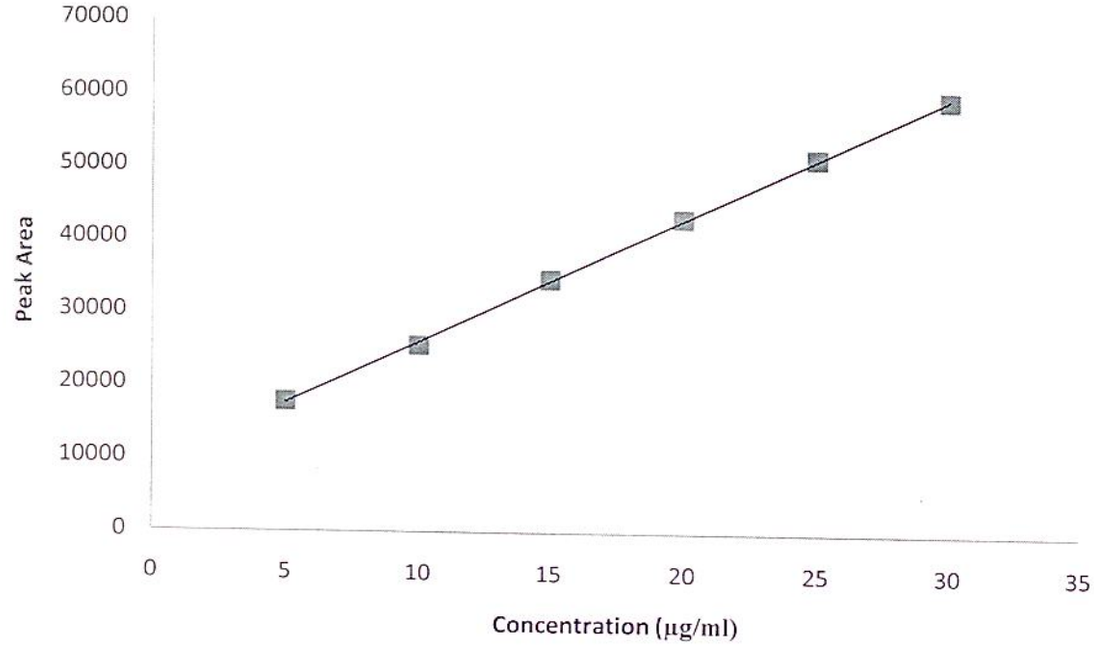


Figure 35: Linear Curve of Gallic Acid

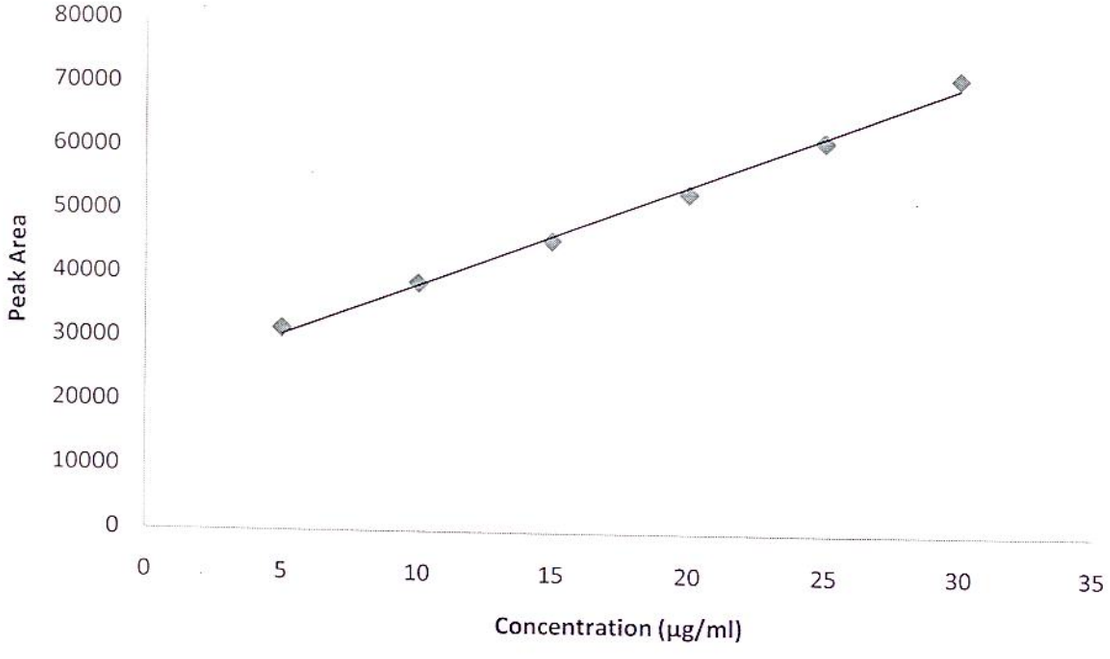


Figure 36: Linear Curve of Chlorogenic Acid

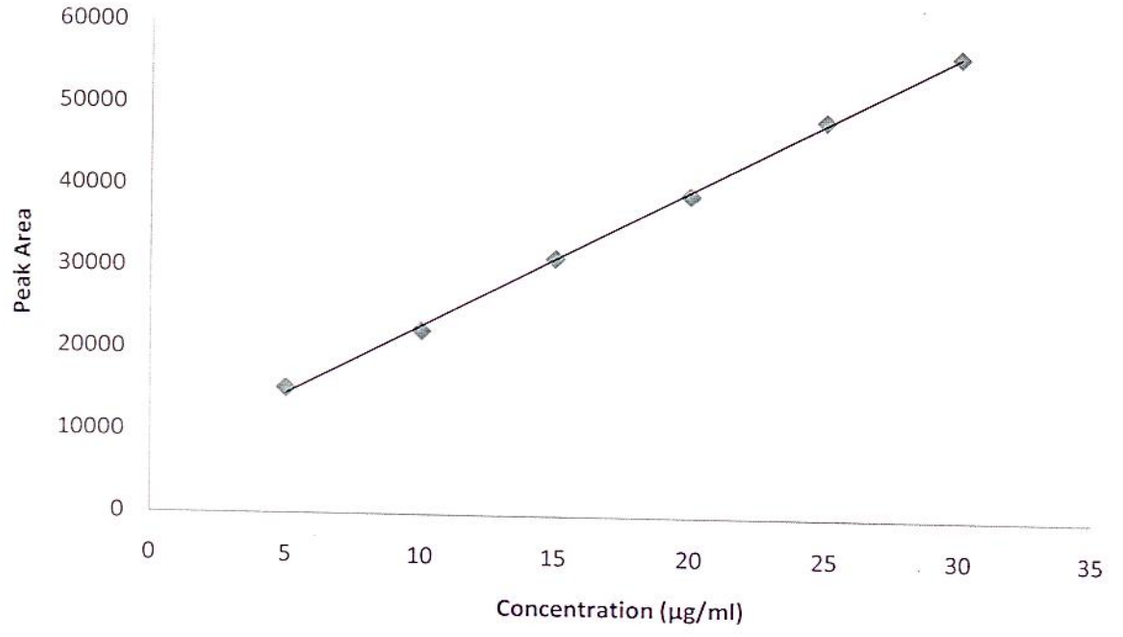


Figure 37: Linear curve of Syringic Acid

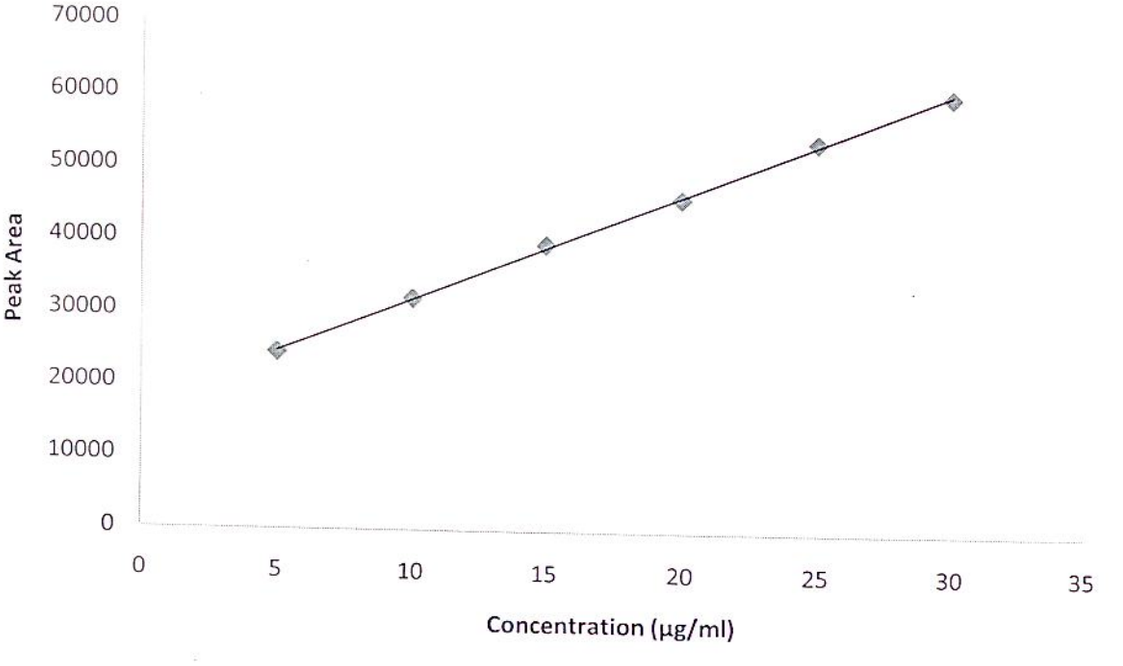


Figure 38: Linear curve of Benzoic Acid

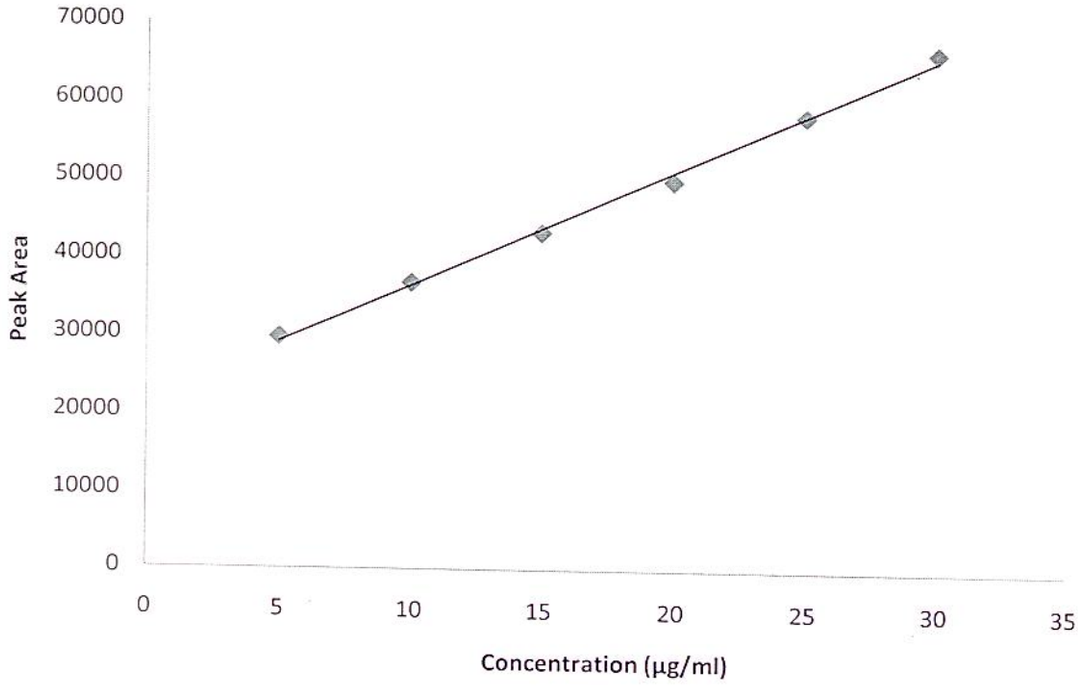


Figure 39: Linear Curve of Vanallic Acid

The limit of detection (LOD) was determined by diluting standard solution of known concentration until the response was three times the noise S/N=3, which was in the range 0.01-0.1µg/mL for all the components while limit of quantification (LOQ) was calculate on the basis of minimum accepted value of S/N=10 and quantification of Phenolic acid were found in the range of 0.20-0.30µg/mL. Bae, *et al.*, (2015) developed HPLC method for quantification of phenolic acids. The LOD and LOQ were reported in the range of 0.06mg/l to 2.92mg/l and 0.21mg/l to 9.72mg/l [221].

The precision was evaluated by analyzing the peak area of each phenolic acid six times on the same sample. Precision was expressed in %RSD were in the range of 1.45-3.11% (n=6) (Table-20). In the study of the recovery of the procedure the leave extract was spiked with each phenolic acid in a known concentration, subjected to the given extraction procedure and analyzed before and after the addition in triplicate. Recoveries

for phenolic acids ranged from 95.5% to 103.1%. Relative standard deviation of recovery was in the range of 0.94 to 3.12%. The recovery rate reported by [221] in developed method for quantification of phenolic acid in cereals within the range of 96.6 to 103.5%. De Paula *et al.*, (2008) also reported the recovery in the range of 98.2 to 104% in spiked sample during method validation. These are closed agreement with our current study [222].

A simple reversed phase high performance liquid chromatographic methods are generally used for the separation of phenolic acids, such a separation are simple rapid and provide high resolution and sensitivity. Irakli *et al.*, (2012) reported the development and validation of HPLC method using DAD for determination of phenolic acid in cereals. These phenolic acids were separated on Nuleosil 100 C18 column, 5um (250mm× 4.6mm) using a linear gradient elution system of methanol and acetified water. The LOD ranged between 0.13 and 0.18µg/g and LOQ varied from 0.39 to 1.60µg/g . by the proposed method six hydrobenzoic acid (4-hydroxybenzoic acid, gallic cid, vanillic acid, protocatechuic, syringic, salicylic acid) and five hydrocinnamic acid (cinnamic, p-coumaric, ferulic, caffeic, sinapic) were quantified in a total run of 65minutes [223]. Sundaram *et al.*, (2012) developed a method for quantification of two phenolic acids in *O. sanctum* by HPLC. The separation system consist C18 column using phosphate buffer/ACN (80:20) and ACN/water/methanol (90:5:5). The flow rate was 1.0mL/minute and detection wavelength was set at 261nm. Two phenolic acid rosmarinic and caffeic acid were quantified in *O. sanctum*. Rosmarinic acid was 1st time reported by Jungmen lee in fresh *O. Sanctum* [224].

By the newly developed method we successfully detected and quantified the three phenolic acids in Ocimum sanctum leaves extract were found in concentration of 2.97, 187 and 1.38µg/mL for gallic, chlorogenic and vanillic acids. (Table-21 and Figure-40), while the Syringic acid and benzoic acids are absent in *O. sanctum* extracts. In *O. basilicum* extract the syringic acid was found instead of Chlorogenic acid. Gallic, syringic and vanillic acid were found 3.86, 1.73 and 1.38µg/mL, while benzoic acid was also absent in *O. basilicum* extract. Four phenolic acids were found in *A. indica* including chlorogenic, syringic, benzoic and vanillic acid, only gallic was found absent. Chlorogenic acid was found 2.31µg/mL, vanillic acid 2.13µg/mL, syringic acid 1.87µg/mL and benzoic acid was 1.27µg/mL.

Table 19: Linerity, LOD and LOQ Values of Phenolic Acids

S.No	Standard	Calibration Equation	Regression	(µg/mL)	
		Y= m χ + C	R^2	LOD	LOQ
1	Gallic Acid	Y= 8370 χ + 6262	0.999	0.05	0.25
2	Chlorogenic Acid	Y= 7899 χ + 22721	0.995	0.01	0.20
3	Syringic Acid	Y= 8408 χ + 9128	0.999	0.10	0.30
4	Benzoic Acid	Y= 7189 χ + 17439	0.999	0.01	0.20
5	Vanillic Acid	Y= 7315 χ + 21810	0.997	0.03	0.20

Table 20: Precision, Recovery and Repeatability of Phenolic Acids

S.No	Standard	Precision RSD%	Retention Time	Repeatability RSD%	Recovery %	RSD %
1	Gallic Acid	1.45	2.05	0.87	94.4	1.34
2	Chlorogenic Acid	2.23	6.45	1.08	95.8	2.76
3	Syringic Acid	1.76	10.15	0.69	103.1	3.34
4	Benzoic Acid	1.60	12.05	1.54	98.5	2.23
5	Vanillic Acid	3.11	21.25	1.85	99.65	1.69

Table 21: Phenolic Acid Concentration in *O. Sanctum*, *O. Basilicum* and *A. Indica* Extracts

S.No	Standard Acid	Concentration µg/mL in leaves Extracts		
		O. sanctum	*O. basilicum*	*A. indica*
1	**Gallic acid**	2.97 ± 0.48*	3.86 ± 0.93	ND**
2	**Chlorogenic acid**	1.86 ± 0.35	ND	2.31 ± 0.67
3	**Syringic acid**	ND	173 ± 0.54	1.87 ± 0.41
4	**Benzoic acid**	ND	ND	1.27 ± 0.36
5	**Vanillic acid**	1.59 ± 0.43	1.38 ± 0.21	2.13 ± 0.56

*The values are in triplicate and ± is its standard deviation

**ND Not Detected

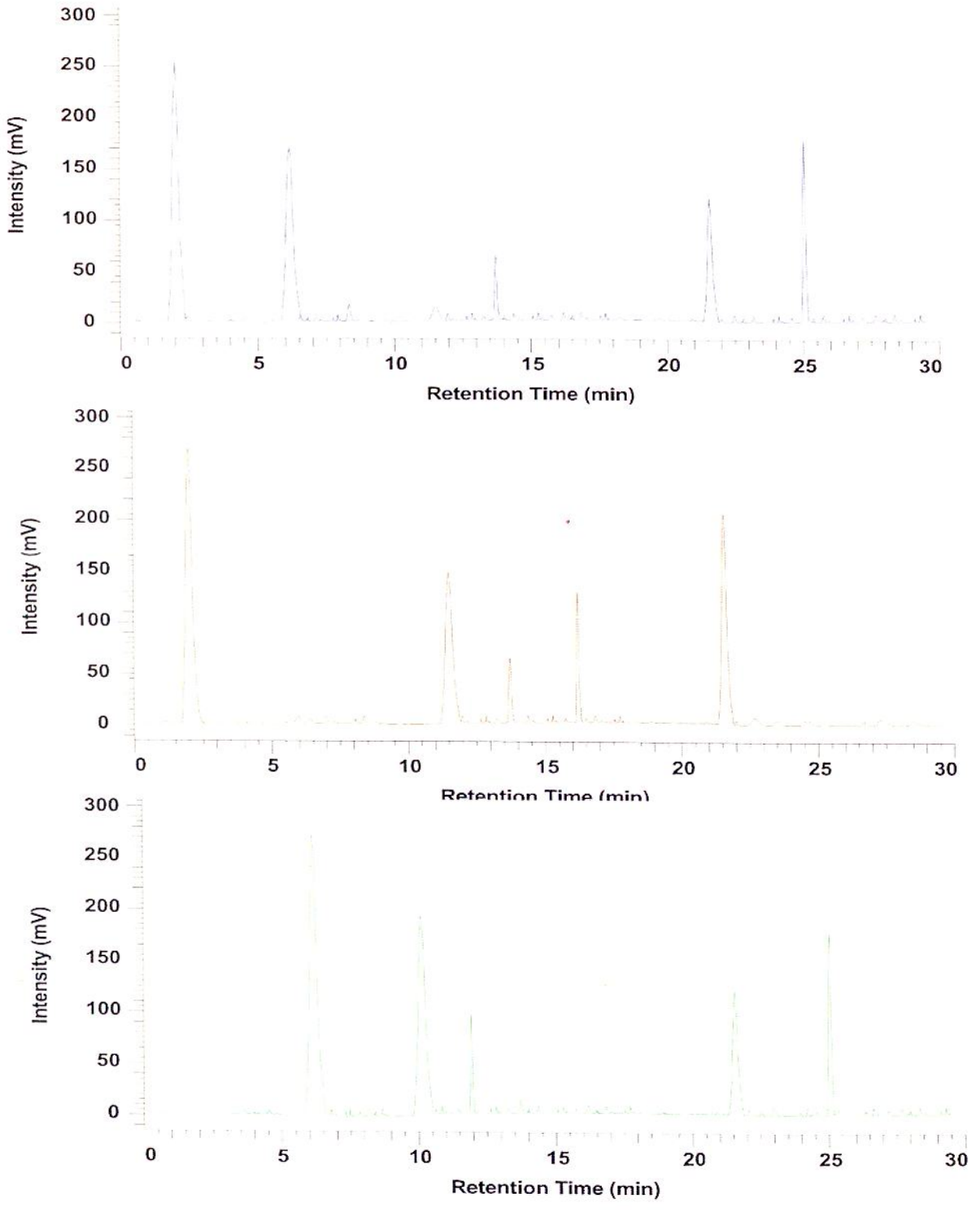


Figure 40: HPLC Chromatogram of Phenolic Acids in *O. Sanctum*, *O. Basilicum* and *A. Indica* Extracts

4.11 HPLC METHOD DEVELOPMENT FOR QUANTIFICATION OF FLAVONOIDS

A novel analytical approach has been developed for quantification of flavonoids in crude extract of *O. sanctum*, *O. basilicum* and *A. indica* by RP-HPLC with analysis time shorter than described in literature. Four common flavonoids including luteoline, Catechins, Kaempferol and quercetin were separated in a single run of 35min using gradient elution system of acidic acid aqueous solution (pH=3) and Acetonitrile as mobile phases with a flow rate of 1.0mL/min. These phenolic compounds were detected by UV-Visible detector. A sequence of other parameters required for method development and analytical validation found with good results.

HPLC (Hitachi-2000) equipped with dual pumps (L-2130) auto injector (L-2200) with analytical column Intersil ODS-3 C18 (GL Sciences Inc. Tokyo Japan 5um, 250+ 4.6mm) in column oven (L-2300) and UV-Vis Detector (L-2400) was used for the method development for quantification of phenolic compounds in plant leaves extracts.

Different analytical techniques for the determination and quantification of flavonoids are summarized in review article [225]. TLC, HPLC and GC-MS techniques are mostly used for identification and quantification of flavonoids. [116, 226-228]. From the previous literature it was found that the most popular techniques used for identification and quantification of flavonoids is HPLC due to fast analysis and isolation of compounds with high sensitivity.

Khan, *et al.*, (2014) developed an analytical procedure using RP-HPLC for the quantification of only phenolic acids [170] while Fang *et al.*, (2007) developed a method for quantification of four flavonoids [229].

The present study was designed to develop an analytical procedure for simultaneous determination of flavonoids in plant extracts by HPLC using UV-Vis detector. UV-Vis detector are commonly used in many laboratories as they can detect a wide array of compounds, other detector also apart of HPLC determination including Photo Diode Array Detector (PDAD), Fluorescent Detector (FLD), Conductivity Detector (CD), Refractive Index Detector (RID), Electro Chemical Detector (ECD), Mass Spectrometer Detector (MSD) and Evaporated Light Scattering Detector (ELSD). The study mainly focused on the optimization of HPLC condition and other prospective during development and validation method. HPLC method validation plays an important role in the discovery, development and validation of pharmaceutical drugs and quantification of active compounds.

4.11.1 CALIBRATION AND METHOD VALIDATION

Linearity, precision, accuracy, specificity, LOD, and LOQ are some of the important parameters for method validation has been studied according to ICH rules. Linearity was determined by a series of five different concentrations (10-50µg/mL) of each standard were injected in triplicate. The peak area was automatically recorded by the integrator (Table-22 and Figure-41).

Peak areas of the calibration standards were plotted against their concentration. A linearity equation was drawn between the different concentration of each compound and peak area and expressed as Y=mx+c (Table-23 and Figures 42-45). The calibration curves were found linear throughout range of study and the co-relation coefficient was found higher than 0.995 for all compounds.

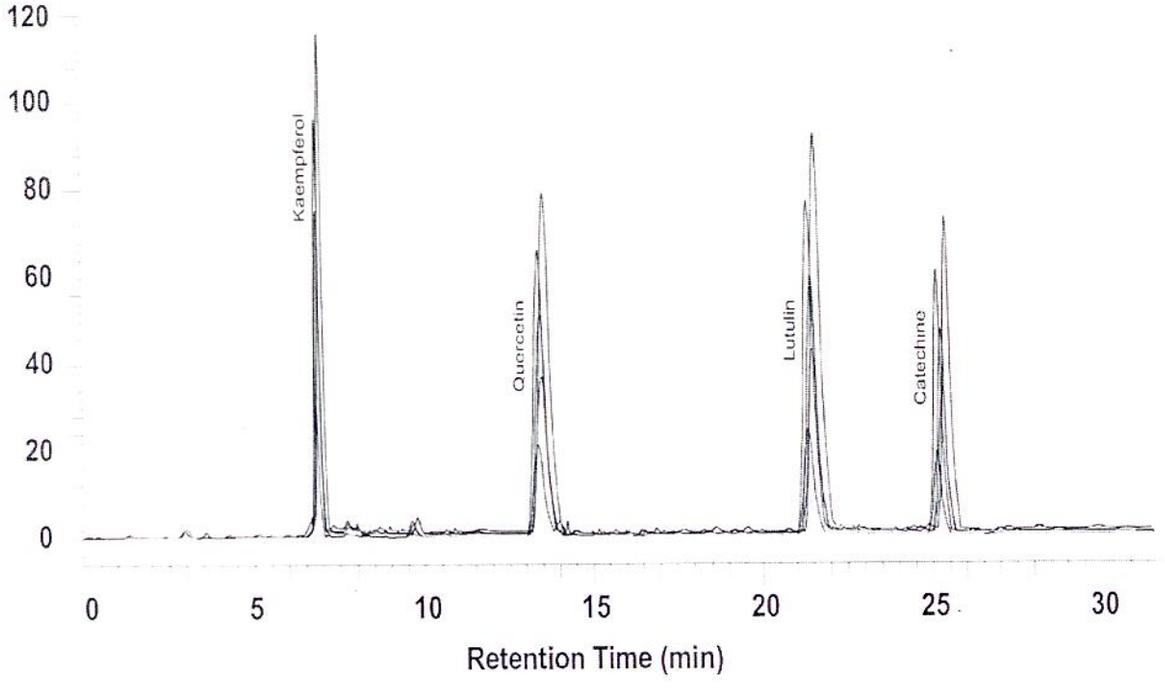


Figure 41: HPLC Chromatogram of Different Concentration of Mixed Standard

Table 22: Peak Area of Each Flavonoids at Different Concentration

S.NO	Standard	Peak Area of Concentration µg/mL				
		10	20	30	40	50
1	Kaempferol	151736	163897	176486	187393	198636
2	Quercetin	123657	135765	143873	157387	163872
3	Luteolin	140764	154872	167320	172383	185723
4	Catechins	98763	108763	129785	129785	147548

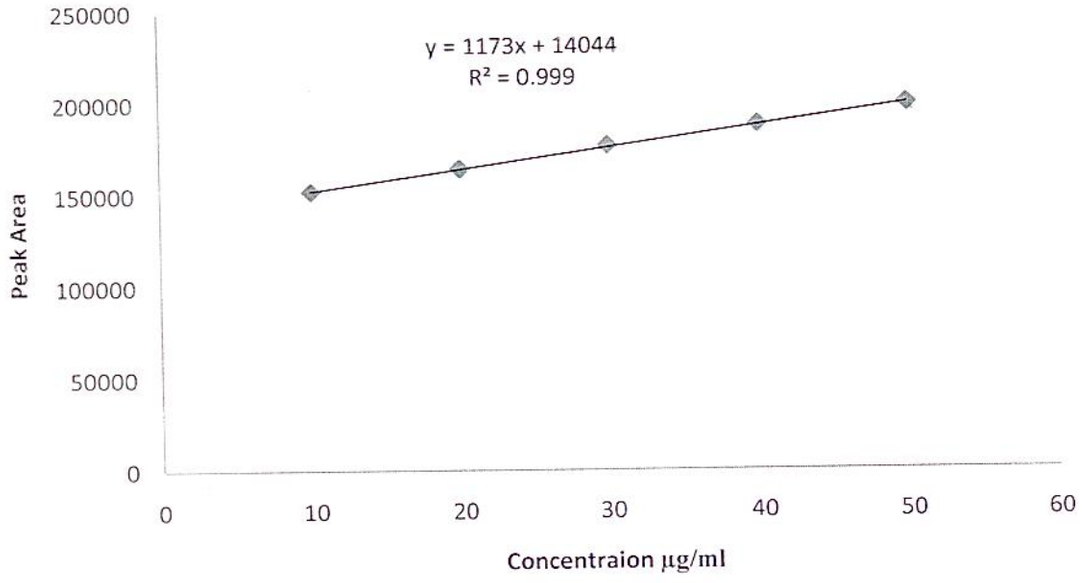


Figure 42: Linear curve of Kaempferol

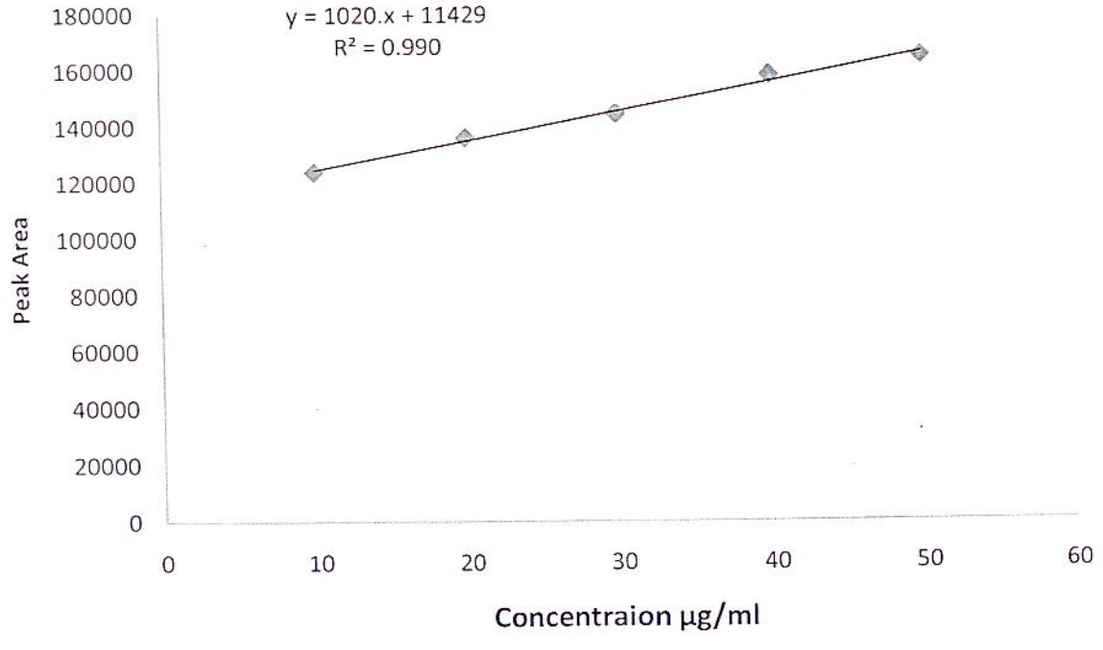


Figure 43: Linear curve of Quercetin

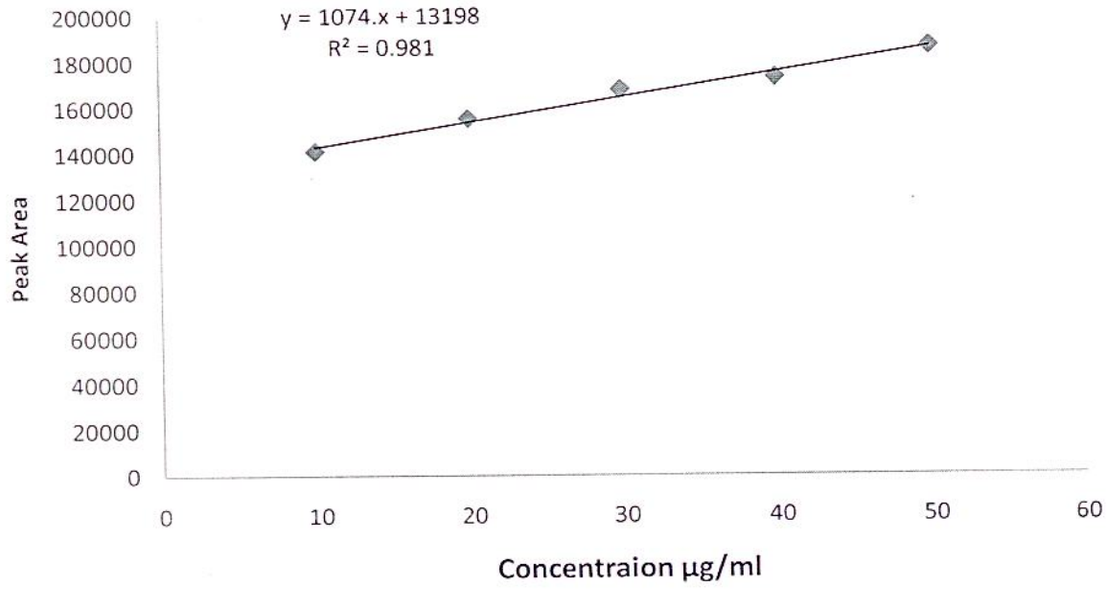


Figure 44: Linear Curve of Luteolin

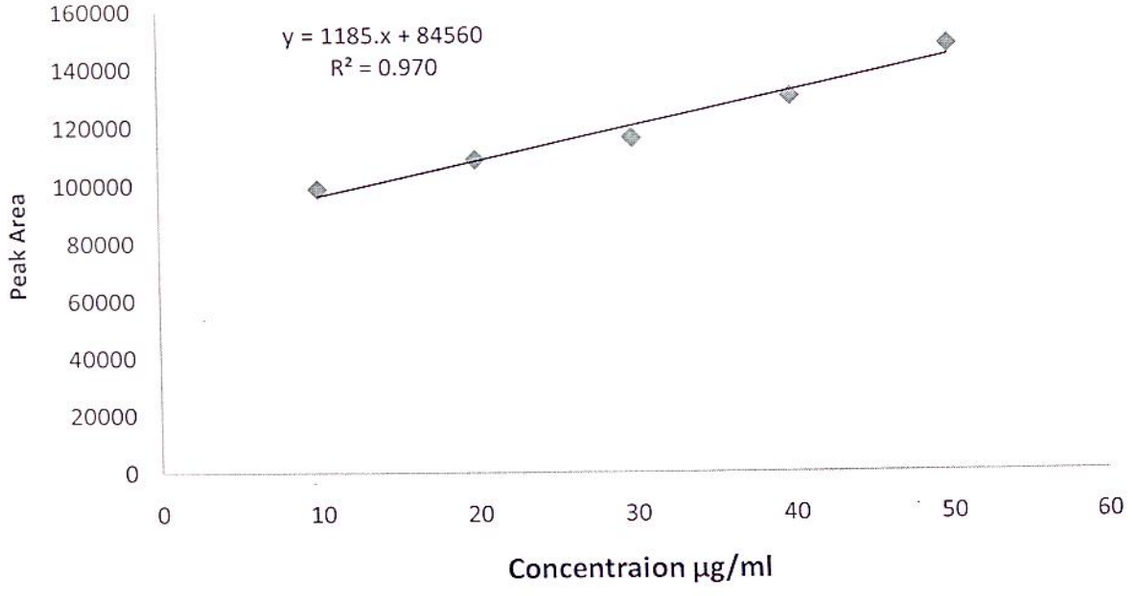


Figure 45: Linear Curve of Catechin

The LOD and LOQ were determined by diluting of each known concentration standard to minimum level at which the analyte can be reliably detected and quantified with accuracy and precision (Table-23). The LOD were consistently below 0.10µg/mL, whereas the LOQ ranged from 0.20 to 1.05 µg/mL for all compounds.

The precision and accuracy of the method were assessed in order to evaluate (within day precision) repeatability and (between day precision) reproducibility of the spiked extract sample with 5µg/mL. (Table: 24) shows excellent percent relative standard deviation (RSD%), lower than 2.61 and 1.32 for all compounds within and between day precision. while the accuracy of method expressed as recovery range form 98-100.2% with less than 2.12% RSD.

By the proposed method we successfully detected and quantified the four flavonoids including kaempferol, quercetin, luteolin and catechins in *O. sanctum*, *O. basilicum* and *A. Indica* (Table-25 and Figure-46). Kaempferol was found 2.97 and 4.86µg/mL in *O. sanctum* and *O. basilicum* while absent in *A. indica* extract. Quercetin was found 8.71µg/mL in *A. indica* and 1.86 in *O. sanctum*, while it was found absent in *O. basilicum*. The lutulin was found in maximum concentration 21.31µg/mL in *A. indica*, while its concentration was 3.65 in *O. basilicum* and absent in *O. sanctum*. The catechin was only found in *A. indica* in concentration of 5.63µg/mL. Hakkinen, *et al.*, (2000) developed HPLC method for phenolic acids using UV-Vis detector. By the proposed method five phenolic acids were identified in *O. sanctum* including gallic, coumaric, benzoic, ferulic, syringic acid [230].

Table 23: Linearity Equation, LOD and LOQ of Flavonoids

S.No	Standard	Calibration Equation	Regression	µg/mL	
				LOD	LOQ
1	Kaempferol	y = 1173x + 14044	0.999	0.05	1.05
2	Quercetin	y = 1020.x + 11429	0.990	0.01	0.20
3	Luteolin	y = 1074.x + 13198	0.981	0.10	0.80
4	Catechins	y = 1185.x + 84560	0.970	0.01	0.50

Table 24: Repeatability, Reproducibility and Recovery of Flavonoids

S.No	Standard	Repeatability RSD%	Retention Time	Reproducibility RSD%	Recovery %	RSD %
1	Kaempferol	2.61	06.9	1.32	99.0	2.11
2	Quercetin	1.37	13.6	0.96	99.8	1.78
3	Luteolin	2.12	21.7	1.26	98.0	1.76
4	Catechins	1.16	25.5	0.73	100.2	1.69

Table 25: Flavonoids Content of *O. Sanctum*, *O. Basilicum* and *A. Indica*

S.No	Standard	Concentration µg/mL in leaves Extracts		
		O. sanctum	*O. basilicum*	*A. indica*
1	Kaempferol	2.97±0.48	4.86±0.61	ND
2	Quercetin	1.86±0.35	ND	8.17±0.82
3	Luteolin	ND	3.65±0.73	21.31±1.41
4	Catechins	ND	ND	5.63± 0.51

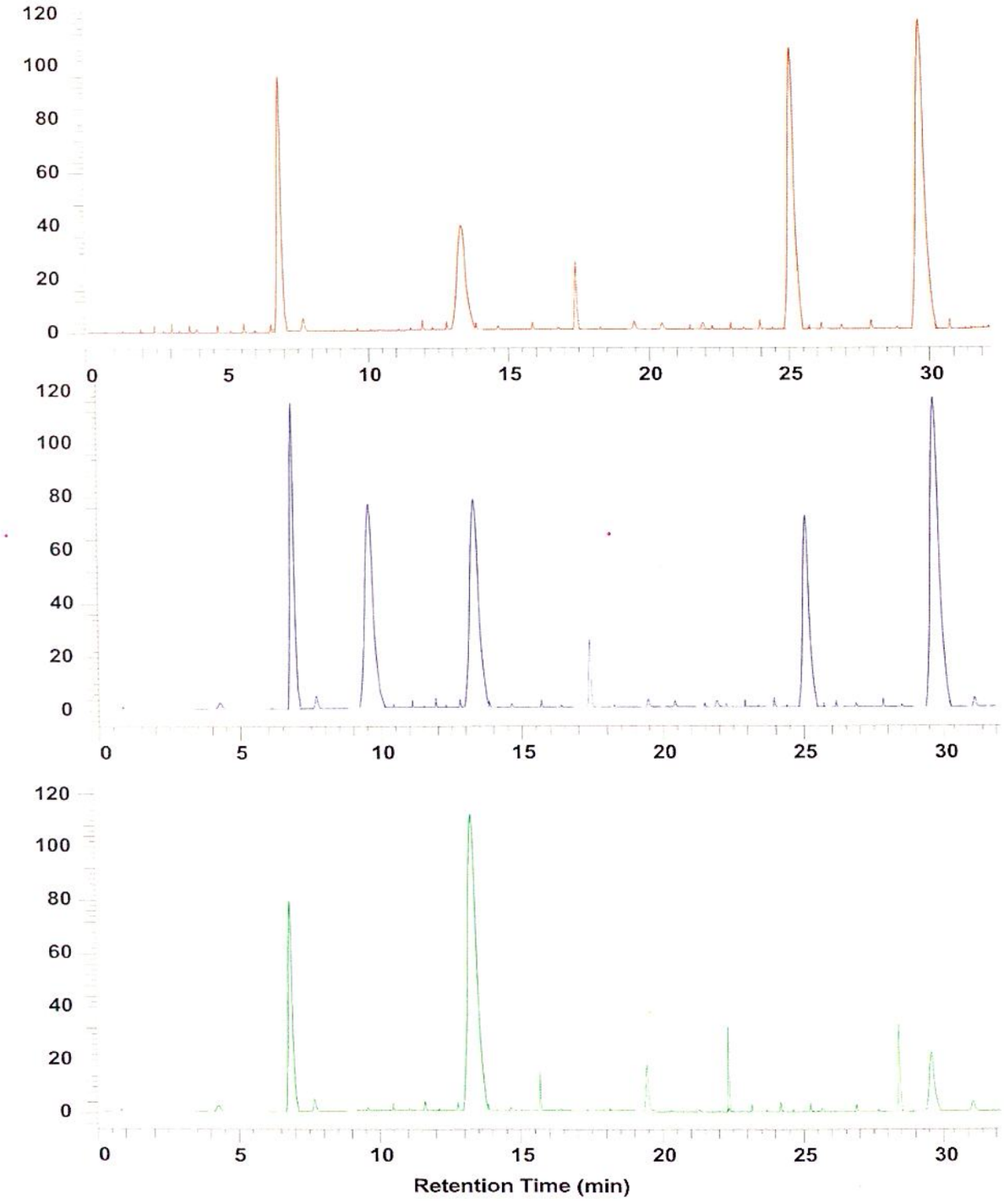


Figure 46: HPLC Chromatogram of Flavonoids of *O.* Sanctum, *O. Basilicum* and *A. Indica*

4.12 EFFECTS OF TEMPERATURE AND STORAGE ON THE ANTIOXIDANT POTENTIAL, POLYPHENOL AND VITAMIN-C CONTENTS OF *A. INDICA* LEAVE AQUEOUS EXTRACT

The aim of the part of this study was to investigate the effects of storage and temperature on the antioxidant potential, vitamin-C contents, total as well as selected individual phenolic acids and flavonoids of fresh aqueous leaves extract of *Azadirachta Indica.* The extracts of *A. indica* have been extensively used to treat various infections and cure various diseases since prehistoric time. Due to its curative power it is used on a large scale in the manufacturing of medicines for gastro intestinal problems, urinary tracks problems, hair problems, oral problems, ulcer, blood pressure, diabetes etc [231, 232]

Fresh young leaves were soaked in water for few hours and the extract was used to cure oxidative disorder, like inflammation and skin diseases, arthritic, rheumatic disorders, and treatment of diabetics, diarrhea, amoebic dysentery and fever. The extract was also used as a bitter tonic to treat malaria fever and stimulate the gastric secretion. The extract also has antimicrobial, anti inflammatory, antiulcer, antioxidant, antipyretic, anti parasitic activities [166, 168, 233, 234]

4.12.1 PREPARATION OF LEAVES EXTRACT

The *Azadirachta Indica* leaves were collected from the Botanical garden of Islamia College Peshawar, Pakistan washed with plenty of water. 500g of leaves were chopped and extracted with 1 litter water at 30°C for 24h at orbital shaker. The

flasks were properly coated by aluminum foil. The mixture was filtered and concentrated to half of its volume by rotary evaporator at 35°C and were stored at 10, 30, and 50°C for duration of 1, 2 and 3 month.

4.12.2 DETERMINATION OF PHENOLIC ACID AND FLAVONOIDS BY HPLC

Determination of phenolic acids and flavonoids constituents in *Azadirachta Indica* extract was carried out by Proestos, *et al.*, (2006) method [120]. 5 mL of extract were further extracted twice with 5 mL of diethyl ether, and centrifuged at room temperature at 5,000 rpm for 10 min. the supernatant was separated and evaporated to dryness with nitrogen gas stream. For the determination of phenolic compounds the residue was dissolved in water-methanol (1:1) and 10µl aliquots injected to HPLC. The chromatographic separations of polyphenol were carried out by analytical column ODS-3 C18 (5µm, 250×4.6mm GL Science Inc. Tokyo Japan). For the separation of phenolic compounds in extract the mobile phase Water: Acidic acid: methanol (88:2:10) as solvent A and Water: acidic acid: Methanol (8:2:90) as solvent B were used in a gradient elution program was run at 0 minute, 100 % A – 0 % B; 15 minutes 85 % A - 15 % B; 25 minutes 50 % A – 50 % B and 34 minutes 30 % A - 70 % B with a flow rate of 1.0 mL/ minute. The phenolic compounds were identified by comparing with the retention time of UV spectra of appropriate standard. Phenolic acids and flavonoids was quantified at 280nm and 360nm using external standard method.

4.12.3 DETERMINATION OF TPC, TFC, VITAMIN-C CONTENTS AND ANTIOXIDANT ACTIVITY

The Antioxidant potential, TPC and TFC of the crude aqueous extract of *Azadirachta Indica* leaves were determined as in section 3.9.1, 3.9.2 and 3.9.3, while the extraction and determination of vitamin-C was carried out by Almeida *et al.*, (2011) method [235].

The % scavenging activity of extract determined by DPPH method was 69% (Table-26), while Almeida, *et al.*, (2011) reported 71% in dry leave crude extract [235, 236]. The TPC and TFC were found 486 and 281mg/L while the value of TPC and TFC reported by Ghimeray *et al.*, (2009) were 2431 and 1543 mg/L in crude organic extract of *Azadirachta Indica* leaves [237].

On the other hand Tsimidou *et al.*, (1992) added that the yield of these compounds depend on the selection of solvent and extraction methods [238]. 121mg/L of vitamin C was found in fresh extract.

Table 26: Changes in Antioxidant Activity, TPC, TFC and Vitamin-C Contents in Aqueous Extract of *A. Indica* Leaves During Storage of 3 Months at 20, 30 And 50°C

Storage (month)	% Scavenging (DPPH)			Content mg/L								
				Vitamin-C			TPC			TFC		
	20°C	30°C	50°C	20°C	30°C	50°C	20°C	30°C	50°C	20°C	30°C	50°C
0		69.3a			121a			486a			281a	
1	68.3a	63.4a	61.7b	101[a]	96.8b	49.3c	461a	434a	381b	264a	259a	231b
2	64.0a	60.7a	57.6b	87.4b	64.3b	33.9d	401a	392a	323c	253b	247b	214c
3	59.3b	56.0b	50.4c	51.8b	23.5d	09.7d	381b	361c	279d	230b	201c	183d

Means with different letters (a-d) are significantly different according of Duncan's multiple rang test at P>0.05.

DPPH scavenging, Vitamin-C, TPC and TFC were determined in triplicate

4.12.4 DETERMINATION OF FLAVONOIDS AND PHENOLIC ACID BY HPLC

By using aforementioned procedure, the phenolic compounds in aqueous extract of *A. Indica* leave were identified and quantified using UV-Vis detector. These compounds showed intense absorbance in UV-Vis region.

[235, 239]. The applied Lachman, *et al.*, (2010) [167] method was found rapid and effective for the quantification of major phenolic compounds. From the data presented (Table: 27) It is clear that among the phenolic acids the gallic acid was found maximum in fresh extract yield 7.89 mg/l, while ferulic, syringic and caffeic were found in the range of 7-2mg/l. The reported method Wijngaard *et al.*, (2012) followed for the content of free phenolic acids due to simplicity [240].

The flavonoids content of aqueous extract of *Azadirachta Indica* leaves are shown (Table: 28). The Hesperitin was found in maximum yield (76.65 mg/l) in fresh extract. Quercetin, luteoline and rutin were found 60.98, 51.62 and 09.98mg/l respectively. From the previous reported data the value of flavonoids and phenolic acids were found too high in dry extract [241, 242]. The yield of phenolic compounds also depends on the extraction solvents and procedures. But the aqueous extraction and procedure applied in current research followed the traditional use of fresh extract.

Table 27: Changes in Contents of Phenolic Acids in *A. indica* Leave Extract During 3 Month Storage at 20, 30 and 50°C

Storage (month)	Phenolic acids (mg/l)											
	Gallic			Ferulic			Syringic			Caffeic		
	20°C	30°C	50°C	20°C	30°C	50°C	20°C	30°C	50°C	20°C	30°C	50°C
0		7.89a			2.71a			2.42a			5.13a	
1	7.83a	7.53a	7.11a	2.55a	3.42b	3.04a	2.42a	2.15a	1.82b	5.03a	4.97a	4.78
2	7.11a	6.46b	6.25b	3.0a	3.36b	3.96b	2.16a	1.63b	1.30c	4.94a	4.54b	4.36c
3	6.36b	5.93c	5.22c	3.98c	4.12d	4.34d	1.72b	1.11c	0.6d	4.76b	4.39c	3.72d

Means with different letters (a-d) are significantly different according to Duncan's multiple rang test at P>0.05

3 determination were done for Gallic, Ferulic, Syringic and Caffeic acids

Table 28: Changes in Content of Flavonoids in Aqueous Extract of Azadirachta indica Leave Extract During 3 Month Storage at 20, 30 And 50°C.

Storage (month)	Flavonoids (mg/l)											
	Quercetin			Luteolin			Rutin			Hesperitin		
	20°C	30°C	50°C	20°C	30°C	50°C	20°C	30°C	50°C	20°C	30°C	50°C
0		60.9a			51.6a			09.9a			76.6a	
1	57.3a	56.5a	52.3b	51.6a	50.7a	43.1b	09.1a	09.3a	09.6a	76.4a	73.9a	73.6a
2	54.4a	51.2b	49.7b	49.8a	46.9b	41.3b	09.5a	08.1a	07.5b	74.3a	70.0a	69.7b
3	48.2b	45.0c	41.8c	40.2c	40.1c	38.6c	08.6a	07.0b	07.0b	68.3b	65.7b	63.8c

Means with different letters (a-d) are significantly different according to Duncan'multiple rang test at P>0.05

3 determination were done for Quercetin, Luteolin, Rutin and Hesperitin

4.12.5 EFFECTS OF STORAGE AND TEMPRATURE ON ANTIOXIDANT AND VITAMIN-C CONTENTS

From the data presented in (Table-26) observed a clear decrease in % scavenging of DPPH, 69% of fee radical of DPPH scavenged by fresh aqueous extract while this scavenging decreased to 18% during storage of 3 month at 50°C. Among the entire tested compounds vitamin-C was found to be most affected by storage and temperature. The results indicated that initial fresh juice contents was 121.2mg/l which was very clearly reduced by high temperature and storage which stored at 20°C reduce the risk of reduction after storage of 3 months. The content of vitamin-C reduces 57, 80, and 91% at 20, 30, and 50°C for storage of 3 months.

The content of Vitamin-C rapidly and strongly effect by the temperature, oxygen, and light. From previous research it also proved that Vitamin-C in different juices sample decrease very rapidly during storage. It also very sensitive to temperature, so temperature also degraded the value of vitamin-C [235].

4.12.6 EFFECT OF STORAGE AND TEMPERATURE ON TPC AND TFC

The TPC and TFC in fresh aqueous extract was 486 and 281mg/l, while these values decreased to 279 and 281 when stored at 50°C for 3 months. The changes in individual phenolic acid and flavonoids were also observed and tabulated in (Table-27) which also showed remarkable changes due to storage and temperature. The

maximum reduction was found 36% followed by 27% in Luteolin, 22% in rutin and 18% Hesperitin during storage for maximum 3 months at maximum temperature of 50°C (Figure-48).

In all phenolic acids decreases was observed accept ferulic acid which increased from 2.71 to 4.34mg/L during storage of 3 month at 50°C (Figure-47). It was result of release from bonded or from their ester forms which are enclosed agreement with data obtained by Tsimidou, *et al.*, (1992) [238].

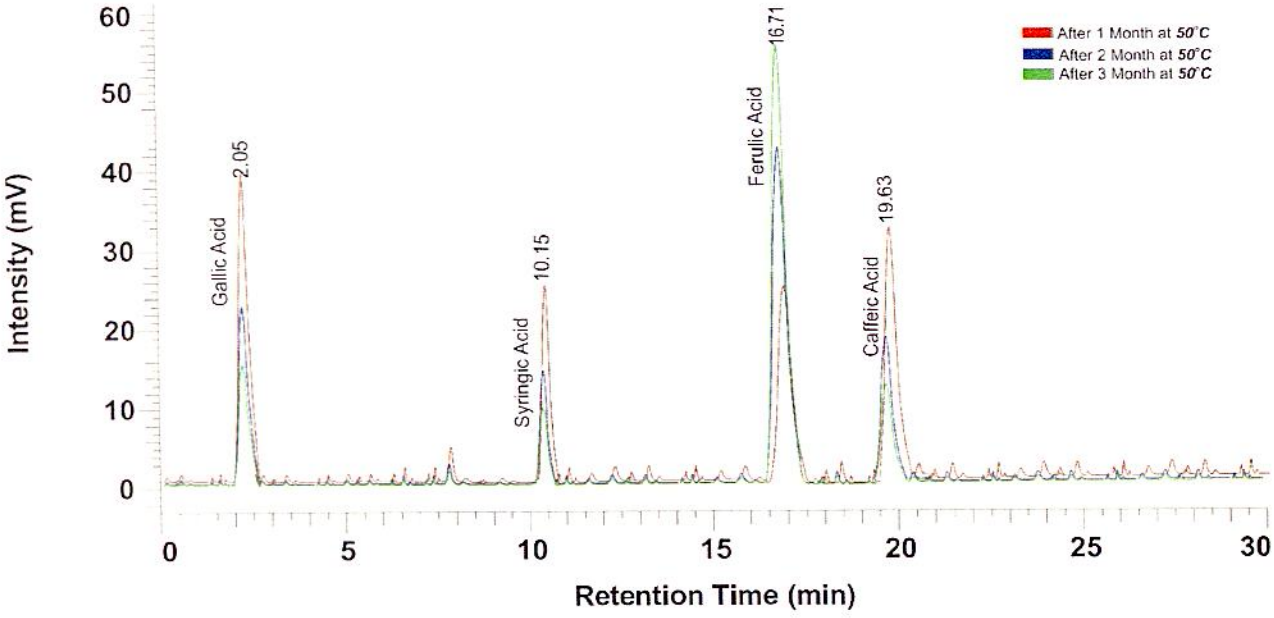


Figure 47: Chromatogram of Phenolic Acids at 50°C at Storage of 1, 2 and 3 Months

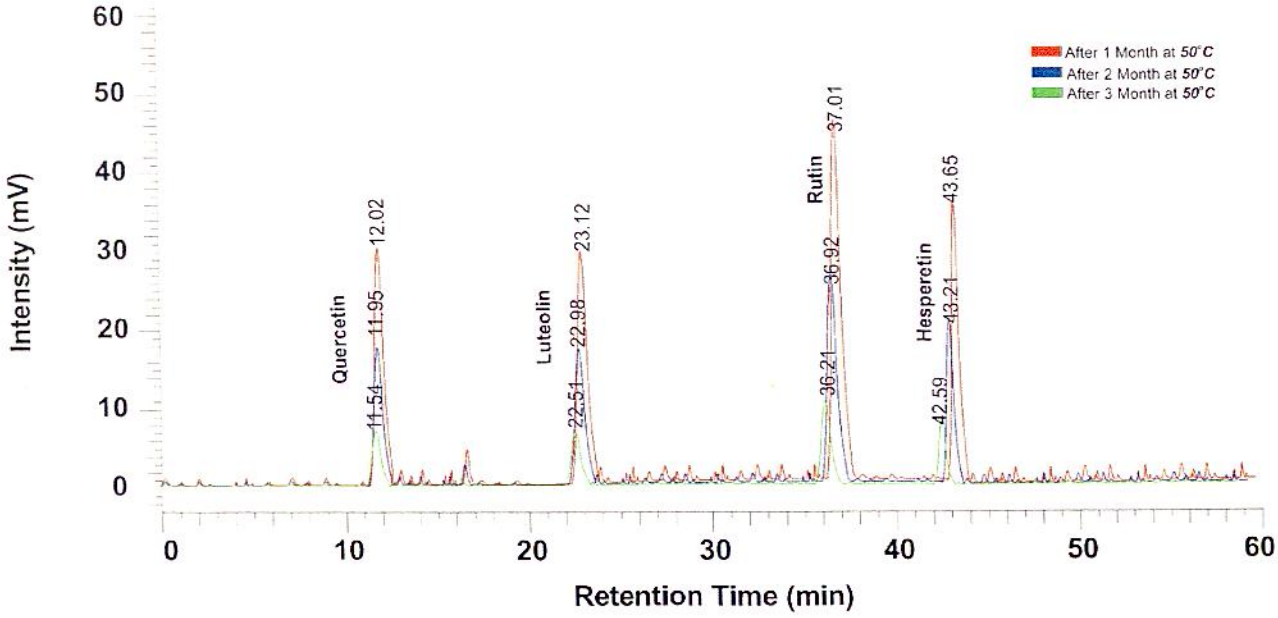


Figure 48: Chromatogram of Flavonoids at 50°C at Storage of 1, 2 and 3 Months

4.13 ANTIFUNGAL ACTIVITY OF PHENOLIC COMPOUNDS

Fungi are the most common microbes living with us in environment which could be pathogenic and sometimes life threatening, these fungi can cause fungal infections which are one of the major problem associated with our daily life. It is well known fact that some of opportunistic fungal infection affects the immunedeficient peoples. Lots of antifungal agents have been discovered so far used as an effective fungicide. In this section the antifungal activity of individual phenolic compounds including quercetin, kaempferol, rutin, catechins, gallic acid and vanillic acid were evaluated against three fungal strains *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus parasiticus*. The zone of inhibition was determined in mm. The results of antifungal assay of six phenolic compounds are presented in (Table-29 and Figure-49). 500µg/mL of each phenolic compound was applied on plates. quercetin, kaempferol and catechins were found active against all tested fungal strains while rutin, gallic acid and vanillic acid were found inactive against all tested fungal strains. The maximum activity was found 24mm of kaempferol against *Aspergillus niger* followed by 23mm against *Aspergillus flavus* and 14mm against *Aspergillus parasiticus*. Quercetin have maximum zone of inhibition 20mm against *Aspergillus flavus* followed by 18mm against *Aspergillus niger* and 17mm against *Aspergillus parasiticus*. Catechins are also found active against *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus parasiticus* and its zone of inhibition found to be 12mm, 13mm and 11mm.

Oka, *et al.*, (1972) studied the effects of phenolic compounds against *Aspergillus flavus*. They found quercetin more effective against *Aspergillus flavus* [128]. Batovska, *et al.*, (2007) found that flavanones possessed activity against the opportunistic pathogen

Candida albicans [129]. Sher, (2004) isolated the flavanols from *Terminalia belerica* fruits has also been shown to posses activity against *Candida albicans* [130]. Chikezie, *et al.*, (2015) isolated two new flavanones from *Artemisia giraldi* have been reported to exhibit activity against *Aspergillus flavus* [131].

The active phenolic compounds quercetin, kaempferol and catechins were quantified for activities using serial dilution method. The MIC value found for Catechins was 480µg/mL for all tested fungal strains (Table-30 and Figure-50). The MIC value recorded for quercetin was 400µg/mL for *Aspergillus niger, Aspergillus flavus and Aspergillus parasiticus.* The MIC of Kaempferol against *Aspergillus niger* and *Aspergillus flavus* was 400µg/mL while 460 against *Aspergillus parasiticus*.

Table 29: Zone of Inhibition of Phenolic Compounds Against Fungal Strains

Phenolic compounds	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus parasiticus*
Quercetin	18	20	17
Kaempferol	24	23	14
Rutin	R	R	R
Catechins	12	13	11
Gallic acid	R	R	R
Vanillic acid	R	R	R

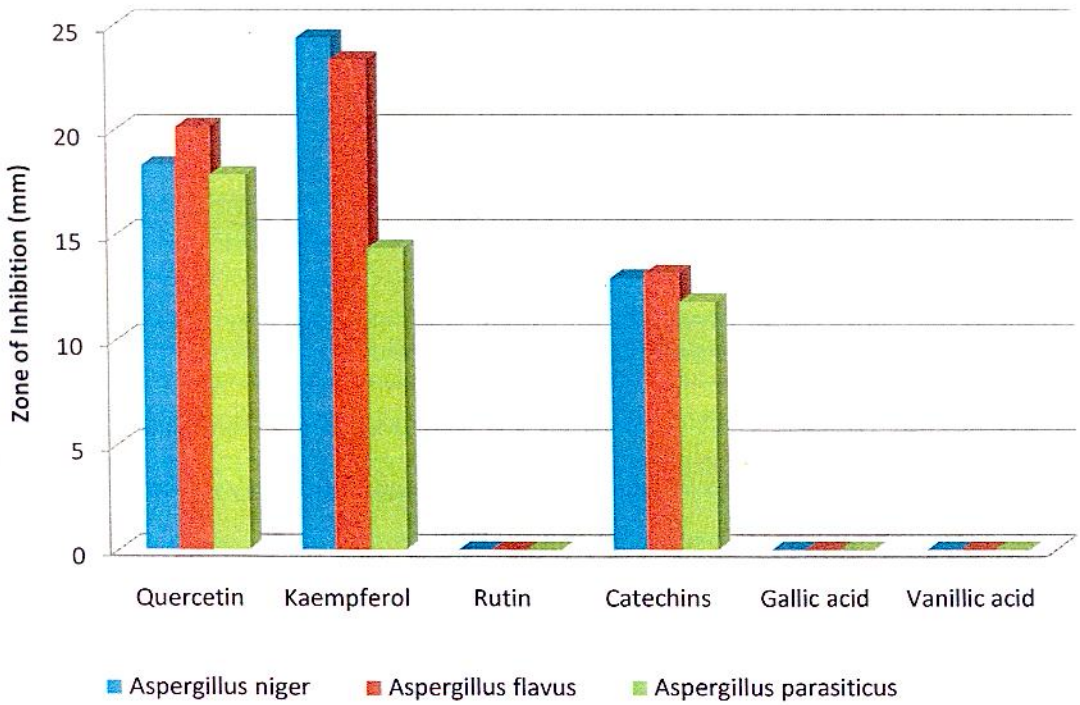


Figure 49: Zone of Inhibition of Phenolic Compound Against Fungal Strains

Table 30: MIC of Phnolic Compound Agaisnt Fungal Strains

Phenolic compounds	Aspergillus niger	Aspergillus flavus	Aspergillus parasiticus
Quercetin	440	440	440
Kaempferol	400	400	460
Catechins	480	480	480

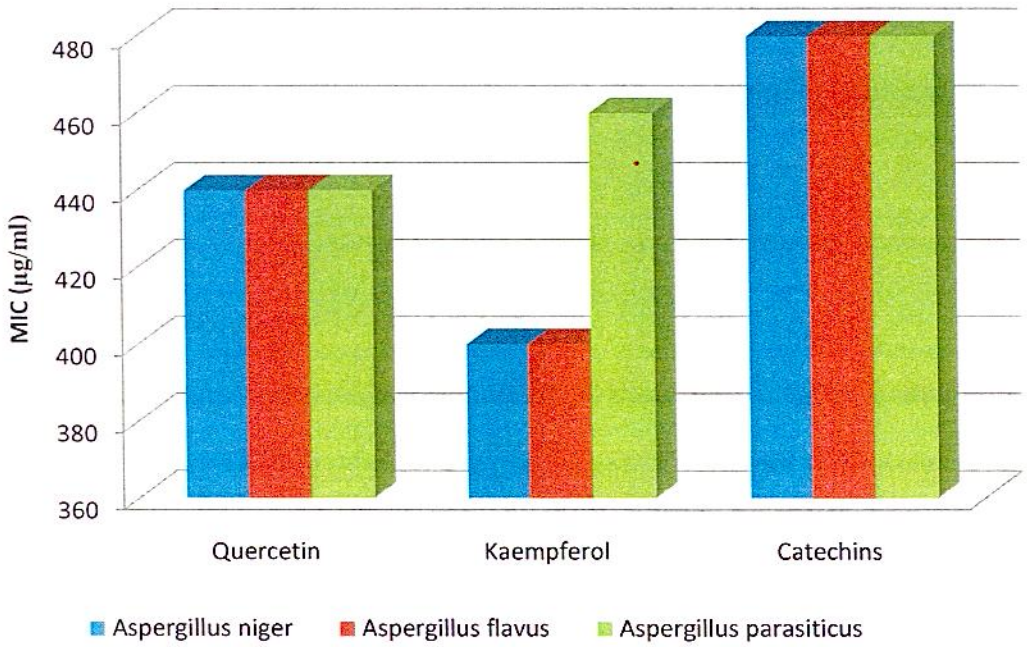


Figure 50: MIC of Phenolic Compounds Against Fungal Strains

Omidbeygi, *et al.*, (2007) evaluated the inhibitory effect of three phenolic compounds of Aspergillus parasiticus. The MIC value of individual phenolic compounds showed that isoeugenol, carvacol and thymol were the most active against *Aspergillus* growth [133].

The inhibitory effects of phenolic compound on different microbial growth could be related to the molecular structure of compounds: non polar part make possible their

passage through the membrane and a hydroxyl group combine with a system of delocalized electron. Bouddine, *et al.*, (2012) evaluated the anti *Aspergillus niger* effect of eugenol, carvacol and thymol. The results revealed that thymol and carvacol proved to have better anti *Aspergillus* effect than eugenol [132] evaluated the anti *Aspergillus niger* effect of eugenol, carvacol and thymol. The results revealed that thymol and carvacol proved to have better anti *Aspergillus* effect than eugenol.

4.14 PHENOLIC COMPOUNDS AGAINST *MRSA*

In vitro assessment the antimicrobial susceptibility tests are used to find the sensitivity and resistance of microbial strain to particular antimicrobial agent. These antimicrobial agents produced naturally by microbes or synthetically in laboratory. Antibiotics, antifungal, antiviral and other plant bioactive compounds such as phenolic used as antimicrobial agents.

Six phenolic compounds including quercetin, kaempferol, rutin, catechins, gallic acid and vanillic acid were tested against standard *S. aurous* and isolated *MRSA*. Quercetin, kaempferol, Catechins and gallic acid were found active against *MRSA* and *S. aureus*, while vanillin acid and rutin were inactive (Table-31). The maximum zone of inhibition shown by kaempferol was 18.5 and 18.0mm against standard and *MRSA* strains followed by gallic acid 14.0 and 14.5, while Catechins and quercetin showed moderately activity against standard and *MRSA* strains. The zone of inhibition of catechins was 11.91 and 11.17mm, while the zone of inhibition of quercetin was 13.56 and 13.42mm against standard *S. Aureus* and *MRSA* strains.

Many research groups isolated the flavonoids from plants sources that possessed antibacterial activity, Such as apigenin by Basile, *et al.*, (1999) [134], galangin by Cushnie et al., (2005) [135], Naringenin by Rauha, *et al.*, (2000) [136], Luteolin by Sato, *et al.*, (2000) [137], quercetin by Cushnie et al., (2005) [135] rutin by Hendra, *et al.*, (2011) [139] and kaempferol by Cushnie et al., (2005) [135] etc. In Pakistan rate of the isolation of *MRSA* is not much different from the rest of the world. Thaver *et al.*, (2009) founded 19.51% isolates were *MRSA* among general population lived in Lahore area. The data reported from hospital in Rawalpindi 42% of isolates are *MRSA* [243]. Cai, *et al.*, (2004) identified some individual phenolic compounds having antimicrobial activity. They found that caffeic acid and chlorogenic acid have demonstrated active against gram negative bacteria [88]. Gülçin, (2006) determined the antimicrobial activity of phenolic compounds and found that caffeic, ursolic and Chlorogenic acid were active against pathogenic bacteria [141].

Nascimento, *et al.*, (2000) found that the caffeic and chlorogenic acid did not demonstrate activity against *MRSA* [142]. Xu and Lee, 2001 reported that kaempferol did not inhibit *Staphylococcus epidermidis* however it demonstrated activity against *MRSA* strains. Rutin did not demonstrate any activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* [143]. The results of Skočibušić and Bezić, (2004) were contradictory to these, where neither kaempferol nor quercetin demonstrated activity against *S. aureus* [144].

The MIC values of active phenols were determined by dilution method. It will refine the usage of antibiotic. The results in (Table-32) showed that the MIC value of Kaempferol 400µg/mL against *S. aureus* and isolated *MRSA*. The MIC value of gallic

acid and quercetin was 450µg/mL against standard and *MRSA* strains. These strains showed resistance below 500µg/mL of Catechins.

Borges, *et al.*, (2013) studied the activity of ferulic and Gallic acid against pathogenic bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus* they found that gallic and ferulic acid were active against all tested pathogenic bacteria the MIC was 500µg/mL for *Pseudomonas aeruginosa* 1500µg/mL for *Escherichia coli* and 1250 for *Staphylococcus aureus* [145].

Table 31: Zone of inhibition of Phenolic compounds against *MRSA*

Phenolic compounds	S. aureus	*MRSA* clinical isolates
Quercetin	13.56 ± 1.09*	13.42 ± 2.14**
Kaempferol	18.62 ± 1.21	18.30 ± 2.54
Rutin	R	R
Catechins	11.91 ± 1.01	11.17 ± 1.86
Gallic acid	14.62 ± 1.26	14.76 ± 1.71
Vanillic acid	R	R

*The values are in triplicate and ± is standard deviation

** Average value of clinical isolates and ± is standard deviation

Table 32: MIC of Phenolic compounds against MRSA

Phenolic compounds	MIC in µg/mL	
	Staphylococcus aureus	*MRSA* clinical isolates
Kaempferol	400	400*
Quercetin	450	450
Catechins	500	500
Gallic acid	450	450

*Average value of clinical isolates

In 2007s *S. aureus* resistance profile revealed 80% against tetracycline and 53% cotrimoxazole and chloramphenicol. In different research reports vancomycin are considered more effective against *MRSA*. The use of vancomycin in *MRSA* infections having high toxicity and the prolong administration is associated with the risk for renal toxicity in patient already infected with *MRSA* [244].

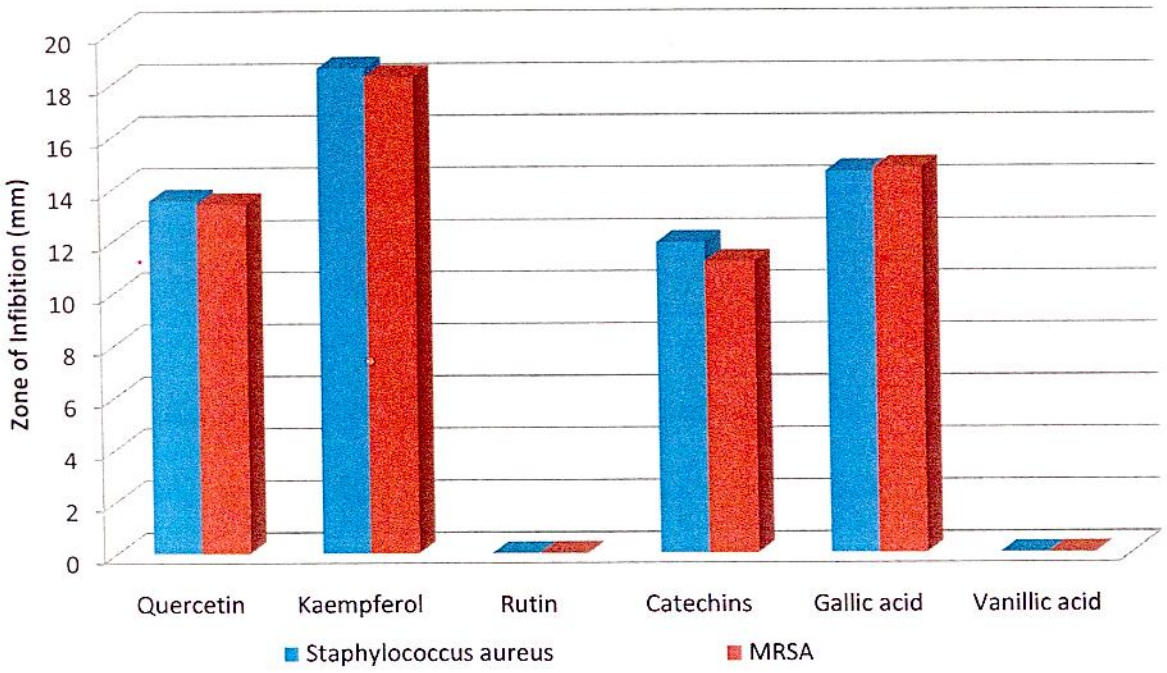


Figure 51: Zone of Inhibition of Phenolic Compounds Against *MRSA*

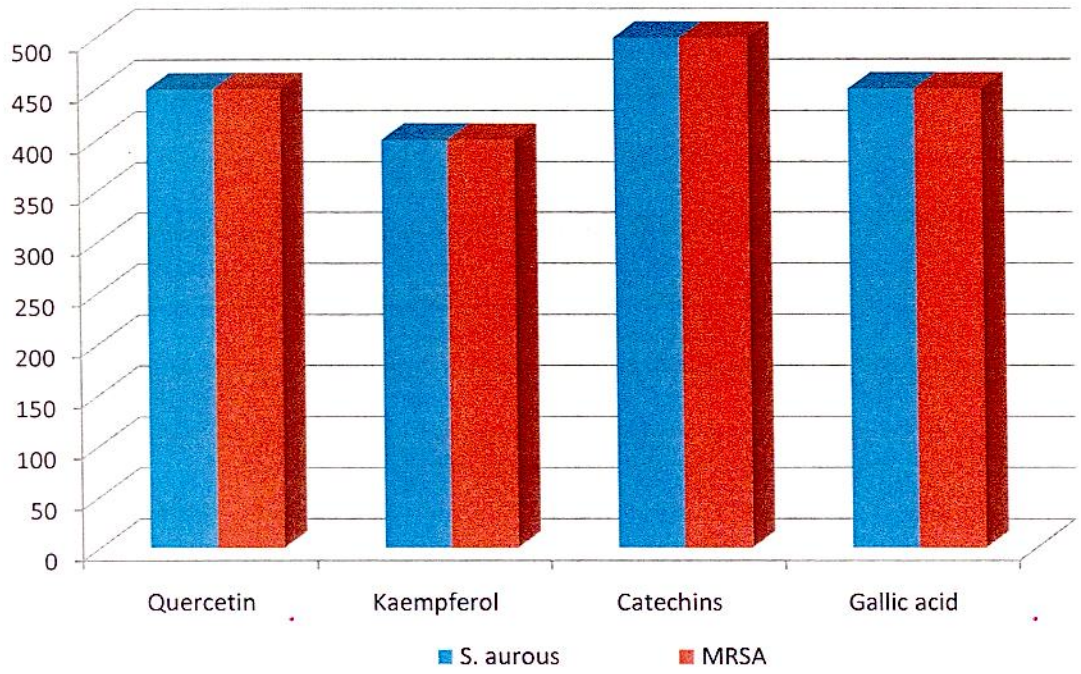


Figure 52: MIC of Phenolic Compounds Against *MRSA*

4.14.1 ANTI-*MRSA* ACTIVITY OF PHENOLIC COMPOUNDS WITH ANTIBIOTIC DRUGS

The four active phenolic compounds (quercetin, kaempferol, catechins and gallic acid) in combination with eight different antibiotic standards (Amoxicillin, Ampicillin, Methicillin, Cephradine, Ciprofloxacin, Erythromycin, Ceftriaxone, and Cefixime) were evaluated against *MRSA*. The antibiotics Amoxicillin, Ampicillin, Methicillin, Cephradine, Ciprofloxacin, Cefixime and Ceftriaxone are found inactive against *MRSA* when tested alone, expressed activity when combine with active phenolic compounds.

The results in (Table-33) showed that the combination of phenolic compounds with Erythromycin neither potentiated nor reduced the activity of drug, while Cefixime in combination with phenolic compounds neither potentiated nor reduced the activity of phenolic compounds. On the other hand Amoxicillin, Ampicillin, Methicillin, Cephradine and Ceftriaxone potentiated the activity of quercetin, kaempferol, catechins and gallic acid, while Ciprofloxacin has observed to reduced the activity of phenolic compound against *MRSA*.

Ali, *et al*., (2012) evaluated the antibacterial activity of two flavonoids orientin and vicenin obtained from *O. sanctum* leaves. The results of both flavonoids showed positive studied against *Escherichia coli* and *Staphylococcus aureus* [146]. Dhayanithi, *et al*., (2011) studied the leaves extract of *A. marina* against isolated multi drug resistance *Staphylococcus aureus* [147]. The results showed activity against different *MRSA* strains, which was resistant against Methicillin, vancomycin and ciprofloxacin. Balakumar, *et al*., (2011) assessed antifungal activity of crude extracts of *O. sanctum* against isolated dermatophytic fungi including *Trichophyton*

mentagrophytes, *Trichophyton rubrum*, *Microspore gypseum*, *Microsporum nanum* and *Epidermophyton flocossum* [148].

4.14.1.1 MIC OF PHENOLIC COMPOUNDS

The MIC of individual phenolic compound was determined to test the susceptibility and resistance of microbial strains. The results in (Table-34) shows the MIC value of active phenolic compounds including quercetin, kaempferol, Catechins and gallic acid against MRSA. The MIC value of quercetin was found 460µg/mL against *MRSA* when combined with resistive drugs *i.e* Amoxicillin, Ampicillin, Methicillin and Ceftriaxone, while 440µg/mL when combine with Cephradine.

The MIC value of kaempferol was found in range 360 to 480µg/mL against *MRSA* in combination with different drugs. Its MIC value was 360µg/mL when combine with Ceftriaxone, 380µg/mL with Amoxicillin and 440µg/mL with Methicillin, 460g/mL with Ampicillin and 480µg/mL with Cephradine. Catechins showed activity in range of 480 to 500µg/mL against *MRSA*. The MIC value of catechins was 500µg/mL against MRSA when combine with Amoxicillin, Methicillin and Cephradine, while 480µg/mL when combine with Ampicillin and Ceftriaxone. The gallic acid was found resistant against *MRSA* below 500µg/mL when combine with Amoxicillin, Ampicillin, Methicillin and Cephradine, while inactive below 480µg/mL when combine with Ceftriaxone.

Table 33: Average Zone of Inhibition of Antibiotic Alone and with Phenolic Compounds

S.No	Antibiotic	Alone	Quercetin	Kaempferol	Catechins	Gallic acid
			With Antibiotic			
1	Amoxicillin	00.0	16.5 ± 0.51*	20.0 ± 0.94	11.0 ± 0.49	09.0 ± 0.41
2	Ampicillin	00.0	17.0 ± 0.57	16.0 ± 0.57	12.5 ± 0.45	11.0 ± 0.43
3	Methicillin	00.0	16.0 ± 0.43	18.5 ± 0.61	09.0 ± 0.31	04.5 ± 0.21
4	Cephradine	00.0	18.0 ± 0.59	13.0 ± 0.48	08.0 ± 0.28	04.0 ± 0.24
5	Ciprofloxacin	10.0	04.5 ± 0.14	07.0 ± 0.26	08.5 ± 0.31	03.0 ± 0.31
6	Erythromycin	21.5 ± 1.42	21.0 ± 0.83	21.0 ± 0.89	21.0 ± 0.94	21.0 ± 1.31
7	Ceftriaxone	00.0	17.5 ± 0.57	22.5 ±1.20	13.5 ± 0.81	11.0 ± 0.73
8	Cefixime	00.0	13.6 ± 0.48	18.2 ± 0.91	12.3 ± 0.61	14.1 ± 0.81

*The values are in triplicate and ± is the standard deviation

Table 34: MIC of Antibiotic with Phenolic Compounds

Antibiotic	Quercetin	kaempferol	Catechins	Gallic acid
	With Antibiotic			
Amoxicillin	460	380	500	500
Ampicillin	460	460	480	500
Methicillin	460	440	500	500
Cephradine	440	480	500	500
Ceftriaxone	460	360	480	480

Liu, *et al.*, (2009) studied the effect of kaempferol on antibacterial resistant bacteria *MRSA* they found that kaempferol showed strong activity against *MRSA* they also found that combination of kaempferol greatly reduced the MIC of fluoroquinolones in *MRSA* [149]. Irfan, *et al.*, (2008) evaluated resistant rated in isolated *MRSA* against Clindamycin, tetracycline, cotrimoxazole, Rifampicin, chloramphenicol, and fusidic acid. They found high resistance rates to tetracycline 82% and Clindamycin 79%, while the resistance to chloramphenicol and fusidic acid was 10 and 9% [150].

Soon, *et al.*, (2011) studied the antibacterial activity of *A. indica* against clinically isolated strains of pathogenic bacteria and two fungal strains. They found the most susceptible strain was s aurous (MIC=16µg/mL), while most resistive was *K. Pneumonia* (MIC=128 µg/mL). The MIC value recorded for *Aspergillus niger* was 32µg/mL [151].

Ferreira, *et al.*, (2010) studied the synergistic activity of flavonoids. The MIC of Norfloxacin calculated 128µg/mL, Ciprofloxacin 8µg/mL, Enoxacin 64µg/mL, Olfoxacin 32µg/mL and Levoflaxacin 2µg/mL against clinically isolated *MRSA*. By addition of Kaempferol the MIC of Norfloxacin reduces to 8µg/mL, Ciprofloxacin 1µg/mL, Enoxacin 8µg/mL and Levoflaxacin 1µg/mL [245].

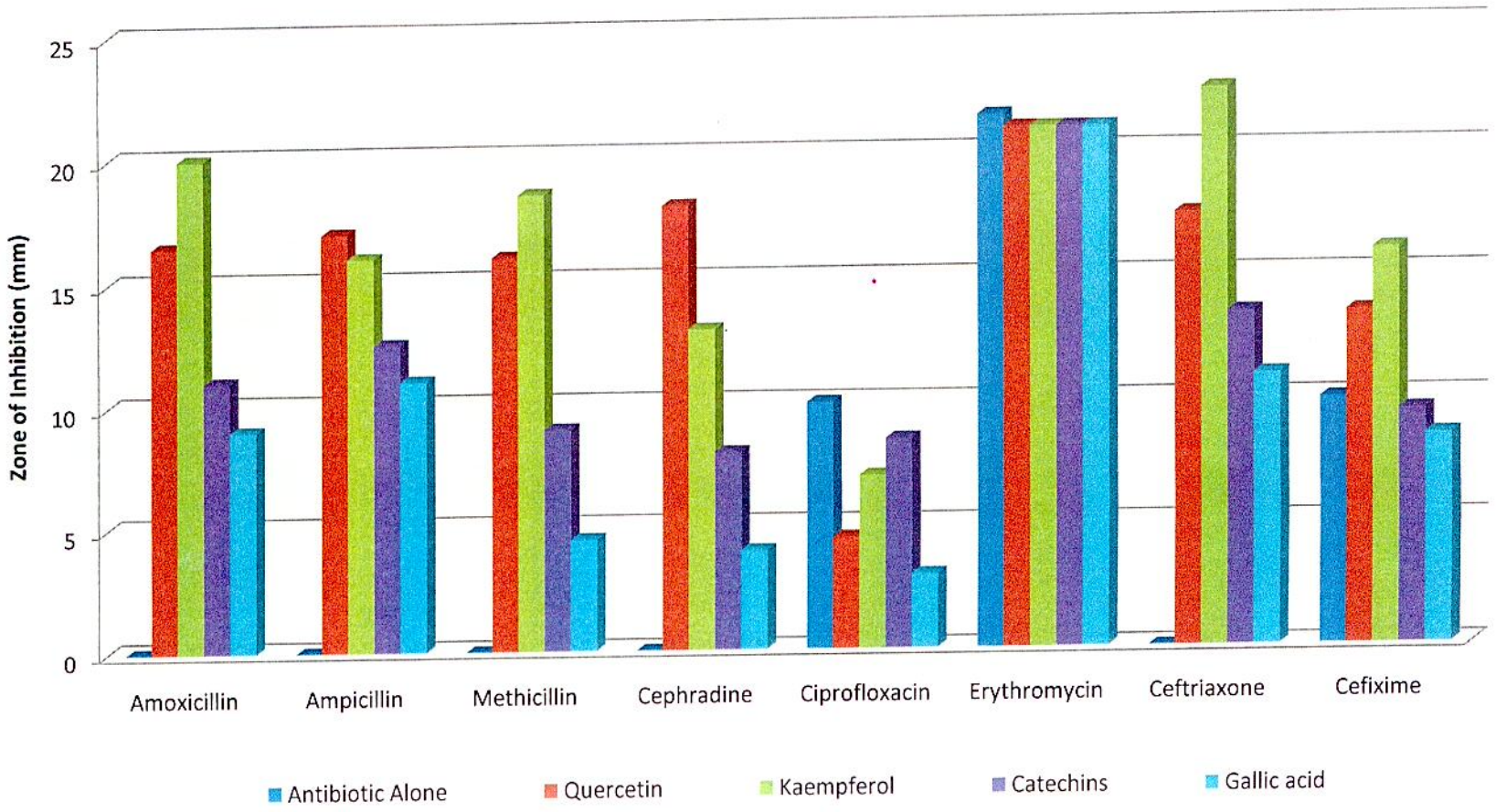


Figure 53: Average Zone of Inhibition Of Antibiotic Alone and with Phenolic Compounds

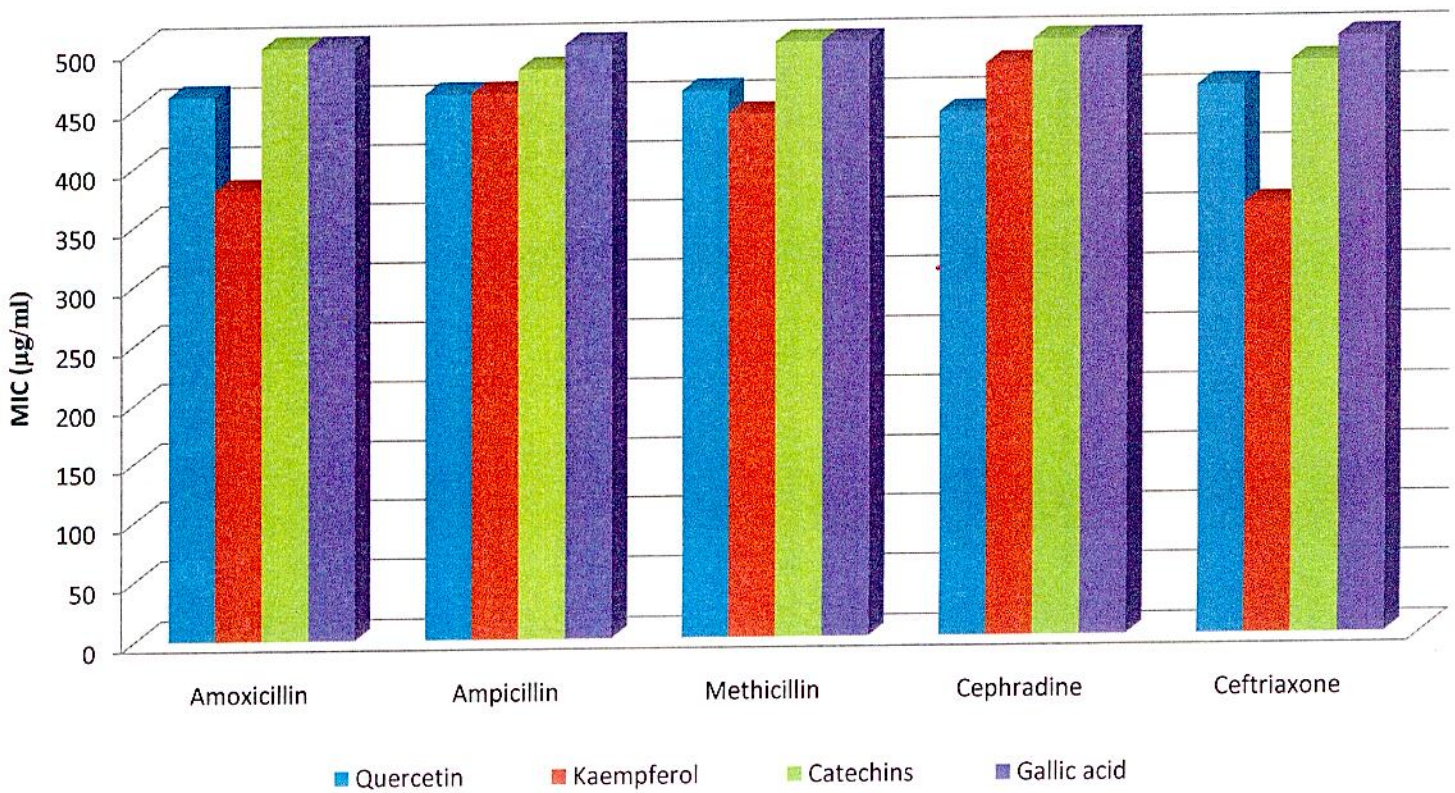


Figure 54: MIC of Antibiotic with Phenolic Compounds

5 CONCLUSION

Form the proximate, minerals and phytochemical analysis it is evident that the leave of *O. sanctum*, *O. basilicum* and *A. indica* plants are good source of nutrients. The antibacterial assay of the crude extracts of *O. sanctum*, *O. basilicum* and *A. Indica* leaves were studied against eight bacterial strains including *staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsilla pneumonia*, *Proteus mirabilis* and *Enterococcus fecalis*. The crude extracts of *A. indica* was found active against all pathogenic strains, while *Salmonella typhi* showed resistance to *O. sanctum* and *O. basilicum* extracts. These crude extracts was also evaluated for their antifungal potential and found active against the selected strains including *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus parasiticus*.

All the crude extracts were evaluated for their antioxidant potential using different extraction procedures and solvents and the crude methanol extract of *A. indica* extracted by ultrasonic extraction procedure showed highest antioxidant activity and highest concentration of TPC and TFC.

The phenolic compounds in plant leaves were characterized using GC-MS by converting to their trimethylsilyl esters by silyation methods. Consequently 10 phenolic compounds in *O. sanctum*, 11 in *O. bsilicum* and 10 in *A. Indica* were identified. A new simple HPLC (UV-VIS) method has been developed and validated according to ICH rules for the detection and quantification of five major phenolic acids including syringic, benzoic, gallic, chloroganic, and vanillic in crude extracts of *O. Basilicum, O. sanctum* and *A. indica*.

The common flavonoids kaempferol, quercetin, Luteolin and Catechins were also quantified by HPLC method.

The antifungal activity of individual phenolic compounds including Quercetin, Kaempferol, Rutin, Catechins, Gallic acid and vanillic acid were also evaluated against *Aspergillus flavus, Aspergillus niger* and *Aspergillus parasiticus*. The results revealed that quercetin, kaempferol and Catechins were active while rutin, gallic acid and vanillic acid were inactive against all tested fungal strains.

These compounds were also tested against 80 isolated *MRSA*, which were collected from the microbiology laboratory of tertiary care hospitals of Peshawar, KPK, Pakistan and standard *S. aureus*. Quercetin, kaempferol, Catechins and gallic acid were found active against *MRSA* and standard *S. aureus*, while vanillin acid and rutin were inactive. The four active phenolic compounds in combination with eight different antibiotic standards (Amoxicillin, Ampicillin, Methicillin, Cephradine, Ciprofloxacin, Erythromycin, Ceftriaxone, and Cefixime) were evaluated against *MRSA*. The antibiotics Amoxicillin, Ampicillin, Methicillin, Cephradine, Ciprofloxacin, Cefixime and Ceftriaxone are found inactive against *MRSA* when tested alone, expressed activity when combine with active phenolic compounds. The activities of active phenolic compound were potentiated when combined with Amoxicillin, Ampicillin, Methicillin, Cephradine and Ceftriaxone.

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