Isolation and Characterization of Flavonoids from *Berberis lycium* Leaves

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A thesis submitted in partial fulfillment of the requirements for the degree of MS Biomedical Sciences

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Declaration

I, Shazia Tahir, certify that this research work titled "Isolation and characterization of *Berberis lycium* leaves" is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources has been properly acknowledged / referred.

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Shazia Tahir

Dedication

I dedicate this piece of work to my parents, sisters and brothers for their immense support and cooperation that led me to this wonderful accomplishment.

Abstract

The production of pharmacological products from medicinal plants have now become one of the leading cause of economic development of countries. Currently, the purification of crude extract and further isolation of bioactive compounds from medicinal plants are gaining special attention by the researchers to obtain natural bioactive formulations. Therefore, to overcome the phenomena of multidrug resistance, the method of using natural products and drugs seems to be an effective strategy against different diseases. This study is carried out to investigate the bioactive flavonoids of *Berberis lycium* leaves on *Escherichia coli*. A 15 % methanol in ethyl acetate as a mobile phase was selected by thin layer chromatography that gave better separation of flavonoids. After the collection of two seventy- six fractions from column chromatography, they were combined based on similar R_f values. Hence thirteen fractions were made. Out of thirteen fractions, five fractions having single spots were selected for antibacterial test against six bacterial strains that included three-gram positive bacteria: *Enterococcus faecalis, Bacillus cereus, Staphylococcus aureus,* and three-gram negative bacteria: *Salmonella typhi, Pseudomonas aeruginosa*, *Escherichia coli*. The separated fractions showed effective antibacterial activity against two bacterial strains; *P. aeruginosa*, and *E. coli*. The characterization was done by FTIR analysis for further confirmation of groups belong to flavonoids in isolated samples.

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

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Chapter 1: INTRODUCTION

Plants are used by human beings to cure different diseases for thousands of years. There is a long history about use of plants and their secondary metabolites constituents in modern 'western medicines' and in certain categories of conventional medicines. According to WHO, the dependency on traditional medicines increases up to 80% among world's population to fulfill their health care needs. The highly available commercial drugs such as atropine, codeine, digitoxin, morphine, quinine and vincristine are obtained from these traditional medicines. The methods like Pharmacological testing, modification, derivatives formation and research on natural products ultimately leads to formation of noval drugs (Sharma & Janmeda, 2017). The development of indigenous medicines from medicinal plants has shown considerable economic benefits to countries (Abdulhamid, Fakai, & Ogwihi, 2017). So, medicinal plants are becoming an important source of formulating drugs for many disorders (Guimarães et al., 2015). Recently, plants used in different forms like their crude extract, spices and dry powder have gaining much popularity for manufacturing of alternative traditional medicine and food supplements (Hossain, AL-Raqmi, AL-Mijizy, Weli, & Al-Riyami, 2013). Infact, to minimize the phenomena of multidrug resistance, it would be a successful strategy of using dietary supplements and drugs with minimal side effects. The one of the leading cause of multidrug resistance is the over expression of multidrug efflux transporters across the cell membrane. Like over expression of P-glycoprotein (P-gp) also known as multidrug resistance protein 1 (MDR1) that causes resistance against many antiepileptic drugs (AEDs). So, to counteract that problem, certain inhibitors are used against these drug efflux transporters to reduce the effect of P-gp-mediated drug resistance. Over the past few decade, the idea of application of several isolated herbal compounds such as flavonoids have thought to be more effective and secure therapy for reversing the P-gp-mediated multidrug resistance. Many in vitro as well as in vivo

studies have been proven the effective potential of flavonoids as an effective P-gp inhibitors. A more favorable safety profile is to use some flavonoids that show same P-gp inhibitory activity like verapamil and cyclosporine. The unsatisfactory results shown by several first-, second- and third-generation of synthetic P-gp inhibitors, demand the investigation of noval type of natural inhibitors. Although there are also side effects of first- and second-generation

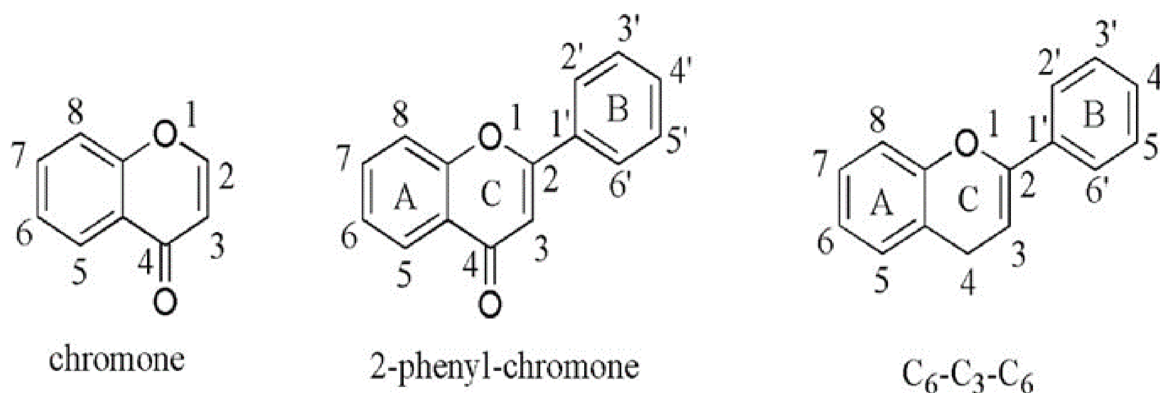


Figure 1 Basic structure of flavonoids nucleus

inhibitors, but they are used in high concentration that causes severe toxicity. Whereas, the main drawback of third-generation P-gp inhibitors like biricodar, elacridar, laniquidar, tariquidar or zosuquidar is as they alter the sites of non-target organs. Thus, to overcome all these negative aspects, the best choice for selecting a non- toxic and highly specific inhibitor are thought to be flavonoids (Ferreira, Santos, Falcão, & Alves, 2018). They receive considerable attention due to its physiological importance**.** They had been very significant in ancient times for the treatment of several diseases. They are structurally characterized by a 15-carbon (C6–C3–C6) backbone having one oxygenated and two aromatic rings (figure 1**).** In the structure, chromane rings are A and C, possesses a second aromatic B ring in position 2, 3 or 4 (Mujwah, Mohammed, & Ahmed, 2010). Majority of flavonoids show antibacterial activity due to the presence of mono and dihydroxyl groups in B ring (Hernández, Tereschuk, & Abdala, 2000).

They are classified together into variety of subclasses but all shared basic common skeleton. The most common groups are flavanols, flavones, flavonols, isoflavones, flavanones,

anthocyanins and chalcones. The most abundantly present groups are flavanols and pro-anthocyanidins (condensed tannins) in nature than other plant phenolics. They have shown the most significant free radical-scavenging and anti-tumor properties among others.

Therefore, isolation of flavonoids is currently highly demanding and important. For this purpose different methods that commonly used are liquid -liquid extraction, column chromatography (silica gel column, polyamide column, sephadex column), macroporous resins, preparative semi preparative reversed- phase liquid chromatography (Di, Zheng, Chen, Huang, & Feng, 2011). The studied plant, *Berberis lycium* is a spiny shrub with an average height of 2-4 meters. They have lanceolate leaves which are alternately arranged on the stem. It has great medicinal importance including enhancing immunity, hemopoiesis as well as anti-oxidation, anti-radiation, anticancer, anti-aging, antimutagenic, hypoglycemic, antihyperlipidemic, pesticidal, and hepatoprotective properties. The plant is also proved to be efficient in healing wounds. Moreover, the alkaloid berberine from this species is confirmed to be antipyretic, antimutagenic, hypoglycemic, antihyperlipidemic, pesticidal, and hepatoprotective. The plant is also proved to be efficient in healing wounds. Moreover, the alkaloid berberine from this species is confirmed to be antipyretic, anti-inflammatory, anti-hypercholesterolemia, antitumor, antidiarrheal and antidiabetic (Ahmed, 2017). This study is initiated for the investigation of antibacterial activity of isolated flavonoids from *Berberis lycium* leaves.

Objectives:

- **Extraction and isolation of flavonoids from *Berberis lycium***
- **Characterization of flavonoids**
- **To perform its antibacterial activity on *E. coli***

Chapter 2: LITERATURE REVIEW

2.1 *Berberis lycium*:

Berberis lycium, a medicinal plant which is abundantly available especially in hilly areas of Pakistan. It is being extensively used for the cure of several human disease. Berberine is a major umbellitine and isoquinoline alkaloid, usually extracted from root bark or other *Berberis* species. Plant fruits are termed as Berries are called as the fruit of these plants which are ovoid or obovoid sub globose in shape. They appear in dark red or purplish colour upon ripening (Sood, Modgil, & Sood, 2012). The fruits are rich source of malic, tartaric, citric acids and tannins. They have numerous applications especially roots of this plant. Severe jaundice and opthalmia can be cure by its root extract. The usage of berberine and plant extract causes reduction in blood glucose level as well as showed significant results on glycosylated hemoglobin, glucose tolerance, serum, lipid profiles and body weight (Gupta, Singh, & Joshi, 2015). The root bark of this plant is used to make an ointment after mixing it with mustard oil for treatment of broken bones, wounds, gonorrhea, curative piles, unhealthy ulcers, acute conjunctive. Its various parts are also used to make bitter tonic astringent, diaphoretic and febrifuge. Further used as a treatment of malaria and intermittent fever, scrofula, fistula and other skin related diseases. It acts as an anti-Inflammatory agent in case of enlargement of liver and spleen (Sood et al., 2012). Traditionally, different preparations are made by using different parts of plants such as its powder is used for treating dysentery, internal wounds and throat pain whereas root bark extract is used against diabetes, pustules, scabies and its root paste is used in site of bone fracture (Shabbir, 2012). So far, many researches have been carried out on isolation of many compounds like alkaloids and steroids (Sabir et al., 2013).

2.1.2 Taxonomical features:

Taxonomically, *Berberis lycium* belongs to kingdom Plantae, Phylum: Tracheophyta, Class: Magnoliopsida, Order: Ranunculales, Family: Berberidaceae, Genus: *Berberis*, Species: lycium, Scientific name: *Berberis lycium*. Among all other angiosperms, it is one of the oldest angiosperm, that has numerous number of discontinuous genera. There are 17 genera and 650 species in this family. In English language *Berberis lycium* is called as barberry, while "sumbhal" is the word used in Urdu for this plant. "Darhald" and "kashmal" are used for roots and fruits respectively. The plant is a native species of Nepal; however, the species has been reported throughout Himalayas Mountains, temperate and semi-temperate localities of

Afghanistan, Bangladesh, India, and Pakistan. In Pakistan, they are found in KPK, Baluchistan, Punjab and Azad Kashmir at an altitude of 900-2900 meters. The plant is a spiny deciduous shrub with an average height of 2-4 meters. It has lanceolate leaves, arranged alternately on the stem. The plant is hermaphrodite, bears bright yellow colored flowers. The flowering season of this species is March to July**.** The ovoid shaped fruit, called as Berries are up to 7-millimeter long. The berries are slightly acidic and juicy. The plant can be grown on different habitats like in sandy, silty or loamy soils. The plant has a hard and branched root with a diameter ranging from 3 to 8 cm. B. lycium has been reported to contain important alkaloids like berberine, berbamine, Palmitine, karakoramine, balauchistanamine, jhelumine, gilgitine, umbellitine and chinabine. The plant is recognized to have Carbohydrates Proteins, Lipids, and Vitamins, hydrolysable tannins, saponins and cardioactive glycosides. Fruits are known to have moisture, phytic acid, hemicelluloses, β carotein, vitamin A, cellulose and anthocyanins. The plant is also recognized to have various minerals including zinc, copper, sulphur, sodium, calcium, iron, lead, magnesium, copper, potassium and phosphorus. B. lycium is found to be effective against microorganisms. It is confirmed to have efficient antifungal and antibacterial activity. The plant is also reported to have anticoccidial and immunostimulant, antimutagenic, hypoglycemic, antihyperlipidemic, pesticidal, hepatoprotective and wound healing property. Moreover, the alkaloid berberine from this species is confirmed to be antipyretic, anti-inflammatory, anti-hypercholesterolemia, antitumor, antidiarrheal and antidiabetic.

2.1.3 Phytochemical constituents:

The phytochemical constituents of this plant include alkaloids, saponins, minerals, and carbohydrates have been reported from this species. Modern technology such as atomic absorption spectrophotometer revealed that the root contains 0.2% zinc (Zn) and 0.2% sulphur (S), while the sulphur (S) and zinc (Zn) contents in fruit were 0.1% and0.8%, respectively. According to a study results, the quantity of Lead (Pb) found in stem and roots of the plant were 7.992 ± 0.106 and 4.360 ± 0.176 ppm, respectively. Result for Copper (Cu) quantity in stem and roots were 5.031 ± 0.176 and 4.360 ± 0.176 ppm, individually. Manganese (Mn) in the stem and plant root was 18.272 ± 0.212 and 15.500 ± 0.212 ppm correspondingly. Atomic absorption spectrophotometer is used to examine elements like Phosphorous, Potassium, Iron, and sodium the fruit of the plant. The results showed the presence of Sodium (14.5± 0.11mg/100 g), Potassium (161.42 ± 0.41 mg/100 g), Calcium (18.272 ± 0.212 mg/100 g), Iron (2.61 ± 0.06 mg/100 g) and Phosphorus (38.0 ± 0.24 mg/100 g) in healthy amount. Leaves

have also been reported to be plentiful in Iron (528.47 ± 0.02 µgg-1), Potassium (4077.00 ± 0.58 µgg-1), Mn (136.12 ± 0.01µgg-1), Phosphorous (1315.00 ± 0.01 µgg-1), Cu (53.41 ± 0.09 µgg-1), Zinc (37.71 ± 0.02 µgg-1), Sodium (79.00 ± 0.01 µgg-1) and Calcium (2389.00 ± 0.04 µgg-1). It was shown in a report that Copper, Zinc, and Sodium were concentrated in root, Potassium in shoot, while Phosphorous, Mn and Calcium in leaves.

B. lycium is also screened for moisture, proteins, sugars, fibers, vitamin and dry matter. According to a report, roots of B. lycium possess moisture (20.5%), sugar (3.5%), dry matter (61.2%), fat (2.6%), protein (4.5%), Vitamin C (0.3%) and fiber (2.5%) in the given percentages. Fruits contain moisture content (12.5%), Vitamin C (0.8%), dry matter (62.5%), fat (1.8%), protein (2.5%), sugar (4.5%) and fiber (1.5%) in the mention percentages. Roots showed the highest amount of fiber and fats while leaves showed highest amount of crude proteins. Phytochemical screening of water extract of B. lycium showed the presence of cardiac glycosides, saponins, hydrolysable tannins, and alkaloids. Analysis of fruit revealed the presence of tannins (8.9 ± 0.15 mg/100g), Phytic acid (2.5 ± 0.04 mg/100 g), β- Carotene (343.0 ± 0.89 µg/100 g), Phytate phosphorus (0.78 ± 0.06 mg/100 g), Hemicellulose (6.01 ± 0.41%), Anthocyanin (82.47 ± 0.29 mg/100 ml juice), Vitamin A (85.65 ± 0.17 µg/100 g) and Cellulose (7.94 ± 0.60%). The concentrations of alkaloids like Berberine and Palmitine are 4.5% and 3.1%, in their roots respectively, while in fruits 2.9% Berberine is present, Berbamine, balauchistanamine, karakoramine, sindamine, gilgitine, jhelumine, punjabine, umbellatine and Chinabine are other alkaloids that have been recognized in B. lycium (Ahmed, 2017).

2.1.4 Nutritional value:

The nutritional value of *Berberis lycium* fruits is remarkably high. The fruit contains 84% water content, about 1.49% crude protein, 1.28% fat and 1.48% fiber. Other than these substances, it also has pectin, vitamin C, β carotene and anthocyanin. They are also rich in various minerals like phosphorus, calcium, iron, sodium, potassium etc. They used as a remedy to get relief in conditions like intestinal infection, pharyngitis, typhoid and fever. Their leaves are used as a tea substitute as well as for treatment of jaundice. Its stem is known as diaphoretic and used to cure rheumatism and skin diseases. The stem bark is very effective for treating ear injury, whooping cough, headache, opthalmia and jaundice etc. Dried and freshly prepared plant material is preferred to use instead of old dried stored material (Intekhab & Aslam, 2009).

The rhizomes of *Berberis lycium* contain a major alkaloid, berberine which has antibacterial effect and used to treat various enteric infections, most commonly bacterial dysentery. In previous studies, different phenolics were detected in methanolic roots extract of *Berberis* lycium. An important chemical of this plant called berberine has proven to have antitumor effect.

There are approximately more than 100 human diseases caused by free radicals. Phenolics including plant flavonoids have proved to exhibit radical scavenging, antioxidant and anticancer property. The leaves of L. barbarum contain many important active components. However, the main flavonoid present in the leaves are still unknown (Sood et al., 2012).

2.2 Flavonoids:

Flavonoids and polyphenolic compounds are considered as the most beneficial bioactive compounds (Ke et al., 2011). They are classified as compounds having chromane-type skeleton, bears a phenyl substituent located in C2 or C3 position. Their main subclasses are shown in Fig. 1. Majority structures have hydroxyl groups present at positions 3, 5, 7, 4 and/or 5 in flavonoids. These hydroxyl groups are also found as methylated, acetylated, prenylated or sulphated forms. Their two forms such as O- or C-glycosides of flavonoids are also common in plants. In both cases, most groups have O bonding as compared to C bonding. The O-glycosides are characterized as sugar substituents bounded to a hydroxyl group of the aglycone at position C3 or C7, but in C-glycosides, the bounding occur at 6th or 8th carbon. Among all, carbohydrates like rhamnose, glucose, galactose and arabinose are most in common. Plants also have flavonoids- diglycosides such as neohesperidose and rutinose that are formed from most common disaccharides glucose and rhamnose shows $1 \rightarrow 6$ linkage and $1 \rightarrow 2$ linkage respectively. The carbohydrates like malonate and acetate, both are acyl residues, often further substituted instead of sugars. Flavonoids are termed as glycosides due to the presence of sugar groups and as aglycones in the absence of sugar group. These are synthesized by phenyl - propenoid pathway (D. Singh & Singh, 2016). The total number of flavonoids that are known today are 4000 approximately, further there are12 subclasses, approximately 3000 flavones and 700 isoflavones found in plants. Therefore, it seems challenging to separate and identify each of them. They receive considerable attention in the literature as they show great biological and physiological importance. Among all secondary metabolites, flavonoids are considered the largest group having great medicinal importance by acting as a signaling compound in reproduction, patho-genesis, defense mechanism as well as in symbiotic association**.** The previous case control statistical **s**tudies had suggested the cause of effective control of

cardiovascular diseases and stroke is flavonoids (Peterson & Dwyer, 1998). During different stress conditions in plants such as exposure of increased UV-B radiations, bacterial infections or herbivore attack, flavonoids play a key role in generating response mechanisms. They are also responsible for development of root nodules in various leguminous plants that serve as nitrogen fixation system for Rhizobium bacteria. They are pigmenting agents to give color in plants to carried out pollination, along with that, they are also involved in different insect's interaction. The antioxidant property of dietary flavonoids contributes to their anticarcinogenic effects (Nishiumi et al., 2011). They are very beneficial for increasing human health due to their antioxidant, antimicrobial and pharmacological properties. They interact with many enzymes that are part of intracellular cell signaling. They especially, effect the activity of protein kinase as it effects the immune functions of host. The major cause of diseases is the alteration in multiple cellular signaling pathways and it has been found that flavonoids effect several mammalian enzymes like protein kinases that is responsible for regulation of multiple cell signaling pathways (M. Singh, Kaur, & Silakari, 2014). Currently, several reviews on their properties clearly depicting their importance in treatment of different diseases.

A main crucial step of flavonoids analysis is to isolate a desired analyte in conjugated or non-conjugated form. They are chemotaxonomic marker compounds due to their significant biological and physiological activities (Cuyckens & Claeys, 2004). The flavonoids in the form of glucuronide and sulphate conjugates are found in biological fluids (serum, plasma and urine). A hydrolysis step is used in most cases total aglycones content is known. Whereas, researchers are more interested to isolate glycosylated flavonoids from plants, to make medicine and food products. For example, in case to classify distinct species of plants, glycosylated flavonoids profile is needed. Currently as the number of target analytes increase so flavonoids analysis become more complicated therefore more sensitive and selective analytical methods are high in demands. Plants that were used to make folk medicines are now selected to make its crude extract for its further screening to check its antibacterial activity during invitro studies**.** Plants species of Hypericum, Capsella and Chromolaena are rich source of flavonoids and their extract have been reported to have antibacterial activity (Cushnie & Lamb, 2005a).

During past five years, there are about three- hundred papers published on quantification as well as characterization of plants constituents for medicinal or taxonomical purpose. And further fifty papers have been published on body fluids analysis in humans as well as in

animals. About 30 papers have been published on flavonoids for showing antioxidant property present in food juices (Sultana, Anwar, & Ashraf, 2009). Many structural features are identified that enhances the antibacterial properties of flavonoids (Cushnie & Lamb, 2011).

2.2.1 Subclasses of flavonoids:

2.2.1.1 Flavonols:

Flavonols are one of the major class of flavonoids, consisting of a 3-hydroxyflavone backbone structure with phenolic- OH group. They are responsible for stress responses in plants. They are considered as the most ancient and well-known group in performing potential physiological activities among another groups of flavonoids (Falcone Ferreyra, Rius, & Casati, 2012). The presence of -OH group in different position gives variety to this class, also enhances its oxidative activity comparable to flavones (figure 2). The most common members include quercetin, myricetin, kaempferol, and galangin. They are commonly present in plant foods. Quercetin and kaempferol are most well-known member of this group. The most predominating flavonol found in leaves of various vegetables is quercetin glycoside. Fruits and leafy vegetables contain kaempferol in vast amount. Some herbs, legumes and root vegetables also have large amount of kaempferol. Onions and-pears contain another member from this class named isorhamnetin. In berries, maize and tea, myricetin is also present. Flavonols and their glycosides are found abundantly in the skin of fruits.

2.2.1.2 Flavanols or flavan-3-ols:

They have fully saturated carbon ring structure belongs to polyphenol group having -OH group at C-3 position (figure 2). They are mostly known as catechins and are significantly present in Camellia sinensis. They have variety of compounds like catechin, epicatechin, epicatechin gallate, and epigallocatechin gallate are also found in green tea leaves. They showed wide range of antifungal activity and approx. 90% growth inhibition occurred in case of C. albicans by green tea.

2.2.1.3 Flavones:

The structure of Flavones can be differentiated due to location of ketone group at C4 and presence of double bond between C2 and C3 (figure 2). Naturally occurring members are Apigenin, luteolin, tangeritin, chrysin, 6-hydroxyflavone, baicalein, scutellarein, and wogonin. They naturally occur in grains likewise parsley and rosemary. Their mechanism of

action is to inhibit the efflux pumps that resulted in cell death or apoptosis. Vitexin and isovitexin is an example of glycosylated flavone. To investigate anti-inflammatory effect of vitexin and isovitexin, cells or mice are usually first treated with lipopolysaccharides, that will give better result (Corcoran, McKay, & Blumberg, 2012).

2.2.1.4 Isoflavones:

They have distinct structure due to phenyl group at C3 instead of C2 position (figure 2). They are considered as plant estrogen due to their similarity in structure with estrogen. The most common antifungal members of this groups are glabridin, isolated from Glycyrrhiza glabra, showing broad-spectrum antifungal activity against several Candida species. Another member like genistein and daidzein show estrogenic activity in animal models so they are also called as phytoestrogens. Szkudelska & Nogowski described the role of genistein for inducing hormonal and metabolic changes in different disease pathways.

2.2.1.5 Chalcones:

They have structure based on aromatic ring along with keto group and two phenyl rings linked by 3- carbon structure (figure 2). They are precursor molecule in biosynthesis of flavonoids. The most common members are 2',4'-dihydroxy-3'-methoxychalcone, 2',4'dihydroxychalcone, and carvacrol. They are also responsible of inhibiting exoenzyme, that promote fungal invasion mechanisms. They are effective against C. albicans with an MIC of 400 µg/ ml is 2',4'-dihydroxy-3'methoxychalcone (Seleem, Pardi, & Murata, 2017).

2.2.1.6 Flavanones:

They are commonly known as dihydroflavones. They are different from flavones due to the presence of single bond between C2 and C3 instead of double bond (figure 2). Citrus fruits like oranges, lemons and grapes have rich source of flavanones. The important member of this group includes hesperidin, naringenin and eriodyctiol. They have free radical scavenging properties that’s why they are very beneficial to health. The bitter taste of citrus fruits is due to the presence of these compounds. These compounds are also responsible for vast pharmacological activity like anti-inflammatory, antioxidant, blood lipid-lowering and cholesterol lowering agents.

They are common in chick peas, cumin, rowanberry, and citrus fruits. Cumin and peppermint contains hesperidin. Berries have narirutin and naringenin glycosides. They cause flavor to citrus fruits.

2.2.1.7 Anthocyanins:

They are pigments that impart colors to plants, flowers and fruits. Many factors contributing their coloring property such as pH and methyl or acyl groups (figure 2). The most commonly studied compounds include cyanidin, delphinidin, malvidin, pelargonidin and peonidin. They are also found in skin of fruits like cranberries, black currants, red grapes, merlot grapes, raspberries, strawberries, blueberries. They have many applications in food industry due to their structural stability and health benefits. They impart blue and red coloration to parts of plants. The extract of grapes contains glucosides, acetyl glucosides and coumaryl glucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin. Their conjugated structures with flavones and metal ions like iron and magnesium are also found in majority of flowers. They are pH dependent and impart red color at 3.5 pH and it becomes blue in color when pH is increases (Panche, Diwan, & Chandra, 2016).

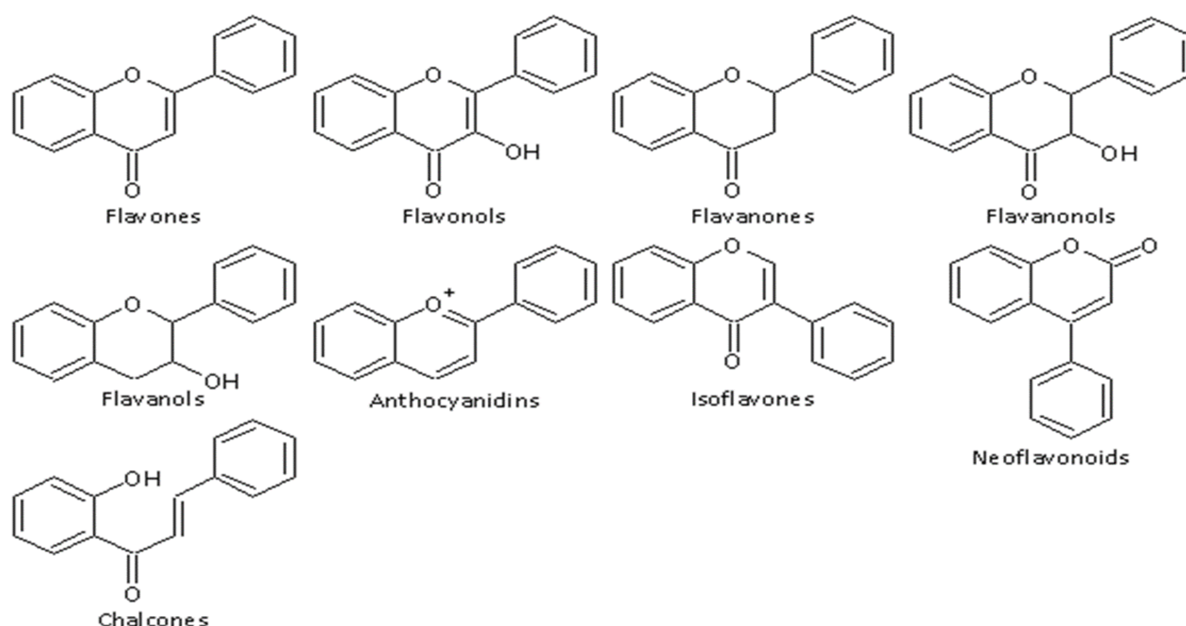


Figure 2 Major classes of flavonoids

2.2.2 Bioavailability and Drug Interactions of Flavonoids:

The factors affecting the bioavailability of flavonoids are due to the presence of fiber, macro- and micronutrients, gastrointestinal transit time, and gut microbiota (Corcoran et al., 2012). They are considered as good therapeutic candidates due to their bioactive nature with low

toxicity. However, they showed poor absorption and decreased cellular uptake due to their low plasma concentrations and cell membrane transporter barriers. The regulation of flavonoids is due to binding of serum albumin that will decrease the plasma concentration of unbound bioactive flavonoids and resulted in delayed release into plasma. Flavonoids bound to albumin show stability against aerobic degradation, leads to greater plasma half- life as well as biological activity. These conditions provide protection against high uptake of flavonoids that ultimately causes decrease in cellular toxicity. The constant rate of flavonoids uptake from the cell is provided by a continuous release by albumin. There is also an interaction of flavonoids with p-glycoprotein and efflux transporters**.** The main efflux transporters such as ATP binding cassette or MRPs causes decrease in net cellular absorption of flavonoids. The transporters located on gateway organs and considered as an epithelial barrier for flavonoids. Thus, due to their low intestinal permeability and high metabolic rate, flavonoids show relatively low permeability. The absorption property of groups such as isoflavones and quercetin glucosides is greater than others like condensed tannins and anthocyanins. The movement of flavonoids are carried out by efflux transporters across intestinal epithelial barriers. The permeability of quercetin is due to pH-dependent transporters whereas not in case of naringenin. It has been proven that the efflux of quercetin is a MRP2 mediated process along with specific ABC inhibitors and not by P-gp. Whereas naringenin is mediated by both MRP2 and P-gp substrate. The biliary excretion of flavonoids is carried out through MRP2 transporters in hepatocytes. Similarly, in case of tea, MRP2 transporters cause the efflux of flavonoids into intestinal lumen. They showed combined or synergistic effects in case of multiple flavonoids interaction like the antiproliferative activity shown by four red wine polyphenols that are resveratrol, quercetin, ethyl gallate, and (+)-catechin. They showed antiproliferative activity stronger than their individual effects in muscle cells. The concentration level of flavonoids as xenobiotics is determine through plasma membrane transporters**.** This provide a future direction to understand their pharmacokinetics, underlying mechanism and signaling pathways. A main focus of future research may on identifying multi- drug targets mechanisms to determine whether structural specificity occur among them or not (Seleem et al., 2017).

2.3 Soxhlet extraction:

It is the most commonly used technique to extract flavonoids from solid samples. The solvents used are aqueous methanol or acetonitrile. The completion time in this process may vary up to 12h. Like in case of plants Tiliaeuropea, Urticadioica, Menthaspicata, and Hypericumperforatum, the reported time is 12 h Soxhlet extraction with methanol. It is very

important to standardize the extraction procedure in order to increase the quality of herbal drug (Brusotti, Cesari, Dentamaro, Caccialanza, & Massolini, 2014). In another study, that extraction method is used to extract phenolics efficiently from seeds of three wild grapevines. For this method a temperature range 60-90°C is required for several hours whereas in maceration it is completed at ambient temperature. This method is simple, economical and gives high yield of phenolics product within limited time duration. Castro-Vargasa et al. found a high yield of phenolic compounds extracted from Guava seed through this method, but with all the advantages, there are some disadvantages, include: (1) the organic solvents used in this technique are hazardous to environmental as well as to health; (2) it may include the degradation of target products due to elevated temperature. These limitations lead to the demand of advance methods to overcome these problems. Some latest techniques to counteract these problems are microwave-assisted extraction (MAE), ultrasound-microwave-assisted extraction (UMAE), supercritical fluid extraction (SFE), and high hydrostatic pressure processing (HHPP). These methods are easy to use as well as minimize the consumption of toxic organic solvents hence leads to reduce toxification in environment. The solvent used normally are methanol or acetonitrile. Many kinds of flavonoids were extracted through this method from Tiliaeuropea, Urticadioica, Menthaspicata, and Hypericumperforatum within12hrs (Sultana et al., 2009).

Solvent extraction is an efficient, convenient and widely applicable technique for preparation of plant extract. The factors such as solvent ratio, polarities, temperature, extraction time chemical and physical properties of sample decide the yield of products. Their solubility also depends on both the nature of plant sample and solvent polarity. Their composition may also vary in samples with different quantitative ratios. In addition, they may also form complexes with others. Therefore, there is no single extraction method implies to extract phenolics from all plant samples. So, there is a need of specific solvent system in which desire mixture of phenolics will dissolve completely and the extraction is made possible from given plant material. There are also some undesired components such as sugar, fats and organic acids. So, the removal of these undesired components, some additional steps should be performed. Most commonly suitable solvents include methanol, acetone, ethanol and ethyl acetate along with different ratio of water. The use of proper solvent will affect the rate and amount of phenolics. Solvent like methanol is widely used to extract low molecular weight polyphenols and for high molecular weight flavanols acetone is required. Another solvent like ethanol is also considered as good as well less hazardous solvent to extract polyphenols. The extraction of anthocyanin-

rich phenolics is mostly carried out through methanol or ethanol. The main advantage of this solvent system is to stabilizes as well as dissolves anthocyanin simultaneously after denaturing of cell membrane. However, during concentration steps, less amount of acid should be used to reduce hydrolyzation of labile, acyl, and sugar residues. The extraction of anthocyanin can be performed by using weak acids like tartaric acid, phosphoric acid, formic acid, acetic acid, citric acid, and low concentrations of strong acids, such as trifluoracetic acid and hydrochloric acid. However, other solvent like sulfured water can be used instead of organic acids to reduce the cost of extraction process. In majority cases, methanol and acetonitrile are considered as best solvents for extraction of phenolics. And RP-C18, Toyopearl, LH-20 and polyamide resin are commonly used sorbents in some conditions. After extraction, the most commonly used eluents include ethanol, methanol and acetone in column chromatography. The Sephadex LH-20 column chromatography can isolate proanthocyanin (condensed tannins) efficiently (Stalikas, 2007).

2.4 Thin-layer chromatography:

This technique has vast applicability for analysis as well as determination of phenolics from plant products since nineteenth century. It provides rapid screening of plant phenolics during investigation of bioactive product before implementing further instrumental techniques. Silica used as a stationary phase and plates are developed by using combination of 2-(diphenylboryloxy) ethylamine and polyethylene glycol or with $AlCl_3$. Soczewinski et al. used a double- development TLC method for separating nine glucosides. An eluent is used to separate highly polar flavonoids that has high solvent strength. And to separate aglycones, weak eluent is used after evaporation of first solvent. This technique does not used for quantification of phenolics. For this purpose, densitometry is used in several studies. Through high-performance TLC, the extraction of quercetin and kaempferol from Ginkgo biloba leaves can be done under the reflectance mode of 254 nm. This procedure gives 94% product. A normal-phase separation is performed to test seven binary eluents whereas in reversed- phase separation only three binary eluents were used. For this purpose, n- hexane is used as a solvent in first step and water in second. As a result, more than twelve spots were identified as flavonoid and phenolics from Flos sambuci extract. Lewis et al. isolated phenolic acids, anthocyanin and flavonoids from potato plant. They reported application of thin layers of cellulose in chromatography by using the following solvents combinations: (i) 15% (v/v) acetic acid in water; (ii) n-butanol, acetic acid, water (4:1:2); and (iii) acetic acid, HCl, water (30:3:10). A two-dimensional TLC used different solvent mixtures like n-butanol, acetic acid,

water followed by acetic acid in water for analysis of various tissue extract by developing cellulose plates. To visualize different spots on TLC, different sprays are used such as ammonia fumes and after that these plates are observed in UV light. Other than simple method, an advance method of high performance TLC densitometric quantify ellagic and gallic acids in plants. This technique is highly precise, repeatable and accurate. MaleD and Medic-Saric used optimized TLC method for flavonoids through silica gel 60 F254. The most suitable mobile phase for phenolics is ethyl acetate-formic acid-water (65:15:20, v/ v/v). The spraying reagents include 5% ethanolic polyethylene glycol 4000 and 1% methanolic diphenylboryloxyethylamine are extensively used for visualization of flavonoids. The compounds show different colors such as flavonoids appeared as orange-yellow bands for flavonoids and phenolic acids as blue fluorescent bands under UV light at k = 366 nm (de Rijke et al., 2006).

2.5. History of flavonoid for antimicrobial treatment:

In the past, formulations that contain flavonoids as a principle component were used by many physicians for the treatment of several human diseases. Such as a plant named Tagetes minuta was used for the cure of many infectious diseases that suffer mankind. That plant contained flavonoid named quercetagetin-7-arabinosyl-galactoside. From the times of ancient Greek (460–377 BC), a balm was prescribed by Hippocrates made from propolis as it has tremendous healing property and used to treat sore and ulcer. That plant showed antimicrobial property due to presence of high flavonoid contents most probably due to flavonoids like galangin and pinocembrin. Another plant derived herbal medicine Huangchin (*Scutellaria baicalensis*) has been used frequently in China from many years for the treatment of oral wounds and periodontal abscesses. A reason behind antimicrobial property of this plant lies also in the presence of a flavone baicalein.

2.6. Toxicity of flavonoids:

As edible plants and folk medicines contain vast distribution of flavonoids so it might be observed that these plants may show some toxicity. However, this diverse group of flavonoids have shown a distinct range of activities in mammals therefore it is very necessary to investigate the possible side effects or toxicity that might be caused by flavonoids during in vivo analysis. That practice would lead to synthesis of new medicines. Different compounds of flavonoids showed distinct effects on different eukaryotic enzymes, so it is necessary to

find out the toxicity of flavonoids on individual basis for the selection of flavonoids with minimal toxicity.

2.7. Antifungal property of flavonoids:

Due to inhibiting property of flavonoids for spore germination of many plant pathogens so they are also used to minimize the fungal attack that infect humans badly. For example, a type of prenylated flavanone showed an antifungal activity against a well-known fungi *Candida albicans* has been isolated from a plant *Eysenhardtia texana*. Some other examples include two new flavones; 6,7,4 -trihydroxy-3 ,5 -dimethoxyflavone and 5,5 - dihydroxy-8,2 ,4 -trimethoxyflavone showed activity against Aspergillus flavus. This fungal species is responsible for progression of disease in immunosuppressed patients. The high content of flavonoids in propolis make it suitable against dermatophytes and Candida spp. The presence of most familiar flavonol Galangin, is also responsible for growth inhibition of Aspergillus tamarii in propolis samples, as well as against other fungal species as well.

2.8. Antiviral property of flavonoids:

Flavonoids achieve great attention due to its inhibitory activity against human immunodeficiency virus (HIV). So far work done in this respect, is the inhibitory action of flavonoids on pandemic HIV1 strain and its enzymes. A flavonoid named baicalin inhibits HIV-1 infection and replication during many in vitro studies. In a study, carried out by Li and coworkers showed the inhibition of entry of HIV-1 into cells that have CD4 and chemokine co-receptors, and flavone O-glycoside effects the activity of HIV-1 reverse transcriptase. Furthermore, the inhibition of HIV-1 reverse transcriptase is also caused by the action of flavonoids like; Baicalein, robustaflavone and hinokiflavone, as well as several catechins also have this property, but they also inhibit the activity of other DNA polymerases and other HIV enzymes, therefore they are considered as non-specific in nature. Some other flavonoids that inhibit HIV- proteinase are like demethylated gardenin A and 3,2 -dihydroxyflavone. Both quercetagetin and myricetin are also non-specific. They are also involved in inhibition of viral transcription e.g. chrysin, acacetin and apigenin both follow this mechanism to prevent HIV-1 activation. Several investigations have been carried out to find out the relationship between the structure of flavonoids and its inhibitory activity against HIV-1 and its enzymes. Many flavonoids show antiviral activity against other viruses as well. In a study by Selway that showed the effectiveness of flavonoids son seven types of viruses, including herpes simplex virus (HSV), respiratory syncytial virus, poliovirus and Sindbis virus. The mechanisms of

action of this antiviral activity indicated the viral enzymes inhibition like polymerases and viral nucleic acid or may also with capsid proteins. The flavonoids named proanthocyanidins, chrysin and kaempferol show antiviral activity especially against HSV, coxsackie B virus and human coronavirus and rotavirus respectively. According to recently published reports the significant antiviral activity is also shown by galangin which is a flavonol against HSV and coxsackie B virus. From 1940s, it was discovered that flavonoids also exhibit antiviral activity but since from 25 years, many research groups struggling hard to achieve improved antiviral activity by chemical modification of distinct types of flavonoids. They achieved success by synthesis of such compound like 6, 4 -dichloroflavan. The other fact about this compound is its inability to show antiviral property in clinical trials. The phenomenon of synergism is also demonstrated in various flavonoids. Both kaempferol and luteolin show synergism against HSV. This phenomenon also exists between flavonoids and other antiviral agents. The enhancement of antiviral activity of acyclovir is carried out by Apigenin against these viruses.

2. 9. Antibacterial property of flavonoids:

2.9.1. Reports of flavonoids possessing antibacterial activity:

The documentation on flavonoids about antibacterial activity is increasing day by day. The screening of many crude extract from plant (that were historically used in folk medicines) is done by many researchers. Many flavonoid rich plant extracts from species of Hypericum, Capsella and Chromolaena were reported to have antibacterial activity. In many other flavonoids rich phytochemical preparations exhibits antibacterial activity. Propolis and other samples rich in flavonoids proved to have antibacterial activity. The further step is taken by many researchers about isolation and identification of exact structure of flavonoids that have antibacterial property. They are also interested in quantification of commercially available flavonoids. Those flavonoids are apigenin, galangin, pinocembrin, ponciretin, genkwanin, sophoraflavanone G and its derivatives, naringin and naringenin, epigallocatechin gallate and its derivatives, luteolin and luteolin 7- glucoside, quercetin, 3-O-methylquercetin and various quercetin glycosides and kaempferol and its derivatives. The identification of flavonoids for having antibacterial activity are also flavone, flavone glycosides, isoflavones, flavanones, isoflavanones, isoflavans, flavonols, flavonol glycosides and chalcones. The synergism between flavonoids and other antibacterial agents can also be seen against many resistant strains of bacteria, such as; epicatechin gallate and sophoraflavanone G. Further modifications are performed on natural flavones for analysis of antibacterial activity. For example, Wang and colleagues had presented that antibacterial activity can be improved after synthesis of

complexes like 5-hydroxy7,4 -dimethoxyflavone with several transition metals. The other group reported same increased antibacterial phenomena after addition of bromine or chlorine substituent in B ring of 3-methyleneflavanones. The in vivo use of flavonoids is described by many researchers. A study done by two scientists Vijaya and Ananthan was about protection of guinea pigs from induced Shigella infection after oral administration of quercetin. Recently, Dastidar and their colleagues reported that intraperitoneal injection of sophoraisoflavone A or 6,8- diprenylgenistein to mice gave significant protection from infection caused by ~9.5 × 108 colony-forming units (CFUs) of *Salmonella typhimurium*.

2.9.2. Discrepancies between reports of related to antibacterial activity of flavonoids:

The results about antibacterial activity of many flavonoids are conflicting when different reports are studied from past few years. For example, a published report showed that apigenin has antibacterial activity against S. aureus when its concentration is taken equal or greater than 128g/mL. Another report showed the inhibiting effect of flavone on 15 strains of MRSA and 5 sensitive strains of *S. aureus* at concentrations between 3.9 g/mLand 15.6g/mL. This is because of application of different assays being used on that specific occasion. These are agar dilution technique, the paper disk diffusion assay, the hole-plate diffusion method, the cylinder diffusion method, the broth macrodilution as well as microdilution technique. The assays that implemented diffusion technique might not be provided a significant antibacterial activity due to low rate of diffusion of flavonoids. In other studies, the results shown by groups in same assays are conflicting. The variations in the assays might be a reason of these conflicting results. Such as in agar dilution method, varied sizes of bacterial inoculum are used by diverse groups. In a susceptibility test, the inoculum size is considered as an important variable by a report of National Committee for Clinical Laboratory Standards (NCCLS). There are also some other factors than inoculum size that are cause of conflicting results in many assays. These factors are incubation period, volume or type of broth or agar, size of wells or paper disks, and strains of a bacterial species. Following methods were published in recent past few years; standard agar dilution, broth macrodilution and microdilution methods. So, it could be possible to minimize the conflicting results related to antibacterial flavonoids. But still there is a need of additional steps like use of solvents for dissolution of flavonoids. There is also a possibility of precipitates formation when flavonoids are dissolved in organic solvents as well as precipitation will form when some flavonoids are diluted in neutral polar solvents or organic solvents. Precipitation causes minimum contact with bacterial cells so leads to false negative reports of antibacterial activity. Moreover, the precipitations formed may also be falsely

interpreted as a bacterial growth resulted in false negative results. Another matter is the change in the structure of galangin when dissolved in alkaline solvents. Flavonoids salts may be formed during a reaction increase/ decrease potency with respect to parent structure resulted in false positive/negative reports of antibacterial activity. Other notable variables include sources which are either natural or commercial.

2.9.3. Structure–activity relationship for antibacterial activity of flavonoids:

Flavonoids perform varieties of functions in eukaryotic system. Different compounds of this class target different components of bacterial cells but the mechanism underlying the antibacterial activity of flavonoids is not well understood. It is found through investigations that there is a presence of common structural features among distinct groups of bioactive flavonoids. However, there is also a possibility of having a multiple target of flavonoids in bacteria rather than one. This structurally common features of flavonoids are might be a cause of gaining proximity into bacterial cell. Tsuchiya and colleagues presented that relation of any kind of activity is due to its specific structure. They performed isolation of different substituted compounds as well as they determine MIC values against MRSA. They concluded from their studies about the cause of anti MARSA activity of flavanone was due to 2 ,4 - or 2 ,6 - dihydroxylation of the B ring and 5,7-dihydroxylation of the A ring in the flavanone structure. The long chain aliphatic group substitution such as lavandulyl (5-methyl-2- isopropenyl-hex-4-enyl) or geranyl (trans-3,7-dimethyl-2,6- octadienyl) at 6 or 8 positions also enhanced this activity. There is another report presented by Stapleton and colleagues, shows that flavan3- ol has antistaphylococcal activity due to substitution with C8 and C10 chains. Osawa et al. investigated the activity of many structurally distinct groups of flavonoids like flavones, flavanones, isoflavones and isoflavanones based on the paper disk agar diffusion assay. They also found that the inhibition of *Streptococcus mutans* and *Streptococcus sobrinus* occurred due to structures like 5-hydroxyflavanones and 5-hydroxyisoflavanones along with additional hydroxyl groups at the 7, 2 and 4 positions. These findings resulted in very similar outcomes with those of Tsuchiya and colleagues. The conclusion made by Osawa and colleagues was that the presence of hydroxyl groups at 7 and 4 positions of 5-hydroxyflavones and 5-hydroxyisoflavones did not inhibit the bacterial growth. Whereas, during the investigations of Sato et al., it had been noticed that isoflavones show inhibitory action against a wide range of streptococcal species due to the presence of hydroxyl groups at the 5, 2 and 4 positions in agar dilution assay. So, it may have suggested that hydroxylation at position 2 is important for this activity. It was also thought that the negative results obtained from findings by Osawa et al.

was because of poor diffusion of flavones and isoflavones in the medium. The earlier findings of Tsuchiya et al. presented the significance of a hydroxyl group at position 5 of flavanones and flavones against MRSA. Further studies also stated that more effective agents against MRSA are chalcones as compared to flavanones or flavones, and that hydroxyl groups at the 2 positions of these compounds are important for the antistaphylococcal activity. The decrease of antibacterial activity is due to the presence of methoxy groups. Sato and colleagues concluded about the presence of -OH group in 2,4,2 -trihydroxy-5 -methyl chalcone and 2,4,2 – trihydroxy chalcone at 2 positions is a contributing factor for inhibition of 15 strains of carcinogenic streptococci. The synthesis of different halogenated derivatives of 3- methylene flavanone was also done by Ward and colleagues. The substitution of chloro groups at C-3 and C-4 whereas bromo group at C-4 in B ring increases the activity of parent compound against *S. aureus*, and four folds more activity against Enterococcus faecalis. Also, the activity against *S. aureus* become four -to eight-fold by 2 ,4 -dichloro derivative and a two to four-fold improvement against *E. faecalis*. Whereas the activity is lessened in 3- methylene-6- bromo flavanone due to halogenation of A- ring. However, it is necessary to add substitutive groups at A ring of chalcones before antibacterial testing whereas both fluorination or chlorination at C-4 position do not affect antibacterial property to those compounds. That's why again it is important to prepare analogues before the substitution of halogen groups to assess antibacterial property.

2.9.4. Nature of flavonoid activity: bacteriostatic or bactericidal?

Many researchers performed majority of experiments to determine whether the activity of flavonoids is bacteriocidal or bacteriostatic. In different experiments, it had been found that flavonoids such as epigallocatechin gallate, galangin and 3-O-octanoyl- (+)-catechin decrease more than 1000 folds inviable counts of MRSA-YK, *S. aureus* NCTC 6571 and EMRSA-16, respectively. So, this shows the bacteriocidal nature of flavonoids. In another case, it has been found that 3-O-octanoyl- (−)-epicatechin causes pseudo multicellular aggregates both in antibiotic-sensitive and antibiotic-resistant strains of *S. aureus*. If this phenomenon is shown by other groups of flavonoids, so that will cause conflict with previous concept of time killing studies. So, it might be concluded as flavonoids kill bacterial cell but cause aggregates hence lead to reduce CFUs in viable counts.

2.10. Mechanisms underlying antibacterial action of various flavonoids:

2.10.1. Inhibition of nucleic acid synthesis:

The studies of Mori and colleagues showed that flavonoids cause inhibition of DNA synthesis in *Proteus vulgaris* whereas RNA synthesis in *S. aureus* respectively. Flavonoids like robinetin, myricetin and (−)-epigallocatechin also performed this type of activity. They showed less inhibitory effect on synthesis of protein and lipid. The inhibitory action of flavonoids is due to the involvement of B ring that effect hydrogen bonding of nucleic acids bases. The screening of 14 flavonoids having various structure for inhibitory action is performed by Ohemeng et against DNA gyrase in *Escherichia coli.* So total seven out of fourteen compounds; quercetin, apigenin and 3,6,7,3 ,4 - pentahydroxy flavone. It was found that 7,8-dihydroxyflavone cause inhibition of *E. coli* DNA gyrase due to involvement of B-ring hydroxylation. So, the cause of inhibitory action of flavonoids is to affect the activity of DNA gyrase in bacterial cell. There may be other mechanisms as compared to enzymes inhibition for antibacterial activity. Another investigation carried out by Plaper and colleagues showed that quercetin bind with GyrB subunit of E. coli leads to inhibition of enzyme's ATPase. The binding of enzymes was explained by measurement of fluorescence labelled quercetin. These findings demonstrated about the antibacterial activity of quercetin against *E. coli* is might be due to inhibition of DNA gyrase hence supported the earlier conclusion of Ohemeng et al. It had been found that glycosylated flavonol rutin was very effective for type II topoisomerase inhibition. The antibacterial activity of this compound also showed effective results on permeable *E. coli strain*. With the help of enzyme assays and SOS chromotest, it was proved that rutin promotes *E. coli* topoisomerase IV-dependent DNA cleavage, and induced the SOS response of the *E. coli* strain. A flavonol galangin has shown increased susceptibility to 4-quinolone-resistant *S. aureus* strain as compared to other 4-quinolonesensitive and -resistant strains. The mechanism of action of galangin is due to the involvement of topoisomerase IV and homologous gyrase enzyme. For further verified results, it is important to work on mutant strains and purified enzymes.

2.10.2. Inhibition of cytoplasmic membrane function:

The intensive antibacterial activity of sophoraflavanone G was shown against MRSA and *Streptococci*. The liposomal model membranes were made to find out about effect of sophoraflavanone G on membrane fluidity and then make its comparison with naringenin that lack 8-lavandulyl and 2 -hydroxyl groups. There was an increase in fluorescence polarization

of liposomes by sophoraflavanone G by taking concentrations nearly equal to their MIC values. The increase level of liposomal polarization is due to the change in membrane fluidity, that further suggested the reducing fluid consistency in outer or inner membranes by sophoraflavanone G. Another study carried out by Naringenin on flavonoids to evaluate membrane effect at high concentrations. So, the antibacterial activity of sophoraflavanone G because of reducing membrane fluidity of bacterial cells is supported by the correlation phenomena between cell membrane fluidity and antibacterial activity. Ikigai and colleagues, took a strong antibacterial catechin; (−)-epigallocatechin gallate found in green tea. The greater tendency towards inhibition of bacterial growth is found more in catechins and effected against Gram-positive than Gram-negative bacteria. In a study, epigallocatechin causes leakage of cell material from the intraliposomal space of liposomes which act as a model bacterial membrane. This liposomal treated compound also showed aggregation. So, the conclusion was made that catechins causes damage to bacterial membranes. The cause of damaged bacterial membrane was not found. But for this purpose, two theories were made. First is, entrance of catechins directly into the lipid bilayer and alter the barrier functions of membrane. Secondly, they may also cause fusion of bacterial membranes leads to leakage of materials of cell and formation of aggregates. The flavonoids epigallocatechin gallate induce leakage of cell contents but when these cells are grown in medium having more negatively charged lipids, this activity was reduced greatly. So, this will support the fact of least susceptibility of catechin towards Gram-negative bacteria as it has lipopolysaccharide membrane. The antibacterial activity is increased by selected flavan-3-ols with substitution at C8 and C10 chains, this was previously demonstrated by Stapleton and colleagues. This group further demonstrated the MRSA clinically isolated cells showed affinity with fluorescent stain propidium iodide when treated with (−)-epicatechin gallate and 3-O-octanoyl- (+)-catechin, respectively. When *S. aureus* cells were grown in the presence of either (−)-epicatechin gallate or 3- O-octanoyl- (−)-epicatechin that leads to the formation of pseudo multicellular aggregates. The work of this group also supported the Ikigai's argument about cause of bacterial damage is due to catechins. Sato and colleagues interpreted the action of chalcone 2,4,2 -trihydroxy-5 -methyl chalcone on causing leakage of 260 nm absorbing substances from *S. mutans*. This shows the antibacterial activity of 2,4,2 -trihydroxy-5 -methyl chalcone by effecting the cell membrane permeability and causes leakage of intracellular material of bacterial cell such as nucleotides. Furthermore, another flavonoid galangin causes leakage of potassium ion causes damage to cytoplasmic integrity in *S. aureus*. When 50g/mL of flavonoids were treated with *S. aureus* and incubated for 12 h, this resulted in 60-fold decrease

in the number of CFUs and 20% more decrease in potassium ion than untreated control bacteria. So, this clearly proved the effect of galangin upon cytoplasmic damage to bacterial cell as well as leakage of potassium ion from these cells. This conclusion further initiated the investigations on the action of galangin may be directly on membrane or may indirectly induce autolysis or osmotic lysis. In an investigation made by Mirzoeva and colleagues that the antimicrobial action of propolis was due to its main constituent flavonoids, quercetin, that causes increase permeability across inner bacterial membrane and disruption in membrane potential. A proper electrochemical gradient of protons across membrane is necessary to maintain concentration of ATP, membrane transport and motility in bacterial cell**.** There is a conclusion by Mirzoeva et al. that the antibacterial property of propolis was due to its effect on membrane permeability as well as membrane potential. A conclusion was also suggested that like tetracycline and ampicillin, propolis and antibiotics also show synergism. It was also seen that disruption of proton motive force across bacterial cell is due to the inhibitory action of quercetin and naringenin that leads to bacterial cells immobility. The mobilization of bacterial cells is thought to be a crucial factor on increasing its virulence as this act guide bacteria to adhere an invade at target sites. Mirzoeva et al. also proposed the cause of inhibition of bacterial pathogenesis and infections was due to antimotility action of propolis components. Mirzoeva and co-workers detected the cause of cytoplasmic membrane activity of quercetin and was due to additional mechanisms as found in other flavonoid compounds that have DNA gyrase- inhibiting activity.

2.10.3. Inhibition of energy metabolism:

Haraguchi and colleagues studied the cause of antibacterial activity of licochalcone A and C (i.e. two retro chalcones) obtained from *Glycyrrhiza inflata* roots. Both compounds showed inhibitory activity against *S. aureus* and *Micrococcus luteus* but not against *E. coli*. A hypothesis made by this research group was about licochalcone that interfere with energy metabolism system as comparable to respiratory-inhibiting antibiotics. As there is a need of energy for active movement and synthesis of metabolites and macromolecules. The antibacterial activity of licochalcone were found greater in *M. luteus* and *S. aureus* except *E. coli* as it targets oxygen consumption of cells. The group also concluded that licochalcone do not inhibit cytochrome c oxidase or NADH-CoQ reductase, it only inhibits NADH-cytochrome c reductase. Therefore, a suggestion was made that these retro chalcones show inhibition sites between CoQ and cytochrome c. It was recently reported by Merck Research Laboratories about the inhibition of macromolecular synthesis in *Bacillus megaterium* was due

to the action of flavanone lonchocarpol A. Several reports indicated that the inhibition of several cell components like RNA, DNA, cell wall and proteins are all inhibited by radioactive precursors of flavonoids compounds at concentrations around their MIC values. All these findings suggested the antibacterial activity flavonoid as they interferes with energy metabolism (Cushnie & Lamb, 2005b).

Chapter 3: METHODOLOGY

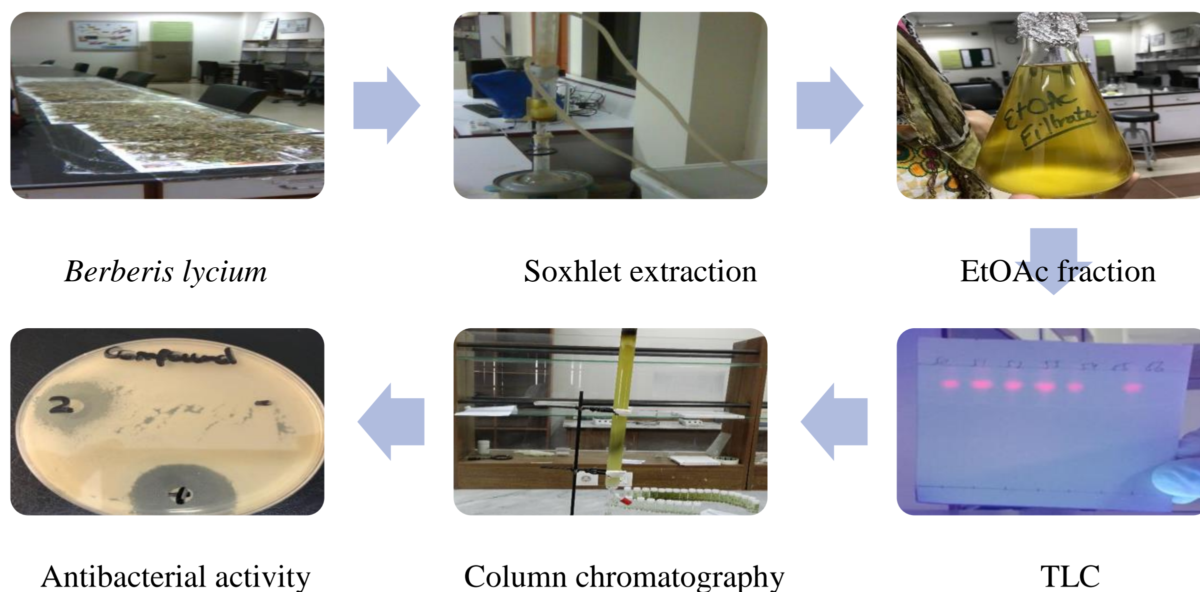


Figure 3 Schematic presentation for isolation and antibacterial activity of flavonoids from B. lycium leaves

3.1. Sample collection:

Plants were collected during the month of July from Ayubia located in Murree, Pakistan. Leaves of plants were separated and thoroughly washed with water for the removal of dirt and dust. After that, they were placed on any absorbent paper for maximum three weeks in a room for shade drying. When the leaves were properly dried, they are finely grinded and forming a proper dust form powder. Weigh and store that powder in air tight sealed bag at room temperature.

3.2. Extraction:

400g of leaves powder is extracted in 80%methanol by using Soxhlet apparatus and then filtered. Then, this methanolic extract was placed in rotary evaporated for the removal of solvent. By following the proposed Subramanian and Nagarajan method (1969), the resultant filtrate was further extracted by using three solvents of varying degree polarity such as petroleum ether, diethyl ether and ethyl acetate stepwise. The above petroleum- ether layer is removed as it contains fatty acids. Free flavonoids are extracted in diethyl layer whereas ethyl acetate layer has conjugated flavonoids. Ethyl acetate fraction was hydrolyzed further with 7% H2So4 for 24 hrs. and then re-extracted with ethyl acetate. After that, washing of fraction with water was performed to neutralize its pH and then weighed. Following is the schematic diagram depicting the further steps that were performed.

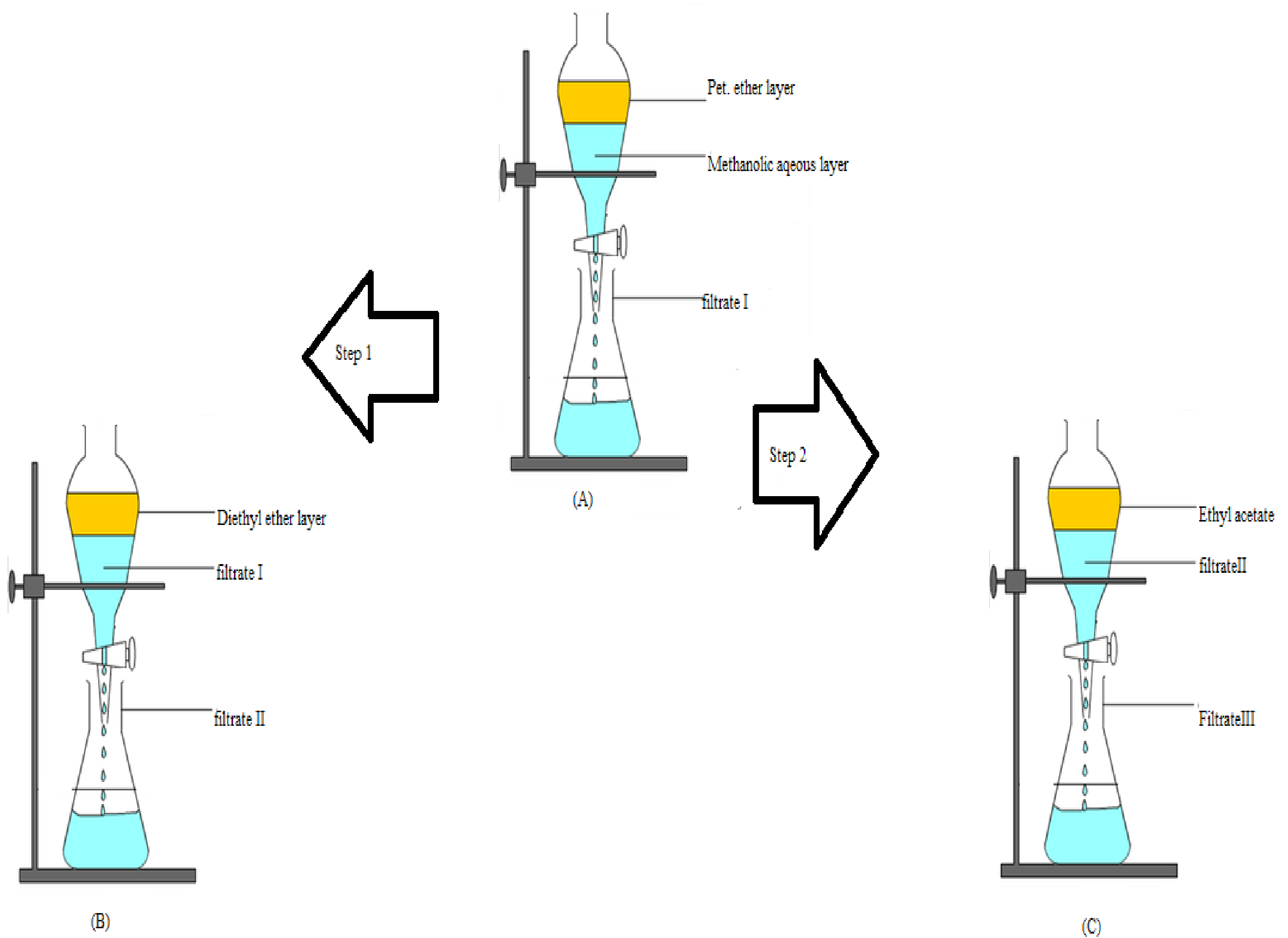


Figure 4 Pictorial representation of solvent- solvent extraction. A) Fatty substances removed by pet. Ether. B) free flavonoids in DEE. C) Bond flavonoids in EtOAc used as sample.

3.3. Colorimetric screening

Flavonoids screening was performed using Shinoda's Test optimized by Mir et al., 2013 and Inalegwu B, Sodipo O, 2013.

3.4. Thin layer chromatography

Silica coated aluminum plates were spotted with ethyl acetate fraction by the help of spotter. After that they were dried for few minutes and placed in chromatographic chamber. When mobile phase was properly run over the plates, they were taken out from the chamber, again

dried and visualized under UV light of 302nm and 364nm respectively. First, we checked the polarity of our sample by taking non- polar solvent; chloroform. The spot of our sample was still on base line after running of mobile phase over it that clearly showed, highly polar nature of our sample. As flavonoids are highly polar compounds so we selected ethyl acetate: formic acid: acetic acid: methanol (80: 10: 10: 20) as mobile phase, that resulted in poor separation. After that we tried another mobile phase ethyl acetate: formic acid: acetic acid: water (100: 11: 11: 26) but again the same result was obtained. We than also tried previous mobile phase by increasing ratio of water by 2, 10, 15 and 20 resulted in little separation. We than totally changed that mobile phase by taking 5% methanol in ethyl acetate, then tried 10%, 15% and 20 % methanol in ethyl acetate, and we get good separation in 15% methanol in ethyl acetate. The mobile phase used for best separation was 15% methanol in ethyl acetate.

3.5. Column Chromatography:

3.5.1. Column packing: 3ft column having 2.5inch diameter along with filtration disc was used. The amount of silica that filled two- third of column was mixed in solvent system that was selected by TLC. That slurry was shifted to column and excess solvent was removed by column tap. Allow that slurry to properly settle down than add properly washed sand on the top making 3cm thick layer.

3.5.2. Sample dilution: 1.5g of sample was diluted in 15ml of solvent system.

3.5.3. Sample loading: Diluted sample was carefully loaded on the sand layer. At that time solvent was in silica layer not in sand layer.

3.5.4. Column running: After sample loading, solvent was filled up to the top of column. Tap was opened and drop wise collection of eluent was made. Three bands were formed on column representing separated fractions.

3.6. Fourier Transform Infrared (FTIR) spectroscopy analysis of flavonoids

The FTIR analysis was done by using an Agilent FTIR. 1mg flavonoids by the help of capillary tube was picked and 300mg of spectral grade potassium bromide (KBr) were compressed together in a dye to form a transparent disc. The FTIR spectrum was set at 4000-500 cm^{-1} using a Perkin Elmer FT-IR spectrometer. The internal element was a ZnSe ATR plate (50 × 20 × 2 mm) with an angle of aperture set at 45°. The sample was scanned 32 times and a spectral graph was generated using EFTIR software.

3.7. Bacterial strains and growth media

Gram positive bacterial strains including Enterococcus faecalis (Clinically isolated), Bacillus cereus (Soil isolated), Staphylococcus aureus (ATCC 6538), whereas gram negative strains including Pseudomonas aeruginosa (ATCC 9027), Salmonella Typhi (ATCC 6539) and Escherichia coli (ATCC 8739) were used. The growth media used for all bacterial strains was Nutrient Agar and Luria Bertani medium (L.B).

3.7.1. In vitro Antibacterial Activity

3.7.1.1. Bacterial cultures

The bacterial pre-cultures were prepared by taking 50µl bacterial glycerol stock solution of 15 mL Luria Bertani broth in culture tube at 30–37°C for 24 h. The Optical density (OD) at 600 nm was also measured which was 0.8–1.0 (equivalent to 108 CFU), now used for antimicrobial testing.

3.7.1.2. Antibacterial Evaluation of flavonoids Using Quantitative Method

The antibacterial activity of free and bound flavonoids was determined according to disc diffusion method. In this method, discs or holes are made as reservoirs containing the solutions of testable substance (Rauha et al., 2000).It is a qualitative method, in order to evaluate the antibacterial activity of antimicrobial material. For this study three gram negative and three gram-positive bacterial species were selected. The selected five aliquots in DMSO (Dimethyl sulfoxide) were prepared. Chloramphenicol of concentration 1 mg/mL and DMSO, both were used as positive and negative controls, respectively. About 20mL nutrient agar was poured to sterilized petri plates. Then, 50µl inoculum from culture tube was taken to petri plate containing solidified agar than spread bacterial culture through Pasteur pipette evenly. Place autoclaved discs of 7mm on this petri plate, load 10µl sample carefully. Positive and negative control was also taken. Allow them for drying, then put them inverted in incubator for 24hours and zone of inhibition was measured after 24 hours at 37°C.

Chapter 4: RESULTS

4.1 Thin layer chromatography

Different spots were obtained on chromatogram after exposure in UV light at λ = 366nm. These spots were shown as one greenish- blue little mixed with next light pink spot at lower side and two dark pinkish fluorescent spots at top and at baseline. depicting separation of different flavonoids through mobile phase (figure.5.). So, they were total five in number. Flavonoids were confirmed by its fluorescence after appearance of orange- yellow four fluorescent bands and one blue fluorescent band on chromatogram (Figure 6). It showed the presence of flavonoids and phenolic acids. The dried TLC was then sprayed with 5% ethanoic $FeCl_3$, bands appeared in bluish grey color. We tried eleven mobile phases but out of these, 15% methanol in ethyl acetate was selected as the most suitable mobile phase because it had the largest discriminating power. Flavonoids were further confirmed by infrared absorption spectrum obtained by FTIR analysis.

Figure 5 Chromatogram of flavonoids on TLC using 15% methanol in EtOAc

After selection of most suitable mobile phase, column chromatography was performed to collect different fractions containing separated flavonoids.

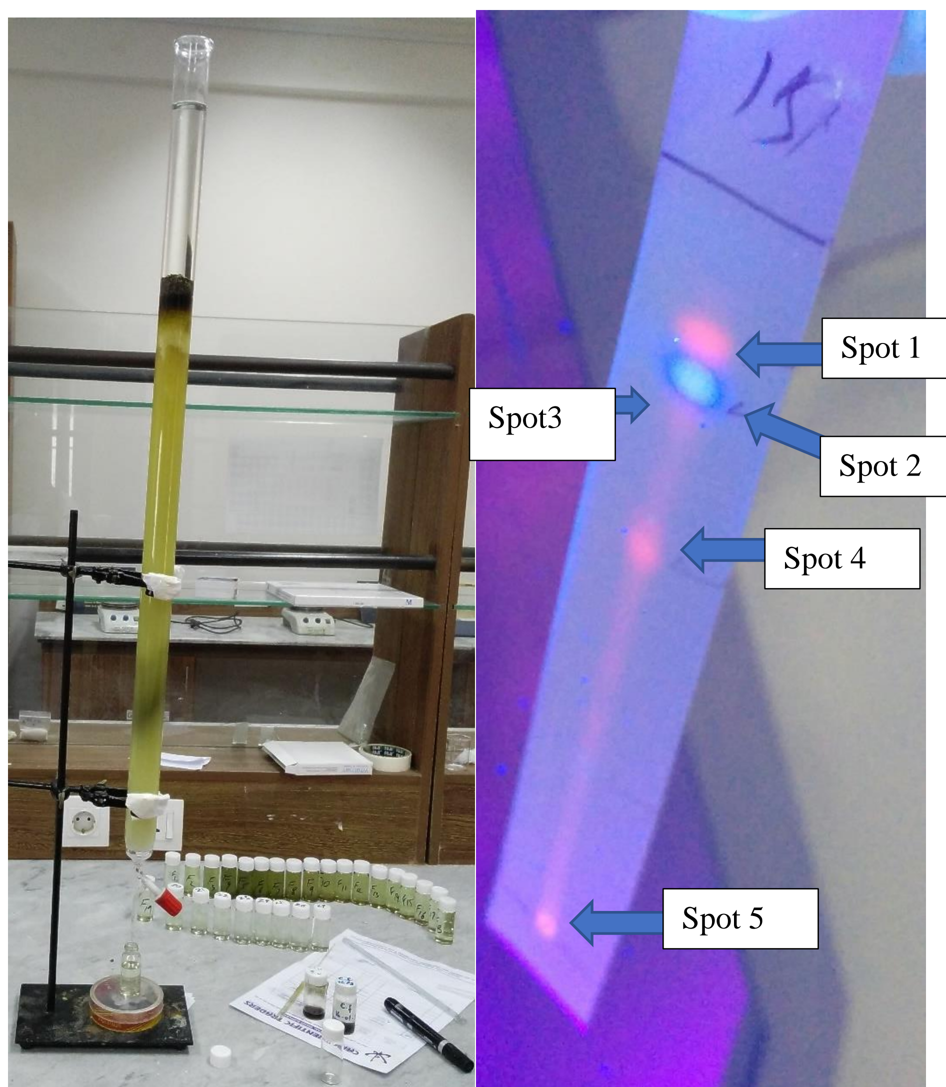


Figure 6 Column Chromatography and TLC indicating different spots

Initially, three rings were appeared in column, at the top and the other two with some minor gap, so both were collected first as their spots were at the top of TLC. The color of whole column was appeared as green in color that depicts the affinity of polar compounds in sample with silica. The highly polar compounds in sample resided at the top of column as selected mobile phase separated whole sample into these components in the form of layers. The total of two seventy-six fractions were collected. These fractions showed different spots on TLC plate having variable R_f value. (shown in fig 6 to 11). All fractions were combined based on similar R_f values. Firstly, five fractions were made F1 to F5 as their TLCs showed single spot. F1 was made by combining fractions 50 to 56, rest F2, F3, F4, F5 were made by combining of fractions 57 to 63, 71 to 76, 77 to 82, 83 to 88 respectively. As all these fractions showed

single spots, so they were subjected to antibacterial test to further confirmation that they are bioactive in nature. After confirmation of antibacterial activity, next step characterization was performed to detect the type of compounds in all fractions. So, for this purpose FTIR was performed that showed almost similar functional groups present in all these five fractions. So, first compound was detected that was depicting as spot 4 in TLC (fig. 5) and proven after finding TLC trend. To find out other four spots, fractions 2f2, 2f4, 2f5 and fb were made by combining fractions 3 to 7, 23 to 28, 29 to 35 and 216 to 230. Some fractions didn't show any spots hence depicting absence of any flavonoids just mobile phase was in these fractions. So, they all were discarded. The standard quercetin was used to compare its FTIR with all isolated fractions for structure elucidation. Some functional groups in all five isolates are common in quercetin as indicated in table 1 and table 4. That shows the structural components are nearly as similar in isolates as in standard quercetin.

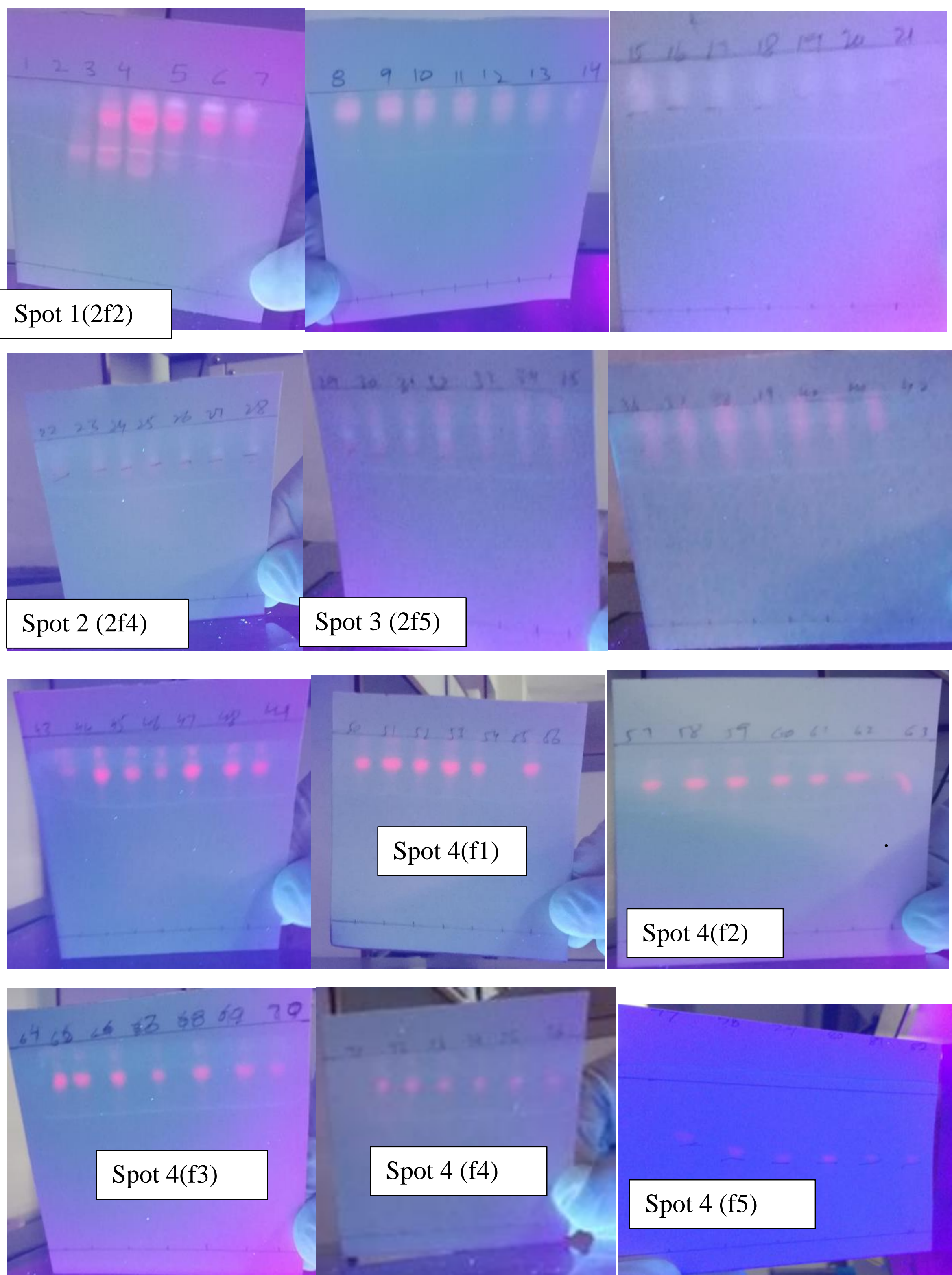


Figure 7 Chromatogram of fractions 1 to 82 on TLC using 15% methanol in EtOAc

Figure 8 Chromatogram of fractions 83 to 141on TLC using 15% methanol in EtOAc

Figure 9 Chromatogram of fractions 142 to 208 on TLC using 15% methanol in EtOAc

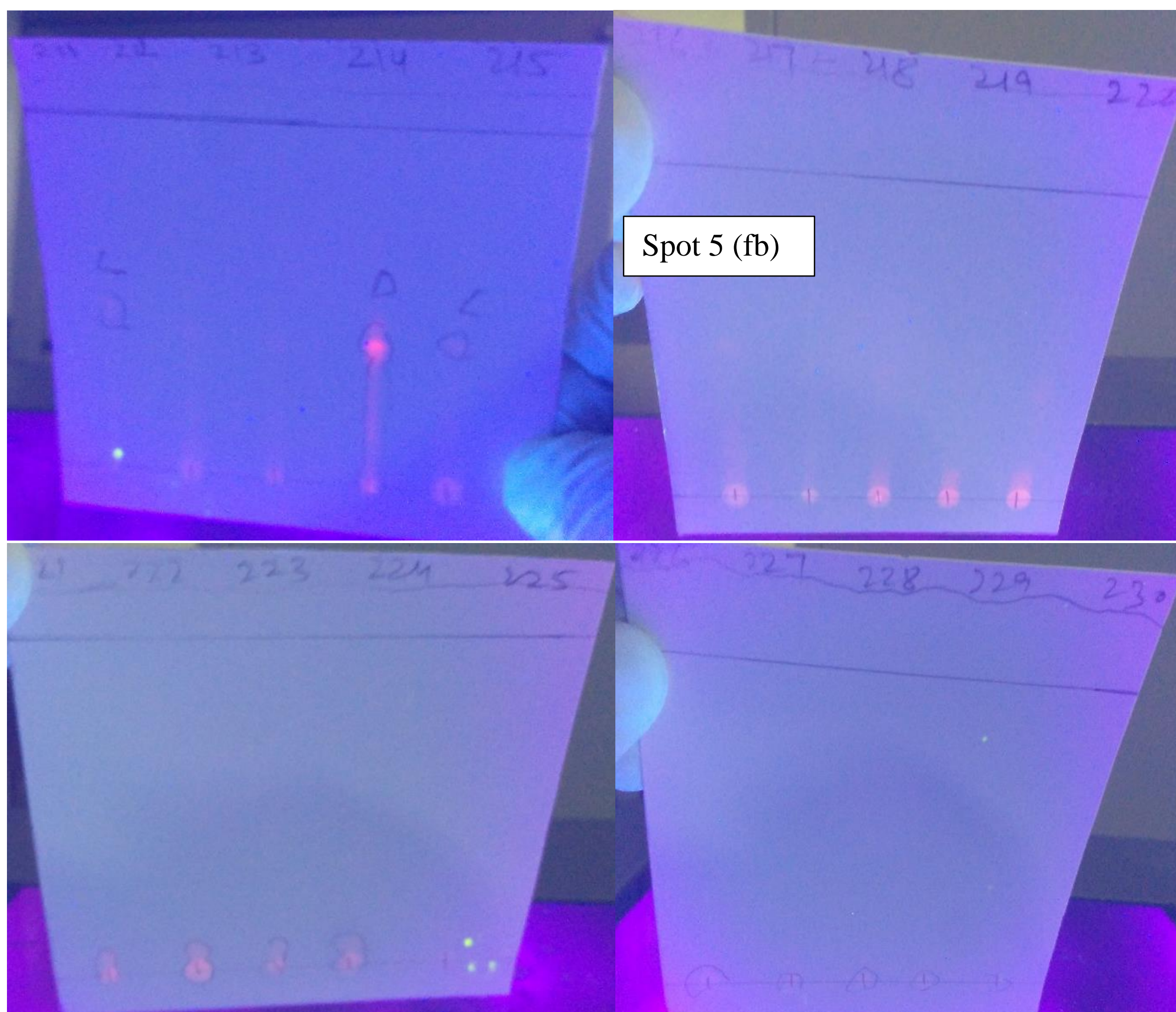


Figure 10 Chromatogram of fractions 211 to 230 on TLC using 15% methanol in EtOAc

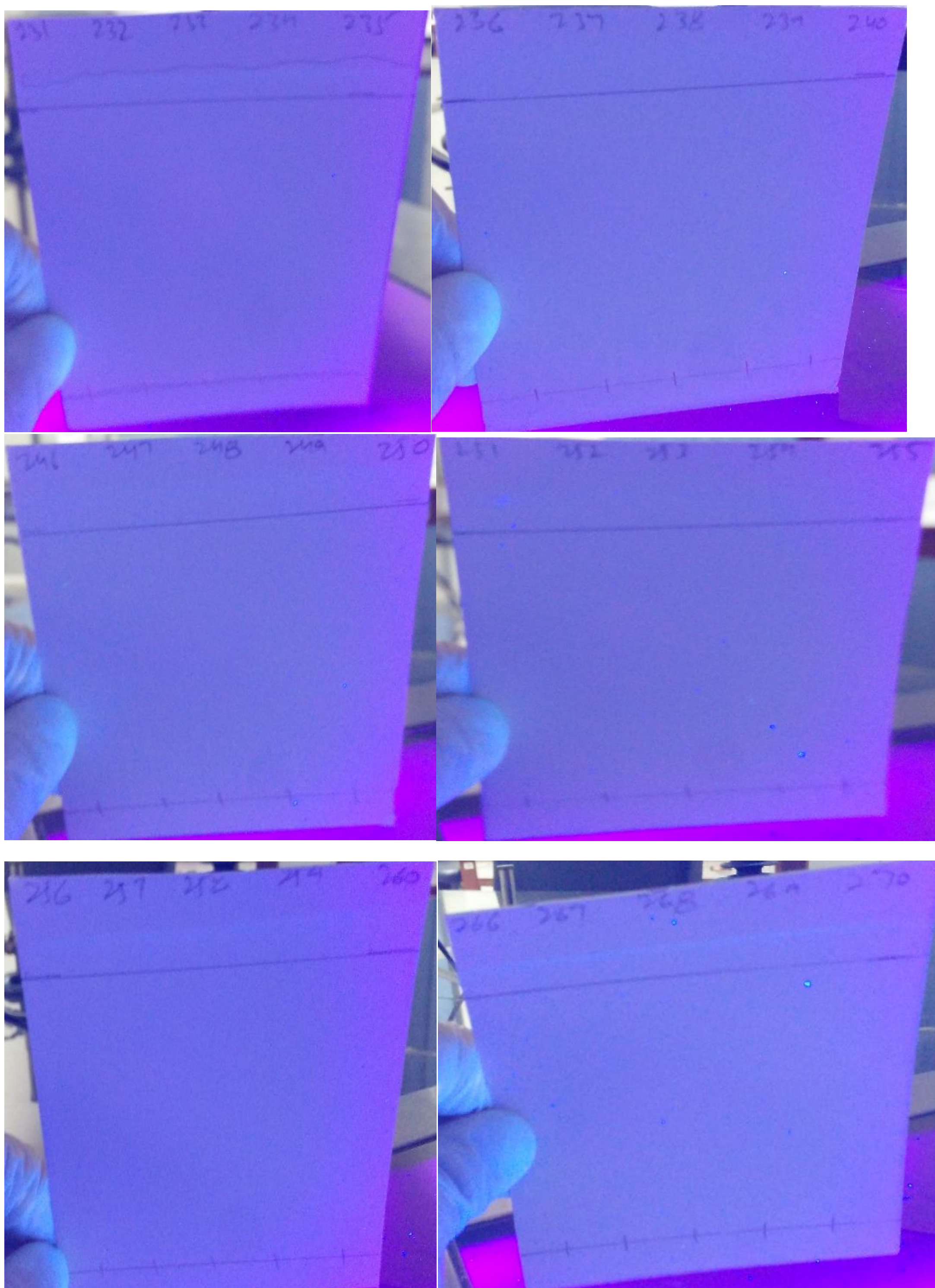
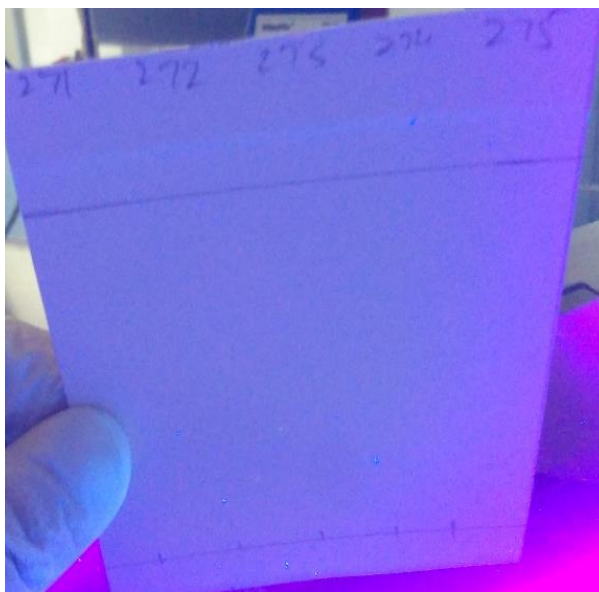


Figure 11 Chromatogram of flavonoids 231 to 270 on TLC using 15% methanol in EtOAc



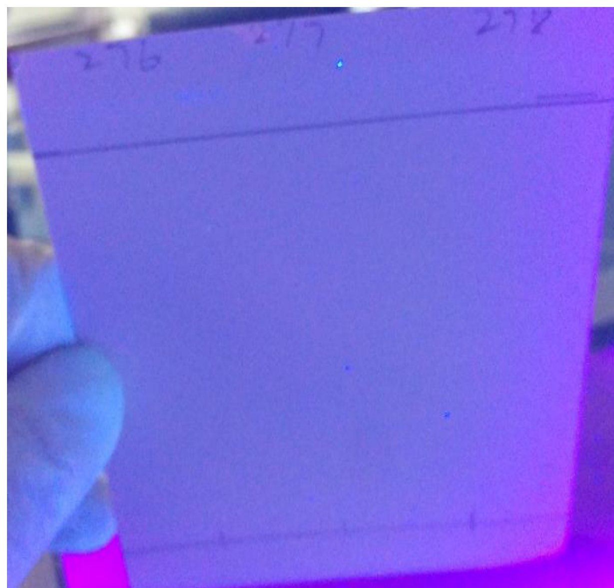


Figure 12 Chromatogram of fractions 271 to 276 on TLC using 15% methanol in EtOAc

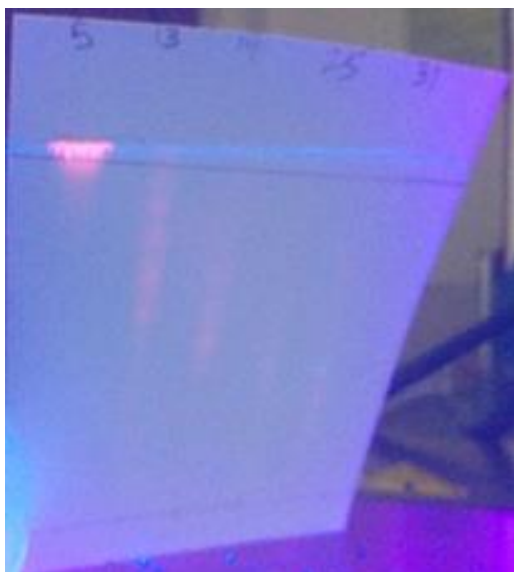


Figure 13 TLC trend of first ten TLC plate by selecting single fraction from each TLC plate.

4.2 Colorimetric screening

Flavonoids were identified by shodina test that was addition of few drops of 1M NaOH into ethyl acetate fraction, that resulted in color change of extract into intense yellow color. The intense yellow color was changed into colorless solution by addition of 80% dilute H_2SO_4, which confirmed the presence of flavonoid (Figure 6).

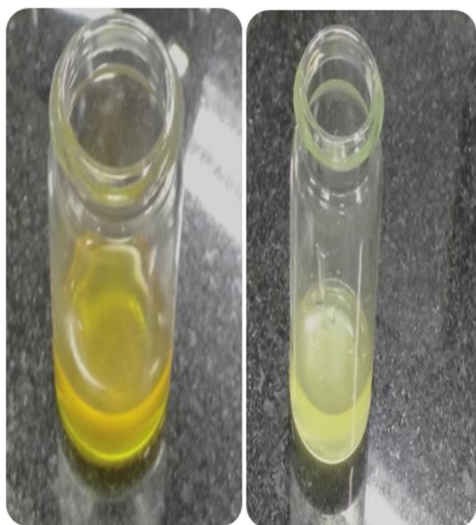


Figure 14 Phytochemical test for flavonoids

4.3 Characterization of flavonoids through FTIR spectroscopy

The analysis of five aliquots containing flavonoids were performed by using FTIR, scale was set in the region of 250 – 3500cm^{-1}. Among them, the absorbance bands showed by first aliquots (F1) at 3413.53 cm^{-1} were assigned for the stretching vibration of – O– H, 2870.90 cm^{-1} (two bands) for C –H stretching vibrations, 1510.80 for – N-H bend, 1248.21 for -C-O stretch- (in aromatic ring), 1097.59 cm^{-1} for -C-O-C- stretch, and 950.30cm^{-1}assigned for =C-H bending vibrations (fig.7). The second aliquot (F2) showed two additional absorbance bands at 829.88 for bending of C-H bond and at 1456.17 cm^{-1} for stretching vibration of C=C (in

aromatic ring), the absorbance band at 3413.53 was not shown in this spectrum (fig. 8). The FTIR spectrum showed almost similar wave form so they all are considered as same spot. The combine spectrum of all five spots (2f2, 2f4, 2f5, f2, fb) are shown in fig. As they all have different functional groups so they might be containing different compounds in all five fractions.

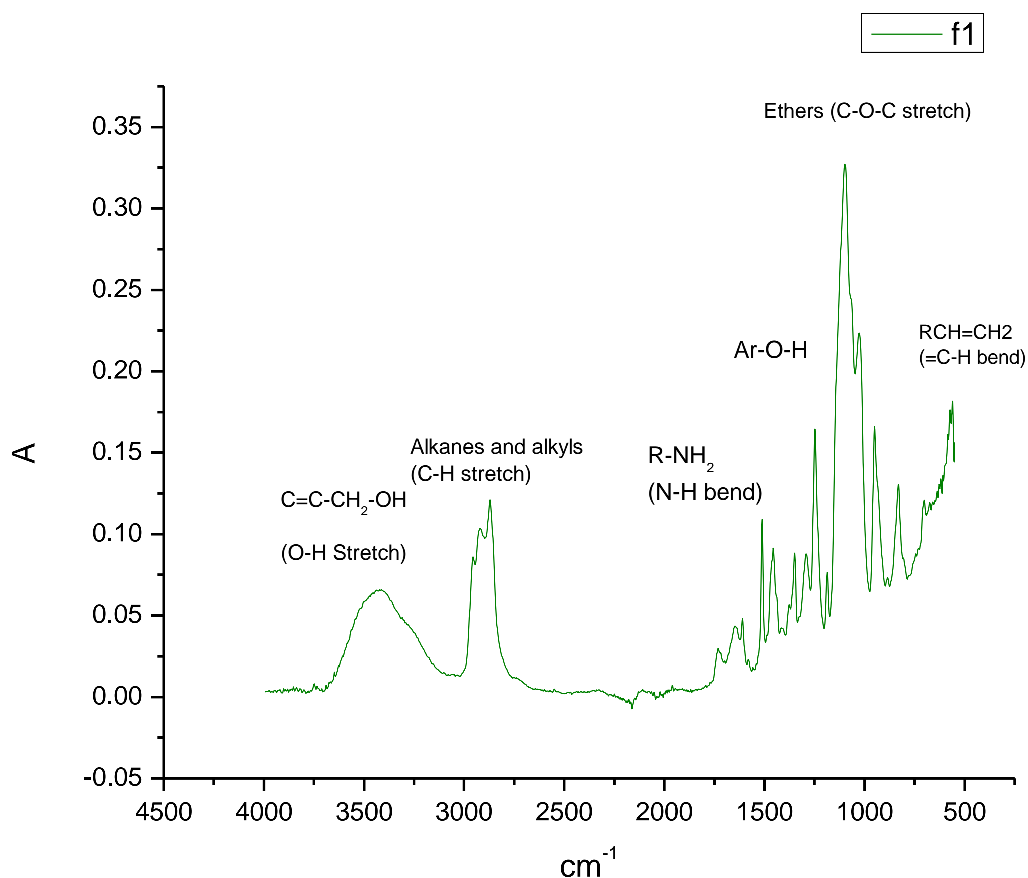


Figure 15 FTIR spectrum of aliquot 1 (fraction 1)

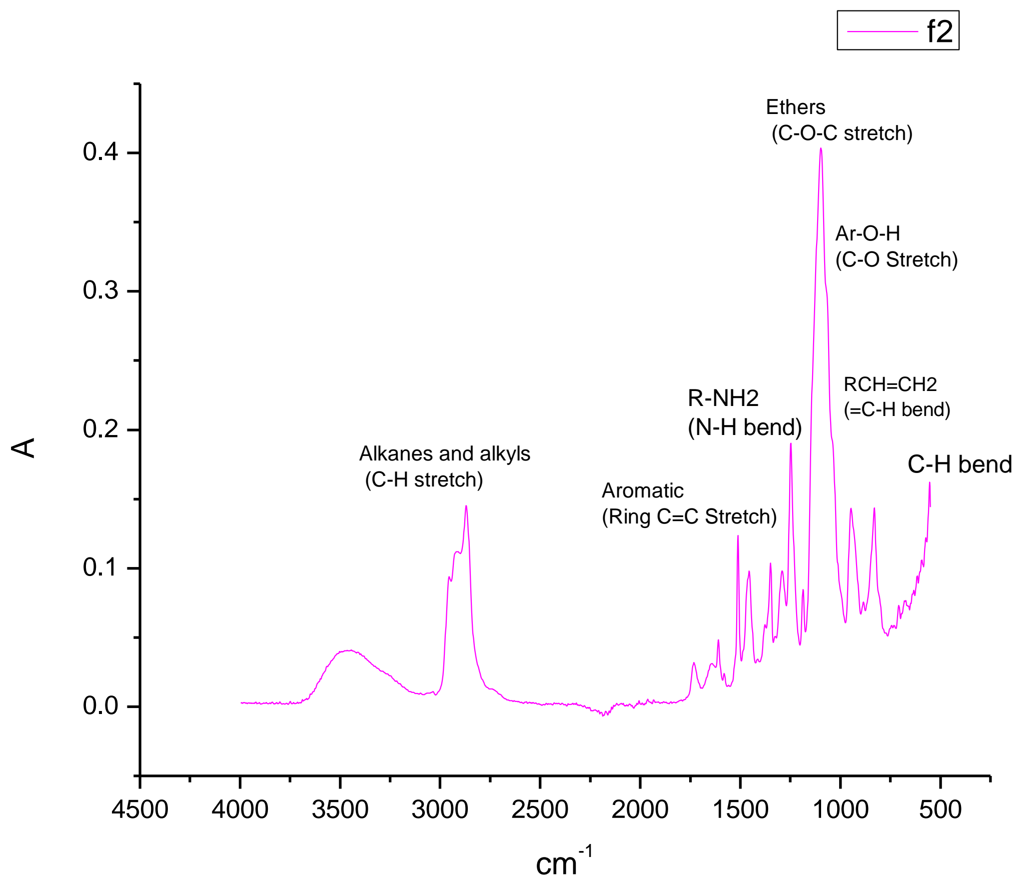


Figure 16 FTIR spectrum of aliquot 2 (fraction 2).

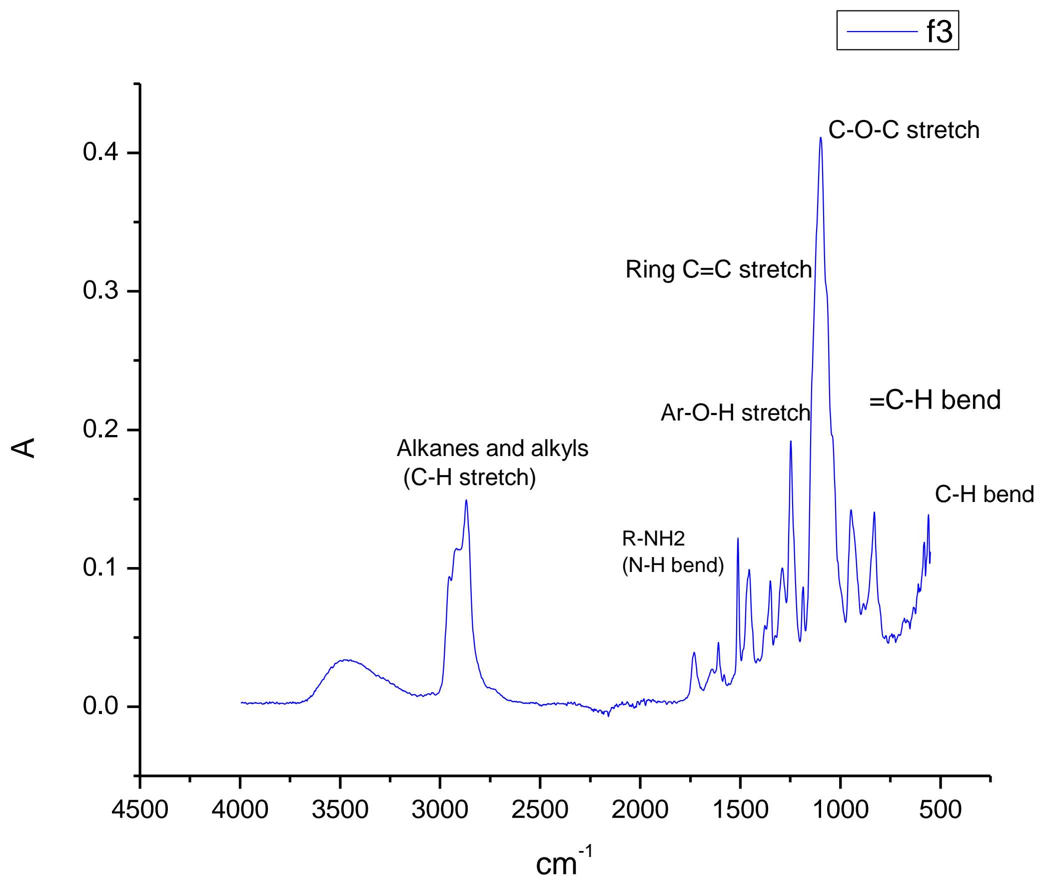


Figure 17 FTIR spectrum of aliquot 3 (fraction 3)

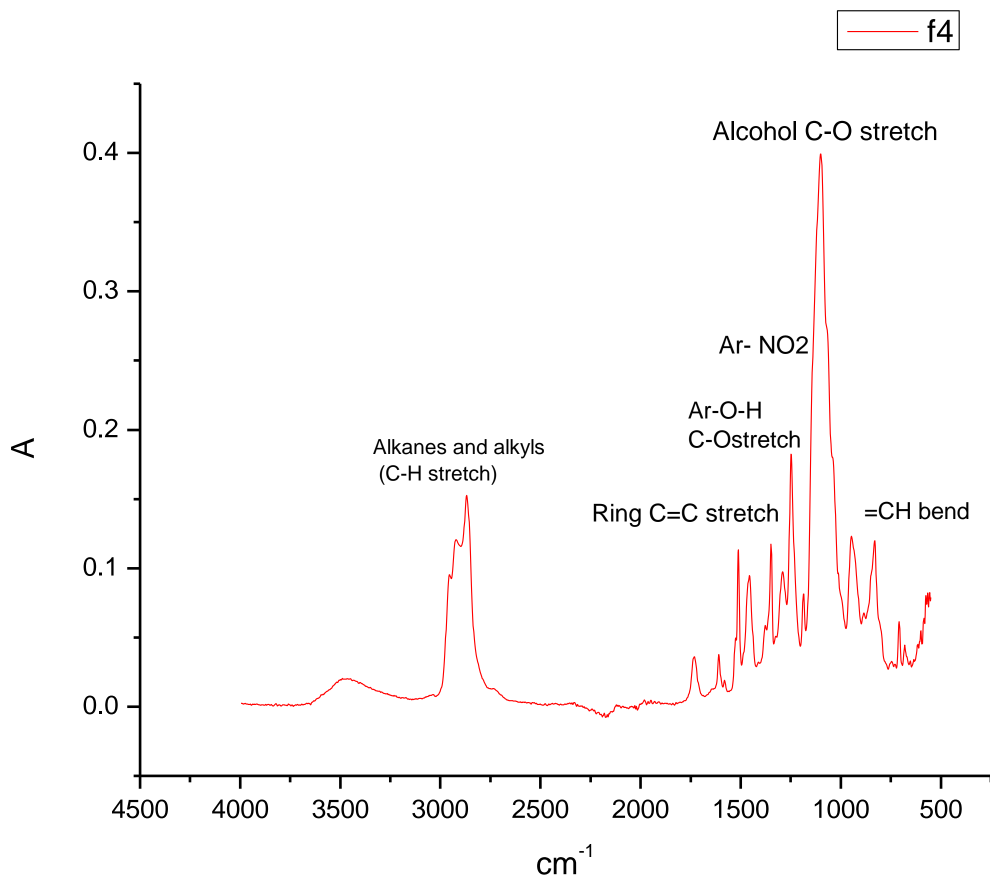


Figure 18 FTIR spectrum of aliquot 4 (fraction 4).

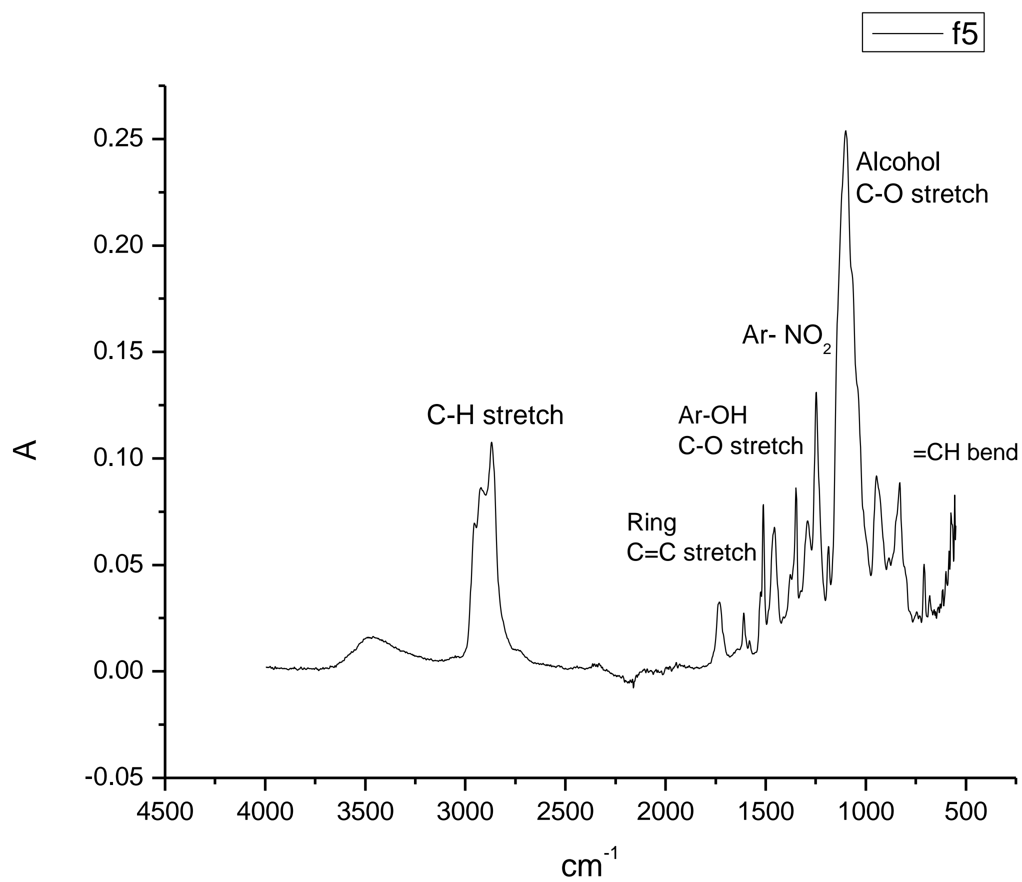


Figure 69 FTIR spectrum of aliquot 5 (fraction 5)

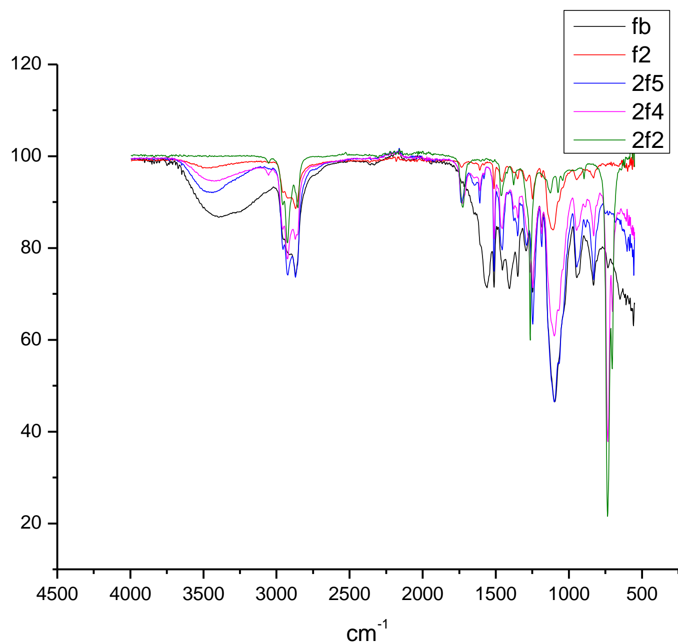


Figure 20 Combine FTIR transmittance spectra of all five spots.

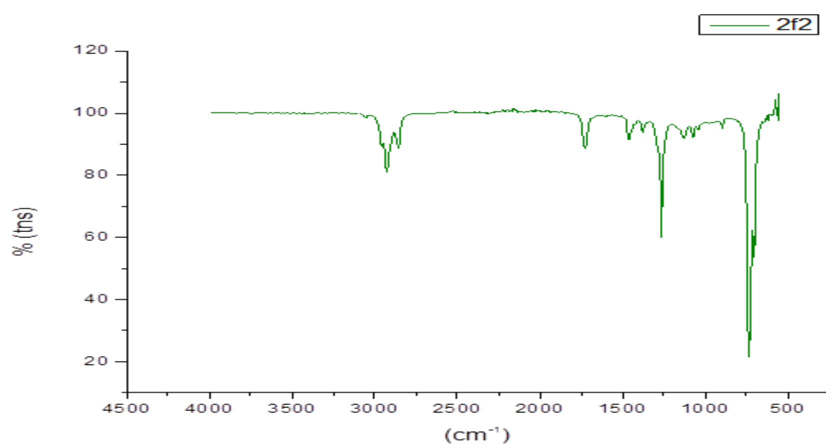


Figure 21 FTIR spectrum of spot 1 (2f2).

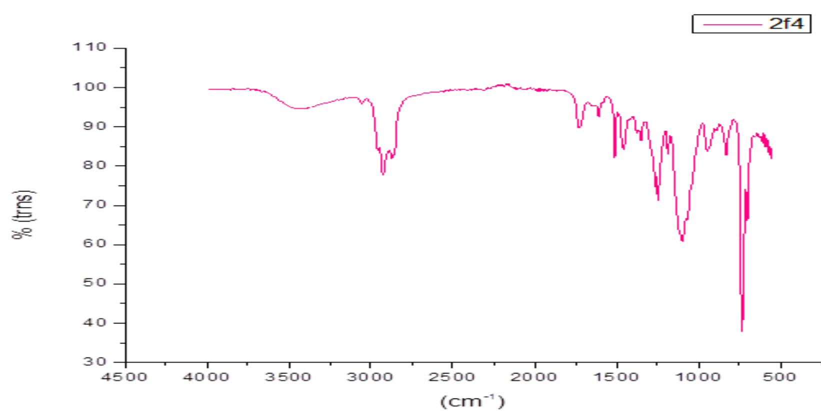


Figure 22 FTIR spectrum of spot 2 (2f4).

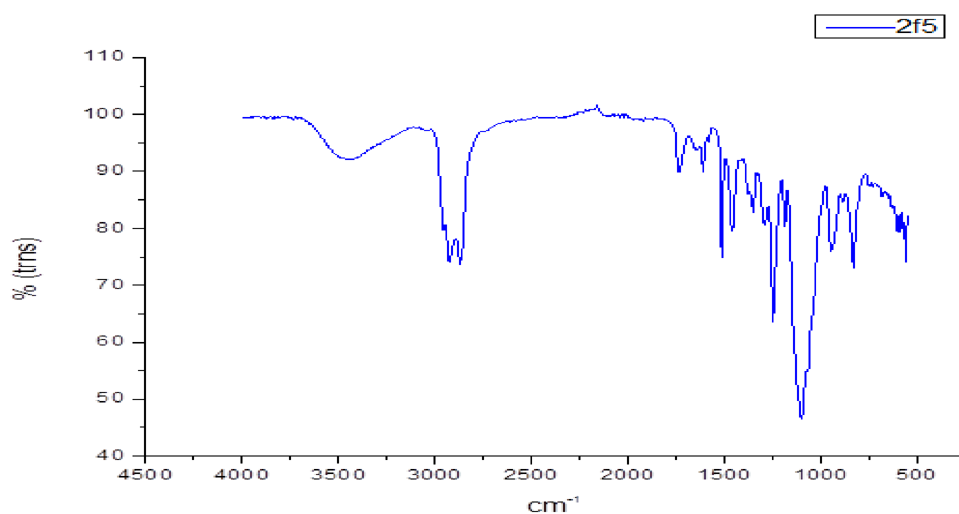


Figure 23 FTIR spectrum of spot 3 (2f5).

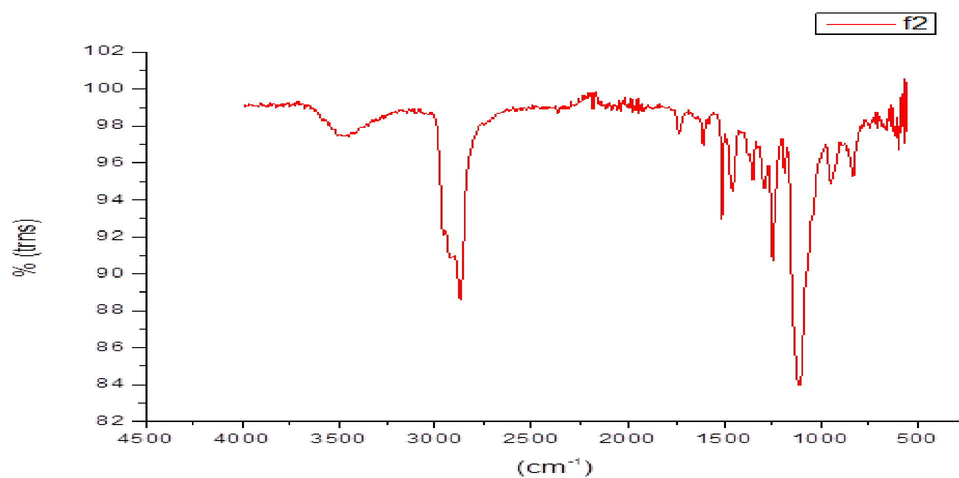


Figure 24 FTIR spectrum of spot 4 (f2).

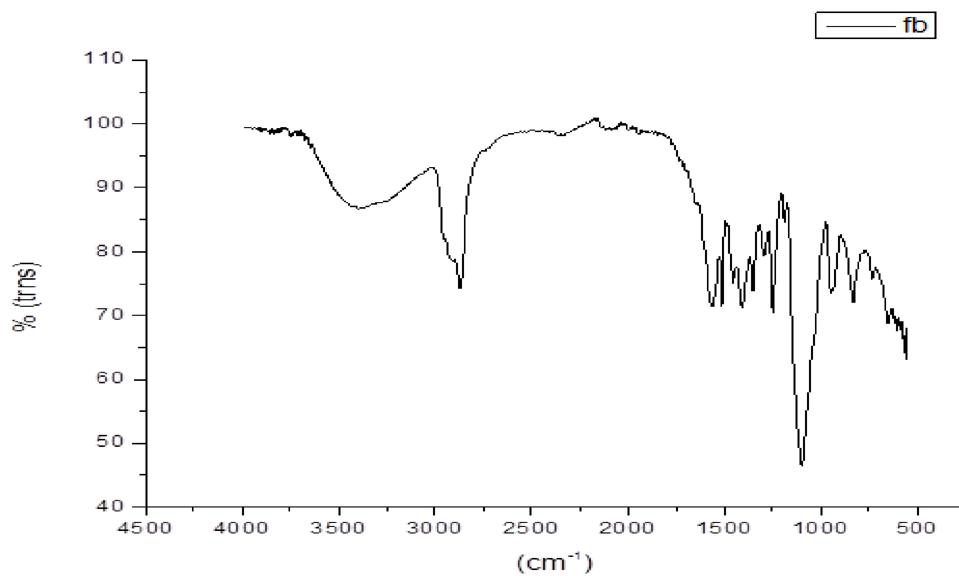


Figure 25 FTIR spectrum of spot 5 (fb).

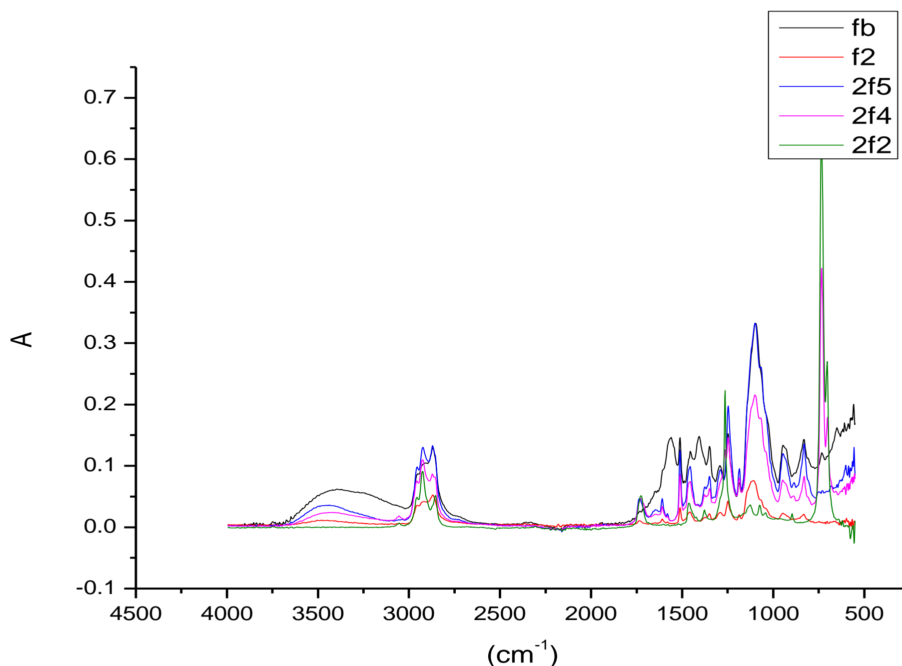


Figure 26 Combine FTIR absorption spectra of all five spots.

Table 1 Different functional groups present in all five spots according to peak values (absorption cm^{-1}) in FTIR spectra.

	Carboxylic acid R-C(O)-OH C=C-C(O)-OH or Ar-C(O)-OH (O- H stretch) (2500-3500cm^{-1})	Alkanes & Alkynes (C-H stretch) (2850-3000 cm^{-1})	Ar-OH (C-O stretch) (1180-1260 cm^{-1})	Aromatic Compounds (C-H bond) (730- 770 cm^{-1})	Alcohol RR'CH-OH or C=C-CRR'-OH (C-O stretch) (1085-1125 cm^{-1})	Amides R-C(O)-NH-R (N-H bend) (1510-1560 cm^{-1} m-s)	Alkanes & Alkynes (C-H bend) (1450-1470 cm^{-1})	Aromatic Compounds (p-disubstituted) (810-840 cm^{-1} C-H bend)	Alkenes (910- 990 cm^{-1}) RCH=CH2 m- s (=C-H bend)
2F2	-----------	2925.56	**1264.10**	734.38	------------	---------------	-----------	----------	-----------
2F4	-----------	2925.29	**1247.58**	733.35	1099.09	**---------------**	-----------	----------	-----------
2F5	-----------	2870.69	**1247.39**	--------------	1098.95	**1510.75**	1456.57	**829.31**	-------
F2	**3413.53**	2870.90	**1248.21**	-------------	1097.59	**1510.80**	----------	-----------	950.30
Fb	--------------	2868.97	**1248.37**	------------	1097.40	**1559.66**	1406.70	---------	--------

4.4 In vitro Antibacterial activity of Flavonoids

The antibacterial activity of five aliquots were performed on six bacterial strains, three gram-positive strains were *S. aureus*, *B. cereus*, *E. fecalis* and three gram-negative strains *S. typhi*, *E. coli* and *P. aeruginosa.* All five aliquots showed significant results on two gram- positive bacterial strains, *P. aeruginosa* and *E. coli* (figure 12, 13). The rest of the bacterial strains were resistant towards all these five aliquots (figure 14). The maximum inhibition zone values showed by all five aliquots on these two bacterial strains were 26mm and 25mm respectively. The ethyl acetate fraction (conjugate flavonoids) not showed as significant result as the aliquots showed. The maximum inhibition zone values of ethyl acetate fraction (conjugated flavonoids) on all bacterial strains were 14mm. These all zone of inhibitions were obtained at higher concentrations of 1mg crude compound ml^{-1}. Chloramphenicol that used as positive control showed the maximum zone of inhibition in the range of 25mm to 30mm against all bacterial strains.

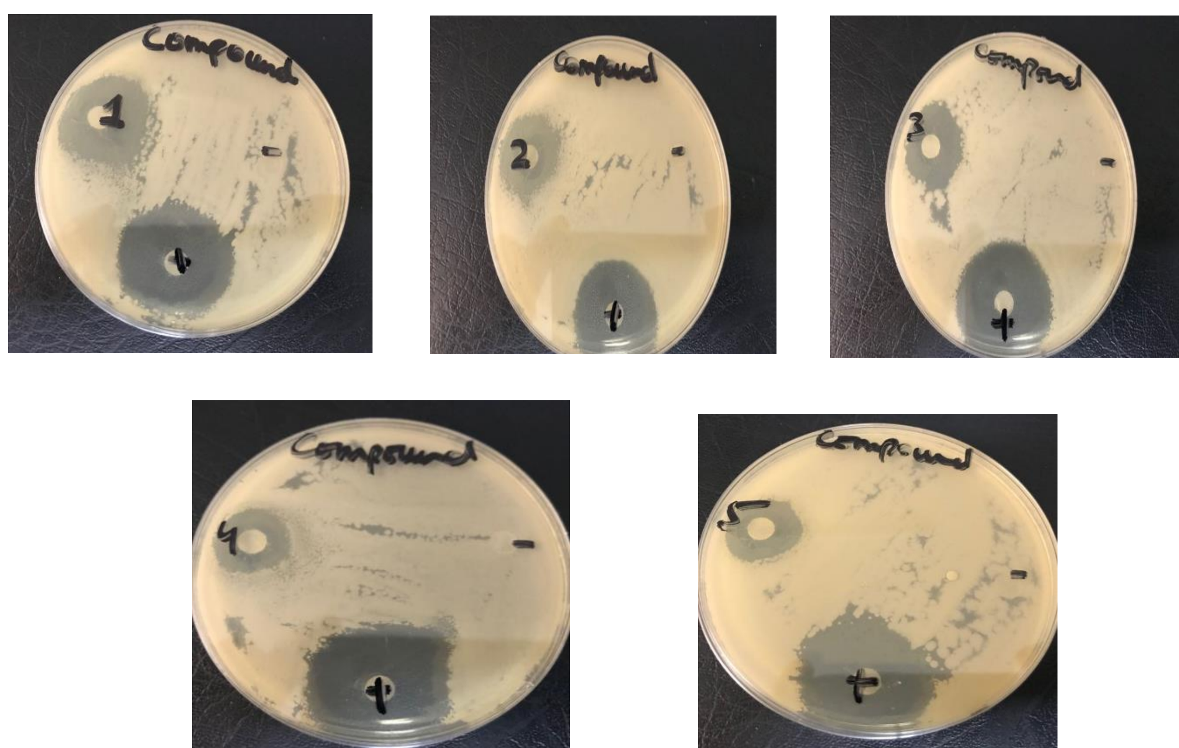


Figure 27 Antibacterial activity of all five aliquots on *P. aeruginosa.*

As in figure 12, the corresponding all five isolated fractions showed maximum inhibition zone on *P. aeruginosa* than other bacterial strains.

Table 2 ZOI (in mm) of isolated fractions on gram negative bacterial strains

	S. typhi	***P. aeruginosa***	***E. coli***
Fraction 1	8	26	26
Fraction 2	7	24	24
Fraction 3	7	23	23
Fraction 4	7	20	23
Fraction 5	7	19	25
+ve control	30	30	30
-ve control	7	7	7

That depicted the bacteriocidal action of all isolated flavonoids fractions. These zones are much greater than the zones showed by crude extract. Following table shows values of ZOI by all isolated fractions on gram negative bacterial strains: The isolated fractions showed highest activity against two-gram negative bacterial strains; *E. coli* and *P. aeruginosa* whereas other bacterial strain *S. typhi* showed resistance towards these isolated fractions.

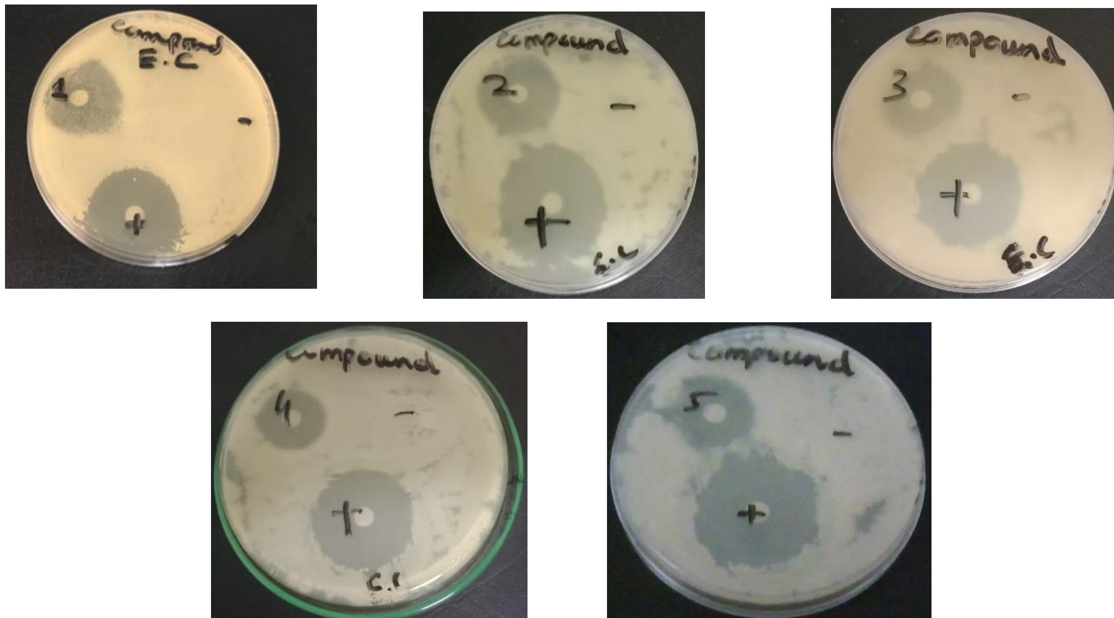


Figure 28 Antibacterial activity of all five aliquots on *E. coli.*

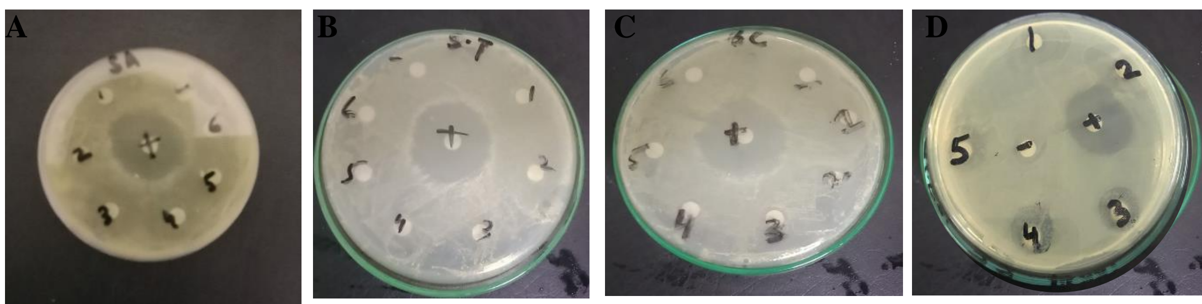


Figure 29 Negative antibacterial results of all five aliquots on bacterial strains. *A) S. aureus B) S. typhi C) B. cereus D) E. fecalis*

Majority classes of flavonoids such as flavones, flavonols, flavanones, and isoflavones along with some of their acylated, methylated and iso-prenylated derivatives have shown vast antibacterial activity. Ali et al reported that chrysin which is a flavone is very much effective on gram negative bacteria *E. coli* and *P. aeruginosa* correspondence to a well-known

antibiotic streptomycin. While Basile et al reported that other flavones apigenin, vitexin, saponarin and lucenin 2-O-glycoside, and luteolin 7-O-gly-coside are effective against gram-negative bacilli: *Proteus vulgaris (P vulgaris)*, *Proteus mirabilis (Pmirabilis)*, *Ps aeruginosa*, *E coli, Klebsiella pneumoniae (K pneumoniae*), and *Enterobacter cloacae (E cloacae*); however, all they showed no activity against the Gram-positive Cocci *S aureus and Enterococcus faecalis (E faecalis).*

Table 3 ZOI (in mm) of isolated fractions on gram positive bacterial strains

	E. fecalis	***B. cereus***	***S. aureus***
Fraction 1	7	8	7
Fraction 2	7	8	7
Fraction 3	8	8	7
Fraction 4	9	8	7
Fraction 5	7	7	7
+ve control	25	30	30
-ve control	7	7	7

In above mention table 2, all fractions showed ineffective results against gram positive bacterial that was obtained after measuring the ZOI values of all respective fractions. That leads to the conclusion that there is a possibility of presence of such flavonoids groups like flavones that shows effective antibacterial activity against gram negative bacteria than gram positive bacterial strains. There might be a presence of those compounds which have at least one or two cis-p-coumaroyl groups, as these groups belong to acylated flavonols. It is

reported in literature that the activity of acylated flavonols is more effective on gram positive than gram negative bacterial strains. The comparative analysis of antibacterial activity of all five fractions on gram negative as well as gram positive bacterial strains is illustrated in fig 15. The activity of both fractions F4 and F5 were seemed to the most effective against *E. coli* among other fractions.

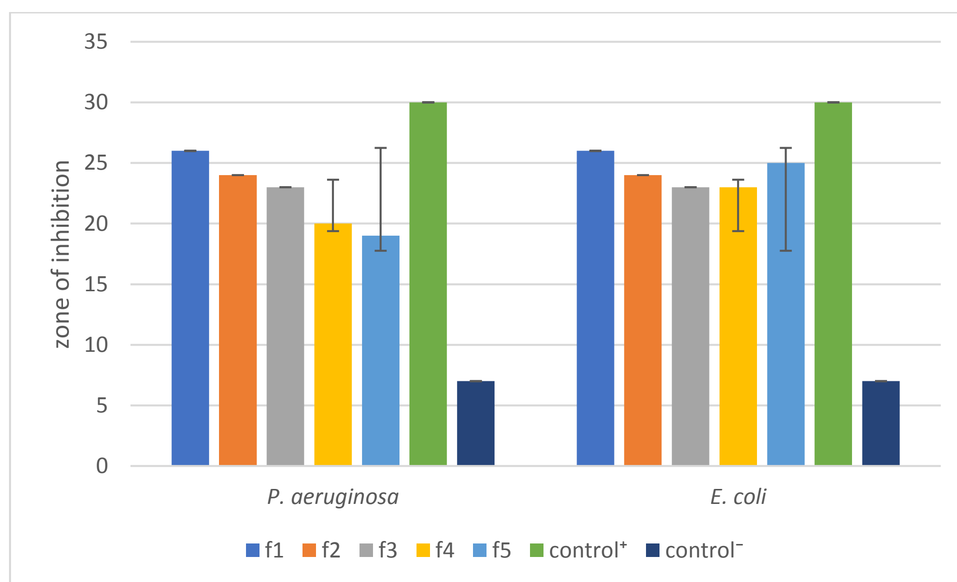
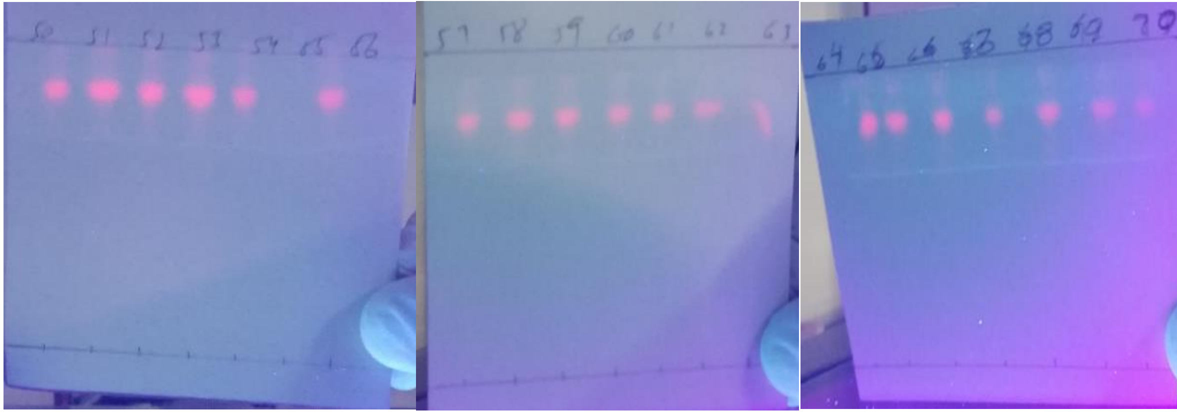


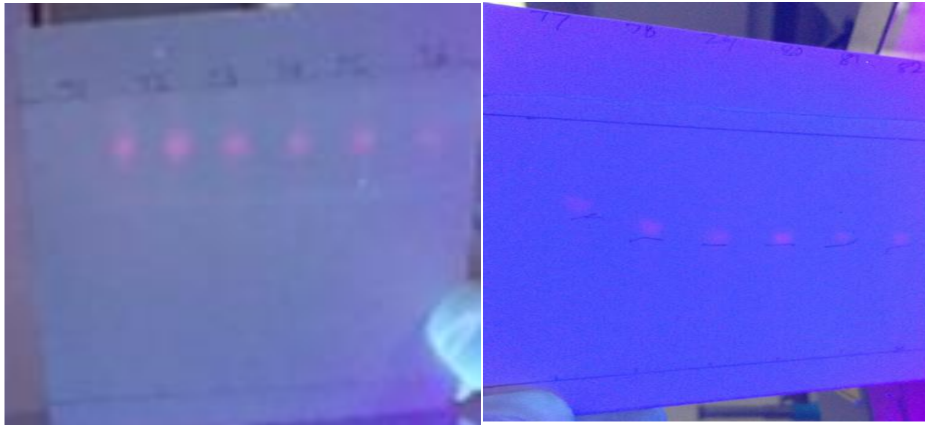
Figure 30 Antibacterial activity of flavonoids in all five fractions against P. *aeruginosa and E. coli*.

4.5 Thin layer chromatography of isolated fractions:

Different spots were obtained from all isolates. The isolates having similar R_f values were combine and fractions were made. Total five fractions were made after combining their isolates. All these five fractions had different R_f values. Based on different R_f values they were further subjected for confirmation of functional groups, that was performed by using FT-IR spectroscopy.



a) R_f value 0.9 **b) R_f value 0.8** **c) R_f value 0.7**



d) R_f value 0.6 **e) R_f value 0.4**

Figure 31 Thin layer chromatogram of all five fractions along with distinct R_f values

Table 4 List of functional groups present in Quercetin standard

Absorption cm^{-1}	Functional groups
3238.65	**Carboxylic acids (O-H stretch)**
1660.33	Alkenes C=C stretch
1603.87	Amines N-H bend
1518.89	**Amides N-H bend**
1446.38	
1378.12	Alkanes and Alkyls $CH(CH_3)_2$ or $CH(CH_3)_3$ bend
1257.07	**Alcohol C-O stretch**
1194.49	Alcohol C-O stretch
1165.18	Alcohol C-O stretch
1012.84	Alkyl halides C-H stretch
863.86	-------
817.95	**Aromatic compounds C-H bend**
637.18	Alkynes C-H bend
601.98	R- Br C- Br stretch

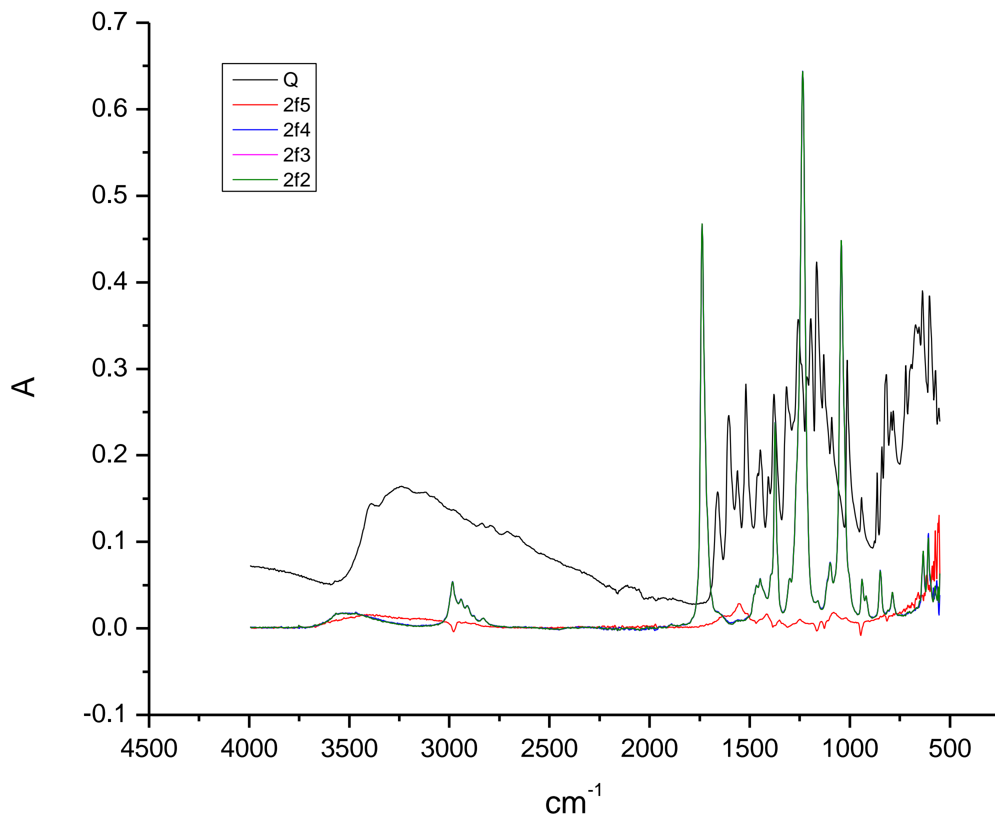


Figure 32 Antibacterial activity of flavonoids in all five fractions against P. *aeruginosa and E. coli*.

CHAPTER 5: DISCUSSION AND CONCLUSION

5.1 Discussion

The most famous multidrug resistant (MDR) organisms includes the pathogenic strains of species such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are considered as dangerous entities to medical practices. There are apparently no antibiotics available for their treatment. That problem evokes a need of development of new antibacterial drugs. These noval antibacterial drugs are currently preferred to obtain from many medicinal plants. Many researches have been done so far on production of drugs those are natural in origin. These plants are used for investigation of causative compounds that show antibacterial activity, leads to ultimately isolation of bioactive compounds for used in many therapeutical purposes. The isolation of bioactive compound is considered as a potential source towards the formation of natural drugs with minimal side effects as well as economical in use. The ability of production of many secondary metabolites is well known in plants. Some of these secondary metabolites are alkaloids, glycosides, terpenoids, saponins, steroids, flavonoids, tannins, quinones and coumarins that give therapeutic property to plants. These secondary metabolites are proved as a source of plant-derived antimicrobial substances (PDAms). They are also highly efficient in the treatment of bacterial infections.

Based on all above-mentioned points, we constructed this study for the investigation of new compounds from natural source to combat against multidrug resistance phenomena. For this purpose, a well-known medicinal plant *Berberis lycium* was selected. As the total number of publications on therapeutical applications of this plant is about 500 provided by the database of National Center for Biotechnology Information (NCBI) PubMed in January 2018 (Bober, Aebisher, & Bartusik-aebisher, 2018). The targeted phytochemical obtained from this plant was flavonoids. Numerous publications have been increasingly documented on antibacterial activity of flavonoids. So, flavonoids were isolated in ethyl acetate solvent as its nature is polar

that's why it can only be separated through a polar solvent. That was decided by the relative increasing polarity order: cyclohexane < pet. Ether < pentane < carbon tetrachloride < benzene < toluene < chloroform < di-ethyl ether < ethyl acetate < ethanol < acetone < acetic acid < methanol. The selected 15% methanol in ethyl acetate as a mobile phase showed the presence of polar flavonoids. As this mobile phase gave better separation of flavonoids. Polar nature of flavonoids depicted the presence of -OH groups in all isolated fractions. This shows the structure-relationship of flavonoids as antibacterial agents. In previous studies, it is concluded that the improved antibacterial activity of flavonoids is due to the presence of hydroxyls at special sites on its aromatic rings (Xie, Yang, Tang, Chen, & Ren, 2014). There is a history of folk medicines manufactured from crude extract of plants for antibacterial activity that leads to further screening of bioactive compounds**.** There have also been reported various other phytochemical preparations containing high flavonoids to elicit antibacterial activity (Cushnie & Lamb, 2011).

FTIR analysis shown by all five fractions depicted various kinds of flavonoids that might be resembles with addition to direct and synergistic antibacterial activity. Various bacterial virulence factors, including enzymes, toxins and signal receptors can be interfered by flavonoids as reported in many findings. This opens the possibility of flavonoids being developed as anti-virulence therapies (Cushnie & Lamb, 2011).

The healing properties of *Berberis* have been known and appreciated for thousands of years. (Elisha, Botha, Mcgaw, & Eloff, 2017). Flavonoids belong to a class of plant constituents that have received increasing interest over the past few years. In many reported studies, the underlying mechanisms behind the antibacterial activity of flavonoids are might be due to its inhibitory action towards synthesis of DNA or RNA, cytoplasmic membrane function, inhibition of biofilm formation and porin attachment on the cell membrane, alteration of the membrane permeability, and attenuation of the pathogenicity (Xie et al., 2014). Due to this

fact, an in vitro antibacterial activity of naturally occurring flavonoids from a medicinal plant *Berberis lycium* was tested against three-gram positive bacterial strains: *Enterococcus faecalis* (Clinically isolated), *Bacillus cereus* (soil isolated), *Staphylococcus aureus* (ATCC 6538), whereas gram negative strains including *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella Typhi* (ATCC 6539) and *Escherichia coli* (ATCC 8739). From the measurement of inhibition diameter, the more susceptible bacterial strains towards all isolated fractions were estimated. The results are summarized in Table 1 & 2**.** The negative results shown by fractions on certain bacterial strains like *E. fecalis, B. cereus, S. aureus, was* might be due poor and slow diffusion of compounds in agar (Nowak, Kopacz, Gruszecka, & Golec, 2013). As some extracts are effective against some specific pathogens. It is may not necessarily important that extracts having high antibacterial activity against gram negative bacteria must show high activity against other gram-negative bacteria compared to gram positive bacteria. This means that dependency of antimicrobial activity is not related to difference in structure of cell wall. It depends on MIC values, as different ranges of these values effect on different strains of same bacterial species. (Elisha, Botha, Mcgaw, & Eloff, 2017). Therefore, results of this study showed the significance of plant extracts comparable with antibiotics, to control resistant bacteria, which are becoming a life-threatening problem day by day. Moreover, the toxic effects of antibiotic resistant bacteria can be lessened by plant extracts at s very low concentration, therefore minimizing the possible toxic effects (Nascimento, Locatelli, Freitas, Silva, & Piracicaba, 2000).

5.2 Conclusion

As the proposed study was conducted to isolate the bioactive flavonoids from *Berberis lycium* leaves. So, flavonoids were successfully isolated from this plants by selecting best mobile phase after several trials through TLC and column chromatography. The antibacterial activity of isolated flavonoids was further tested that showed effective results on two human

pathogenic bacterial strains; *E. coli and P. aeruginosa*. Hence proved the therapeutical potential of this plant. The characterization through FTIR indicated the presence of flavonoids in our separated aliquots. This study further insights towards the synthesis of natural bioactive drugs that are effective against diverse types of bacterial strains.

Future perspective

- To perform structure elucidation of noval compound and enhance its antimicrobial activity by structural modification of these isolated compounds.
- To determine the regulatory gene in metabolic pathway of antimicrobial compound, and to increase the production of antibacterial substances by application of genetic engineering technology.
- In vivo screening in Balb/c mice model, for evaluating the prospective toxicity screen.
- Optimal potency or selectivity of isolated compound.
- Selection of modified characterization techniques for proper structure elucidation of novel compounds.

REFERENCES

Abdulhamid, A., Fakai, I. M., & Ogwihi, O. J. (2017). Phytochemical Screening , Chromatographic Studies and Antibacterial Activity of Carica Papaya Leaves Extracts, *3*(1), 11–15. https://doi.org/10.20431/2454-7999.0301002

Ahmed, S. (2017). Evaluation of different parts of Berberis lyceum and their biological activities: a review. *Pure and Applied Biology*, *6*(3). https://doi.org/10.19045/bspab.2017.60095

Bober, Z., Aebisher, D., & Bartusik-aebisher, D. (2018). Fundamentals of the use of Berberis as a medicinal plant, *1361*(March), 41–46. https://doi.org/10.15584/ejcem.2018.1.7

Brusotti, G., Cesari, I., Dentamaro, A., Caccialanza, G., & Massolini, G. (2014). Isolation and characterization of bioactive compounds from plant resources: The role of analysis in the ethnopharmacological approach. *Journal of Pharmaceutical and Biomedical Analysis*, *87*, 218–228. https://doi.org/10.1016/j.jpba.2013.03.007

Corcoran, M. P., McKay, D. L., & Blumberg, J. B. (2012). Flavonoid Basics: Chemistry, Sources, Mechanisms of Action, and Safety. *Journal of Nutrition in Gerontology and Geriatrics*, *31*(3), 176–189. https://doi.org/10.1080/21551197.2012.698219

Cushnie, T. P. T., & Lamb, A. J. (2005a). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents*, *26*(5), 343–356. https://doi.org/10.1016/j.ijantimicag.2005.09.002

Cushnie, T. P. T., & Lamb, A. J. (2005b). Antimicrobial activity of flavonoids, *26*, 343–356. https://doi.org/10.1016/j.ijantimicag.2005.09.002

Cushnie, T. P. T., & Lamb, A. J. (2011). Recent advances in understanding the antibacterial properties of flavonoids. *International Journal of Antimicrobial Agents*, *38*(2), 99–107. https://doi.org/10.1016/j.ijantimicag.2011.02.014

Cuyckens, F., & Claeys, M. (2004). Mass spectrometry in the structural analysis of flavonoids. *Journal of Mass Spectrometry*, *39*(1), 1–15. https://doi.org/10.1002/jms.585

de Rijke, E., Out, P., Niessen, W. M. A., Ariese, F., Gooijer, C., & Brinkman, U. A. T. (2006). Analytical separation and detection methods for flavonoids. *Journal of Chromatography A*, *1112*(1–2), 31–63. https://doi.org/10.1016/j.chroma.2006.01.019

Di, D. L., Zheng, Y. Y., Chen, X. F., Huang, X. Y., & Feng, S. L. (2011). Advances in application of high-speed countercurrent chromatography in separation and purification of flavonoids. *Fenxi Huaxue/ Chinese Journal of Analytical Chemistry*, *39*(2), 269–275. https://doi.org/10.1016/S1872-2040(10)60419-7

Elisha, I. L., Botha, F. S., Mcgaw, L. J., & Eloff, J. N. (2017). The antibacterial activity of extracts of nine plant species with good activity against Escherichia coli against five other bacteria and cytotoxicity of extracts, 1–10. https://doi.org/10.1186/s12906-017-1645-z

Falcone Ferreyra, M. L., Rius, S. P., & Casati, P. (2012). Flavonoids: biosynthesis, biological functions, and biotechnological applications. *Frontiers in Plant Science*, *3*(May). https://doi.org/10.3389/fpls.2012.00222

Ferreira, A., Santos, A. O., Falcão, A., & Alves, G. (2018). In vitro screening of dual flavonoid combinations for reversing P-glycoprotein-mediated multidrug resistance: Focus on antiepileptic drugs. *Food and Chemical Toxicology*, *111*(November 2017), 84–93. https://doi.org/10.1016/j.fct.2017.11.004

Guimarães, C. C., Oliveira, D. D., Valdevite, M., Saltoratto, A. L. F., Pereira, S. I. V., França, S. de C., … Pereira, P. S. (2015). The glycosylated flavonoids vitexin, isovitexin, and quercetrin isolated from Serjania erecta Radlk (Sapindaceae) leaves protect PC12 cells against amyloid-β25-35peptide-induced toxicity. *Food and Chemical Toxicology*, *86*, 88–94. https://doi.org/10.1016/j.fct.2015.09.002

Gupta, M., Singh, A., & Joshi, H. C. (2015). Berberis Lycium multipotential medicinal application : An overview, *3*(4), 10–13.

Hernández, N. E., Tereschuk, M. L., & Abdala, L. R. (2000). Antimicrobial activity of flavonoids in medicinal plants from Tafí del Valle (Tucumán, Argentina). *Journal of Ethnopharmacology*, *73*(1–2), 317–322. https://doi.org/10.1016/S0378-8741(00)00295-6

Hossain, M. A., AL-Raqmi, K. A. S., AL-Mijizy, Z. H., Weli, A. M., & Al-Riyami, Q. (2013). Study of total phenol, flavonoids contents and phytochemical screening of various leaves crude extracts of locally grown Thymus vulgaris. *Asian Pacific Journal of Tropical Biomedicine*, *3*(9), 705–710. https://doi.org/10.1016/S2221-1691(13)60142-2

Intekhab, J., & Aslam, M. (2009). Isolation of a flavonoid from Feronia limonia. *Journal of Saudi Chemical Society*, *13*(3), 295–298. https://doi.org/10.1016/j.jscs.2009.10.012

Ke, M., Zhang, X. J., Han, Z. H., Yu, H. Y., Lin, Y., Zhang, W. G., … Wang, T. J. (2011). Extraction, purification of Lycium barbarum polysaccharides and bioactivity of purified fraction. *Carbohydrate Polymers*, *86*(1), 136–141. https://doi.org/10.1016/j.carbpol.2011.04.023

Mujwah, A. A., Mohammed, M. A., & Ahmed, M. H. (2010). First isolation of a flavonoid from Juniperus procera using ethyl acetate extract. *Arabian Journal of Chemistry*, *3*(2), 85–88. https://doi.org/10.1016/j.arabjc.2010.02.003

Nascimento, G. G. F., Locatelli, J., Freitas, P. C., Silva, G. L., & Piracicaba, U. M. De. (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic-, 247–256.

Nishiumi, S., Miyamoto, S., Kawabata, K., Ohnishi, K., Mukai, R., Murakami, A., … Terao, J. (2011). Dietary flavonoids as cancer-preventive and therapeutic biofactors. *Frontiers in Bioscience (Scholar Edition)*, *3*(November 2014), 1332–1362. https://doi.org/229 [pii]

Nowak, D., Kopacz, M., Gruszecka, J., & Golec, K. (2013). Pharmacology comparative study on the antibacterial activity of some flavonoids and their sulfonic derivatives, *70*(3), 567–571.

Panche, A. N., Diwan, A. D., & Chandra, S. R. (2016). Flavonoids: An overview. *Journal of Nutritional Science*, *5*. https://doi.org/10.1017/jns.2016.41

Peterson, J., & Dwyer, J. (1998). Flavonoids: Dietary occurrence and biochemical activity. *Nutrition Research*, *18*(12), 1995–2018. https://doi.org/10.1016/S0271-5317(98)00169-9

Rauha, J.-P. P., Remes, S., Heinonen, M., Hopia, A., Kähkönen, M., Kujala, T., … Kahkonen, M. (2000). Antimicrobial effects of Finnish plant extracts containing flavoniods and other phenolic compounds. *International Journal of Food Microbiology*, *56*(1), 3–12. https://doi.org/10.1016/S0168-1605(00)00218-X

Sabir, S., Tahir, K., Rashid, N., Naz, S., Masood, B., Shah, M. A., & Sualeh, M. (2013). Phytochemical and antioxidant studies of Berberis lycium. *Pakistan Journal of Pharmaceutical Sciences*, *26*(6), 1165–1172.

Seleem, D., Pardi, V., & Murata, R. M. (2017). Review of flavonoids: A diverse group of natural compounds with anti-Candida albicans activity in vitro. *Archives of Oral Biology*, *76*, 76–83. https://doi.org/10.1016/j.archoralbio.2016.08.030

Shabbir, A. (2012). Berberis lycium Royle: A review of its traditional uses, phytochemistry and pharmacology. *African Journal of Pharmacy and Pharmacology*, *6*(31), 2346–2353. https://doi.org/10.5897/AJPP12.927

Sharma, V., & Janmeda, P. (2017). Extraction, isolation and identification of flavonoid from Euphorbia neriifolia leaves. *Arabian Journal of Chemistry*, *10*(4), 509–514. https://doi.org/10.1016/j.arabjc.2014.08.019

Singh, D., & Singh, V. (2016). Isolation and Characterization of Flavonoids in Urena lobata Leaves. *European Journal of Medicinal Plants*, *11*(1), 1–6. https://doi.org/10.9734/EJMP/2016/21502

Singh, M., Kaur, M., & Silakari, O. (2014). Flavones: An important scaffold for medicinal chemistry. *European Journal of Medicinal Chemistry*, *84*, 206–239. https://doi.org/10.1016/j.ejmech.2014.07.013

Sood, P., Modgil, R., & Sood, M. (2012). Berberis lycium a Medicinal Plant with Immense Value. *Pharmaceutical & Biological Research*, *1*(1), 1–9.

Stalikas, C. D. (2007). Extraction, separation, and detection methods for phenolic acids and flavonoids. *Journal of Separation Science*, *30*(18), 3268–3295. https://doi.org/10.1002/jssc.200700261

Sultana, B., Anwar, F., & Ashraf, M. (2009). Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. *Molecules*, *14*(6), 2167–2180. https://doi.org/10.3390/molecules14062167

Xie, Y., Yang, W., Tang, F., Chen, X., & Ren, L. (2014). Antibacterial Activities of Flavonoids : Structure-Activity Relationship and Antibacterial Activities of Flavonoids : Structure-Activity Relationship and Mechanism, (September). https://doi.org/10.2174/0929867321666140916113443